INHIBITORY EFFECT OF PLANT NATURAL PRODUCTS ON THE BREAST CANCER DEVELOPMENT AND PROGRESSION

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CERTIFICATE

This is to certify that the thesis entitled **"INHIBITORY EFFECT OF PLANT NATURAL PRODUCTS ON THE BREAST CANCER DEVELOPMENT AND PROGRESSION"** is a bona fide record of research work carried out by **Ms. Gilcy George K.**, under my guidance and supervision at Amala Cancer Research Centre, Thrissur, Kerala, India, and no part thereof has been presented for the award of any other degree, diploma or other similar titles of any University or Institute. 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 **"INHIBITORY EFFECT OF PLANT NATURAL PRODUCTS ON THE BREAST CANCER DEVELOPMENT AND PROGRESSION"** is based on the original research work carried out by me at Amala Cancer Research Centre, Thrissur, Kerala, India, under the guidance and supervision of **Dr. Girija Kuttan, Ph.D.,** Professor, Dept. of Immunology, Amala Cancer Research Centre. This thesis has not previously formed the basis for the award of any degree, diploma or other similar titles of any other university.

Gilcy George K

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CONTENTS

Introduction

Nature has been the rich source of medicinal products for the past millions of years. With the advent of many useful drugs from plant sources, plants have formed the basis of indigenous traditional medicine systems, dating from around 2600 BC in Mesopotamia. Egyptian record on medicinal plants "Ebers Papyrus", the Chinese "Materia Medica", the documentation of the Indian Ayurvedic system "Charaka Samhita and Sushruta Samhita" and the contributions of Greeks, Romans and Arabs all points out the importance of plants and plant derived products in the ancient traditional medical practices, that formed the basis for treatments still used today (Cragg and Newman, 2013). Research on natural products, that to be used in the treatment of cancer is on high demand, especially with the focus on those derived from plant species. Naturally derived compounds are considered to have less toxic side effects compared to current treatments (Greenwell and Rahman, 2015).

In 2030 the number of cancer deaths is projected to increase 11.5 million globally. In order to manage or arrest the carcinogenic process the use of medicinal plant products as an alternative or in combination to conventional allopathic medicine is highly encouraged. Several herbal products have already been evaluated in clinical studies and are still under investigation to find out the tumouricidal properties against different types of cancers. The Indian Ayurvedic system of medicine incorporates herbs into its treatment regime. The holistic approach in the Ayurveda treatment claimed to provide an effective alternative to individual plant isolates in the treatment of cancer. The well known Ayurvedic classics Charaka and Sushruta samhitas, describe cancer as inflammatory or non-inflammatory swelling and mention them as either Granthi (minor neoplasm) or Arbuda (major neoplasm) (Desai et al., 2008).

Recent statistics shows that globally breast cancer is the most commonly occurring cancer among women. Breast cancer comprises twenty three percent of the approximate 1.7 million female cancers that are newly diagnosed each year. In the last 5 years ~ 6.2 million women were diagnosed with breast cancer, making breast cancer the single most prevalent cancer around the globe (Braithwaite et al., 2016). The TNM system, where T is the size of tumour, N denotes whether or not the tumour has spread to lymph nodes and M the

metastatic status of the tumour to distant sites of the body is used to assess breast cancer staging. Stage zero is a marker condition or precancerous condition. In stage one to three there is the presence of tumour within breast and lymph nodes. The stage four is the most severe condition where it has already reached the blood stream and metastatic progression to distant organs has occurred. At this stage the cancer is managed by combinations of all treatments such as surgery, chemotherapy, radiation therapy and targeted therapies. The survival rate is about ten percent with treatment and five percent without treatment (Carlson et al., 2009).

At the time of diagnosis most cancer patients already have disseminated disease either in the form of undetectable micrometastases. The features of primary tumour cells will persist in the metastases. To survive the metastatic cascade the cancer cells should have the crucial characteristics like heterogeneity, plasticity and genetic instability. The metastatic cells evolve from the primary tumours and may develop resistance to current therapeutic approaches and establish themselves in a new microenvironment. This process is of central importance from a drug discovery perspective (Lehembre and Regenass, 2012).

In the last few years Complementary And Alternative medicine (CAM) has been developed as one of the major aspects of cancer therapy in order to alleviate the side effects of drugs and for relieving the pain in breast cancer patients (Mansky and Wallerstedt, 2006). It is essential to find other adjuvant methods for breast cancer therapy and CAM may be valuable for optimizing the conventional therapy. To find out the effect of CAM in breast cancer patients several studies were already been done. Medicinal plants can be used in combination with conventional medicine as a supportive therapy to improve Health-Related Quality of Life (HRQoL). It has been shown that the use of some types of CAM in breast cancer patients has dramatically increased and is gaining in popularity. For example as an anthroposophical medicine, mistletoe is one of the most important herbal drugs and is potentially effective against cancer. Using mistletoe extract for cancer therapy especially breast cancer is recommended due to its minimal side effects and the fact that these side effects are not life threatening (Marvibaigi et al., 2014). The natural products used in the mainstay

of cancer therapy are nature selected. Mother Nature has taken about three billion years to refine her chemistry and today we are only scratching the surface in exploring Nature's molecular diversity (Cragg and Newman, 2013).

Relevance and objectives of the study

Agents that are able to block metastatic process of tumour cells have wide potential as anticancer agents therefore, it is essential to search for novel antimetastatic agents with minimal side effects. Hence there is an incitement to find out newer drugs with less toxic effects to prevent metastasis (George and Kuttan, 2016). In the present study we have taken an effort to find out the effects of selected natural products from the medicinal plants on the breast cancer development and progression in order to assess the therapeutic efficacy of these products in a catastrophic complication like metastatic cancer progression using syngeneic mouse 4T1 breast tumour model, in mice with normal immune function. To our knowledge, when introduced orthotopically this tumour model has the capacity to metastasize in a way that is similar to or can mimic the breast cancer metastasis in humans and is found to be the only system of its kind (Tao et al., 2008).

There was a strong literature background for the selection of the natural products used in the study. Source plants of these selected natural products are well renounced for its uses in the traditional medicines. These plants are already been in use for various disease treatments including cancer in the indigenous systems of traditional medicine including Ayurveda system in India. In the present study we also tried to analyse the effect of these selected products in the allied aspects of cancer treatment like anti-inflammatory, antiangiogenic, immunomodulatory or the usefulness in combination with conventional radiotherapy and chemotherapy.

We analysed the effect of selected plant products on the immune system that are not previously been examined for its immunomodulatory effects because the functional involvements of immune system in resisting or eradicating budding

neoplasias, late-stage tumours, and micrometastases have great impact on tumour growth and development (Hanahan and Weinberg, 2011).

Other important aspects we analysed were inflammation and angiogenesis that are closely related to tumour initiation and progression. Inflammation plays decisive roles in every single step of tumourigenesis, from initiation through tumour promotion, all the way to metastatic progression with significant effects on different stages of tumour development like malignant conversion, invasion etc (Grivennikov et al., 2010). Formation of new blood vessels from pre-existing vasculature is an indispensable process in the tumour initiation, invasion and metastasis (Bhat and Singh, 2008).

As part of our study we also tried to find out whether any of the selected plant products can be used in combination to the existing therapeutic approaches. Radiation therapy is one of the prime treatment modality of breast cancer. But the cancer initiating tumour cells present in the hypoxic tumour environment will form a major hurdle for the therapeutic success. Likewise chemotherapy using agents like cyclophosphamide in cancer treatment, has been shown to have bladder toxicity in the form of hemorrhagic cystitis, immunosuppression, alopecia and at high doses cardiotoxicity. Agents those are able to alleviate these negative results of conventional therapy will be of great significance in the battle against cancer.

Chapter 1

Review of literature

1.1. Role of immune system in cancer development and progression

Immune system functions as an open integrated system. The link between many herbal products and various aspects of immunity is well established; therefore immune-related therapeutic potential of herbal products cannot be underestimated (Majdalawieh and Fayyad, 2015). Theory of immune surveillance proposes that cells and tissues are constantly monitored by an ever alert immune system and is responsible for recognizing and eliminating the vast majority of incipient cancer cells and thus nascent tumours. Solid tumours that are able to avoid detection or have been able to limit the extent of immunological killing can still come into sight by evading eradication (Hanahan and Weinberg, 2011). For the maintenance of homeostasis, immune responses are well coordinated in multicellular organisms. To bestow maximum protective immunity there is an integration of various branches of innate and adaptive immune systems with proper recognition, processing, and presentation of antigenic agents (Baker et al., 2014).

Immunomodulators act either by stimulating the humoral antibody responses or by modifying other cell-mediated immune responses. The antigenic stimulation induces specific antibody synthesis and the production of active effector cells. The nature and magnitude of this response are determined by an array of modulatory processes (George and Kuttan, 2015). Many traditional cancer therapies work in a way to provide anticancer immunity by inducing tumour cell death either by their immunostimulatory action or by the modulation of tumour-induced immunosuppression (Nowak et al., 2003). A major drawback of current cancer therapeutic practices such as chemotherapy and radiation therapy is bone marrow suppression, resulting in cytopenia and subsequent suppression of humoral and cellular as well as nonspecific and specific cellular responses (Devasagayam and Sainis, 2002).

Studies shows that deficiencies in the function of Natural killer (NK) cells or CD8+ cytotoxic T lymphocytes (CTLs) can lead to increase in tumour incidence (Teng et al., 2008). Cytotoxic T lymphocytes (CTLs) are important in the defence against tumours, and a major population of cytotoxic lymphocytes were

CTLs (Boon et al., 1994). CTLs accomplish target tumour cell destruction by recognizing and reacting against the antigen on the target cell (Zhang et al., 2005). NK cells are important class of innate immune cells that play a significant role in mediating the antitumour immune response. NK cells are effector lymphocytes and they control several types of tumours by stemming their growth and dissemination (Langers et al., 2012). The defining functional feature of NK cells remains their intrinsic ability to conduct "natural killing" of cellular targets without prior sensitization (Chester et al., 2015). Cytokines also play a major role in tumour cell killing. Interferon-γ (IFN-γ) is an important immunoregulatory molecule with antitumour and immunomodulatory properties (Blankenstein and Qin, 2003). Interleukin-2 (IL-2) has many immunopotentiating effects, such as augmentation of cytotoxic ability of T-cells, proliferation of T cells, B cells, natural killer (NK) cells, monocytes, etc. (Asano et al., 1997).

An increasing body of evidence has indicated a role for the immune system as a substantial barrier to tumour formation and progression. Indeed, mice that were deficient for various cells of the immune system for example CD8+ cytotoxic T lymphocytes, CD4+ T helper 1 cells or natural killer (NK) cells developed neoplastic formation more frequently and/or showed increased tumour growth compared with immune-competent animals. This evidence indicates that, at least in some experimental models, the innate and adaptive cellular components of the immune system contribute to immune surveillance and tumour suppression (Hanahan and Weinberg, 2011). A number of studies have shown that the presence of intratumoural NK cells correlates with delayed tumour progression and improved outcomes (Rusakiewicz et al., 2013; Mamessier et al., 2011; Navarro et al., 2015). As soon as the balance between inhibitory and activating signals within NK cells are skewed toward activation, NK cells are capable of forming synapses with target cells, allowing the release of perforin and granzyme to lyse the target cells (Bryceson et al., 2006).

Fc receptor–expressing immune cells with cytotoxic ability consist of neutrophils, natural killer (NK) cells, monocytes, and macrophages (Egmond and Bakema, 2013). NK cells effectively induce apoptosis in target cells via

antibody-dependent cellular cytotoxicity (Hatjiharissi et al., 2007). Antibody-dependent cell-mediated cytotoxicity (ADCC) involves degranulation of effector cells resulting in the lysis of target cells. ADCC is predominantly attributed to NK cells, although it was proposed the involvement of cells other than NK in the process (Hubert et al., 2011). In ADCC NK cells can express FcγRIIIA and/ or FcγRIIC, which can bind to the Fc portion of immunoglobulins, transmitting activating signals within NK cells. Once activated through Fc receptors by antibodies bound to target cells, NK cells are able to lyse target cells without priming, and secrete cytokines like interferon gamma to recruit adaptive immune cells. This antibody-dependent cell-mediated cytotoxicity of tumour cells is utilized in the treatment of various cancers over expressing unique antigens, such as neuroblastoma, breast cancer, B cell lymphoma, and others. FcγRs function as receptors for the Fc portion of IgG immunoglobulins, and in doing so serve as a link of the innate immune system to the humoral system (Wang et al., 2015).

1.2. Inflammation and cancer

Various epidemiological, clinical and experimental studies have not only demonstrated a link between chronic inflammation and cancer onset but also shown that immune cells from the bone marrow such as tumour-infiltrating macrophages significantly influence tumour progression (Stockmann et al., 2014). Inflammation also affects immune surveillance and responds to the therapy. Recent studies unambiguously show that metastasis requires close collaboration between cancer cells, immune and inflammatory cells and stromal elements.

Tumour-associated inflammation drives tumour growth and angiogenesis and can perpetuate itself through an extensive network of cytokines and chemokines, which are produced by immune, stromal and malignant cells in response to diverse signals (Grivennikov et al., 2010). The microenvironment for tumour growth and development will be unfriendly for the cancer stem cells. The inflammatory and immune cells make this hostile environment favourable for the growth of tumour by the production of cytokines. Cytokines are small

secreted proteins that have specific effect on the interactions between cells and provide malignant cells with a continuous supply of growth and survival signals and act as tumour promoting agents. In most cases, tumour-promoting cytokines act in a paracrine manner, yet several types of cancer cells produce their own cytokines, including interleukin (IL-6), to achieve the same effect (Gao et al., 2007).

An inflammatory microenvironment is an essential component of all tumours (Mantovani et al., 2008). Continuous exposure to tobacco smoke will lead to lung cancer in mice, underlying this type of tumour promoting effects are inflammatory mechanisms (Takahashi et al., 2010). Inhaled asbestos or silica particles without any mutagenic effects can give rise to lung cancer by triggering inflammation through effects on prointerleukin-1β (IL-1β) processing by the inflammasome (Dostert et al., 2008), and this may mediate their tumourigenic activity. The tumour endorsement by mediators of inflammation can occur in an early or late stage of tumour development and this will escort the activation of dormant premalignant lesions. Inflammation shape tumour promotion by numerous mechanisms, in addition to increased proliferation and enhanced survival, can also involve the so-called angiogenic switch, which permits a small dormant tumour to receive the blood supply necessary for the next growth phase (Lewis and Pollard, 2006).

The process of metastasis can be divided somewhat into four major steps. Epithelial-mesenchymal transition is the first step in the metastatic progression, in which cancer cells acquire fibroblastoid characteristics that increase their motility and invade epithelial linings/basal membranes thereby reach efferent blood vessels or lymphatics (Kalluri and Weinberg, 2009). Intravasation of cancer cells into blood vessels and lymphatics will occur in the second stage of tumour progression. Inflammation plays a significant role in promoting this process by the production of mediators that increase vascular permeability. In the third step, metastasis initiating cells that stay alive in the hostile environments will travel throughout the circulation. Among the cancer cells that enter into circulation, the percentage of cells that survive is only about 0.01% and these cells are able to give rise to the so called micrometastases (Joyce and

Pollard, 2009). Finally, single metastatic progenitors interact with immune, inflammatory, and stromal cells and start to proliferate (Polyak and Weinberg, 2009).

Some of these cells may already be targeted to the premetastatic niche in response to tumour-generated inflammatory signals prior to the arrival of metastasis-initiating cancer cells (Kaplan et al., 2005). These inflammatory signals lead to macrophage activation and production of the metastasis promoting cytokines like Tumour necrosis factor (TNF)-α (Kim et al., 2009). IL-1, TNF-α, and IL-6 promote matrix metalloproteinase (MMP) expression, invasiveness, and metastasis. Intravasation is regulated by prostaglandins (which are produced in a cyclooxygenase (COX)-2 dependent manner and act on the epithelium), by cytokines and by MMPs (which clear the way for the migration into capillaries) (Nguyen et al., 2009). Systemic inflammation enhances attachment of circulating cancer cells to hepatic sinusoids, and this process is governed by neutrophil dependent upregulation of adhesion molecules (McDonald et al., 2009). Several proinflammatory cytokines that are elevated in the circulation of cancer patient's up regulate expression of adhesion molecules on the endothelium or in target organs and thereby increase the probability of metastatic cell attachment (Mantovani et al., 2008).

The interplay between neoplastic cells and their microenvironment has a crucial role in cancer development and progression (Li et al., 2013). In this regard, the tumour surroundings can divert the inflammatory reaction in a way that foster the survival, proliferation and migration of cancer cells (Balkwill and Mantovani, 2001; Yegutkin et al., 2011). This link between inflammation and neoplasia was later extended to include wound healing, which is a process that is also mediated by inflammatory cells. A tumour represents tissues that are undergoing continual wound healing, which is supported by activated inflammatory cells and angiogenesis (Dvorak, 1986).

In tumours that arise in the context of underlying inflammation or in advanced tumours containing inflammatory infiltrates, the net effect of the immune system (both innate and adaptive) is stimulation of tumour growth and progression.

However, cancer cells represent an ‘‘altered self’’ and express ‘‘non-self’’ antigens in the context of stress and danger signals that can promote antigen presentation. Thus, even growing tumours may be subject to immune surveillance and killing by activated T and NK cells (Dunn et al., 2004). It is likely that immunosurveillance and tumour-promoting inflammation can coexist even in the same tumour (Bui and Schreiber, 2007). In spite of such limitations, there are anti-inflammatory drugs that are able to diminish tumour occurrence when used as prophylactics. These anti-inflammatory products can also decelerate cancer progression and reduce mortality when used as therapeutics (Gupta and Dubois, 2001).

The process of inflammation has effect on every aspect of tumour development and progression as well as the response to therapy. In the past years, there is lots of learning about different mechanisms by which cancer and inflammation interconnect, and it’s time to apply much of the fundamental knowledge gained thus far and to use this in cancer therapeutics. Targeting every single facet of cancer biology will help us to fight against this currently incurable disease. In addition to a combination of anti-inflammatory approaches that target the tumour microenvironment with more sophisticated and selective tumouricidal drugs, future therapies should also take notice of the natural genetic variation that affects inflammation and immunity. Such considerations are extremely important in the design of new preventive approaches for the reduction of cancer risk in relatively healthy individuals. Prevention is a much better and more economical way to fight cancer than treating an already advanced and often stubborn disease, as is done currently (Grivennikov et al., 2010).

1.3. Process of neovascularisation in tumour expansion

Tumour associated inflammation induce uninterrupted cell replenishment and proliferation therefore tumours have been referred to as ‘‘wounds that do not heal’’ (Dvorak, 1986). Angiogenesis, the formation of new blood vessels, as well as inflammation with massive infiltration of leukocytes are hallmarks of various tumour entities (Stockmann et al., 2014). Vasculature modulated by inflammatory triggers, displays increased leakiness and enhanced leukocyte

adhesiveness, resulting in endothelial cell activation, proliferation and vascular sprouting (Cines et al., 1998; Folkman, 1995). Tumours have to establish new blood supply in order to advance, and tumour angiogenesis is critical in the process of tumour expansion. Tumour microenvironment and infiltrating immune cell subsets play significant role in regulating the process of tumour angiogenesis. These infiltrates involve the adaptive immune system including several types of lymphocytes as well as cells of the innate immunity such as macrophages, neutrophils, eosinophils, mast cells, dendritic cells and natural killer cells. Besides their known immune function, these cells are now recognized for their crucial role in regulating the formation and the remodelling of blood vessels in the tumour (Stockmann et al., 2014).

Tumour blood vessels are characterized by increased density and various structural and functional abnormalities including irregularities in size and shape, the absence of the typical vessel hierarchy or the distinct organization in arterioles, capillaries and venules (Ribatti et al., 2007). The tumour vasculature also exhibit decreased mural cell coverage and/or abnormal basement membrane sleeves. This structurally aberrant and functionally defective vasculature is the result of the increased proliferation rate of the endothelial cells that constitute the vascular bed of tumours in comparison to the normal endothelial cells. Increased permeability of this vasculature allows the passage of tumour cells into the circulation (Jain, 2005). Appropriate distribution and positioning of immune cells within dynamic tissue microenvironments is the key process in a mounting immune response. This is controlled by the vascular network and its interactions with circulating immune cells, particularly during pathological circumstances such as inflammation (Cook-Mills and Deem, 2004; Danese et al., 2007).

The same molecular events trigger inflammation and angiogenesis (Costa et al., 2007). Pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) can be prometastatic or proangiogenic and their deregulated expression directly correlates with the metastatic potential of several human carcinomas (Huang et al., 2015). The proinflammatory response can be directed towards tumour

promotion via stimulation of the angiogenic process (Ferrara et al., 2003). Antiangiogenic therapy that target vascular growth within the tumour is now widely accepted to treat various tumours, because the agents used in this treatment modality is with lesser side effects due to the quiescent nature of the blood vessels in adults. The genetic instability of tumour cells is the main cause of the failure of systemic chemotherapies. But the endothelial cells of tumour stroma are genetically stable and believed to respond antivascular therapy because they are unable to become drug resistant. Vascular endothelial growth factor (VEGF) is a potent mitogen, a survival factor for endothelial cells, and also mediates vessel permeability and migration of endothelial progenitor cells from the bone marrow. VEGF is a rational therapeutic target because it has a limited role in adults, often VEGF is secreted by tumours (Yadav et al., 2015).

Metastasis supporting neovasculature should possess certain structural characteristics allowing the process of tumour cell intravasation or an active entry of cancer cells into the vessel interior. Development of tumour vessels with lumens of a distinctive size ~ 10–30 μm in diameter and support of these vessels by a discontinuous pericyte coverage constitute critical microarchitectural requirements to: provide accessible points for vessel wall penetration by primary tumour cells; provide enough lumen space for a tumour cell or cell aggregate upon intravasation; and allowing sufficient rate of blood flow to carry away intravasated cells from the primary tumour to the next, proximal or distal site (Deryugina and Quigley, 2015).

Extracellular membrane (ECM) provides necessary contacts between endothelial cells (EC) and surrounding tissue and thus prevents vessels from collapsing. ECM plays a key role in tissue architecture and homeostasis. The principal proteinaceous components of the ECM are collagens that are secreted by a variety of stromal cells, of which fibroblasts are major contributors. Endothelial cell invasion during angiogenesis is a key process that involves degradation of the basement or extracellular matrix barriers to allow free mobility of the cells required for the formation of new blood vessels. This is accomplished by the production of lytic enzymes that are able to digest the specific matrix components and permit cell invasion (Deryugina and Quigley, 2015). There is a

correlation between Matrix metalloproteinase (MMP) expression and tumour invasion. MMPs induce angiogenesis by degrading ECM along with the release of angiogenic mitogens stored in the matrix (Yadav et al., 2015). Tissue inhibitor of matrix metalloproteinase (TIMPs) regulates MMPs through endogenous protease inhibition; high levels of TIMP were always associated with inhibition of endothelial cell migration. Progression of invasive and metastatic tumours showed a decreased level of TIMPs (Wajner et al., 2014). Along with the inhibition of MMP activity TIMPs suppress ECM turnover. TIMPs inhibit MMP activity when inserted into the zinc activity pocket (Brew et al., 2000).

MMPs that are characterized by different substrate specificities, e.g. collagenases and gelatinases, and that originating from different cell types, e.g. tumour cells, inflammatory leukocytes and cancer-associated fibroblasts that can induce within primary tumours the development of a distinct angiogenic vasculature capable of sustaining tumour cell growth. Major biochemical mechanisms that operate in MMP mediated development of the intravasation-sustaining vasculature are proteolytic remodeling of the tumour matrix and the linked release of angiogenic factors like VEGF. In this regard, the majority of accumulated evidence indicates that the pro MMP-9 delivered by tumour-influxing neutrophils possesses the highest biochemical and physiological potentials, such as rapid release, high local concentration, TIMP-free status, high rates of activation and efficiency of substrate catalysis, to digest tumour matrix and release matrix-sequestered VEGF and Basic Fibroblast Growth Factor (bFGF) into the tumour microenvironment. In turn, the released and functionally induced VEGF and bFGF directly regulate the microarchitecture and functions of the intratumoural vasculature, including its ability to sustain tumour cell intravasation and metastasis (Deryugina and Quigley, 2015).

Novel therapeutic approaches target endothelial cells involved in the process of angiogenesis, due to its genetic stability over the rapidly mutating drug resistant cancer cells. Tumour vasculature is considered as a prime prognostic marker of tumour grading (Bhat and Singh, 2008). Most blood vessels in the adult organism remain quiescent but have the capability to divide in response to

proper stimulus and results in angiogenesis. The antiangiogenic therapy is a highly effective strategy for destroying tumour development because it directly affects the vascular supply, the basic requirement for tumour growth. The agents that target angiogenesis can be effectively used in therapy because of its more specific nature compared to chemo and radio therapies. These agents are also less toxic and can be used for long term without the development of drug resistance in the target cells (Yadav et al., 2015). Plants constitute a major source of highly effective conventional drugs for the treatment of different types of cancers.

1.4. Metastatic tumour progression

During the course of malignancy, tumour cells invade neighbouring tissues, stimulate angiogenesis, remodel ECM, undergo Epithelial-to-mesenchymal transition (EMT), and metastasize (Sims Mourtada et al., 2015). The prevalence rate of breast cancer, the most common cancer among females worldwide is over 1.6 million cases per year (Torre et al., 2015). The mortality rate of breast cancer is more than 0.5 million every year, and 90% of these patients die of metastasis, which is when cancer cells depart from their tumours of origin, spread systemically and colonize at distant organs. When the metastasis lesions invade vital organs there is deterioration of patient's health. Metastatic lesions with multiple foci are challenging to surgically remove and often develop resistance to present systematic therapies (Jin and Mu, 2015). It is well known from clinical observations that different tumour types display distinct organ tropisms in metastatic patterns (Chiang and Massague, 2008). Breast cancer displays distinct tropisms depending on the subtypes (Kennecke et al., 2010). Bone, lung, liver, and brain are the common target organs for breast cancer metastasis, in addition to distant lymph nodes (Jin and Mu, 2015).

What underlies the metastasis tropism has been a heated topic (Valastyan and Weinberg, 2011). The spreading pattern of blood flow can explain some tumour types. For example, the primary site of colon cancer metastasis is the liver and the second site is the lung. This is explained by the massive cell trapping in the liver capillary after mesenteric circulation and then in the lung after cancer cells

come out of circulation from liver (Gupta and Massague, 2006). This general rule is not applicable in the case of breast cancer. Paget in the 19th century proposed an alternative view, who proposed that disseminated cancer cells and the so called seeds can form metastases as they reach a microenvironment called soil that is congenial enough for their survival and proliferation (Paget, 1989). This seed-and-soil hypothesis (Fidler, 2003) has received extensive support with the identification of gene mediators that contribute to metastasis formation (Jin and Mu, 2015).

The metastasis cascade requires an intricate, coordinated action of multiple gene programs, one may expect that it should be easy to disrupt this delicate cascade and inhibit metastasis by abolishing whichever gene mediator of the process. When it comes in the matter of a single cell this view is true. Actually metastasis involves cell populations. Heterogeneity that is intrinsic to most of the metastases renders the metastatic traits versatile, redundant and complicated for targeting. Analysis of single clones from the metastatic subpopulations toward a certain organ suggests that different clones within the aggressive subpopulation do not necessarily upregulate every metastatic gene, but rather harbour different subsets (Kang et al., 2003; Minn et al., 2005[a]; Minn et al., 2005[b]). Metastasis remains the biggest hurdle for curing breast cancer. How to combat drug resistance via developing novel combination therapy of chemotherapy, small molecule therapy, and immunotherapy is a solution for attaining long-lasting therapeutic efficacies (Jin and Mu, 2015).

Breast carcinomas may directly move into the skin and muscle, via lymphatics and other lymph nodes or via the blood stream to other organs. Whatever the route of metastasis, the tumour cells must first migrate through the local tissues. Non invasive breast cancers remain within the basement membrane of the terminal duct lobule. Invasive breast cancers involve the dissemination of cancer cells outside the basement membrane of the duct and the lobules into the surrounding adjacent normal breast tissue stroma (Michael Dixon, 2006). Modifications in the malignant cells are often accompanied by alterations in the supporting environment of myoepithelium and stromal cells, due to a

combination of events leading up to the invasion of the stroma, neovascularisation and ultimate penetration into the lymphatics or blood vessels.

MMP activity facilitates ECM remodelling a prerequisite to ductal progression and by removing or breeching the basement membrane and stromal matrix (Fata et al., 2000). Based on substrate specificity MMPs can be divided into four groups: the interstitial collagenases, the gelatinases, the stromelysins, and the membrane type MMPs. The gelatinases, for example, MMPs 2 and 9, are type IV collagenases that degrade gelatin (denatured collagen) and types IV, V, VII, IX, X collagens. Type IV collagen is particularly abundant in basement membranes (Lebeau et al., 1999). In a study using mammary tumour bearing mice there was a dramatic upregulation of MMP 9 secretion by splenic and tumour infiltrating T-lymphocytes suggesting that tumour cells may use inflammatory cells to make contributions to the tumour phenotype (Owen et al., 2004). Elevated serum levels have been found to be associated with tumours and correlate with cancer invasion and metastasis (Stuelten et al, 2005). Cell contact between breast cancer cells results in the rapid release of inactive membrane associated MMP 2. MMP 2 is abundantly expressed at tumour leading edges in breast cancer and contributes to cell migration across collagen type I. Once released MMP 2 may then associate with other MMP complexes facilitating its activation and subsequent invasion of normal tissues by malignant cells (Saad et al., 2002).

Epithelial-mesenchymal transition (EMT) is a development process in which epithelial cells take on the characteristics of invasive mesenchymal cells (Radisky and Radisky, 2010). EMT impart epithelial tumour cells the ability to migrate, invade stroma and disseminate. EMT like changes correlates with a more aggressive phenotype (Slattery et al., 2013). Elevated levels of MMPs in the tumour microenvironment can directly induce EMT in epithelial cells. Cancer cells undergoing EMT can then produce more MMPs facilitating cell invasion and EMT can generate activated stromal like cells which drive cancer progression via further MMP production (Radisky and Radisky, 2010).

Future research may one day target the rate limiting steps in the malignant conversion of breast cancer cells. Focusing only on these mechanisms renders healthy tissues uninterrupted, and tumour cells remain constrained within the local tissues. The important diagnostic criteria for malignancies, tumour invasion may not be there and by definition the tumour would no longer be malignant. As breast cancer is the largest cause of deaths in women aged 35–55, this would be a ground breaking achievement that would significantly reduce mortality and morbidity associated with breast cancer (Davies, 2014).

4T1 mouse breast tumour model

Availability of appropriate metastatic models that represent *in vivo* metastatic progression is a major hurdle in the study of tumour development and progression. Immunocompromised mice with human tumour cell incorporation may act as xenograft models. This type of tumour models has been used widely, for the validation of specific gene products as targets of specific drugs in cancer therapy. Some of these models may be successful in representing primary tumour growth, while replication of the process of metastasis is very difficult (Bibby, 2004; Eccles et al., 1994; Hoffman, 1999). Metastasis of human tumour cells occurs scarcely in mice and when they occur unanticipated results may obtain. On the contrary murine tumour cell models can metastasize and mimic conditions as shown in human patients (Vernon et al., 2007). This is not surprising while considering factors like tumour microenvironmental conditions and tumour-host interactions that effect tumour cell performance.

Fred Miller and co workers of Karmanos Cancer Institute originally isolated 4T1 mammary carcinoma cell line (Miller et al., 1983; Miller, 1983). Orthotopic induction of 4T1 cells will lead to metastasis to the organs like those affected in breast cancer (Yoneda et al., 2000; Aslakson and Miller, 1992; Eckhardt et al., 2005). The extent and kinetics of metastasis to organs affected in human breast cancer indicated extensive colonization of lungs and liver in most animals within a six week period with lower efficiency of metastasis to bone, brain and other sites. Innate and adaptive immune responses were shown to play important roles in growth and metastasis of the lines in BALB/c mice (Tao et al., 2008).

Presence of tumour cells in lymph nodes lying nearby to primary tumour suggest 4T1 cells metastasize via lymphatic system, there is also the presence of hematogenous spreading (Aslakson and Miller, 1992; Tao et al., 2001).

In the 4T1 mouse mammary tumour model, the 4T1 mouse mammary carcinoma cells were injected orthotopically into the mammary fat pad of the female BALB/c mice directly. Due to the high aggressivity and metastatic characteristics of 4T1 cells, it is easy to establish metastasis (Pulaski and Rosenberg, 1998). 4T1 is an animal model for stage IV human breast cancer, which is able to spontaneously produce highly metastatic tumours that are known to metastasize to the lung, liver, lymph nodes etc. in BALB/c mice (DuPre et al., 2007). An acquired immune response was found to play an important role in regulating 4T1 tumour growth and metastasis. To our knowledge, the 4T1 model is the only system that has the capacity to metastasize to all organs affected in breast cancer in humans when introduced orthotopically (Tao et al., 2008).

1.5. Conventional cancer therapy

1.5.1. Ionizing radiation and hypoxia

One of the prime treatment modality for breast cancer is radiotherapy (Aravindan et al., 2013). The poor outcome of this treatment modality is always associated with the reduced ability of ionizing radiation to produce DNA damage in the absence of oxygen. In solid tumours like breast carcinoma the oxygen tension may reduce below the normal level because of an unbalanced condition in the oxygen delivery and oxygen consumption. Abnormal vascularisation in the malignant tissue is one of the reasons for this type of tissue hypoxia (Lundgren et al., 2007). In nearly 40% of tumour malignancies there are regions with O_2 concentrations below that is required for half maximal radiosensitivity (Vaupel et al., 1991). This hypoxic tumour cells are capable of active proliferation, invasion, metastasis and neovascularisation (Ruan et al., 2009). Specific targeting of hypoxic process will be the future arena of novel cancer therapies.

The survival of cells from normoxia (~ 21% O_2) to hypoxia (~1% O_2) is regulated by the hypoxia inducible factor -1 (HIF-1). This HIF-1consists of HIF 1α subunit which is hypoxically inducible and another subunit HIF-1β that is constitutively expressed. The stabilization and translocation of HIF-1α from cytoplasam to nucleus occurs during hypoxic condition. In nucleus it dimerizes with HIF-1β and form transcriptionally active HIF-1 complex (Kallio et al., 1998). This HIF complex then associates with Hypoxia response elements (HRE) in the target genes and induce gene expression (Lando et al., 2002). More than 2% of all tumour genes are directly or indirectly regulated by HIF-1 in arterial endothelial cells (Manalo et al., 2005).

Resistance to radiation therapy is often associated with an increased cellular invasion and metastatic potential (Postovit et al., 2004). HIF-1α contributes to tumour aggressiveness, invasiveness and resistance to radiotherapy and chemotherapy (Diaz et al., 2005). Hypoxic cells are two to three fold more resistant to radiation than well-oxygenated cells because the biological effect of radiation is greatly influenced by the presence or absence of molecular oxygen at the time of irradiation (Hall, 1994; Gray et al., 1953). Many studies have demonstrated that the growth of solid tumours and their metastases are dependent on angiogenesis, which is regarded as a critical event in tumour development (Bergers et al., 2003). Over expression of HIF-1α is correlated with vascular density in tumours, indicating that HIF-1α is a key initiator of angiogenic activity (Sivridis et al., 2002).

Hypoxic microenvironment stimulates VEGF via its primary regulator HIF-1 and that plays a key role in tumourigenic process (Stacker et al., 2001). VEGF is produced by tumour cells, and its binding with the VEGF receptor Flk-1 (which is expressed on vascular endothelial cells) leads to the proliferation and migration of endothelial cells (Ferrara, 2004; Liu et al., 2005). VEGF directly participate in neovascularisation by recruiting endothelial cells into hypoxic and avascular areas (Conway et al., 2001). Again VEGF receptors expressssed on tumour cells lead to tumour proliferation in an autocrine manner involving interaction with VEGF produced by tumour cells themselves (Kyzas et al., 2005).

Hypoxia also induces genes like Matrix metalloproteinases (MMPs) that are involved in matrix metabolism and vessel maturation (Ben-Yosef et al., 2002). Degradation of extra cellular matrix (ECM) by MMPs secreted by tumour cells occurs at the time of tumour cell invasion (Basset et al., 1997). Enzymes that play a key role in tumour cell invasion and metastasis are type IV collagenases, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) (Björklund and Koivunen, 2005). These enzymes are secreted in an inactive pro enzyme form and they are activated by proteolytic cleavage (Mignatti et al., 1986). Ionizing radiation was found to accelerate MMP activation and subsequent invasion, neovascularisation and metastasis in malignant tissues (Kaliski et al., 2005).

The plant derived compounds are a tremendous source of active therapeutic agents with fewer side effects that can be used alone or in combination with other therapeutic methods. The curative potential of radiotherapy is often limited by the radioresistant tumour cells and hypoxia is such a barrier that reduces its therapeutic success. In this context there is a great importance in the natural product based inhibition of hypoxia inducible factor (Nagle et al., 2006).

1.5.2. Chemotherapy and toxic effects

Therapy using chemical substances or chemotherapy in cancer treatment may be done alone or in combination with other therapeutic approaches like radiation therapy. Surgery and radiation therapy is conventionally used in an early stage of tumour detection and chemotherapy is the treatment of choice for advanced tumours (Sakthivel and Guruvayoorappan, 2015). Antiangiogenic agents can enhance the effectiveness of chemotherapy. VEGF and VEGF receptor expression by tumour cells can increase in response to chemotherapy. This will result in increased mobilization of circulating endothelial progenitor cells thereby promoting angiogenesis. An antiangiogenic agent can counteract this response and enhance the antiproliferative action of chemotherapy. Also antiangiogenic agents claimed to be efficient in inhibiting tumour cell repopulation in between the chemotherapy cycles this phenomenon further increase efficacy of chemotherapy (Arjaans et al., 2016).

Cyclophosphamide (CP) is a chemotherapeutic agent used in the treatment of cancers like breast cancer. It is an alkylating agent with immunosuppressive effects and act by cross linking the DNA strands. The adverse effects of CP are the induction of oxidative stress and neutrophil infiltration in tissues. This oxidative stress is shown by increased lipid peroxides (LPOs) or malondialdehyde (MDA) levels together with decreased antioxidants activities or concentrations. In many studies, CP decreased superoxide dismutase (SOD) as well as glutathione (GSH) concentration (Merwid-Ląd et al., 2012).

Urinary bladder is the major site of action of the toxic metabolites of CP. Because bladder is the storage organ of urine the concentration of toxic metabolites of CP is likely to be higher in bladder as compared to other organs. The urological side effects of CP vary from transient irritating voiding symptoms to invasive bladder cancer and hemorrhagic cystitis (Bhatia et al., 2006). Cyclophosphamide induces urotoxicity characterized by the development of cystitis, which involves bladder over activity and inflammation. Tissue damage caused by cyclophosphamide administration leads to the release of several inflammatory and hyperalgesic mediators, including chemokines. It is now well established that cyclophosphamide induced cystitis is characterized by marked bladder oedema and haemorrhage and urothelial damage (Korkmaz et al., 2007). The urotoxicity is produced by the severe oxidative stress due to CP administration. The urotoxicity of CP is due to the formation of 4-hydroxy metabolites, in particular acrolein, which is formed from enzymatic hydroxylation of CP by hepatic microsomal cytochrome P450. Acrolein reacts with reduced glutathione (GSH) thereby decreasing its level. Since GSH is a primary cellular antioxidant, this interaction disrupts overall glutathione cycle and antioxidant defence of the bladder. Therefore, it is desirable to mitigate or minimize overall imbalance in redox cycling of the bladder caused by CP to increase its efficacy in cancer treatment (Bhatia et al., 2006). There is good evidence that cytokines, such as TNF-α and IL-1β are critical mediators of the inflammatory process in cyclophosphamide-induced cystitis (Dornelles et al., 2014).

However, CP-induced urotoxicity is probably not only because of the direct contact of acrolein with bladder mucosa but also related to the increased production of Reactive nitrogen species (RNS) and Reactive oxygen species (ROS). The coupling of O2 •– and NO results in the formation of peroxynitrite at the site of inflammation, which is mainly responsible for the detrimental damage to the bladder. As a result of these two mechanisms, a significant reduction in the levels of all the antioxidants along with an increase in the lipid peroxidation (LPO) was observed in the urinary bladder in CP-treated animals (Bhatia et al., 2006)

Various natural and synthetic compounds, mixtures of compounds and herbal extracts have been investigated as modulators of CP induced toxicity. CP in combination with agents having significant antioxidant activity like blue green algae, *Spirulina* (Sinanoglu et al., 2012) taurine (Abd-Allah et al., 2005) and flavanoids (Ozcan et al., 2005) found to reduce or eliminate the severe toxic effects of CP. Several traditionally used natural products were found to be ameliorative agents of oxazaphosphorines urotoxicity. This include some of the works on *Ipomoea obscura* (Hamsa and Kuttan, 2011), *Withania somnifera* (Davis and Kuttan, 2000) and S-allylcysteine (Bhatia et al., 2008).

1.6. Plant natural products

Traditional systems of medicine have always encouraged the use of plant-derived therapeutics in the treatment of many chronic ailments. Most of these agents are free from adverse effects and many of them are a source of diet (George and Kuttan, 2015). Throughout the history various plants and plant products have been incorporated as part of the traditional medicine of almost all human cultures for the prevention or treatment of diseases. The gentle, nourishing, and synergistic action of herbal remedies make them an excellent treatment of choice. Whether a treatment is approached using natural herbs or chemical drugs, whose synthesis is based on properties and actions of medicinal herbs that have played a major role in the development of modern, conventional medicine, and they will remain, like a historical treasure, as source of therapy (Majdalawieh and Fayyad, 2015).

There is an urgent need to explore agents that will be effective in preventing and treating metastasis of breast cancer. For centuries, nature has provided us with rich source of compounds for various disease treatments. Such naturally-derived molecules have been utilized in formal drug discovery platforms of the pharmaceutical industry. Plants have proven to be a rich source of lead compounds or the basis for synthetic drugs. The complexity and variation of plant structures indicates that their evolution has naturally completed the screening process, and that the creation of potent compounds makes them more likely to survive (Tobin et al., 2013).

1.6.1. *Emilia sonchifolia* (L.) DC and its major sesqueterpene γ-humulene

Emilia sonchifolia (L.) DC, is a widely distributed medicinal herb used mainly in the indigenous Ayurvedic system of medicine in India. This plant is one among the ten sacred plants of Kerala state in India, collectively known as Dasapushpam (George and Kuttan, 2016). Dasapushpam constitutes a group of 10 sacred medicinal plants (Sanskrit word *Dasa* means "ten" and *pushpam* means "flower"), typically utilized in the Ayurvedic system of medicine and are specially associated with Kerala tradition and culture. They have been used even from the time immemorial and are referred in classical Ayurvedic books such as *Arogya kalpadruma*, *Vaidya manorama*, etc. The herbal preparations of whole plants or their different parts or the polyherbal combinations possess remedial potential to rejuvenate the body and to cure various diseases and are added as ingredients in various Ayurvedic formulations. These Dasapushpas are consumed to improve health and to resist diseases in the monsoon season. Other members of Dasapushpa include *Aerva lanata* (L.), *Biophytum sensitivum* (L.) DC, *Cardiospermum halicacabum* Linn., *Curculigo orchioides* Gaetrn, *Cynodon dactylon* (Linn.) Pers, *Eclipta Alba* Hassk, *Evolvulus alsinoides* Linn., *Ipomoea sepiaria* koen. Ex Roxb, and *Vernonia cinerea* (Linn.) Less (George and Kuttan, 2015).

Emilia sonchifolia (E. sonchifolia), the Lilac tassel flower belonging to the family Asteraceae with the local name of Muyalchevian in Kerala state of India, is an edible plant used in the Ayurvedic system of medicine for the treatment of

gastropathy, diarrhea, ophthalmia, nyctalopia, cuts and wounds, intermittent fevers, pharyngodyma and asthma (Nair and Chopra, 1996). *E. sonchifolia* is used in the folklore medicine for treating tumour and inflammation (Shylesh et al., 2005). Previous studies conducted on this plant revealed its anti inflammatory (Muko and Ohiri, 2000; Nworu et al., 2012) and anti tumour (Shylesh and Padikkala., 2000) properties. There are reports on *E. sonchifolia* that evince its protective effect on oxidative stress and modulation of selenite cataract (Lija et al., 2006) and antinociceptive effect (Couto et al., 2011). Studies on the apoptotic activity of this plant on cancer cells (shylesh et al., 2005; Lan et al., 2011; Lan et al., 2012) further proved its anticancer potential.

Various biological activities of *E. sonchifolia* covering a gamut of beneficial properties have been reported. Recently, we have done a complete phytochemical screening of the plant, and its antimetastatic effect was analyzed using the most active solvent fraction containing the major active principle γ-humulene ($C_{15}H_{24}$) (George and Kuttan, 2016). Plants constitute a major source of highly effective conventional drugs for the treatment of different types of cancer. A large number of the sesquiterpenes obtained from medicinal plants that are used in traditional medicine show anticancer activity by inhibition of inflammatory responses, prevention of metastasis, and angiogenesis (George and Kuttan, 2016). The anti-inflammatory properties of α humulene and transcaryophyllene that share close similarity with γ humulene has already been reported (Fernandes et al., 2007).

1.6.2. Punarnavine an alkaloid from the plant *Boerhaavia diffusa*

Boerhaavia diffusa is a perennial herb and it is ascribed the name *punarnava*. *Boerhaavia diffusa* (Punarnava) is widely used in Ayurvedic system of medicine for jaundice, hepatitis, oedema, oligurea, anaemia, inflammations, eye diseases, etc. Pharmacolgists and clinicians have investigated 'Punarnava' for all these activities and support the existing clinical uses (Chude et al., 2001). It has a long history of use in indigenous as well as tribal medicine and also in Ayurvedic or natural herbal medicines (Dhar et al., 1968). The alkaloidal fractions of *Boerhaavia diffusa* found to possess immune modulatory activity (Mungantiwar

et al., 1999). Punarnavine is a quinolizidine alkaloid, isolated from the plant *Boerhaavia diffusa* (Manu and Kuttan, 2009), which is a member of Nyctaginaceae family. There were only a few studies to explore the potential of this quinolizidine alkaloid. These studies revealed the antimetastatic (Manu and Kuttan, 2009), antiangiogenic (Saraswati et al., 2013), antiapoptotic (Manu and Kuttan, 2009[a]), antigenotoxic (Aher et al, 2013), antidepressant (Dhingra and Valecha, 2014), cell mediated immune response (Manu and Kuttan, 2007) and immunomodulatory (Manu and Kuttan, 2009[b]) properties of punarnavine.

1.6.3. Harmine

Harmine is a beta-carboline alkaloid originally isolated from the seeds of the plant *Peganum harmala* (Family: Zygophyllaceae), which is used as a folk anticancer medicine (Li et al., 2015). Studies have shown the potent anti-metastatic and anti-invasive effects of harmine using the highly metastatic murine B16F10 melanoma cells (Hamsa and Kuttan, 2011). Harmine played a significant role in the apoptosis of B16F-10 cells by activating both intrinsic and extrinsic pathways of apoptosis and by regulating some transcription factors and pro-inflammatory cytokines (Hamsa and Kuttan, 2011[a]). Results of the study on *in vivo* anti-angiogenic activity of harmine in C57BL/6 mice revealed a decreased capillary growth, validating its inhibitory effect on the tumour directed neovascularization (Hamsa and Kuttan, 2010). Harmine showed inhibitory effects on cell proliferation against CCD18Lu, transformed HeLa, C33A and SW480 cells (Herraiz et al., 2010). Harmine reduced the proliferation and differentiation of HL60 cells, alone or in combination with ATRA and G-CSF, in a dose and time dependent manner (Zaker et al., 2007). Harmine also showed cytotoxicity against HL60 and K562 cell lines (Jahaniani et al., 2005). Harmine induced apoptosis in HepG 2 cells via mitochondrial signaling pathway (Cao et al., 2011), and further showed down-regulation of cyclooxygenase-2 expression in gastric cancer (Zhang et al., 2014). In addition to these harmine exhibits various other types of pharmacological activities such as antimicrobial, antifungal, antitumour, cytotoxic, antiplasmodial, antioxidant, antimutagenic, antigenotoxic and hallucinogenic properties (Patel et al., 2012).

Chapter 2
Materials and methods

2.1 Plant materials used in the study

1) Punarnavine, quinolizidine alkaloid, isolated from the plant *Boerhaavia diffusa* (Manu and Kuttan, 2009).
2) Harmine, beta-carboline alkaloid purchased from Sigma Chemicals, St. Louis, USA.
3) Genuine whole plant methanolic extract of *Emilia sonchifolia* (L.) DC. (George and Kuttan, 2016).
4) γ-hum, the active *n*-hexane-ethyl acetate fraction of *Emilia sonchifolia* (L.) DC. enriched with 71% of the major sesquiterpene γ-humulene (Gilcy and Kuttan, 2016).

2.2. Animals used in the study

Healthy adult BALB/c mice, Swiss albino mice and C57BL/6 mice were maintained in well-ventilated rooms at 24±2 °C and 50%–60% relative humidity, with a 12-h light-dark cycle. They were accommodated in individual ventilated cages fed with normal mice chow and water *ad libitum*. All the animal experiments were carried out with the prior approval of the Institutional Animal Ethics Committee and were conducted strictly adhering to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

2.3. Cell lines used in the study

4T1 mouse mammary carcinoma cell line were maintained in Dulbecco' s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. Human umbilical vein endothelial cells (HUVECs) isolated from human umbilical cord veins (Jaffe et al., 1973). HUVECs were grown in medium 199 supplemented with 20% FBS, 100units/ml penicillin, 100μg/ml streptomycin, and 2ng/ml Vascular endothelial growth factor (VEGF) and fibroblast

growth factor (FGF) at 37°C in 5% CO_2 atmosphere. L929 mouse lung fibroblast cells, B16F10 metastatic mouse melanoma cells, K562 leukemic cells and EL4 thymoma cells were obtained from National Centre for Cell sciences, Pune, India.

2.3.1. Cell and tissue culture

2.3.1.1 Sterilization

All the culture wares used for cell and tissue culture purposes were thoroughly washed and rinsed with distilled water (DH_2O) and dried in a hot air oven. After drying these were autoclaved at 15 lb/inch^2 pressure for 15 minutes, again placed in hot air oven for drying and used for cell and tissue culture experiments.

2.3.1.2. Preparation of culture media

All the cell culture media were prepared in autoclaved double distilled water (DDH_2O) and pH was adjusted to 7.2 using sodium bicarbonate. Medium was supplemented with L-glutamine (2mM) and filtered under negative pressure using a 0.22μm cellulose filter. Sterility of the medium was tested using fluid thioglycollate medium. For this 1ml of the filtered medium was inoculated into 10ml of sterile thioglycollate and incubated at 37^{o}C for 7 days and checked for visible contamination. Broad spectrum antibiotics such as penicillin (100units/ml) and streptomycin (100μg/ml) and FBS (10%) were added to the medium prior to use.

2.3.1.3. Maintenance of adherent cell lines

Confluent cell culture flasks were washed with phosphate buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA). 100μl of trypsin solution (0.2%) was added and kept for sometime and then tapped in order to dislodge the cells. Complete culture medium (containing 10% FBS and antibiotics) was then added to these culture flasks. By repeated pipetting cells were made into single cell

suspension and from this an aliquot of cell suspension was added to fresh tissue culture bottles containing 10ml of complete medium and incubated at 37^0C in 5% CO_2. The subculturing of cells was done as soon as when the culture bottles become confluent.

2.3.1.4. Maintenance of cell lines in suspension

By repeated pipetting the clumps of cells in suspension culture bottles were dispersed. Cells were counted and required number of cells were seeded into fresh culture flasks containing 10ml of complete culture medium and incubated at 37^0C in 5% CO_2 and these cells were then subcultured at required time intervels when it become confluent.

2.3.1.5. Isolation and maintenance of Human Umbilical Vein Endothelial Cells (HUVECs)
(Jaffe et al., 1973)

According to the protocol of Jaffe and colleagues endothelial cells were collected in a sterile manner from human umbilical cord vein. All the reagents, collecting devices and culture conditions should be sterile. Soon after birth the umbilical cord was collected in cord buffer placed in a sterile container. This was then kept at 4^0C upto the time of processing. Using a tie one needle was secured on one end of the cord. Using a syringe 100ml of cord buffer was perfused through the vein to wash out blood. Soon after washing the other end was also tied. Then one to two milliliter of 0.2% collagenase in cord buffer was infused through the cord vein and the cord was placed in a water bath containing cord buffer at 37^0C for 15 minutes. This collagenase solution was then flushed out from the cord using 30ml cord buffer. The effluent was collected in a sterile centrifugal tube containing medium 199 with 20% FBS. This was then centrifuged and the cell pellet was resuspended in fresh medium and poured into gelatin pre-coated tissue culture flasks for incubation at 37^0C in 5% CO_2. In addition to the normal cell culture reqirements 2ng/mL VEGF

and fibroblast growth factor (FGF) were supplemented to the culture medium and cells were used for experiments from third to sixth passage.

2.3.1.6. Preparation of cells for *in vitro* studies

70-80% confluent cultures were used for *in vitro* studies. As soon as the the cells become monolayered culture flasks were washed three times with PBS. Using a cell scrapper the cells were dislodged from the flask. The cell number was adjusted according to the requirement and the viability of cells was checked by trypan blue dye exclusion method (Kuttan et al., 1985). For experimental purposes cell suspension with more than 95% viability was used.

2.3.1.7. Determination of cell viability (Trypan blue dye exclusion method) (Kuttan et al., 1985)

Cell suspension was mixed with 100 µl of 1% trypan blue, kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the dye and appear in blue colour while viable cells will not take the dye and appear as normal. The number of stained and unstained cells was then counted separately.

$$\% \text{ Dead cells} = \frac{\text{Number of dead cells}}{\text{Number of viable cells} + \text{Number of dead cells}} \times 100$$

2.3.1.8. MTT assay
(Cole, 1986; Campling et al., 1991)

The cells in log phase of the culture were used for this experiment. These cells were incubated for 24hours at 37°C in 5% CO_2 atmosphere in 96-well flat bottom tissue culture plates (5000 cells/well) containing 200µl complete medium. Various concentrations of the test compounds were added after incubation by removing 100µl of the medium. The medium was then made upto 200µl and the cells were

further incubated for 48 hours. 20µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/ml) was added to each well four hours before the completion of incubation. Culture supernatant was discarded after centrifugation. To the attached layer of cells 100µl of dimethyl sulphoxide (DMSO) was added and in incubated at room temperature for 15 minutes. Optical density (OD) was measured at 570nm.

$$\% \text{ of dead cells} = \left(1 - \frac{\text{OD of treated}}{\text{OD of control}}\right) \times 100.$$

2.4. Detailed experimental procedures

2.4.1. Hematological parameters

2.4.1.1. Determination of total count of white blood cells (WBC) (Cheesbrough and McArthur, 1976)

The blood was diluted in a diluting fluid. The glacial acetic acid in the fluid will lyse red blood cells and crystal violet will stain the white blood cells.

Procedure

20µl of blood was mixed with 380µl of diluting fluid (acetic acid-1.5ml; 1% crystal violet-1ml in methanol; DH_2O-98ml). Kept for about 2-3 minutes in room temperature (RT). After gentle mixing, loaded on to the haemocytometer. The number of cells in the large four corner squares of the haemocytometer was counted under a 10X objective of the microscope.

$$\text{Total white blood cell counts/mm}^3 = \frac{\text{Number of cells counted (N) x dilution factor x depth factor}}{\text{Area counted}}$$

Dilution factor = 20; Depth factor = 10 mm; Area counted = 4mm^2

Therefore, Total white blood cell counts /mm^3 = $\frac{N \times 20 \times 10}{4}$ = N x 50

2.4.1.2. Differential count of white blood cells (DC)

A thin smear of blood was prepared by placing one drop of blood at one end of a glass slide and spreading this drop evenly using a glass spreader. Film was airdried and stained with Leishman's stain (150mg in 100ml methanol). After keeping for about 3 minutes stain was diluted with DH_2O and again kept for seven minutes, washed with running tap water and again airdried. Slides by keeping under a 100x oil immersion objective various cell types were counted based on their morphology in this manner a total of hundred cells were counted.

2.4.1.3. Determination of Haemoglobin
(Drabkin and Austin, 1932)

When combined with haemoglobin ferricyanide in drabkin's solution forms methaemoglobin. Cyanides can convert this methamoglobin to cyanmethaemoglobin with absorption at 540nm.

Procedure

20µl of blood was mixed with 5ml of Drabkin's solution (Agappe diagnostics Ltd, kerala, India) and kept for 5 minutes at room temperature. Haemoglobin content was calculated using the formula,

Haemoglobin content =
(OD of the test/ OD of standard) x (251x Concentration of standard/1000)

2.4.2. Immunological parameters

2.4.2.1. Collection and preparation of sheep red blood cells (SRBC) (Mehera and Vaidya, 1993)

Sheep blood was collected in sterile Alsever's solution and stored at 4^0C (maximum storage time is one week). Cells were washed three times with PBS (pH7.2) and pellet was suspended in Hanks balanced salt solution (HBSS). For trypsinization of SRBC ten parts of 4% SRBC and one part of 1% trypsin solution were incubated at 37^0C for 30 minutes, washed twice in PBS (pH7.2) and resuspended.

2.4.2.2. Spleen, thymus and bone marrow processing

All the procedures were done under sterile conditions. After sacrificing the animals spleen and thymus was removed devoid of any adherent tissue, cut into small pieces and mashed over a stainless steel mesh. Clumps were allowed to settle, supernatant was collected washed with HBSS and resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium as per the concentration requirements. For bone marrow cell collection the skin and flesh overlying the limbs of the mice were removed and the femur was separated from both ends and the bone marrow was flushed out of the cavity by passing medium with 2% FBS through the ends using a 26G needle and syringe. By repeated pipetting single cell suspension of bone marrow cells were made and suspended at required cell concentrations in RPMI-1640 medium.

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

Esterases are enzymes which are capable of hydrolyzing esters. The esterase activity split α-naphthyl acetate releasing α-naphthole, this then combines rapidly

with the coupling agent (pararosaniline) to produce an insoluble azo-dye at the site of enzyme activity.

Procedure

Bonemarrow cells were collected as explained in section 2.4.2.2. Cell number was determined and a thin smear was prepared on the glass slide. This smear was then Air dried and fixed. Slides were incubated in a reaction buffer containing pararosaniline, sodium nitrate and α-naphthyl acetate for 45 minutes. Slides were counterstained with haematoxylin for one to two minutes. α–esterase positive cells will be coloured and were counted under the microscope using oil immersion objective.

2.4.2.4. Hemagglutination assay

(Singh et al., 1984)

Agglutinated SRBC cells will settle down as a diffused 'mat' and the nonagglutinated ones will be seen as a clear 'button'. The antisera with maximum dilution that gives clear agglutination gives the titre of the antibody.

Procedure

100µl of anti-sera was serially diluted in 96-well round bottom tissue culture plates containing 100µl of PBS/well (pH 7.2). 100µl of trypsinized SRBC was then added to each well, mixed and incubated for 3 hours. The dilution of the antisera at which clear agglutination observed was recorded.

2.4.2.5. Determination of antibody forming cells

(Jerne and Nordin, 1963)

Lymphoid cells of SRBC immunized animals will produce antibody that can cause lysis of SRBC in the presence of complement and form clear areas (plaques).

Procedure

0.5% of 500µl agarose was poured into tubes at 45^0C. To this 50µl of SRBC (7%) and 50 µl spleen cells ($8x10^6$ cells/ml) were added and mixed well. The contents in each tube were then poured over separate glass slide and allowed to solidify. An incubation rack was filled with fresh rabbit serum (1:10 diluted with PBS, pH 7.2) that act as a source for complement and incubated for 1hour at 37^0C. Using a colony counter the clear areas or the plaques formed were counted and represented as plaque forming cells/10^6 spleen cells.

2.4.3. Biochemical parameters

2.4.3.1. Estimation of Hydroxyproline
(Bergman and Loxley, 1970)

Chloramine T oxidizes the hydroxyproline present in the sample. The high concentration of isopropanol stabilizes the coloured product of the reaction.

Reagents

1. Oxidant solution

$C_2H_3NaO_2$	- 5.7g
$Na_3C_6H_5O_7$	- 3.75g
$C_6H_8O_7$	- 0.55g
C_3H_8O	- 38.5ml
DH_2O	- 61.5ml

2. Ehrlisch's reagent

Para dimethyl amino benzaldehyde	- 4.4g
$HClO_4$	- 10.2g (60%)
C_3H_8O	- 25ml (Final volume)

3. Chloramine T - 1.75g/25ml oxidant solution was prepared on the day of use

Procedure

Using 4ml isotonic saline 1g of lung tissue was homogenized. Hydrolysation was done in a sealed hydrolyzing tube by using 6N HCl with incubation at 110^0C for 24 h. 1ml of the hydrolysate was neutralized and make up to 5ml with H_2O. To 500µl of the sample 2.5ml isopropanol and 1ml oxidant solution was added and kept at RT for 4 minutes. To this 2ml Ehrlisch's reagent was added and incubated at 60^0C in water bath for 21 minutes. Tubes were kept at RT for 1h. The absorbance was recorded at 560nm.

2.4.3.2. Estimation of uronic acid

(Schiller et al., 1961; Bitter and Muir, 1962)

Reagents

Reagent 1	- 0.952g sodium tetraborate in 100ml of Conentrated sulphuric acid (0.025M)
Reagent 2	- 0.125g carbazole in absolute alcohol
Reagent 3 (0.1M)	- Solution A - 0.2M CH_3COOH Solution B - 0.2M $C_2H_3NaO_2$

Procedure

The lung tissue (10mg) was digested using crude papain in 5ml of reagent 3 (pH 5.5) containing 0.005M cysteine and 0.005 M disodium salt of EDTA at 65^0C for 24h. 5ml of reagent 1 was taken in tubes and cooled at 4^0C for some time. 1ml of sample or standard glucuronicacid lactone solution layered on it. Ground glass stoppers were used to close the tubes and placed on a rack which was shaken first gently and then vigorously. These tubes were then kept in a boiling shaking-water bath for 10 minutes and cooled. 200µl of reagent 2 was added to these tubes and heated in a boiling water bath for 15 minutes and cooled. The colour developed was read at 530nm.

2.4.3.3. Estimation of Hexosamine
(Elson and Morgan, 1933)

Reagents

2ml of $C_5H_8O_2$ in 100ml of 0.5M sodium carbonate.

Ehrlisch's reagent –

1.33g of p-dimethyl aminobenzaldehyde (PDAB) was dissolved in 100ml of 1:1, ethanol: concentrated HCl.

Procedure

5mg of the lyophilized tissue samples were hydrolyzed with 5ml of 2N HCl at 100^0C for 6h. HCl was removed by evaporation; Residue was dissolved in water and made up to a known volume. Aliquots were treated with 1ml of freshly prepared 2% acetyl acetone in capped tubes and kept in boiling water bath for 15minutes. After cooling in tap water, 5ml of 95% ethanol and 1ml of Ehrlisch's reagent were added and mixed thoroughly. The colour developed was read after 30 minutes at 530nm.

2.4.3.4. Estimation of serum sialic acid
(Skoza and Mohos, 1976)

By acid hydrolysis serum sialic acid was liberated which forms a coloured compound with thiobarbituric acid.

Reagents

H_2SO_4	- 0.2N
H_5IO_6	- 25µM in 62.5 mM H_2SO_4
$NaAsO_2$	- 0.2% in 0.5M HCl
Thiobarbituric acid	- 6% (DH_2O) (pH 9.0)

Procedure

200µl of serum sample was mixed with equal volume of 0.2N H_2SO_4 and hydrolyzed for 1h at 80^0C. To this 50µl H_5IO_6 was added and incubated for 30 minutes at 37^0C. 50µl of $NaAsO_2$ was added to this mixture followed by 100µl of thiobarbituric acid and was heated in a boiling water bath for ~7 minutes. 400µl of DMSO was added to intensify the colour.

2.4.3.5. Estimation of γ-glutamyl transpeptidase
(Szasz, 1976)

The transfer of γ-glutamyl moiety of a γ-glutamyl donor to a variety of acceptors is catalysed by γ-glutamyl transpeptidase.

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) all these reagents along with 25µl sample. p-nitroaniline release was measured at 410 nm.

2.4.3.6. Determination of superoxide dismutase (SOD) activity
(Mc Cord and Fridovich, 1969).

Superoxide radical was generated by autooxidation of riboflavin in the presence of light. This superoxide radical will reduce Nitro blue tetrazolium (NBT) to formazan (blue coloured)

Procedure

100µl of the homogenate was mixed with 200µl of 0.1M EDTA (containing 1.5mg sodium cyanide/100ml), 100µl NBT (1.5mM), 0.05ml riboflavin and phosphate buffer (0.06M, pH7.8) in a total volume of 3ml. Tubes without any enzymes was kept as control. After adding 0.05ml of riboflavin, the absorbance of the solution was measured against distilled water at 560nm. All the tubes were illuminated uniformly for 15min and absorbance of the blue color formed was measured again at 560nm. Percent of inhibition was calculated after comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). The volume of the sample required for scavenging 50% of the generated superoxide anion or reduction of NBT was considered as 1 unit of enzyme activity and expressed in units/mg protein.

2.4.3.7. Determination of catalase (CAT) activity

(Aebi, 1974)

Catalase catalyzes the decomposition of H_2O_2. The decomposition of H_2O_2 can be followed directly by the decrease in extinction at 240nm.

Procedure

At 240nm the absorption of diluted peroxide solution (34µl/10ml) will be recorded against 0.5M phosphate buffer (pH7) and initial OD was noted. After addition of 100µl of tissue the decrease in absorbance was measured for one minute at 15 seconds interval. Molar extinction coefficient for H_2O_2 is 43.6/ minute. Activity of catalase was calculated using micromoles of H_2O_2 decomposed/min/mg protein.

2.4.3.8. Determination of glutathione peroxidase (GPx) activity (Hafemann et al., 1974)

GPx activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and sodium azide.

Procedure

Tissue homogenate 100µl was incubated with 100µl of 0.2µM GSH, 500µl of 0.25mM H_2O_2, 100µl of 1mM sodium azide and phosphate buffer (0.4mM, pH7) in a total volume of 2.5ml and incubated at 37^0C for 6min. The reaction was stopped by adding 2ml of 1.67% metaphosphoric acid, centrifuged at 800rpm for 15 minutes. 2ml of the supernatant was mixed with 2ml 0.4M disodium hydrogen phosphate and 1ml of 1mM dithionitrobenzene (DTNB). Incubated for 10min at 37^0C and absorbance of the colored complex was measured at 412nm.One unit of enzyme activity is the decrease in log GSH by 0.001/ min after substraction of the decrease in log GSH/min for non enzymatic reaction, and expressed as units/mg protein.

2.4.3.9. Determination of reduced glutathione (GSH) (Moron et al., 1979)

The acid soluble sulfhydryl groups form yellow colored complex with DTNB. The absorbance of the colored complex was measured at 412nm.

Procedure

125µl of 25% TCA was added to 100µl of tissue homogenate, kept on ice for few minutes. Mixture was further diluted with 600µl of 5% TCA, centrifuged for 5 minutes. 300µl of the supernatant was mixed with 700µl of 0.2M sodium phosphate buffer (pH7) and 2ml of 0.6mM DTNB (prepared in 0.2M phosphate buffer, pH8). The yellow color formed was measured after 10min at 412nm. A standard graph

was prepared using different concentrations of GSH. Using this standard graph GSH content was determined and expressed as nmols/mg protein.

2.4.3.10. Determination of lipid peroxidation
(Ohkawa et al., 1979)

The tissue malondialdehyde (MDA) was allowed to react with thiobarbituric acid (TBA). The level of malondialdehyde (MDA) was estimated by extracting MDA-thiobarbituric acid (TBA) adduct to the organic layer and its subsequent measurement at 532nm.

Procedure

Reaction mixture containing 400µl of tissue homogenate, 1.5ml of 0.8% TBA, 1.5 ml of acetic acid (20%, pH3.5) was made upto 4ml using distilled water and kept for 1h in a boiling water bath. The reaction mixture was then cooled and 1ml of distilled water was added. 5ml of pyridine: butanol mixture (15:1) was added to the reaction tube, mixed and centrifuged at 3000rpm for 10min. Absorbance of the clear supernatant was measured at 532nm against butanol: pyridine mixture.

2.4.4. RNA isolation from tissue
(Chomczynski and Mackey, 1995)

All the glass wares and plastic wares used for RNA isolation should be rinsed with DEPC treated water, autoclaved twice and dried in hot air oven at 40^0C. Tissues (100mg) were collected from the animals, washed with PBS and minced well, and disperse the cells in cold conditions. Trizol reagent (1ml) was added, dispersed well by pipetting and incubated at room temperature for 5min. The solution was aspirated out by pipetting and transferred to a centrifuge tube placed on ice bath. The same step was repeated with 250µl of trizol reagent and the cell suspension was collected in the same tube. The suspension was centrifuged at 10,000rpm for 10min at 4^0C. The supernatant was collected in another tube and kept at room temperature.

Chloroform (300µl) was added to the supernatant and mixed well for 3min at room temperature. The resulting milky pink solution is then mixed by inverting the tube and it was centrifuged at 10,000rpm for 10min at 4^0C. The upper aqueous layer was carefully collected to an appendorf tube and 600µl of ice cold isopropanol was added to it. The solution was mixed well and allowed to stand at room temperature for 10min. After incubation, the tubes were centrifuged at 12,000rpm for 10min at 4^0C. The supernatant was discarded; the pellet was dissolved in 70% ethanol (prepared in DEPC-treated water) and kept at room temperature for 10min. It was then centrifuged at 13,000rpm for 10min at 4^0C. The supernatant was discarded; the pellet was dried and dissolved in 50µl of DEPC-treated water and stored at -20^0C. The qualitative checking of RNA samples were done by agarose gel electrophoresis.

2.4.5. cDNA synthesis from RNA
(Sambrook and Russel, 2001)

An aliquot of RNA sample containing 4µg of RNA was used for cDNA synthesis. The reaction mixture consists of the following components.

RNase free water	-4.4µL
AMV RT buffer	-1µL
Oligo (dT)	-0.6µL
dNTPs	-1µL
RNA sample	-1µL (vary depending up on the concentration of the RNA sample)
Total	=8µL

The reaction mixture was incubated at 65^0C for 5min. This was followed by cooling at room temperature for 5min. DTT (100mM, 1µl) was added, followed by addition of 1µL of AMV RT. The tubes were kept at 42^0C for 30min. The prepared cDNA

was stored at -20^{0}C. Amplification of genes using specific primers were done by varying temperature and time duration depending up on the primers used.

2.4.6. Detection of PCR products by agarose gel electrophoresis.

The PCR product (10µL) was resolved in 1.5% agarose gel. The electrophoresis apparatus was cleaned well and the edges of the gel tray were sealed. The gel comb was placed appropriately. 1.5% agarose gel was prepared in 1X TAE buffer and 0.5µg/mL ethidium bromide (1µL) was added to it. The gel was mixed well without air bubbles; carefully poured into the gel tray and allowed to solidify. Then the comb was carefully removed and the electrophoresis tank was filled with 1X TAE buffer until the gel is fully immersed in the buffer. The molecular weight marker as well as the amplified samples (10µl) were separately mixed with the gel loading dye (2µl) and loaded into individual wells of the gel. Electrophoresis was carried out at 70V until the dye front reaches 3/4th portion of the gel. Afterwards, the gel was photographed under UV illuminator chamber using the gel documentation system.

2.5. Statistical analysis

The data were analyzed using Graphpad InStat software (San Diego, CA) and expressed as mean± SD. Statistically significant differences between groups were calculated by the application of an analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Values of $P< 0.05$ were considered as significant.

2.6. Solutions and Reagents

2.6.1. 4T1 organ specific metastasis

2.6.1.1. Collagenase type I cocktail

Collagenase type I (50mg) was dissolved in 25ml of 1X HBSS. The solution is filter sterilized and stored as aliquots containing 2.5ml per tube at -20^{0}C. The aliquots were thawed in a 37^{0}C water bath, when needed (Pulaski and Rosenberg, 2001).

2.6.1.2. Collagenase type IV/elastase cocktail

Collagenase type IV (50mg) was dissolved in 25ml of 1XHBSS. The solution is filter sterilized and stored as aliquots containing 2.5ml per tube at -20^{0}C. The aliquots were thawed in a 37^{0}C water bath, when needed, and 30 units elastase was added per aliquot (Pulaski and Rosenberg, 2001).

2.6.1.3. Hyaluronidase cocktail

Hyaluronidase (50mg) and BSA (0.025g, preservative for storage) was dissolved in 25ml 1XHBSS; filter sterilized and stored as 2.5mL aliquots at -20^{0}C. The aliquots were thawed in 37^{0}C water bath, when needed (Pulaski and Rosenberg, 2001).

2.6.1.4. 6-Thioguanine

6-thioguanine (0.1g) was dissolved in 10ml of 0.01M NaOH to make the 60mM stock solution and stored at 4^{0}C. The solution was thawed in 37^{0}C water bath, when needed, and diluted to 60µM in culture media (Pulaski and Rosenberg, 2001).

2.6.2. Detection of PCR products

5X TAE buffer

Tris-base	- 24.2g
Acetic acid	- 5.71mL
0.5M EDTA	- 10mL

Add 800ml of double distilled water adjust the pH to 8.3 and made up to 1L.

2.6.3. Gelatin zymography

(Billings et al., 1991)

2.6.3.1. Preparation of gels

Resolving gel

11% Polyacrylamide gels with 0.1%SDS and 0.6% gelatin

29.2% acrylamide + 0.5% bisacrylamide	-11ml
0.1M Tris-HCl, pH8.8	-1.2ml
20% SDS	-0.15ml
20% APS	-0.10ml
Gelatin (180mg/2ml distilled water)	-2ml
DH_2O	-6.505ml
TEMED	-0.045ml

Mix and pour at room temperature.

Stacking gel

29.2% acrylamide + 0.5% bis acrylamide	-1.67ml
0.1M Tris-HCl, pH8.8	-1.75ml
20% SDS	-0.10ml
20% APS	-0.10ml
DH_2O	-6.36ml
TEMED	-0.02ml

Mix and pour, on above the resolving gel at room temperature

Sample buffer (2X)

Glycerol	-1ml
1M Tris-HCl, pH6.8	-0.25ml

20% SDS -1ml

Bromophenol blue (Tracking dye) -1.65mg

Made up to 5ml with DH_2O

Running buffer

Tris base -3g

SDS -2g

Glycine -14.2g

Made up to 1L with DH_2O

2% Triton X-100

Triton X-100 - 2ml

0.1M Tris HCl, pH 7.8 - 100ml (Final volume)

10mM EDTA solution

EDTA- Na_2 - 372.24mg

0.1M Tris-HCl, pH7.8 - 1000ml (Final volume)

2.7. Other Reagents

2.7.1. Phosphate Buffered Saline (PBS)

NaCl -8.00g

KCl -0.20g

$Na_2HPO_4.2H_2O$ -1.44g

KH_2PO_4 -0.20g

Distilled Water -1000ml

pH was adjusted to 7.2 with 1N HCl or NaOH

2.7.2. Cord buffer

NaCl -8.176g

KCl -0.298g

Dextrose -2.028g

Dissolved in one litre 0.001M phosphate buffer pH-7.2.

2.7.3. PBS-EDTA solution

EDTA -20mg

PBS -100ml

2.7.4. Trypsin solution

Trypsin -200mg

Glucose -20mg

PBS-EDTA -100ml

Sterilized by filtration

2.7.5. Alsevier's solution

Dextrose -2.05g

Sodium citrate -0.80g

NaCl -0.42g

Distilled water -100ml

pH adjusted to 6.1 with 10% citric acid.

2.7.6. Griess Reagent

A. 0.1% N- (1-Naphthylethylene diamino dihydrochloride) (NNED)

B. 1% Sulfanilic acid in 5% H_3PO_4

A+B= 1:1

2.7.7. Scintillation Fluid

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

2.8. Stains and kits

Trypan blue stain

Trypan blue	-100mg
Saline (0.9%)	-100ml

Trypan blue was dissolved in saline by overnight stirring. Any suspended particles were removed by filtration

Crystal violet stain

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

Kits for Alkaline phosphatase (ALP), Alanine aminotransferase (ALT/GPT), Blood urea, Total protein, Creatinine: - Span diagnostic Ltd, Surat, India.

DAB system: - Bangalore genei

ELISA: - Specific quantitative sandwich ELISA kits for mouse IL-2, IFN γ, IL-1β, IL-6, TNF-α and GMCSF were obtained from Pierce Biotechnology, Rock ford, IL, USA.

ELISA kits for VEGF and TIMP was purchased from R&D Systems (Minneapolis, Minn., USA).

2.9. Misellaneous

Tissue culture flasks, culture dishs and titre plates (Tarson, India.)
Modular incubator chamber (Billupus Rothenburg, USA.)
Modified Boyden Chamber (Nucleopore, Cambridge.)
Medium filtering assembly (Millipore, USA.)
Polycarbonate membrane filter (Millipore, USA.)

All the culture media, Collagenases, Collagen, Fibroblast growth factor (FGF), 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TNP 470, chloramine T, hyaluronidase, 6-thioguanine, Diethylpyrocarbonate (DEPC), Dithiothreitol (DTT), Ethidium bromide **(**EtBr), Hydroxyproline, glucuronic acid lactone, oligonucleotide primer sequences, Harri's hematoxylin, pararosaniline hydrochloride, α-napthyl acetate, lipopolysaccharide (LPS), phytohemagglutinin (PHA), concanavalinA (Con A), pokeweed mitogen (PWM), and mitomycin were purchased from Sigma Aldrich, Bangalore, India.

Anti CD31 antibody (Abcam, USA.)

N-Acetyl neuraminic acid, PPO, POPOP, Papain, Naphthalein (Sisco Research Laboratory, Mumbai, India.)

Radioactive ^{3}H-thymidine and $Na_2{}^{51}CrO_4$ (Board of Radiation and Isotope Technology, Mumbai, India.)

L-glutamine, Trypsin, Fluid thyoglycollate medium, HBSS, cysteine, carbazole (Hi media laboratories, India.)

FBS (biological industries, Israel.)

Trizol reagent, RNase free water, AMV RT buffer, AMV RT, Oligo (dT), loding dyes, molecular weight markers, dNTPs (Genei, India.)

All other chemicals used were of analytical reagent grade.

2.10. Instruments

Rack Beta fluid scintillation counter (Wallac 1209; Pharmacia, Uppsala, Sweden.)
Automatic Gamma Counter (PerkinElmer, USA.)
CO_2 Incubator (Napco, Canada.)
Deep Freezers, High speed cooling centrifuge (Remi, Chennai, India.).
Disc electrophoresis unit (Balaji Scientific Service, Chennai.)
ELISA plate reader (ThermoLabsystems, USA.)
Electronic Balance (Schimadzu, Japan.)
Gel Documentation system and Trans Transilluminator (Vilber Lourmat, France.)
Minicycler – Thermocycler (MJ Research, USA.)
Inverted Microscope (Leica, Germany.)
Lyophilizer (Labconco Inc, USA.)
Spectrophotometer (Elico, India.)
Spinwin Microcentrifuge (Tarson, India.)
Submerged electrophoresis unit (Genei, India)
Tissue homogenizer (Yorco Scientific Industries, Delhi.)
Hot air oven (Amur instrumentation, India.)
Autoclve, Laminar air flow, waterbath (Kemi, India.)

Chapter 3

Emilia sonchifolia (L.) DC: Effect on the immune system

3.1. Introduction

Immune system acts as a vital defence system against tumours, cancerous growth, and infectious diseases. Many of the plant based products are capable of positively altering the host immune response by triggering the defence cells of the immune system, and thereby act as potent immunomodulatory agents. In some disease treatments immunostimulatory agents are required to overcome the immunosuppression induced by drugs or environmental factors, and in others when there is undesired immunepotentiation, immunosuppressants are required. Immunomodulatory agents that can improve the immune system are necessary to quash the immunosuppressive effects or in situations where there is an impaired immune responsiveness. Immune response modulatory effects of medicinal plants can provide additional support to conventional therapeutic approaches like chemotherapy (Lin et al., 2015). Study on the immune response modulatory effect of *E. sonchifolia* (George and Kuttan, 2015) was done in order to provide a scientific basis for the conventional use of this plant in the traditional Indian Ayurvedic medicine possibly through modulation of the host immune defence. Previous investigations revealed the antitumor and anti-inflammatory potential of *E. sonchifolia* (Shylesh et al., 2005; Shylesh and Padikkala, 2000; Muko and Ohiri, 2000; Nworu et al., 2012). Thus, the plant will be a better target to be subjected to more studies, which can reveal its multifunctional roles in the physiological system. In the present study, we have focused on analyzing the immunomodulatory effects of the plant on humoral and cell-mediated immune responses in experimental animal models, which could contribute additional evidences to validate the pharmacological uses of the plant. The modification of immune response favourably by enhancement of immunological and nonspecific host defences is an exciting development in the field of immunopharmacology (George and Kuttan, 2015).

3.2. Materials and Methods

Plant material: - *E. sonchifolia* whole plant methanolic extract 25mg/kg b.wt., resuspended in 1% gum acacia.

Dosage: - Plant material was intraperitoneally (i.p) administered for five consecutive days.

Animals: - BALB/c and C57BL/6 mice (4-6 weeks old)

Cell lines: - B16F10 melanoma cells, EL4 thymoma cells and K562 leukemic cells.

3.2.1. Toxicity study

E. sonchifolia in different concentrations (200, 100, 50 and 25mg/kg b.wt.) were administrated intraperitoneally in BALB/c mice for 14 days. Animals were observed for mortality, behavioural changes, and change in body weight. On 15^{th} day, all the animals were sacrificed and selected organs such as liver, spleen, thymus, kidney and lungs were dissected out and weights were recorded. Blood was collected by heart puncture; serum separated and used for the analysis of hepatic and renal functions. Liver function markers, such as alkaline phosphatase (ALP) (King, 1965), glutamate pyruvate transaminase (GPT) (Bergmeyer and Bernt, 1980), and kidney function markers such as creatinine (Toro, 1975) and blood urea (Murray, 1984) were determined.

3.2.2. Hematological parameters

Blood was collected from the caudal vein of all the animals (BALB/c mice, n= 6/group) prior to the administration of *E. sonchifolia*, which was continued every third day for one month. The various parameters such as total white blood cell (WBC) count (hemocytometer), differential count (DC) (Leishman's stain), and hemoglobin content by cyanmethmoglobin method were assessed. In addition, change in body weight was also recorded.

3.2.3. Relative organ weights, bone marrow cellularity, and α-esterase activity

Animals (BALB/c mice, n= 6/group) were weighed 24 h after the last dose of *E. sonchifolia* administration and were sacrificed. Weights of vital organs such as liver, kidney, lungs, spleen, and thymus were recorded and expressed as relative organ weights. Bone marrow cells from the femur were collected and the cell number was determined and expressed as total live cells/femur (Sredni et al., 1992). The numbers of α-esterase-positive cells were determined by the azodye coupling method (Bancroft and Cook, 1984). The numbers of α-esterase-positive cells were expressed out of 4000 cells.

3.2.4. Hemagglutinating antibody titer

The BALB/c mice (n=6/group) were immunized with SRBCs (2.5×10^8 cells/animal, i.p.) along with the last dose of *E. sonchifolia* administration. Blood was collected from the caudal vein every third day for a period of 30 days. Serum was separated and heat inactivated at 56^0C for 30 min. Antibody titer was determined by hemagglutination assay using SRBC as antigen (Singh et al., 1984).

3.2.5. Plaque forming cell assay

BALB/c mice (n=21/group) immunized with SRBC (2.5×10^8 cells/animal, i.p.) along with the last dose of *E. sonchifolia* administration. The animals were sacrificed on different days starting from the third day up to the ninth day. Spleens were collected and processed into single cell suspension and the numbers of plaque forming cells (PFCs) were determined by the Jerne's Plaque assay (Jerne and Nordin, 1963).

3.2.6. Blastogenesis assay

Mitogens can stimulate resting lymphocytes to undergo a series of changes and are converted to blast-like cells. This process leads to cell division and can be quantified by ^{3}H thymidine incorporation assay. The animals (BALB/c mice, n=6/group) were sacrificed 24h after the last dose of *E. sonchifolia* administration. Lymphoid organs such as spleen, thymus, and bone marrow were collected and processed aseptically into single cell suspension. Then, 5×10^{4} cells were cultured in a 96-well round-bottomed titer plate in the presence and absence of various mitogens such as phytohemagglutinin (PHA) 2.5µg/ml, concanavalin A (Con A) 10µg/ml, pokeweed mitogen (PWM) 10µg/ml, and ipopolysaccharide (LPS) 10µg/ml in a humidified 5% CO_2 atmosphere at $37^{0}C$ and incubated for 48h. All the cells were labelled with 1µCi of ^{3}H-thymidine and further incubated for 18h. After incubation, DNA was precipitated using 10% ice-cold perchloric acid, centrifuged and the pellets were dissolved in 0.5 ml of 6 N NaOH and transferred to 5ml scintillation fluid. Radioactivity was measured using Rack Beta fluid scintillation counter.

3.2.7. Determination of the effect of *E. sonchifolia* on the cell-mediated immune response by cytotoxic T lymphocytes (CTL) production

Winn's neutralization test: CTL activity was assessed using Winn's neutralization test according to the method of (Kobayashi et al., 1992). Briefly, 1ml (1×10^{7}) of effector cells (spleen cells) was mixed with the same volume containing 5×10^{5} EL4 cells (target cells). The cells were incubated for 1h at 37 ^{0}C in 5% CO_2 atmosphere, and 0.2ml of this mixture was injected intraperitoneally to BALB/c mice. The animals were observed for survival time period. The survival times of treated animals were compared with those animals that received tumour cells alone. Increase in survival days was directly related to the CTL activity.

Alloimmunization was carried out by injecting spleen cells (2×10^{7}) from C57BL/6 mice, subcutaneously to BALB/c mice. Two systems were used to generate effector cells from the spleen cells of BALB/c mice.

System A

Spleen cells (effector) were obtained 7 days after alloimmunization of BALB/c mice with spleen cells from C57BL/6 mice as described above. Four groups of BALB/c mice (n = 10/group) were used. Group I received EL4 cells alone (5×10^5/0.1 ml), group II animals received *E. sonchifolia* along with EL4 cells. Group III animals received EL4 cells incubated with normal spleen cells (0.2ml) from the BALB/c mice and group IV animals received EL4 cells incubated with spleen cells from *E. sonchifolia*-treated alloimmunized mice. All the animals were observed for survival. Blood was collected from the caudal vein of each animal 2 days after the last dose of *E. sonchifolia* administration, and levels of IL-2 and IFN-γ were determined using ELISA kits according to the manufacturer's protocol.

System B

Effector cells were produced by a 5 day mixed lymphocytes culture (MLC) of spleen cells (responder cells) from *E. sonchifolia* treated and untreated BALB/c mice and mitomycin treated (50μg/ml) spleen cells (stimulator cells) from C57BL/6 mice. Three groups of animals (n= 10/group) were used. Group I animals received EL4 cells alone, group II animals received EL4 cells cocultured with effecter cells generated using normal spleen cells and group III animals received EL4 cocultured with effector cells generated using spleen cells from *E. sonchifolia* treatment.

3.2.8. Determination of the effect of *E. sonchifolia* on cell mediated immune response (CMI) in B16F10 melanoma bearing animals

Mice (C57BL/6) were divided into 3 groups (n=36/group). Group I animals received B16F10 cells ($1x10^6$ cells/animal) intravenously through lateral tail vein and kept as untreated control, group I1 animals were treated with *E. sonchifolia* for 5 consecutive days and group III animals received B16Fl0 cells ($lXl0^6$ cells/animal) intravenously after the last dose of *E. sonchifolia*

administration. Starting from 24 hour after tumour induction three animals from each group were sacrificed; spleen and blood were collected on different time intervals. Spleen cells were processed to single cell suspension and used as effector cells for Natural Killer (NK) cell activity and antibody dependent cellular cytotoxicity (ADCC) by ^{51}Cr release assay (Kim et al., 1980). ^{51}Cr binds to cytoplasmic proteins after diffusing through the cell membrane and is released only when the cell membrane is sufficiently damaged. Serum was used for the estimation of antibody dependent complement mediated cytotoxicity (ACC) by trypan blue exclusion method (Talwar, 1983).

Labelling of target cells

Target cells K562 (10^6) and SRBC (10^7) was done by adding 100µCi of $Na_2{}^{51}CrO_4$ and incubation for 1 hr at 37^0 C on a shaker. Cells were washed and further incubated at 4^0C for 1hr.

$$\text{Percentage of cell lysis} = \frac{\text{Experimental release - Spontaneous release}}{\text{Total releases - Spontaneous release}} \text{ X100}$$

Spontaneous release (SR) - wells contained only target cells and medium.
Total release (TR) - wells contained target cells, medium and 100 µl of 1N HCl.

A. Determination of NK cell mediated cytotoxicity

100µl of labelled target cells K562 (lxl0^4 cells/ml) were mixed with effector spleen cells in 96-well round bottom titre plates to yield an effecter: target ratios of 100:1. Final volume was adjusted to 200µl and incubated at 37^0C for 4 h and ^{51}Cr release assay was performed. For this titre plates were centrifuged for 15 minutes, 100µl of supernatant was collected and radioactivity measured using gamma counter. Control tubes were kept for each experiment.

B. Determination ADCC

By mixing 100µl of labelled SRBC (lxl0^4 cells/ml) that act as target cells and spleen cells (effector cells) in an effector-target ratio of 100:1. 100µl of anti-sera against SRBC was added to this and incubated at 37^0C for 4h, and ^{51}Cr release assay was done as explained above.

C. Determination ACC

ACC was done using the heat inactivated (56^0C for 30 min) serum. Fresh rabbit serum was used as a source of complement for the reaction. Serum samples were mixed with 100µl of B16F10 (1×10^4 cells) and 50µl of complement. Final volume was made up to 2ml and incubated at 37^0C for 3h. Cytotoxicity was assessed by trypan blue exclusion method

3.3. Results

3.3.1. Toxicological evaluation of *E. sonchifolia*

The no-observed-adverse-effect level (NOAEL) of *E. sonchifolia* was 50mg/kg b.wt. *E. sonchifolia*, when administered at doses of 25 and 50 did not produce any mortality and change in behaviour, body weight, relative organ weight, hepatic and renal functions when compared with control group. Meanwhile, administration of 100mg/kg b.wt. produced a nonsignificant increase in the serum GPT and blood urea when compared to control and 50mg/kg b.wt. groups. *E. sonchifolia* at 200mg/kg b.wt. showed observable toxicity showing behaviour changes, hypoactivity, weight loss, and decrease in the relative organ weight with altered hepatic and renal functions (table 3.1). Hence, the immunomodulatory study was conducted using three different concentrations of *E. sonchifolia* (10, 25, and 50mg/kg b.wt.), which are observed to be nontoxic. Since *E. sonchifolia* 25 and 50mg/kg showed similar effects on the immune parameters and also the concentration of 10mg/kg did not show any significant

Table 3.1. Toxicity profile of *E. sonchifolia*

	Concentrations of *E. sonchifolia* (mg/kg body weight)				
	Control	25	50	100	200
Mortality (D/T)	None	None	None	None	None
Behavioural change	None	None	None	None	Hypo activity
Change in body weight (g)	+2.01±0.2	+2.08±0.8	+2.05±1.1	+1.76±0.6	-0.98±0.5
Relative organ weights (g/100 g body weight)					
1. Liver	5.39±0.23	5.38±0.17	5.34±0.19	5.07±0.18	4.77±0.25
2. Spleen	0.39±0.02	0.41±0.02	0.40±0.02	0.36±0.02	0.31±0.03
3. Thymus	0.12±0.01	0.11±0.01	0.11±0.01	0.10±0.01	0.09±0.02
4. Kidney	1.34±0.15	1.34±0.13	1.30±0.12	1.27±0.12	1.23±0.07
5. Lungs	0.61±0.02	0.62±0.02	0.60±0.04	0.59±0.03	0.57±0.03
Serum ALP (U/ml)	13.37±0.42	13.31±0.5	13.96±0.78	14.42± 0.78	24.57±2.01
Serum GPT (U/ml)	58.1±3.62	54.9±2.6	58.72±3.22	67.96±7.10	129.28±2.7
Blood urea (mg/dl)	42.88±1.17	43.06±1.1	43.93±1.12	50.17±2.38	53.22±1.07
Serum creatinine (mg/dl)	0.92±0.01	0.91±0.02	0.92±0.03	0.95±0.03	1.37±0.15

Values are the mean ± standard deviation.

Abbreviations: D/T, dead/treated mice; ALP, alkaline phosphatase;

GPT, glutamate pyruvate transaminase.

“None” means that no toxic symptoms were seen during the observation period.

effects, the data of minimum dose, which is nontoxic but immunologically effective (25mg/kg b.wt.), were shown.

3.3.2. Effect on hematological parameters

The total WBC count of *E. sonchifolia* treated normal BALB/c mice were increased significantly ($p<0.001$) to 9995±535 cells/mm^3 on the sixth day compared to control animals (figure 3.1). There was no significant difference in the DC, hemoglobin content, and body weight (data not shown) of the animals after treatment with *E. sonchifolia* compared to control groups.

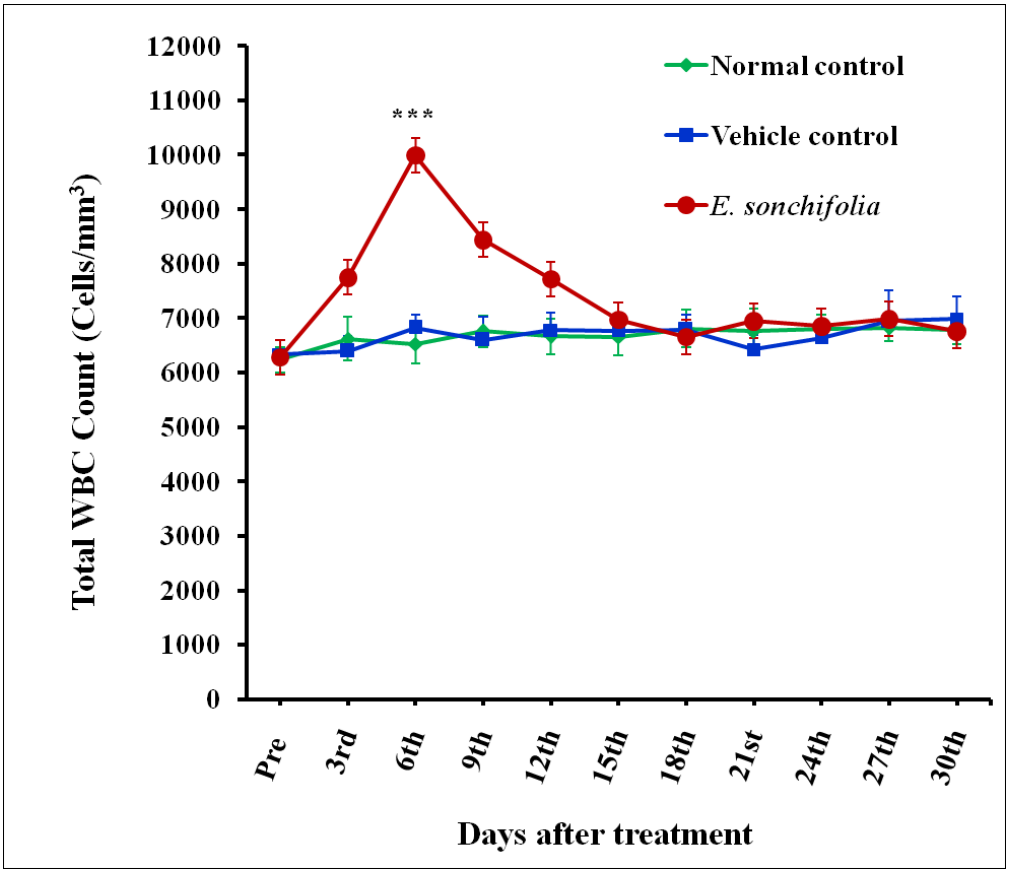
3.3.3. Effect on relative organ weights, bone marrow cellularity, and α-esterase activity

There was a significant ($p<0.001$) increase in the weight of thymus and spleen after administration of *E. sonchifolia* when compared with the control groups. There was no significant change in the weight of other vital organs such as liver, kidney, and lungs (table 3.2). This shows the stimulatory effect of *E. sonchifolia* on the lymphoid organs such as spleen and thymus. The effect of *E. sonchifolia* on the bone marrow cellularity and the increase in the number of α-esterase positive cells were also significant ($p< 0.001$) compared to control groups (table 3.3).

3.3.4. Effect on hemagglutinating antibody titer and PFC assay

Emilia sonchifolia administration produced a titer value of 1024, up to which the agglutination of the antibody formed against SRBC antigen was recorded. This value was obtained in the serum collected on the 12th day. The control animals showed a maximum antibody titer value of 128 on the same day (figure 3.2). There was a significant ($p<0.001$) increase in the number of PFCs, which showed a clear area of lysis of SRBC in the *E. sonchifolia* treated group (257±23 PFC/10^6 spleen cells) on the sixth day compared to the control groups of animals (149.3±9.4 PFC/10^6 spleen cells) (figure 3.3).

Figure 3.1. Total WBC count of animals treated with *E. sonchifolia*



*****p < 0.001**

Figure 3.2. Hemagglutinating antibody titer of animals treated with *E. sonchifolia*

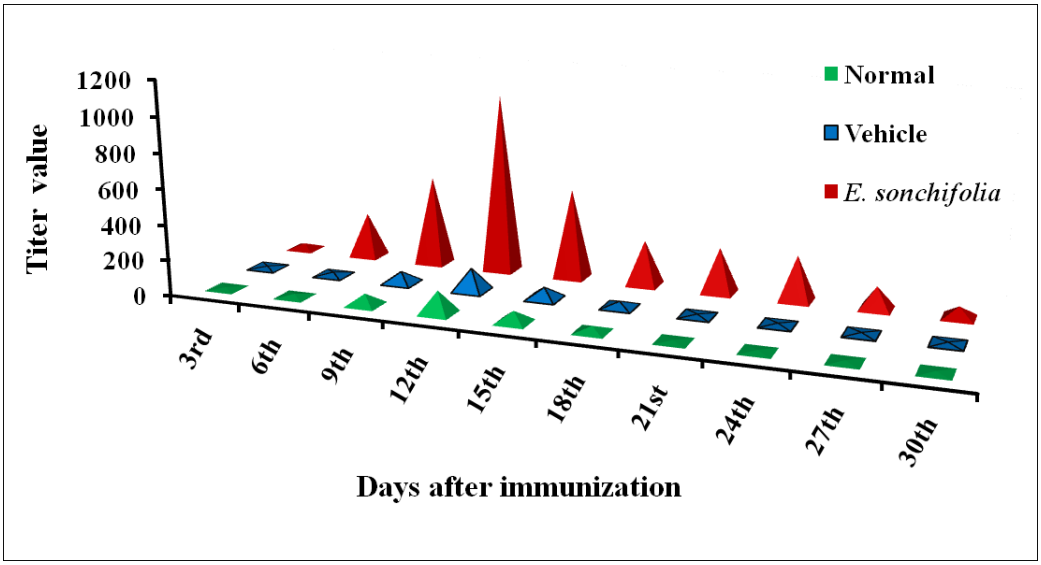
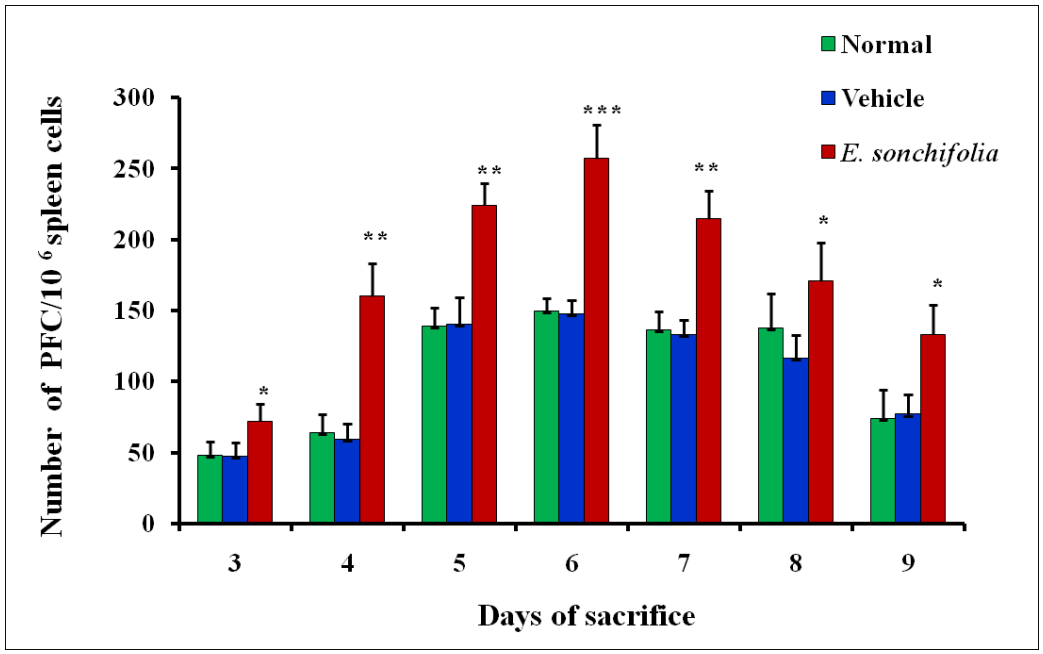


Figure 3.3. Plaque forming cell assay of animals treated with *E. sonchifolia*



***p < 0.001, **p < 0.01, *p < 0.05**

Table 3.2. Effect of *E. sonchifolia* on the relative organ weights (g/100g body weight)

Treatment	Spleen	Thymus	Liver	Kidney	Lungs
Normal	0.43±0.01	0.13±0.01	5.76±0.12	1.56±0.07	0.63±0.03
Vehicle	0.44±0.02	0.12±0.02	5.73±0.11	1.56±0.06	0.61±0.04
E. sonchifolia	0.52±0.01***	0.18±0.01***	5.75±0.10	1.54±0.04	0.64±0.02

Values are mean ± SD. *** $p < 0.001$ compared with control groups.

Table 3.3. Effect of *E. sonchifolia* on the Bone marrow cellularity and the number of α-esterase positive cells

Treatment	Bone marrow cellularity ($1x10^6$Cells /femur)	α-esterase positive cells (No of +ve cells/4000 cells)
Normal	16.08±0.74	817.00±25.13
Vehicle	17.08±0.38	825.33±29.87
E. sonchifolia	22.92±1.69***	1186.00±118.24***

Values are mean ± SD. *** $p < 0.001$ compared with control groups

3.3.5. Effect on blastogenesis assay of splenocytes, thymocytes, and bone marrow cells

Significant ($p<0.05$) enhancement in the rate of proliferation of spleen, thymus, and bone marrow cells was observed in the *E. sonchifolia* treated group of animals compared with control groups. Administration of *E. sonchifolia* also enhanced significantly ($p<0.01$) the mitogenic potential of various mitogens such as LPS, PWM, PHA, and Con A in spleen and bone marrow cell proliferation. In *E. sonchifolia*-treated group of animals, the rate of thymocytes proliferation was increased significantly ($p<0.001$) in the presence of mitogens excluding LPS (table 3.4).

3.3.6. Effect on CTL production

System A

Effect of *E. sonchifolia* on *in vivo* CTL generation is given in table 3.5A. There was a 44% increase in the lifespan of animals treated with five doses of *E. sonchifolia* when compared with the untreated tumour bearing group (EL4 alone). However, in animals injected with EL4 cells incubated with normal alloimmunized effector cells, the percentage increase in lifespan was only 23%. But when the animals received EL4 cells incubated with *E. sonchifolia* treated alloimmunized spleen cells, the number of survival days was significantly ($p< 0.001$) increased to 69%, showing the *in vivo* generation of CTL that can destroy sensitive thymoma cells (EL4) and decrease mortality due to tumour burden. The effect of *E. sonchifolia* on the production of IL-2 and IFN-γ by EL4-bearing animals incubated with *in vivo* generated effector cells is presented in table 3.5B. Administration of *E. sonchifolia* significantly increased the level of cytokines, which are involved in immunomodulation, when compared to administration of tumour alone and tumour plus normal alloimmunized spleen cells.

Table 3.4. Effect of *E. sonchifolia* on spleen, thymus and bone marrow blastogenesis

	Counts per minute (CPM)				
	Without mitogens	Con A	PHA	PWM	LPS
Splenocytes					
Normal	1736±113	4582±127	3487±123	3745±176	4062±208
Vehicle	1709±104	4668±101	3545±130	3697±145	4048±227
E. sonchifolia	1940±98*	4857±128**	3850±163**	4128±176**	4469±128**
Thymocytes					
Normal	1686±96	3648±105	3026±108	3524±102	-
Vehicle	1720±121	3765±110	3181±103	3652±108	-
E. sonchifolia	1896±103*	4680±125***	4201±213***	4881±101***	-
Bone marrow cells					
Normal	1325±101	1472±104	2835±126	2165±128	3396±161
Vehicle	1346±109	1502±105	2869±159	2121±158	3438±129
E. sonchifolia	1524±139*	1765±134**	3156±103**	2492±190**	3712±108**

Values are mean of the rate of proliferation in Counts per minute (CPM) ± SD.

$^{***}p< 0.001$, $^{**}p< 0.01$, $^{*}p< 0.05$ compared with control groups

Table 3.5A. Effect of *E. sonchifolia* on the CTL generation and survival

Treatment	Number of days survived	% ILS
System A		
EL4 alone	31± 2.3	
EL4 + *E. sonchifolia*	48.6± 2.1[a]	44
EL4 + normal alloimmunized effector cell	39.3 ± 3	23
EL4 + *E. sonchifolia* treated alloimmunized spleen cells	53.1 ± 3.8[b]	69
System B		
EL4 alone	32.3 ± 2.3	
EL4 + normal cocultured spleen cells	39.6 ± 2.4[a]	20
EL4 + *E. sonchifolia* treated cocultured spleen cells	53.2 ± 2.6[b]	62

System A: All data are expressed as means ± SD. [a] $p<0.001$ compared with EL4 alone [b] $p<0.01$compared with EL4 + normal alloimmunized effector cell. System B: All data are expressed as means ± S.D. [a] $p<0.001$, compared with EL4 alone, [b] $p<0.001$compared with EL4 + normal co-cultured spleen cells.

Table 3.5B. Effect of *E. sonhifolia* on the cytokine production System A

Treatment	IL-2 (pg/mL)	IFN-γ (pg/mL)
Normal	11.9 ± 1.8	2957.4± 222.86
EL4 alone	7.04 ± 0.23	1362.6± 101.4
EL4 +*E. sonchifolia*	19.9 ± 2.38[a]	3452.43 ± 138.12[a]
EL4 +normal alloimmunized effector cell	8.4 ± 1.31	1356.84 ± 92.81
EL4 +*E. sonchifolia*-treated alloimmunized spleen cells	28.96 ± 2.68[b]	3690.49 ±104.63[b]

Results were expressed as means ± S.D. [a] $p<0.001$ compared with EL4 alone. [b] $p<0.001$ compared with EL4 + normal alloimmunized effector cell.

System B

The effect of *E. sonchifolia* on the generation of CTL by system B is given in table 3.5A. There was a 20% increase in the lifespan of animals that received EL4 cells incubated with effector cells from cocultured normal spleen cells. When EL4 cells were incubated with *E. sonchifolia* treated alloimmunized effector cells, the lifespan of animals was increased by 62%.

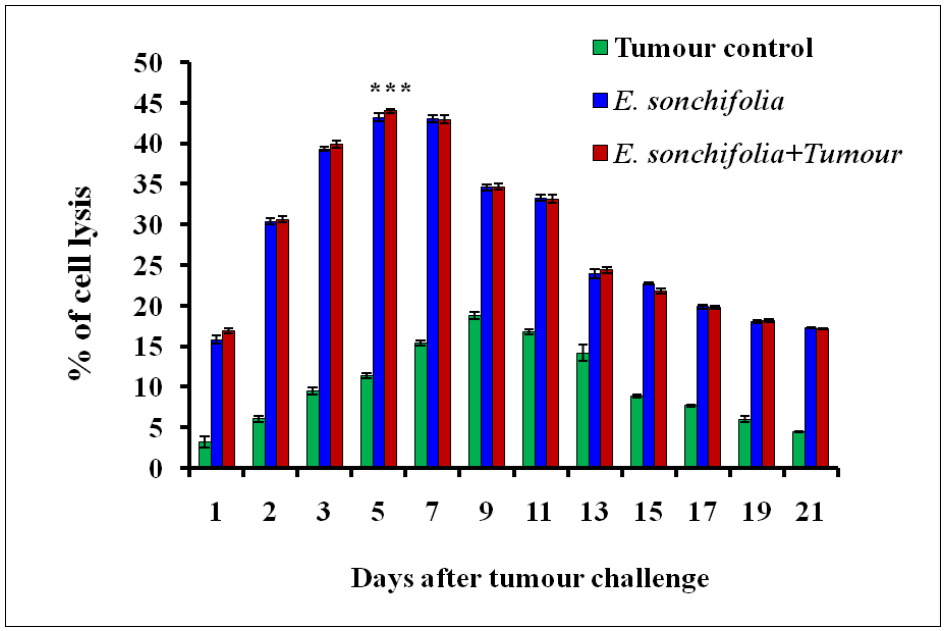
3.3.7. Effect on NK cell activity, ADCC and ACC

E. sonchifolia administration enhanced NK cell activity ($p < 0.001$) in normal as well as in tumour bearing animals as shown in figure 3.4A. On 5^{th} day after tumour induction maximum lysis of target cells 43.2% and 44% was seen in *E. sonchifolia* treated, and in *E. sonchifolia* treated tumour bearing mice respectively. At the same time in untreated tumour bearing control animals, the maximum cell lysis of 18.8% was observed only on day 9. Effect of *E. sonchifolia* on ADCC is represented in figure 3.4B. *E. sonchifolia* treatment augmented ADCC ($p < 0.001$) in tumour-bearing as well as in normal animals. On day 9 in *E. sonchifolia* treated normal as well as in tumour-bearing animals 37% maximum lysis of target cells was seen compared to untreated tumour-bearing control animals that showed a peak lysis of only 9.6%. *E. sonchifolia* administration significantly ($p < 0.001$) enhanced ACC in tumour-bearing as well as in normal animals as shown in figure 3.4C. Maximum activity was observed on day 15 with 21.7% and 22.1% of cell death in normal and tumour bearing *E. sonchifolia* treated mice. Whereas in untreated tumour-bearing control animals, the peak activity of 13.6% cell death was observed on day 17.

3.4. Discussion

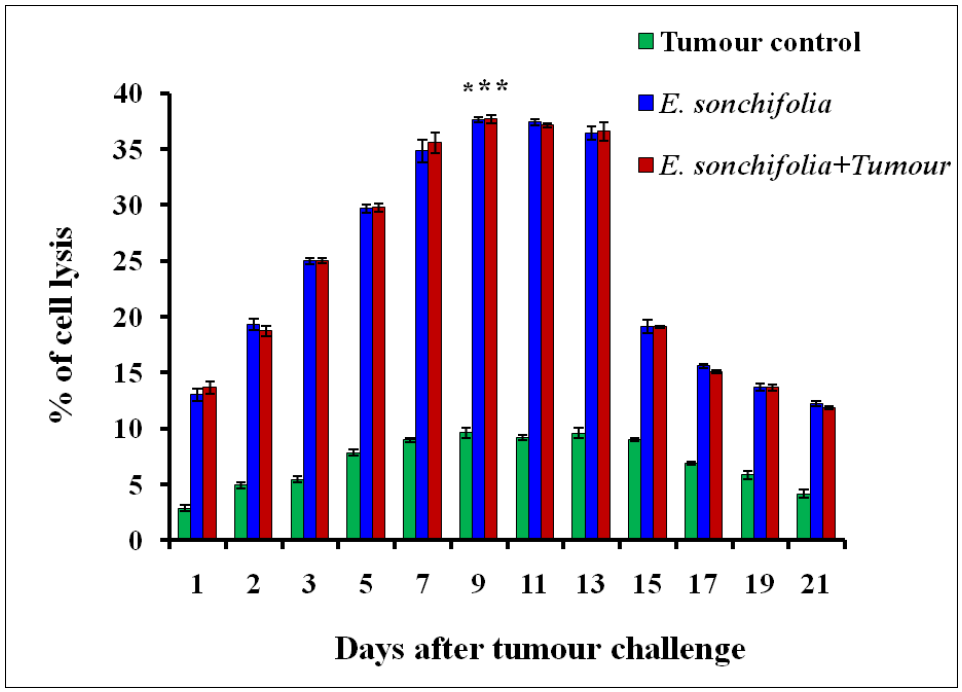
Maintenance of health and prevention of and recovery from diseases are important functions attributed to a healthy immune system whose activities are well regulated by cross-linked pathways of cytokines. During pathological conditions such as cancer, the immune tolerance induced by tumour microenvironment helps tumour cell proliferation, survival, and migration

Figure 3.4A. Effect of *E. sonchifolia* on NK cell activity



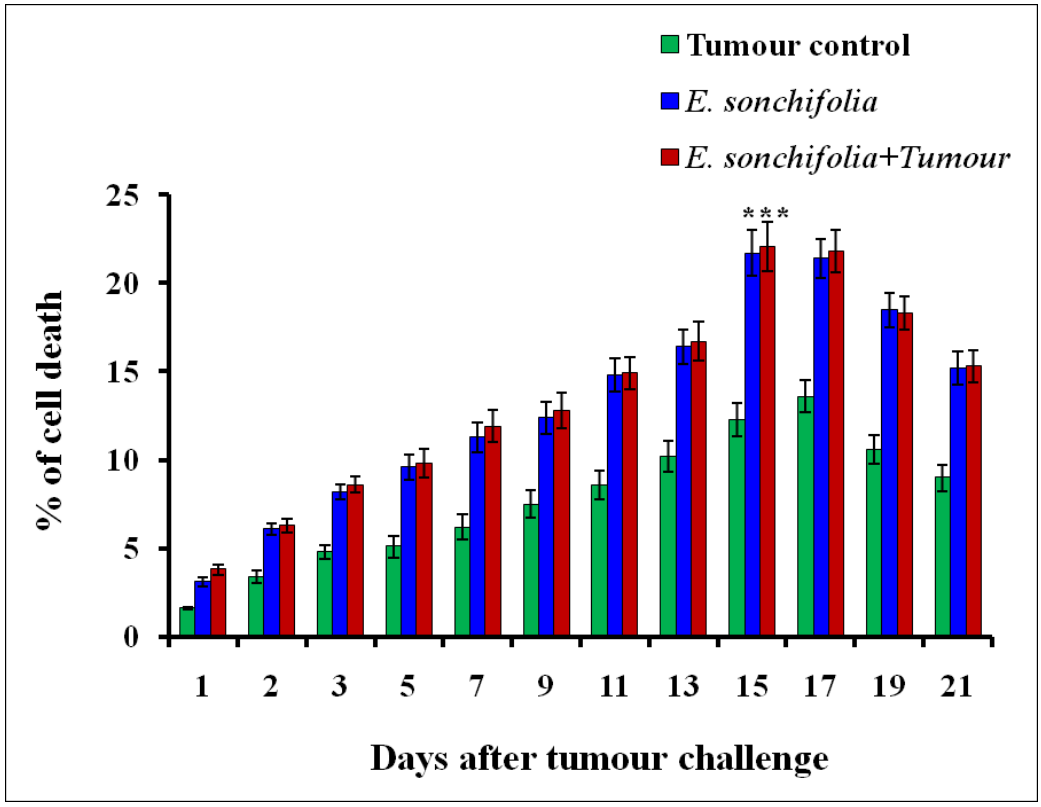
*****p < 0.001**

Figure 3.4B. Effect of *E. sonchifolia* on ADCC



*****p < 0.001**

Figure 3.4C. Effect of *E. sonchifolia* on ACC



***p < 0.001

without being attacked by the immune system (Zou, 2005). Hence, the immune cells should be boosted to get activated to maintain an antitumor defence state, thus enabling the physiological system to overcome the immune suppressive tumour microenvironment (Kwon et al., 2009). The investigations on novel antitumor substances with immunomodulatory effects should be encouraged, and this type of immune response modulation can be considered as an alternative against the currently known toxic side effects of cancer treatment (Xu et al., 2009).

The hematopoietic stem cells present in the bone marrow can give rise to myeloid and lymphoid progenitor cells. These cells then differentiate into various blood cells of corresponding lineages. The preliminary observation itself regarding an increased production of total WBC by *E. sonchifolia* treatment thus signifies the stimulation of the hematopoietic system. Analysis of the treatment effects on the production of bone marrow cells further confirmed the above results, where the bone marrow cells were found actively increased along with the increased rate of their subsequent differentiation in the bone marrow. In order to check whether or how treatment with *E. sonchifolia* could influence the lymphoid organs, lymphoid organ weights as well as their mitogen-induced proliferation were studied. The results were in fact promising, which strongly suggest that *E. sonchifolia* stimulated the proliferation of the cells of lymphoid organs, which in turn caused an increase in organ weights. Since the lymphoid cells (B and T cells) are the major effectors of the immune system, these results directly correlate to the immune stimulatory effects of *E. sonchifolia.* These regulatory effects on the immune cells can be utilized in therapy, especially when the immune system must be alert, as in the case of infections or immune suppressed conditions.

Antibody functions as the effector of humoral immune response by binding to the antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. To evaluate the effect of *E. sonchifolia* on humoral immune response, sheep erythrocyte antigen specific hemagglutinating antibody (HA) production was assayed in SRBC treated mice. In mice treated with *E. sonchifolia,* enhancement in HA titer was

obtained, with a peak value on the 12th day, reflecting an overall elevation in humoral immune response. The PFC assay is considered to be one of the most highly predictive single assays used to assess potential modulation of the humoral immune response, which quantifies the number of B cells producing SRBC specific IgM (Wilson et al.,1999) Treatment with *E. sonchifolia* markedly increased the number of PFCs, indicating its enhancing effect on humoral immunity. The radioactive ^{3}H-thymidine incorporation assay clearly showed a significantly enhanced proliferation of both B and T lymphocytes in the presence and absence of specific mitogens. The increased proliferation of these lymphoid cells specially points out the influence of *E. sonchifolia* in the modulation of cell mediated immune response. This effect of *E. sonchifolia* on proliferation may be due to the reduction in the threshold levels for the mitogens needed to induce cell proliferation or by unmasking of the mitogen receptors on cell surface. From these results, it is confirmed that *E. sonchifolia* can stimulate the humoral arm of the immune system via B lymphocytes and cell-mediated arm via T lymphocytes.

Cell-mediated immune responses are the major immune effectors during pathogenesis of tumour, which might be mediated mainly by T cells, including NK cells. These cells will produce many factors like macrophage mobile factor, lymphotoxin, and interferon, which will lead to the proliferation and differentiation of immune cells and to macrophage phagocytosis and increase the capacity of killing target cells, and all these will have active participation in preventing the tumour (Kim et al., 2001). Alloimmunization of mice will produce CTLs, resulting in the augmentation of the cytotoxic activity of NK cells (Suzuki et al., 1985). In the present study, Winn's neutralization assay was used for the generation and activity determination of CTLs. The increased lifespan of treated animals compared with control groups suggests that *E. sonchifolia* could increase CTL production and thereby cause cytotoxicity to the tumour cells. Mixed lymphocyte culture (MLC) is an *in vitro* test of lymphocyte recognition and proliferation. The experimental analysis using MLC reaction also showed that *E. sonchifolia* augmented T-cell-mediated immune response by modulating CTL activity. It was reported that the activated T cells in MLC will produce cytokines, especially IL-2, which was responsible for the differentiation

of precursor into mature effector CTL (Kern et al., 1981). Since cytokines are the key intermediators in the regulation of immune response, we also assessed the effect of *E. sonchifolia* on the production of specific cytokines, such as IL-2 and INF-γ. The proliferating CD4+ will secrete IL-2, which stimulates the proliferation of CTL and helper T lymphocytes, and potently augment the cytolytic activity of NK cells, lymphokine-activated killer cells, and macrophages, all of which can participate in immunological antitumor mechanisms (Mizuno et al., 2000). NK cells, CD8+T cells, and the Th1 subclass of CD4+T cells produce IFN-γ, which is responsible for antitumor activity either by promoting the apoptotic death of target cells through the Fas/Fas ligand pathway or through secretion of perforin and granzymes (Tanaka et al., 2004; Wang et al., 2004). Analysis of serum from tumour bearing mice after treatment with *E. sonchifolia* revealed an increased secretion of IL-2 and IFN-γ. These observations indicated that these cytokines have a key role in regulating the increased CTL response, which in turn results in cytotoxicity to the tumour cells (Kelly et al., 2002; George and Kuttan, 2015). NK cells mediate nonspecific target cell killing, without prior sensitization and they do not need to recognize antigen/MHC on the target cell (Cooper et al., 2001). Fc receptors on NK cell surface allow them to kill antibody-coated target cells by ADCC, thus providing another form of immune defence (Qu and Li, 2010). Treatment with *E. sonchifolia* significantly enhanced the NK cell activity in normal as well as tumour bearing animals along with an enhancement in ADCC and ACC altogether proved the activation of cell mediated immune system.

Chapter 4

Emilia sonchifolia (L.) DC: Evaluation of the anti-inflammatory effects

4.1. Introduction

About 150 years ago the relationship between inflammation and cancer was first hypothesized by Rudolph Virchow. Inflammation in the tumour microenvironment has many cancer-promoting effects and aids in the proliferation and survival of malignant cells and further promotes angiogenesis and metastasis. Epidemiologic evidence suggests that approximately 25% of all human cancers worldwide are associated with chronic inflammation, chronic infection, or both (Morrison, 2012). Inflammation is the localized protective response of the tissue to irritation, injury, or infection, characterized by pain, redness, swelling, and sometimes loss of function. Now it was realized that inflammation has a profound role in a wide variety of diseases, including cancer. While acute inflammation is a part of the defence response, chronic inflammation can lead to cancer, diabetes, cardiovascular, pulmonary and neurological diseases (Aggarwal et al., 2006). Macrophages in particular are important tumour infiltrating cells that affect tumour growth and metastasis (Morrison, 2012). Inflammatory cells and mediators are an essential component of the tumour microenvironment. Inflammatory circuits can differ considerably in different tumours in terms of cellular and cytokine networks and molecular drivers. However, macrophages are a common and fundamental component of cancer promoting inflammation. Dissection of the diversity of cancer related inflammation is instrumental to the design of therapeutic approaches that target cancer-related inflammation (Balkwill and Mantovani, 2012). The plant *E. sonchifolia* is used in the folklore medicine for treating tumour and inflammation (Shylesh et al., 2005). Previous studies conducted on this plant revealed its anti inflammatory (Muko and Ohiri, 2000; Nworu et al., 2012) and anti tumour (Shylesh and Padikkala, 2000) properties. This study is a pioneer attempt to find out the anti-inflammatory effect of an active fraction from the medicinal plant *Emilia sonchifolia*, enriched with the major compound γ-humulene (γ-hum).

4.2. Materials and Methods

Plant material: - An active fraction from *E. sonchifolia*, enriched with the major sesqueterpene γ-humulene (71%) (γ-hum).

Dosage: - Different concentrations of γ-hum was intraperitoneally (i.p) administered for five consecutive days.

Animals: - Male BALB/c mice (6–8 weeks old)

4.2.1. Toxicity study

γ-hum in different concentrations (5, 10, 15, 20mg/kg b.wt.) was administered intraperitoneally to mice (n=8) for 14 days. Animals were observed for mortality, behavioral changes, and change in body weight. On 15^{th} day, all the animals were sacrificed and selected organs such as liver, spleen, thymus, kidney, and lungs were dissected and weights were recorded. Blood was collected by heart puncture, the serum was separated and used for the analysis of hepatic and renal functions. Liver function markers, such as alkaline phosphatase (ALP) (King, 1965), glutamate pyruvate transaminase (GPT) (Bergmeyer and Bernt, 1980), and kidney function markers such as creatinine (Toro, 1975) and blood urea (Murray, 1984) were determined.

4.2.2. Carrageenan induced paw edema model

Mice were divided into five groups (n=6/group).

Group I: Carrageenan alone treated (Control)
Group II: Carrageenan+γ-hum (1mg/kg b.wt.)
Group III: Carrageenan+γ-hum (2.5mg/kg b.wt.)
Group IV: Carrageenan+γ-hum (5mg/kg b.wt.)
Group V: Carrageenan+diclofenac (10mg/kg b.wt.)

One hour, after the last dose of γ-hum or diclofenac administration, paw edema was induced by carrageenan (30μg/animal) administration on sub-plantar region of the hind paw of all mice (Winter et al., 1962). The paw thickness was measured using vernier calipers before carrageenan injection and continued at 30 min intervals for 8 hours followed by 24 hour.

4.2.3. Dextran induced paw edema model

Mice were divided into five groups (n=6/group).

Group I: Dextran alone treated (Control)
Group II: Dextran+γ-hum (1mg/kg b.wt.)
Group III: Dextran+γ-hum (2.5mg/kg b.wt.)
Group IV: Dextran+γ-hum (5mg/kg b.wt.)
Group V: Dextran+diclofenac (10mg/kg b.wt.)

One hour, after the last dose of γ-hum or diclofenac administration, paw edema was induced by dextran (30μg/animal) administration on sub-plantar region of the hind paw of all mice (Maity et al., 1998). The paw thickness was measured using vernier calipers before dextran injection and continued at 1 hour intervals for 6 hours followed by 24 and 48 hour.

4.2.4. Formalin induced paw edema model

Mice were divided into five groups (n=6/group).

Group I: Formalin alone treated (Control)
Group II: Formalin+γ-hum (1mg/kg b.wt.)
Group III: Formalin+γ-hum (2.5mg/kg b.wt.)
Group IV: Formalin+γ-hum (5mg/kg b.wt.)
Group V: Formalin+diclofenac (10mg/kg b.wt.)

One hour, after the last dose of γ-hum or diclofenac administration, chronic inflammation was induced by sub-plantar injection of freshly prepared 2%

formalin in sterile water on the hind paw of all mice (Chang and lewis, 1989). The paw thickness was measured using vernier calipers before and after formalin injection and was continued up to fifteen days.

In all the above three models the percentage inhibition of paw thickness was calculated using the formula;

% inhibition of paw thickness =

$$\frac{(tCn - tC0) - (tTn - tT0)}{(tCn - tC0)}$$

Where, tCn = paw thickness at particular time point of control animal; tC0= paw thickness before induction of control animal; tTn = paw thickness at particular time point of treated animal; and Tt0 = paw thickness before induction of treated animal.

4.2.5. Determination of the effect of γ-hum on proinflammatory cytokines, CRP and NO

Mice were divided into three groups (n= 6/group). To elicit macrophages 0.1 ml of 5% sodium caesinate was injected intraperitoneally to all the animals. Group I was kept as normal control; Group II were treated with single dose of Lipopolysaccharide (LPS) (250μg/animal) intraperitoneally and group III animals received single dose of LPS two hours after the last dose of γ-hum (5mg/kg b.wt) treatment. After 6 hours, all the animals were sacrificed. Blood was collected by heart puncture, serum was separated and used for the estimation of inflammatory mediators like interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α) and C-reactive protein (CRP) using ELISA kits according to manufacturer's instructions. The estimation of nitric oxide was done by Griess assay (Green et al., 1982). For this an equal volume of griess reagent was added to the serum collected, incubated at room temperature for 10 minutes and optical density was measured at 540nm.

4.2.6. Determination of the effect of γ-hum on TNF-α Production by macrophages

Macrophages were collected from the peritoneal cavity of all the above three (section 4.2.5.) groups of animals after injecting 5 ml of phosphate buffered saline (PBS) followed by aspiration. Macrophages (2×10^5) were plated on to 96-well titer plates and incubated for 2h at 37^0C in RPMI medium. Non-adherent macrophages were removed after incubation and fresh medium was added and again incubated for 24h at 37^0C in CO_2 atmosphere. The plates were centrifuged after incubation and the medium (100µl) from each well was added to L929 cells (5×10^3cells/well). Plates were incubated for 48 hours and the cells were evaluated morphologically by fixing and staining with crystal violet and the cell density was assessed by MTT assay.

4.2.7. Determination of the effect of γ-hum on iNOS, COX-2 gene Expression in LPS-activated Macrophages

Macrophages were elicited in mice by injecting 5% sodium caesinate intraperitoneally. Two hours after the last dose of γ-hum administration mice were treated with single dose of LPS (250µg/animal). After six hour all the animals were sacrificed macrophages were collected and cDNA was synthesized. cDNA was then amplified using specific mouse primers of inducible nitric oxide synthase (iNOS); Cycloxygenase-2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was selected as a stable reference gene under the experimental conditions in this study.

4.3. Results

4.3.1. Toxicological Evaluation

The toxicity study (table 4.1) revealed the no observed-adverse-effect level of γ-hum as 15mg/kg body weight. The doses of 5, 10, and 15mg/kg body weight, administered for 14 days, did not produce any mortality, change in behavior,

Table 4.1. Toxicity profile

	Concentrations of γ-hum (mg/kg body weight)				
	Control	5	10	15	20
Mortality (D/T)	None	None	None	None	None
Behavioural change	None	None	None	None	None
Change in body weight (g)	+2.13±1.3	+2.12±0.6	+2.06±0.4	+2.01±0.2	+1.92±1.5
Relative organ weights (g/100g body weight)					
1.Liver	5.32±0.32	5.38±0.16	5.23±0.18	5.19±0.18	4.97±0.15
2.Spleen	0.38±0.03	0.41±0.02	0.36±0.02	0.39±0.02	0.40±0.02
3.Thymus	0.11±0.01	0.11±0.01	0.10±0.01	0.12±0.01	0.11±0.01
4.Kidney	1.36±0.24	1.34±0.13	1.34±0.16	1.34±0.15	1.31±0.12
5.Lungs	0.60±0.03	0.61±0.02	0.60±0.03	0.61±0.02	0.57±0.03
Serum ALP (U/ml)	13.5±0.40	13.4±0.42	14.21±0.8	15.8± 1.08	14.5±2.01
Serum GPT (U/ml)	58.35±3.7	55.77±3.0	59.97±4.6	64.17±7.0	63.91±6.1
Blood urea (mg/dl)	43.38±1.5	42.81±1.6	44.31±0.8	43.55±2.7	43.22±1.0
Serum creatinine (mg/dl)	0.91±0.01	0.9±0.02	0.93±0.02	0.99±0.05	0.98±0.13

Abbreviations: D/T, dead/treated mice; ALP, alkaline phosphatase; GPT, glutamate pyruvate transaminase.

Values are the mean ± standard deviation. All the treated animals were carefully examined for 14 days for any signs of toxicity (behavioural changes and mortality).

"None" means that no toxic symptoms were seen during the observation period.

body weight, relative organ weight, and hepatic and renal functions when compared with untreated animals. Administration of γ-hum at 20mg/kg body weight produced slight weight loss. Based on these results and some preliminary screening on biological activity, we selected the nontoxic lowest dose of 5mg/kg b.wt. for further studies (Gilcy and kuttan, 2016).

4.3.2. Effect of γ-hum on carrageenan induced paw edema

In mice carrageenan administration produced an increase in paw thickness. This increase was very high in the control group of animals compared to γ-hum and diclofenac treated group of animals. At the third hour this increase was reduced ($p<0.001$) by about 61% in γ-hum 5mg/kg b.wt. treated group. There was a reduction of about 47% and 37% in γ-hum 2.5mg/kg b.wt. and γ-hum 1mg/kg b.wt. treated group of animals respectively. Administration of the standard drug diclofenac produced 53% inhibition in the paw thickness at the same time point (figure 4.1).

4.3.3. Effect of γ-hum on dextran induced paw edema

Dextran administration also produced an increase in paw thickness. In the dextran alone treated control group the increase was high compared to γ-hum and diclofenac treated group of animals. At the third hour this increase was reduced ($p<0.001$) by about 60% in γ-hum 5mg/kg b.wt. treated group. There was a reduction of about 45% and 31% in γ-hum 2.5mg/kg b.wt. and γ-hum 1mg/kg b.wt. treated group of animals respectively. The standard drug diclofenac produced 52% inhibition in the paw thickness (figure 4.2).

4.3.4. Effect of γ-hum on formalin induced paw edema

Formalin induced chronic inflammation produced an increase in paw thickness in control group. When γ-hum was administered at concentrations 5mg/kg b.wt., 2.5mg/kg b.wt. and 1mg/kg b.wt., there was a reduction in the paw thickness ($p<0.001$) in these three groups 58%, 47% and 39% respectively. These results

Figure 4.1. Effect of γ-hum on carrageenan induced paw oedema formation

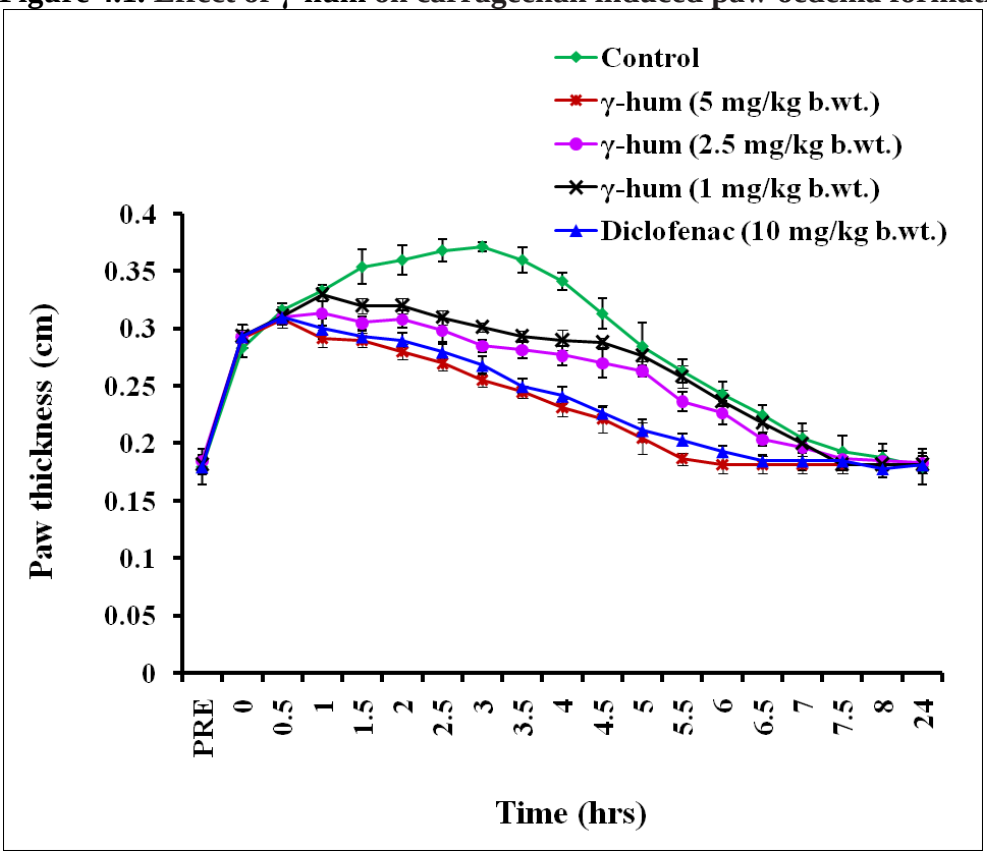
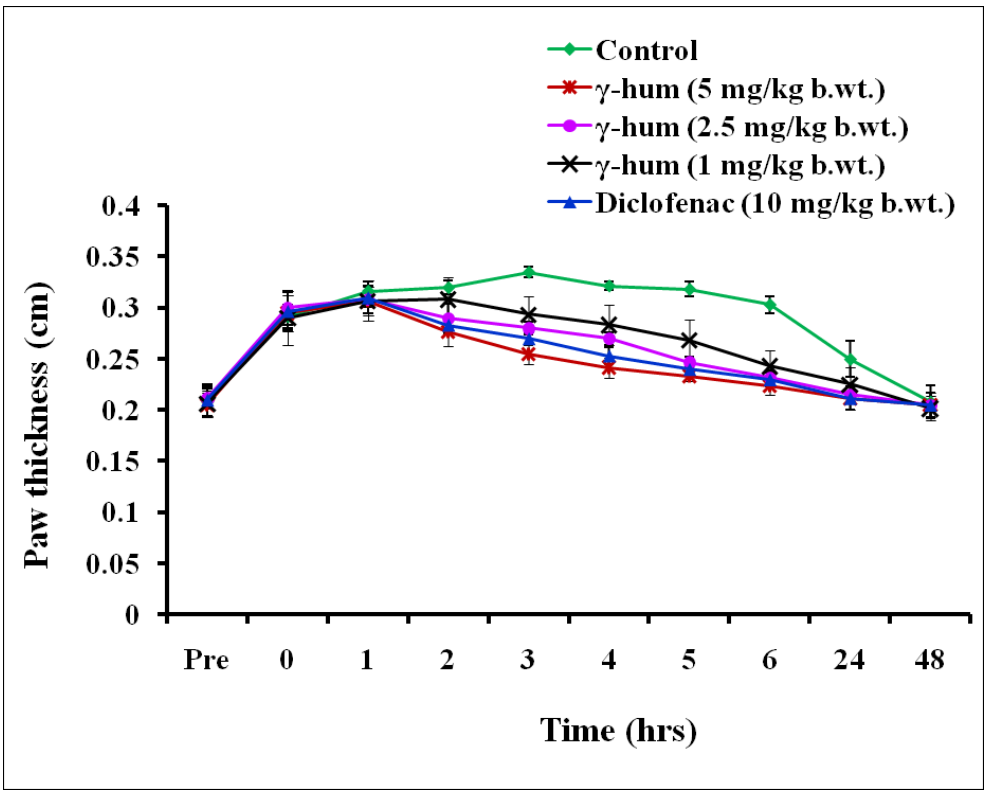


Figure 4.2. Effect of γ-hum on dextran induced paw oedema formation



were comparable to the results obtained by the standard drug diclofenac (55%) (figure 4.3).

4.3.5. Effect of γ-hum on proinflammatory cytokines, CRP and NO

There was a significant increase in the level of proinflammatory cytokines TNF-α (547.95±35pg/ml), IL-1β (119.51±12pg/ml), IL-6 (356.38±39pg/ml) in the sera of LPS induced animals compared to normal animals without any treatment. This increase was found to be significantly $p<0.001$ inhibited by the administration of LPS+γ-hum (5mg/kg b.wt.) the values are TNF-α (149.18±21pg/ml), IL-1β (59.31±7.8pg/ml), IL-6 (125.41±13pg/ml) (Figure 4.4). The serum C- reactive protein (CRP) and Nitric oxide (NO) level after treatment with γ-hum (5mg/kg b.wt.) in LPS stimulated animals was significantly reduced ($p<0.001$) in comparison with LPS alone treated group (table 4.2).

4.3.6. Effect of γ-hum on TNF-α production by macrophages

Peritoneal macrophages were elicited by injecting 200µl of 5% sodium caesinate solution. On the day of sacrifice 5ml of PBS or HBSS was injected into the peritoneal cavity of the mice. The peritoneal fluid containing macrophages was aspirated and the cells were washed and suspended in the culture medium at desired cell concentrations. Bio assay for TNF-α level was done using L929 cell line because this cell line is TNF sensitive and the inflammatory cytokine TNF-α can produce direct cytotoxicity to these cells. When normal macrophage culture supernatant without LPS induction was added to L929 cells it will not produce any cytotoxicity. But culture supernatant of macrophages with LPS induction was added to L929 cells it produced 100% toxicity. When the cuture supernatant of macrophages collected from mice with γ-hum (5mg/kg b.wt.) + LPS induction it produced only 14% cytotoxicity with almost normal cell morphology (figure 4.5) indicating the inhibition of TNF-α production by γ-hum treatment.

Figure 4.3. Effect of γ-hum on formalin induced paw oedema formation

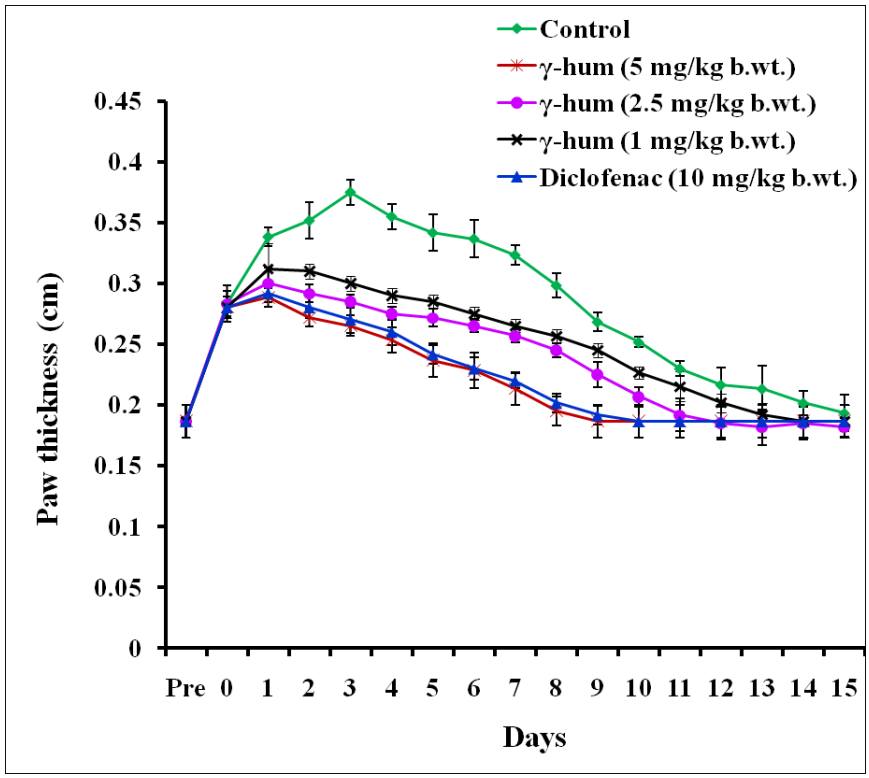
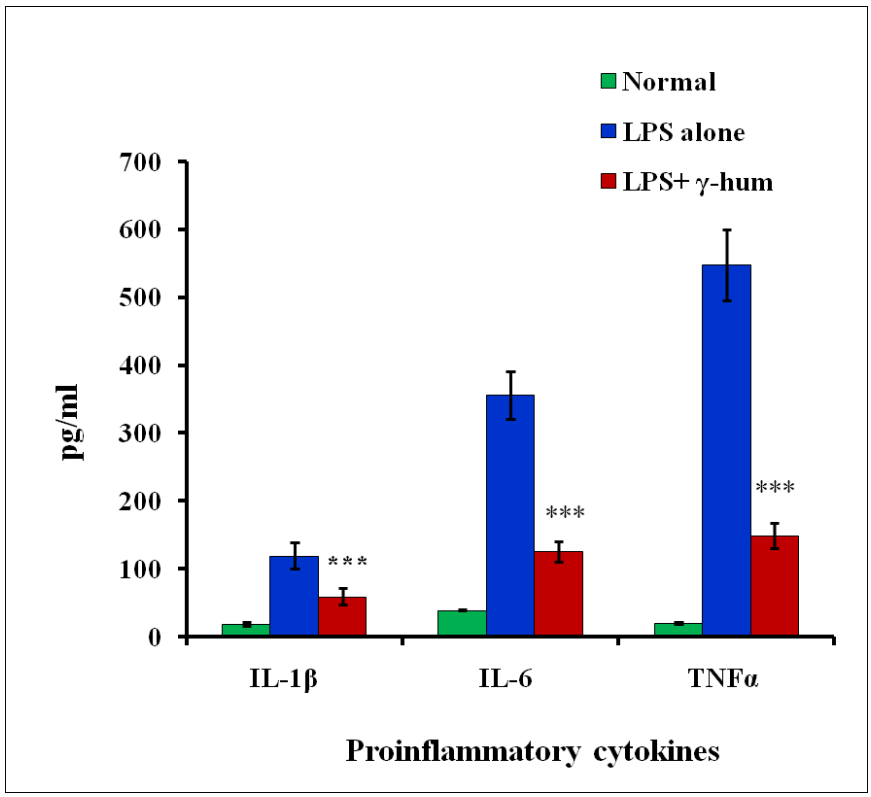


Figure 4.4. Effect of γ-hum on the proinflammatory cytokine levels in LPS induced animals



***p < 0.001

Table 4.2. Effect of γ-hum on LPS induced serum CRP and NO level

Treatment	CRP (µg/ml)	NO (µmol)
Normal	547±21	20.05±1.41
LPS alone	4388±192	77.46±9.98
LPS+ γ-hum (5mg/kg b.wt.)	1383±182***	32.33±4.44***

Values are mean ± SD. ***p< 0.001 compared with LPS alone control

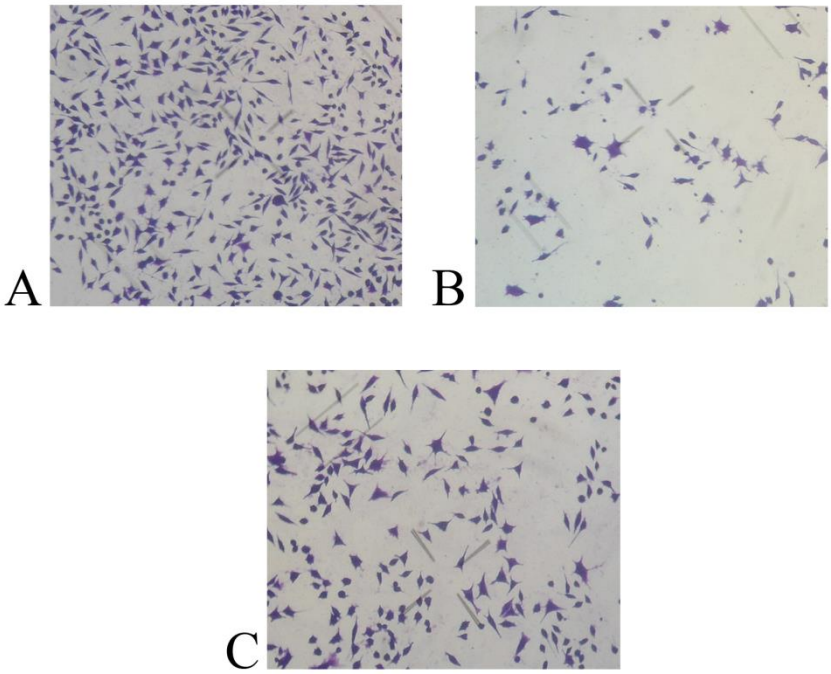
4.3.7. Effect of γ-hum on iNOS, COX-2 gene Expression in LPS-activated Macrophages

According to the gene expression analysis in unstimulated macrophages neither iNOS nor COX-2 expression were detectable. In response to LPS stimulation the level of iNOS and COX-2 were increased markedly. Treatment with γ-hum (5mg/kg b.wt.) reduced both iNOS and COX-2 expression in LPS stimulated macrophages. The inhibition in the expression of iNOS and COX-2 was evident from the band intensity (figure 4.6).

4.4. Discussion

In the present study, anti-inflammatory effect of γ-hum was assessed by two acute inflammatory models of paw edema induced by carrageenan and dextran and one chronic inflammatory model induced by formalin. The mucopolysaccharide carrageenan is used for liberating inflammatory and proinflammatory mediators like prostaglandins, leukotrienes, histamine, bradykinin and TNF-α. The biphasic carrageenan induced inflammation consists of two phases, the first phase starts with the release of histamine, serotonin, and kinins (Bhukya et al., 2009) and the second phase is related to the release of prostaglandin like substances (Brooks and Day, 1991). Histamine and serotonin released by mast cells were considered to be responsible for dextran induced inflammation. Vasodilation, increased permeability and an increase of blood flow ultimately leading to an increase in paw size is the result of the marked vascular changes induced by these inflammatory mediators (Igbe et al., 2010). From the results it was evident that γ-hum is able to reduce the acute inflammation induced by these two agents and the results were comparable to the standard drug diclofenac. The chronic inflammation induced by formalin closely resembles human arthritis. The effect of formalin is also biphasic the second phase of reactions is the result of chemical insult which results in tissue damage and the tissue mediated response is with the involvement of histamine, prostaglandins and bradykinin (Wheeler and Cowan, 1991).

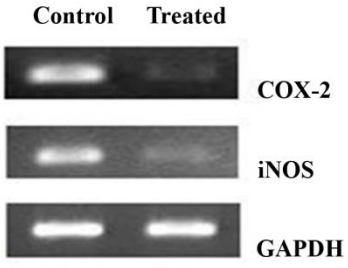
Figure 4.5. Effect of γ-hum on the production of TNF-α by the macrophages in LPS induced animals



Bioassay of L929 cells

A. L929 cells treated with normal macrophage culture supernatant
B. L929 cells treated with LPS alone treated macrophage culture supernatant
C. L929 cells treated with LPS+γ-hum treated macrophage culture supernatant

Figure 4.6. Effect of γ-hum on COX-2 and iNOS gene expression in LPS stimulated macrophages



LPS is a component of the bacterial cell wall, which stimulates the inflammatory response by activating the production of inflammatory mediators such as TNF-α, IL-1β, IL-6, CRP and NO. The proinflammatory cytokines TNF-α, IL-1β and IL-6 recruit additional immune cells to sites of infection or tissue injury (Bosca et al., 2005). In the present study, administration of γ-hum could inhibit significantly the LPS induced production of proinflammatory cytokines, CRP and NO. This may be due to the regulatory effect of γ-hum on the immune cell response in a state of inflammation. Macrophages are known to play a major role in the innate immune defence system and represent one of the main cellular sources of COX-2 expression upon exposure to different stimuli. The Cycloxygenase-2 (COX-2) expression is induced by a wide range of stimuli including LPS, pro-inflammatory cytokines such as IL-1β and TNF-α and growth factors such as epidermal growth factor (Steele et al., 2003). COX-2 is responsible for the increased production of prostaglandins, the key mediators or the main culprit responsible for inflammation (Firdous et al., 2015).

The release of NO by activated macrophages is an important cytotoxic/cytostatic mechanism of nonspecific immunity. In macrophages, NO is generated from l-arginine and molecular oxygen by iNOS. Induction of iNOS activity and the subsequent NO synthesis in macrophages may be caused or modulated by activators of these cells, including such bacterial products as LPS, muramyl dipeptide and especially some cytokines such as IFN-γ, TNF-α, and IL-1β. Activation of the iNOS results in the production of reactive nitrogen species like nitric oxide that damage DNA and cell membranes (Ohshima and Bartsch, 1994). γ-hum inhibits the gene expression of iNOS and COX-2 in LPS stimulated macrophages.

For the proper functioning of the immune system there should be a balance between the proinflammatory and anti inflammatory mediators. The regulatory effect of γ-hum involves the apt moulding of the body defences with the proper level of expression of the participating genes and the resulting concentration of its products. The anti-inflammatory properties of α humulene and transcaryophyllene that share close similarity with γ humulene has already been reported (Fernandes et al., 2007). γ-humulene is a major phytochemical

ingredient of some of the non drug anti-inflammatory products available today like CannaFX®21™, MedFX®Alpha™ (Norwood, MA,USA). The results obtained in the present study strongly indicate the anti-inflammatory effect of γ-hum obtained from *E. sonchifolia,* the plant with proven anti-inflammatory effects.

Chapter 5

Emilia sonchifolia (L.) DC: Evaluation of the antiangiogenic effects

5.1. Introduction

The requirement of tumour cells for the supply of oxygen and other nutrients is fulfilled by the formation of new blood vessels. The induction of neovessel formation starts with the release of various molecules by the tumour cells. These molecules will send signals to the surrounding normal tissue and activate genes for making proteins involved in the process of angiogenesis (Yadav et al., 2015). Tumour cell dissemination in the process of metastasis requires the development of angiogenic blood vessels. Neovasculature should possess certain structural characteristics to sustain active intravasation of tumour cells and their dissemination to secondary sites (Deryugina and Quigley, 2015). The relatively delicate process of neovessel formation can be a target for antiangiogenic therapy in cancer treatment because of the genomic stability of endothelial cells compared to cancer cells. Cancer cells require less generation time and have more genetic instability, whereas endothelial cells require long generation time and have higher genomic stability. The rapidly proliferating cancer cells often undergo mutations and would likely acquire drug resistance (Bagri et al., 2010).

Neovessel formation is initiated by the secretion of various growth factors, especially vascular endothelial growth factor (VEGF), which can pursue capillary growth into the tumour. VEGF is a powerful inducer of angiogenesis, stimulates growth and proliferation of endothelial cells, acts as a survival factor of endothelial cells, prevents their apoptosis, and also regulates vascular permeability. The degradation of ECM components surrounding the tumour tissue is mainly brought about by the gelatinases MMP-2 and MMP-9 (Deryugina and Quigley, 2015). The family of natural inhibitors of metalloproteinase are TIMPs (tissue inhibitors of metalloproteinases) and TIMP expression is related to tumour metastasis and angiogenesis (Gong et al., 2014). Interactions between malignant and inflammatory cells present in the stroma could be closely associated with angiogenesis and tumour progression (Lin and Karin, 2007). Traditionally used natural compounds and herbal extracts have been investigated as antiangiogenic agents to prevent neovascularization of developing tumours. These include some

of the works on *Andrographis paniculata* (Sheeja et al., 2007), (+)-catechin (Guruvayoorappan and Kuttan, 2008) vernolide-A (Pratheeshkumar and Kuttan, 2011) and ursolic acid (Kanjoormana and Kuttan,2010). The present study was designed to explore the inhibitory effect of the active fraction from *E sonchifolia* (γ-hum) on tumour-specific neovessel formation, using both *in vivo* and *in vitro* models (Gilcy and kuttan, 2016).

5.2. Materials and Methods

Plant material: - An active fraction from *E. sonchifolia*, enriched with the major sesqueterpene γ-humulene (71%) (γ-hum).

Dosage: - γ-hum 5 mg/kg body weight was intraperitoneally (i.p) administered for five consecutive days.

Animals: - C57BL/6 mice (4-6 weeks old)

Cell Lines: - Human umbilical vein endothelial cells (HUVECs) and B16F10 melanoma cells

5.2.1. Experimental design

Three groups of C57BL/6 mice (n=8/group) were used for the study. Angiogenesis was induced in all groups of animals by injecting B16F10 melanoma (10^6 cells/animal) intradermally on the shaven ventral skin of each mouse.

Group I animals were kept as control. Group II and group III animals were treated with 5 consecutive doses of γ-hum (5mg/kg body weight) and TNP-470 (30 mg/kg body weight), respectively, starting simultaneously with tumour challenge. For *in vitro* experiments three nontoxic concentrations of γ-hum (2.5, 1, and 0.5 μg/ml) were resuspended in 0.1% dimethyl sulfoxide. Dimethyl sulfoxide alone

when used as vehicle in 0.1% and lesser concentrations showed no inhibitory effect on neovessel formation in the experimental conditions.

5.2.2. Determination of the effect of γ-hum on tumour specific capillary formation

The angiogenesis induced animals were sacrificed on the ninth day after tumour challenge. The skin from the ventral side was dissected out, washed with phosphate-buffered saline, and the number of tumour-directed capillaries were counted using a dissection microscope at 20× magnification.

5.2.3. Determination of serum IL-1β, IL-6, TNF-α, GM-CSF, VEGF, and TIMP-1 levels

Blood was collected from the caudal vein of all groups of angiogenesis induced animals at 2 time intervals days 2 and 9 after tumour induction. Serum was separated and used for the estimation of IL-1β, IL-6, TNF-α, GM-CSF, VEGF, and TIMP-1 levels using ELISA kits according to the manufacturer's instructions, and the readings were taken using an ELISA plate reader.

5.2.4. Cell viability by MTT Assay

The viability of cultured cells was determined by assaying for the reduction of MTT to formazan. HUVECs were seeded (5000 cells/well) in a 96-well culture plate and incubated for 24 hours at 37^0C in 5% CO_2 atmosphere. Different concentrations of γ-hum (0.5-100μg/ml) were added and incubated further for 48 hours. Four hours before the completion of incubation, 20μl of MTT (5mg/ml) was added. The percentage of viable cells was determined using an ELISA plate reader.

5.2.5. ^{3}H-Thymidine incorporation assay

HUVECs (5000 cells/well) were seeded on a 96-well culture plate and incubated at 37^{0}C in 5% CO_2 atmosphere. After 24 hours, various concentrations of γ-hum (2.5, 1, and 0.5μg/ml) were added and further incubated for 48 hours. ^{3}H-thymidine was added to each well (1μCi/well) and incubation was continued for an additional 18 hours. After completing incubation DNA was precipitated using 10% ice-cold perchloric acid, and pellets were dissolved in 0.5 ml of 6 N NaOH and transferred to 5 ml scintillation fluid. Radioactivity was measured using Rack Beta fluid scintillation counter.

5.2.6. Rat aortic ring assay

The rat aortic ring assay is used as an *in vitro* angiogenesis experimental model. Dorsal aorta from a freshly sacrificed rat was taken out in a sterile manner and rinsed in ice-cold phosphate-buffered saline. It was then cut into approximately 1mm thick sections, and each ring was placed in a collagen precoated 96 well plate. The rings were incubated for 24 hours at 37^{0}C in complete medium and then replaced with conditioned medium from B16F10 melanoma cells and incubated with different concentrations of γ-hum (2.5, 1, and 0.5μg/ml). Controls were kept without γ-hum treatment. The rings were analyzed by phase-contrast microscopy for microvessel outgrowth on the sixth day and the sections were photographed.

5.2.7. Determination of the effect of γ-hum on endothelial cell migration

HUVECs were seeded on a collagen-precoated 96-well plate at a density of 2×10^{5}cells/well and incubated for 24 hours at 37^{0}Cin 5% CO_2 atmosphere. The monolayers of cells were scraped with a narrow tip by applying suction. The scraped out cells were removed by washing with serum free medium. Different concentrations of γ-hum (2.5, 1, and 0.5μg/ml) were added along with 2ng/ml VEGF and further incubated for 24 hours. After incubation the cells were fixed in formalin and stained with crystal violet and photographed (Guo et al., 2002).

5.2.8. Determination of the effect of γ-hum on endothelial cell invasion

The invasion assay was carried out in modified Boyden chambers as described by Albini et al.,1987. The lower compartment of the chamber was filled with serum-free medium and a polycarbonate filter membrane coated with 25μg type I collagen was placed above this. HUVECs (10^5cells/150μl medium 199) were then seeded on to the upper chamber in the presence and absence of γ-hum (2.5, 1, and 0.5μg/ml) along with 2ng/ml VEGF and FGF and incubated at 37^0Cin 5% CO_2 atmosphere for 10 hours. After incubation, the membranes were removed, fixed with methanol, and stained with crystal violet. Cells migrating to the lower surface of the polycarbonate membrane were counted under a microscope. The results were expressed as percentage inhibition of invasion.

5.2.9. Gelatin zymography

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed by incorporating 0.1% gelatin in the separating gel (Billings et al.,1991). Subconfluent HUVECs were incubated with serum-free medium for 24 hours at 37^0Cin 5% CO_2 atmosphere. The conditioned medium was then collected and subjected to zymographic analysis. Samples equivalent to 100μg protein were activated with 5μl trypsin solution (75μg/ml) in the presence and absence of γ-hum (2.5, 1, and 0.5μg/mL) in 0.1 M Tris-HCl, 10 mM $CaCl_2$ buffer (pH 8.0), and incubated for 1 hour at room temperature. Samples were mixed with an equal volume of 2× sample buffer and loaded on to 11% polyacrylamide gels containing 0.1% gelatin. Electrophoresis was carried out at 4°C with a constant current of 2 mA/tube until the tracking dye reached the periphery. The gels were then washed with 2% Triton X-100 in 0.1 M Tris-HCl, 10 mM $CaCl_2$ at 37^0Cfor 18 hours followed by staining with Gelcode Blue stain reagent for 2 hours. The clear areas on the gels were then visualized.

5.3. Results

5.3.1. Effect of γ-hum on tumour specific capillary formation

The effect of γ-hum on tumour directed capillary formation is shown in figure 5.1. γ-hum treatment produced significant (P>0.001) inhibition (57%) of tumour-directed capillary formation along with a noticeable reduction in tumour size. The result obtained as shown in the figure was comparable to the well known antiangiogenic compound TNP-470 used as reference in this study.

5.3.2. Effect of γ-hum on the level of proinflammatory cytokines, VEGF, and TIMP

γ-hum treatment significantly inhibited the elevated levels of serum IL-1β, IL-6, TNF-α, and GM-CSF, as shown in figure 5.2. The elevated level of angiogenesis promoting VEGF in tumour bearing animals was also lowered significantly by γ-hum treatment compared to control animals. At the same time the level of tissue inhibitor of MMPs was increased by the treatment (figure 5.3).

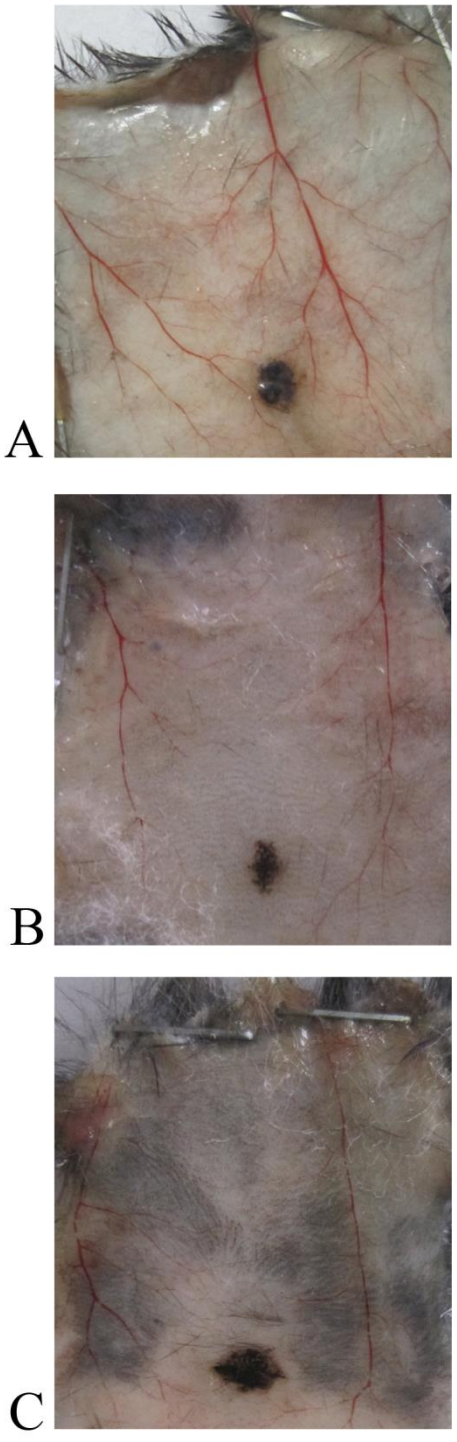
5.3.3. Effect of γ-hum on cell viability

The percentage viability of HUVECs after treatment with γ-hum is shown in Figure 5.4. The concentration needed for 50% growth inhibition (IC50) of HUVECs was found to be 80.8μg/ml. The cells were 100% alive at 0.5, 1, and 2.5 μg/ml concentrations of γ-hum, and these concentrations were used for further *in vitro* experiments.

5.3.4. Effect of γ-hum on endothelial cell proliferation

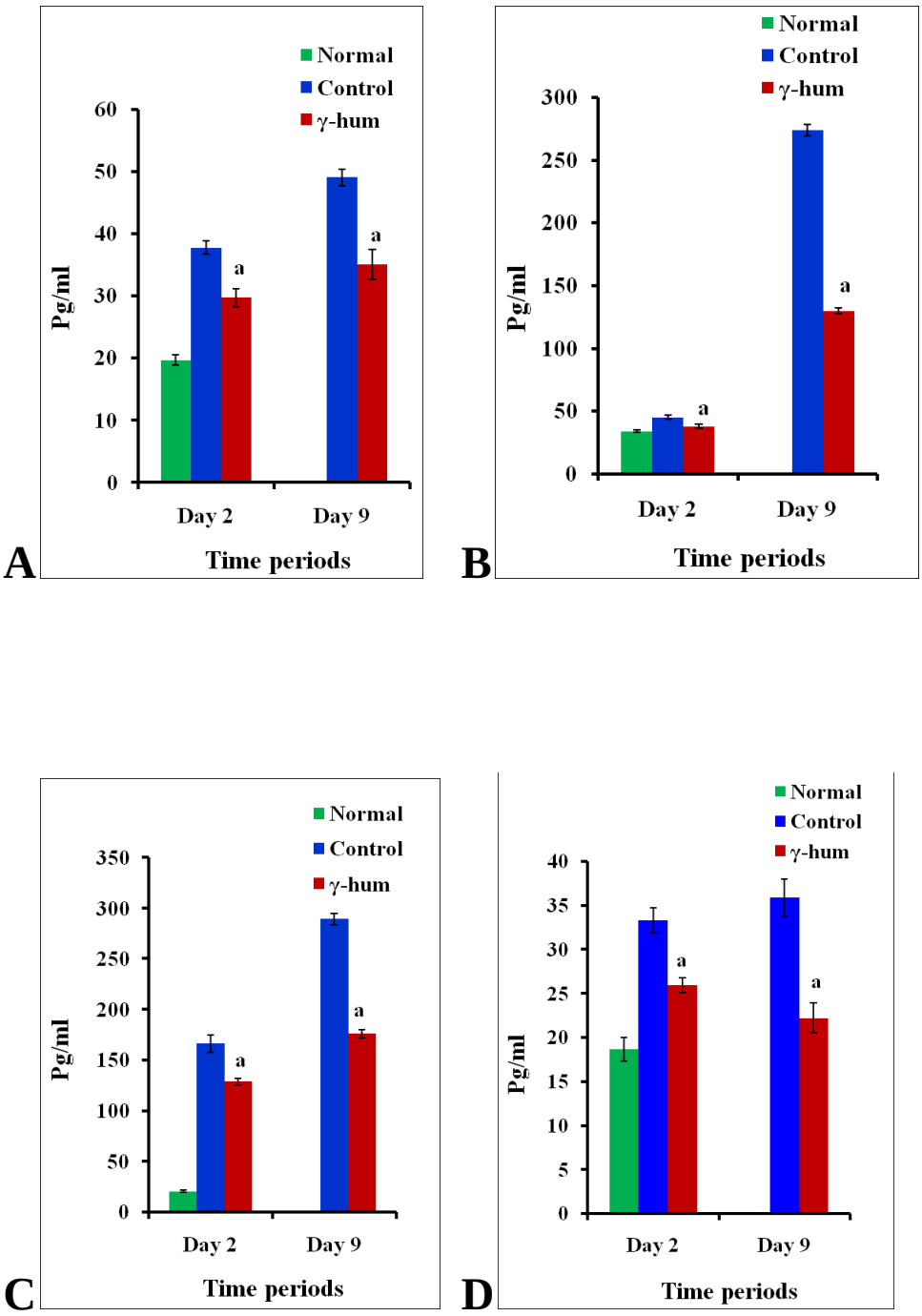
Rate of proliferation of HUVECs was determined by ^{3}H-thymidine incorporation into the cellular DNA. Proliferation was expressed as radioactive counts per minute (cpm). HUVECs showed very high rates of proliferation (4313±110cpm) when

Figure 5.1. Effect of γ-hum on *in vivo* angiogenesis



A. Control without any treatment
B. γ-hum treated
C. TNP-470 treated

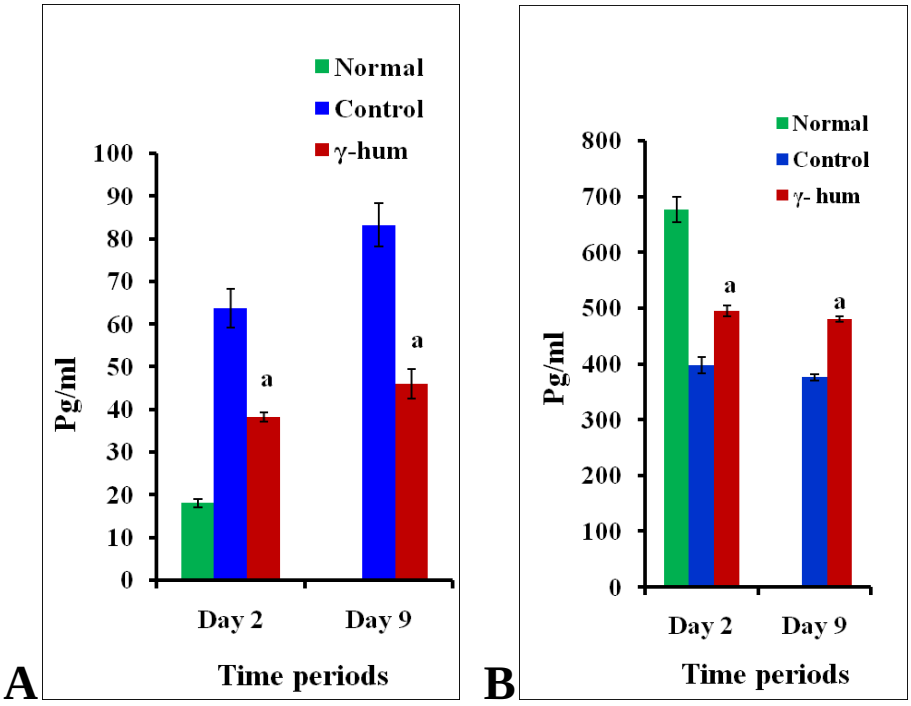
Figure 5.2. Effect of γ-hum on serum cytokine levels



A. IL-Iβ; B. IL6; C. TNF-α; D. GMCSF

$^{a}p < 0.001$

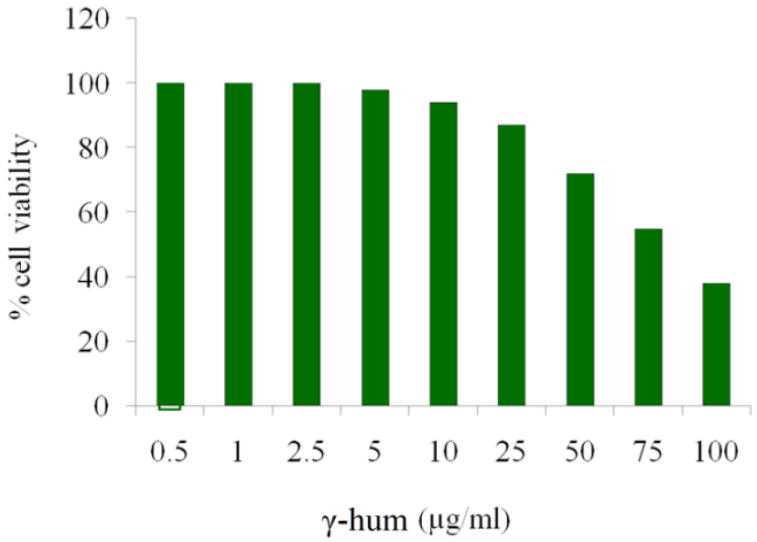
Figure 5.3. Effect of γ-hum on serum VEGF and TIMP levels



A. VEGF; B. TIMP

[a]p < 0.001

Figure 5.4. MTT assay showing viability of HUVECs following treatment with the indicated concentrations of γ-hum



stimulated with VEGF. Administration of γ-hum at a concentration of 2.5μg/ml showed a significant inhibition (55%, 1940±40cpm) of VEGF induced proliferation of HUVECs. Considerable inhibition of proliferation was also observed when γ-hum was administered at concentrations of 1μg/ml (48%, 2236±95 cpm) and 0.5 μg/ml (22%, 3349±105cpm).

5.3.5. Effect of γ-hum on the microvessel outgrowth from the rat aortic ring

The microvessel outgrowth from the rat aortic ring was induced by treatment with conditioned medium from B16F10 melanoma cells. Treatment with different concentrations of γ-hum significantly inhibited the microvessel outgrowth in a dose-dependent manner compared to the control group without any treatment (figure 5.5).

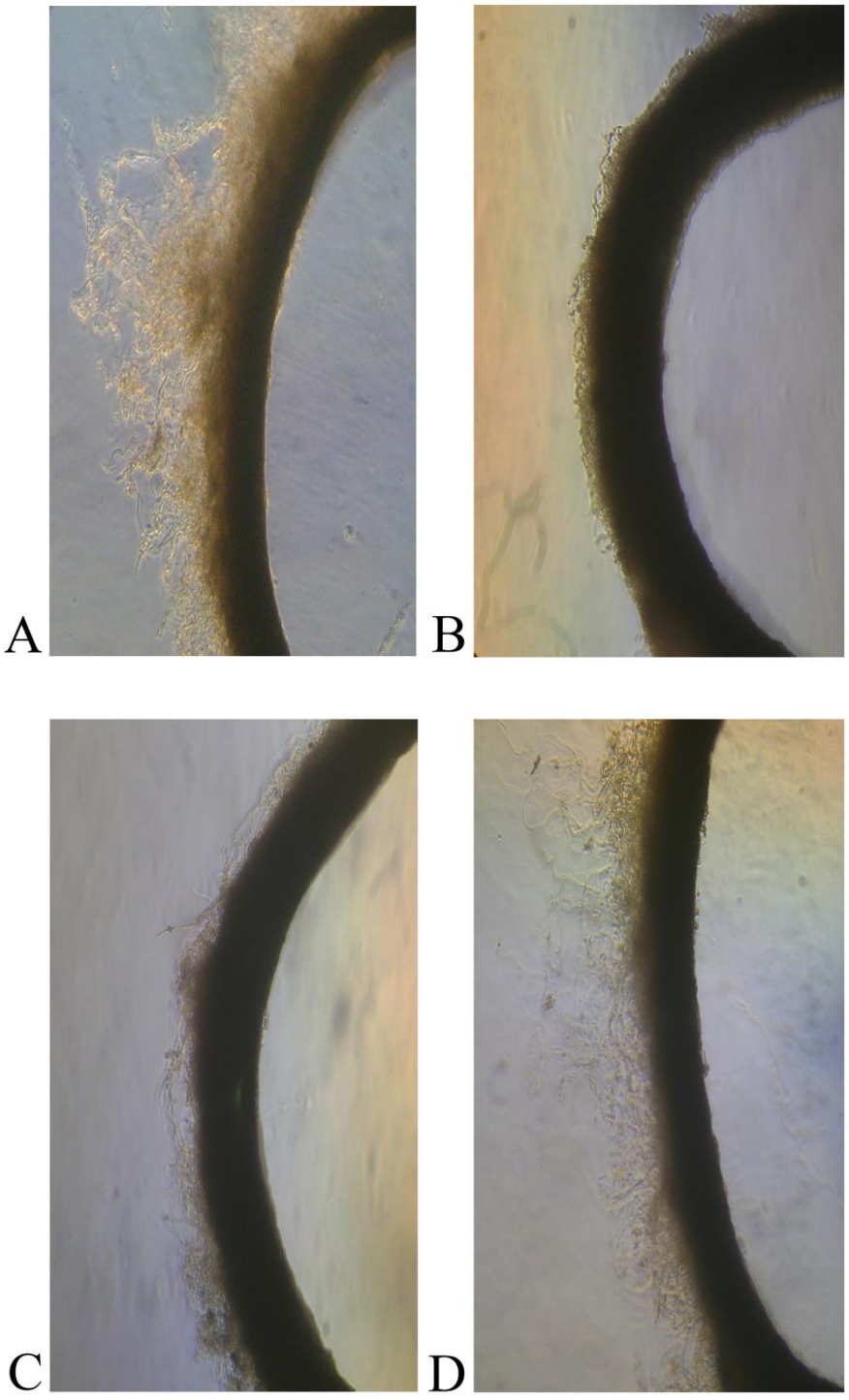
5.3.6. Effect of γ-hum on endothelial cell migration

Effect of γ-hum on the migration of HUVECs is shown in Figure 5.6. HUVECs migrated into the scraped clear area when stimulated with VEGF as shown in the control without any treatment. γ-hum significantly inhibited the VEGF-induced migration of endothelial cells in a dose-dependent manner and maximum inhibition of endothelial cell migration was observed at 2.5μg/ml.

5.3.7. Effect of γ-hum on endothelial cell invasion assay

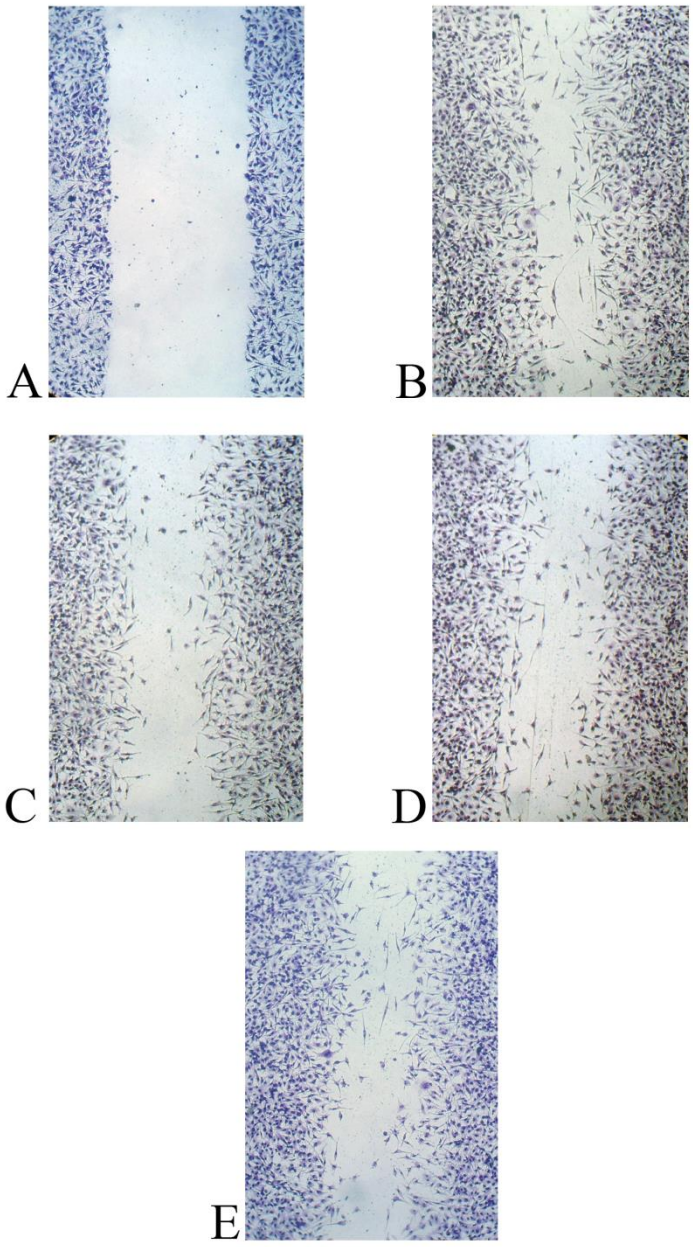
The invasive property of HUVECs through the collagen matrix is very high when stimulated with VEGF and FGF. The administration of γ-hum produced significant inhibition in the invasion of cells in a dose-dependent manner. The number of cells found on the lower surface of the polycarbonate membrane at a concentration of 2.5μg/ml γ-hum was very low. The treatment of HUVECs with higher concentration of γ-hum produced an inhibition of invasion by 61%, whereas at 1μg/ml and 0.5μg/ml, the inhibition of invasion was found to be 52% and 39%, respectively (figure 5.7).

Figure 5.5. Effect of γ-hum on *in vitro* angiogenesis



A. Control with conditioned medium alone
B. Conditioned medium + γ-hum (2.5 microgram/ml)
C. Conditioned medium + γ-hum (1 microgram/ml)
D. Conditioned medium + γ-hum (0.5 microgram/ml)

Figure 5.6. Effect of γ-hum on HUVECs migration



A. Control "0" hour incubation
B. Control after 24-hour incubation in medium without γ-hum
C. γ-hum (2.5 microgram/ml)
D. γ-hum (1 microgram/ml)
E. γ-hum (0.5 microgram/ml)

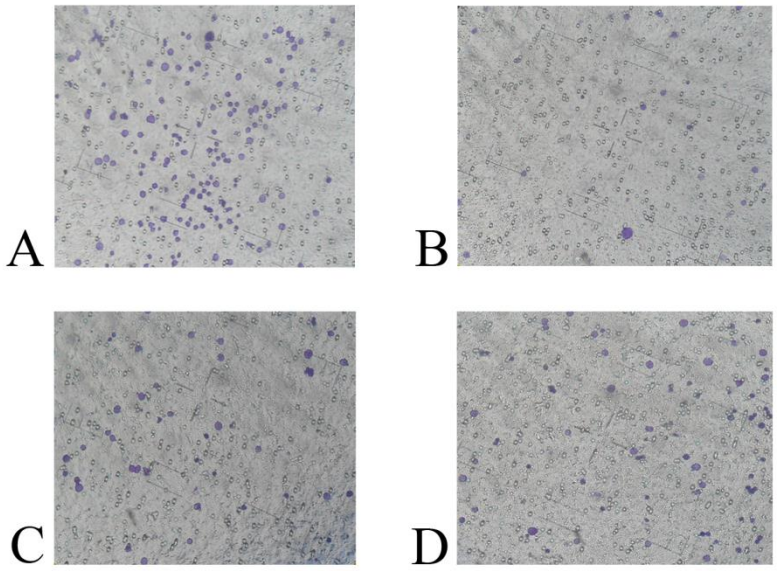
5.3.8. Gelatin Zymography

MMP-2 and MMP-9 secreted by the cells into the conditioned medium are mainly proenzymes that require activation. Conditioned medium after trypsin activation showed digested clear areas at 92 kD and 72 kD, which were identical to MMP-9 and MMP-2 activity. The inactive form of the enzyme did not show clear degradative areas when the gels were loaded by conditioned medium without trypsin activation. EDTA inhibits the proteolytic activity by chelating Ca^{+}, and the enzyme responsible for degradation is metalloproteinase, which is evidenced when gels loaded with trypsin-activated conditioned medium were incubated with 10 mM EDTA, which did not produced clear degradative areas. When conditioned medium was treated with γ-hum at concentrations 2.5 and 1 μg/ml during trypsin activation, it did not show any clear bands suggesting that γ-hum inhibited the activation of proenzyme to active enzyme of metalloproteinases. But γ-hum at 0.5 μg/ml showed an activation of metalloproteinases as shown in figure 5.8.

5.4. Discussion

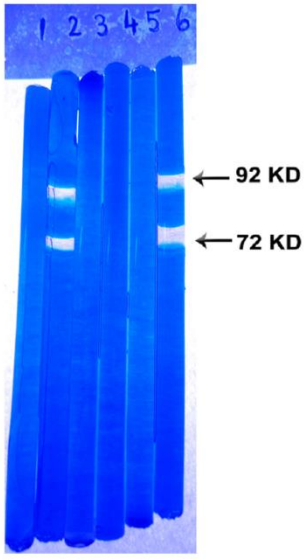
A large number of the sesquiterpenes obtained from medicinal plants that are used in traditional medicine show anticancer activity by inhibition of inflammatory responses, prevention of metastasis, and angiogenesis. In this study, an active fraction containing the major sesquiterpene γ-humulene from the plant *E sonchifolia*, with renowned anti-inflammatory and antitumour effects, showed significant reduction in tumour-directed capillary formation, with a simultaneous inhibition in the proliferation, migration, and invasion of endothelial cells, the crucial processes of neovessel formation. The inhibition of capillary formation toward the developing tumour by γ-hum treatment revealed its antiangiogenic effect in an *in vivo* experimental condition. γ-hum significantly halted the endothelial cell proliferation, invasion, and migration, further proving its targeted activity toward the cells involved in neovascularization. The *in vitro* experiment of microvessel outgrowth from the aortic ring is strong direct evidence for the

Figure 5.7. Inhibitory effect of γ-hum on HUVECs invasion through collagen matrix



A. Untreated control
B. Treatment with γ-hum (2.5 microgram/ml)
C. Treatment with γ-hum (1 microgram/ml)
D. Treatment with γ-hum (0.5 microgram/ml)

Figure 5.8. Effect of γ-hum on MMP-2 and MMP-9 production by HUVECs



1. Condition medium from untreated HUVECs without trypsin activation
2. Condition medium from untreated HUVECs after trypsin activation
3. Condition medium from untreated HUVECs after trypsin activation+EDTA
4. Condition medium from pretreated HUVECs (2.5microgram/ml γ-hum)after trypsin activation
5. Condition medium from pretreated HUVECs (1microgram/ml γ-hum)after trypsin activation
6. Condition medium from pretreated HUVECs (0.5microgram/ml γ-hum)after trypsin activation

inhibitory effect of γ-hum on the endothelial cell-dependent angiogenic process. The metastasis-supporting vasculature is shaped by continuous proteolytic modification of ECM and MMPs. MMP-mediated regulation of VEGF induced tumour vascularization was completely inhibited by MMP blockage and reduced the volume of angiogenic vasculature (Deryugina and Quigley, 2015).

The matrix degrading enzymes are produced in inactive proenzymatic forms, and the enzyme trypsin can activate pro-MMPs to active MMPs. The gelatinases A and B (MMP-2 and MMP-9) were found to be overexpressed in invasive tumour cells (Gong et al., 2014). MMP-9 not only readily digests denatured collagens and gelatins but also plays its particular role in angiogenesis since it increases the bioavailability of proangiogenic factors. γ-hum treatment showed a significant inhibition in the invasion and migration of the collagen matrix by HUVECs in a dose-dependent manner, and zymography analysis showed an inhibition in the activation of the proenzyme form to the active form of metalloproteinases; both these processes are mediated by the involvement of matrix-degrading enzymes. These results denote the regulatory effect of γ-hum on MMPs. The TIMP-free status of pro-MMP-9 is an important biochemical characteristic required for unencumbered and rapid activation and high angiogenic capacity of naturally produced pro-MMP-9 (Zajac et al., 2013). The enhancement in the level of TIMP-1 by γ-hum again pronounces its antiangiogenic effect and the ability to block MMPs via positively modulating the level of its tissue inhibitors.

The prime target of VEGF is endothelial cells, and cancer cells release VEGF to induce tumour angiogenesis. The VEGF stimulates VEGF receptors on the tumour endothelium or in the tumour cell itself. VEGF therapy supports tumour growth by inducing angiogenesis and also by direct action via VEGF receptor expression by tumour cells (Lee et al., 2015). Administration of γ-hum reduced the elevated level of serum VEGF in angiogenesis-induced animals, indicating the efficiency of γ-hum in targeting VEGF and thereby reducing the VEGF-assisted migration and survival of endothelial cells. By lowering the level of VEGF, γ-hum can also inhibit the neovessel formation induced by tumour cells for their growth

and development. The role played by TNF-α can be linked to almost all steps involved in tumourigenesis, including proliferation, invasion, angiogenesis, and metastasis (Aggarwal et al., 2006). TNF-α acts as a macrophage-activating factor and activates these cells to secrete angiogenic factors (Yadav et al., 2015). IL-1β is required for invasion and metastasis of cancer cells and promotes tumour growth and angiogenesis (Saijo et al., 2002). IL-6 produced by endothelial and tumour cells is a potent proangiogenic cytokine and promotes MMP-9 activation and VEGF release from these two types of cells (Fan et al., 2008). IL-6 is also capable of increasing endothelial permeability and stimulating proliferation of endothelial cells (Kim et al., 2009). GM-CSF enhances tumour cell proliferation, migration, and angiogenesis. It stimulates angiogenesis by endothelial cell proliferation and migration (Gutschalk et al., 2013). Lowering of the elevated levels of TNF-α, IL-1β, IL-6, and GM-CSF further confirmed the antiangiogenic effect of the active fraction from *E sonchifolia* with already proven anti-inflammatory effects. By lowering the level of these cytokines, γ-hum was involved directly or indirectly in the inhibition of tumour directed neovessel formation by blocking the main culprits like MMP and VEGF that actively take part in the process. The present study clearly proved the inhibitory effect of the active fraction containing the major active principle γ-humulene on tumour angiogenesis by efficiently decreasing MMPs, VEGF, and proinflammatory cytokines while at the same time increasing the level of TIMP. The retarding effect of γ-hum was clear cut from the decreased capillary formation and prevention of microvessel outgrowth from the aorta. Additional evidence for the inhibitory effect of γ-hum is the impediment on endothelial cell proliferation, invasion, migration, and also hampering of the activation of proenzyme to active enzyme as evident by the gelatin zymographic analysis.

Chapter 6

Evaluation of the inhibitory effect of selected plant isolates on the development and metastatic progression of mouse 4T1 breast tumour

6.1. Introduction

To win the battle against breast cancer we have to give prime importance for combating metastasis. Metastasis is the leading cause of breast cancer-associated deaths. 30-40% of patients may eventually suffer from distant relapse and succumb to the disease, even though there is a significant improvement in current therapies in extending patient life. Deeper understanding of the metastasis biology is very important in developing better treatment strategies and achieving long-lasting therapeutic efficacies against breast cancer (Jin and Mu, 2015). To study metastasis the *in vivo* metastatic models that mimic human breast cancer metastasis are limited, when xenograft models are used the exact replication of the human metastasis is difficult and these models only represents the primary tumour growth (Bibby, 2004; Eccles et al., 1994; Hoffman, 1999). The syngenic mouse 4T1 breast tumour model metastasizes in mice with normal immune function. This type of immunocompetent models is necessary for the study of cancer therapeutics because immune system also play a significant role in tumour development and progression. 4T1 mouse breast tumour model has many advantages to be used as suitable animal model that represent human mammary carcinoma. First of all 4T1 primary tumour will develop on the anatomically correct site. Secondly metastasis will develop spontaneously from primary site. Finally the pattern of metastatic spread is similar to the human condition (Pulaski and Rosenberg, 2001).

In order for its departure from primary site and to recolonise in different organs cancer cells need to undergo a series of changes. This metastasis cascade (Nguyen et al., 2009) comprises: cancer cells becoming locally invasive and migratory, reaching the blood vessel and intravasating into the circulation, circulating via the blood flow, arresting and extravasating to the distant organ, surviving the initial hostile stress, and reinitiating outgrowth and co-opting the distant stroma. Thus, it is more and more recognized that survival and outgrowth in foreign tissue are the rate-limiting steps for metastasis colonization and constitute the bottleneck of the metastasis cascade (Vanharanta and Massague, 2013). In the present study we analyzed the effect of some selected plant isolates on the organ specific tumour progression in a drastic breast cancer metastatic

condition in murine 4T1 model. Instead of going for a blind selection of test materials, we evaluated the plant products with strong literature background on pulmonary antimetastatic effects in the spontaneous metastatic B16F10 mouse model, punarnavine (Manu and Kuttan, 2009), Harmine (Hamsa and Kuttan, 2011) and an active fraction from *Emilia sonchifolia* (George and Kuttan, 2016; Gilcy and Kuttan, 2016), enriched with γ-humulene (71%) (γ-hum).

6.2. Materials and Methods

Plant materials:-

- Punarnavine an alkaloid from *Boerhaavia diffusa*
- Harmine β-Carboline alkaloid from *Peganum harmala*
- An active fraction from *Emilia sonchifolia*, enriched with the sesqueterpene γ-humulene (71%) (γ-hum).

Dosage:-

Punarnavine - 40mg/kg body weight
Harmine - 10mg/kg body weight
γ-hum - 5mg/kg body weight

Administration - Intraperitoneal (i.p) for ten consecutive days.

Animals: - Female BALB/c mice (8 weeks old)

Cell line: - 4T1 mouse breast carcinoma cell line
Site of injection subcutaneous to the abdominal mammary fat pad

6.2.1. Tumour volume and rate of survival

Mice were divided into four groups (22 animals/group). All animals were injected with a cell suspension of 0.1 ml, containing 7×10^4 4T1 cells into the fat pad of the 4^{th} mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight.

Group III animals were treated with harmine 10mg/kg body weight. Group IV animals were treated with γ-hum 5mg/kg body weight. The administration of the test materials were done intraperitoneally for 10 days starting from 7^{th} day of tumour induction. On every 7^{th} day solid tumour development was measured with vernier calipers and the tumour volume was calculated using the formula

$V = LW^2/2$ (V, tumour volume; L, length; W, width).

The remaining 14 animals in each group were observed for their survival.

6.2.2. Organ specific metastatic tumour progression

Mice were divided into four groups. All animals were injected with a cell suspension of 0.1 ml, containing 7×10^4 4T1 cells into the fat pad of the 4^{th} mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight. Group III animals were treated with harmine 10mg/kg body weight. Group IV animals were treated with γ-hum 5mg/kg body weight. Administration of the test materials were done intraperitoneally for 10 days starting from 7^{th} day of tumour induction. Animals were sacrificed on particular time point's, organs were collected and metastatic cell density was assessed. For histopathological analysis specific organs from each group of sacrificed animals on the particular day of organ collection were fixed in 10% formalin, dehydrated in different concentrations of alcohol and embedded in paraffin wax. Sections were stained with eosin and hematoxylin (Pulaski and Rosenberg, 2001).

6.2.2.1. Metastatic tumour progression to lymphnode

On the 18^{th} day of tumour inoculation eight animals from each group were sacrificed. The draining lymphnodes were collected and placed in a tissue culture dish containing 10ml culture medium (DMEM) supplemented with 60µM 6-thioguanine. The samples were dissociated by forcing through a cell strainer. The cells were evenly distributed by swirling the dish. The dissociated cells were then incubated in 37^0C with 5% CO_2. When the cells were attached

medium with unattached cells were removed. Cell density was assessed and photographed (Pulaski and Rosenberg, 2001).

6.2.2.2 Metastatic tumour progression to lungs

On the 25th day of tumour inoculation sixteen animals from each group were sacrificed. Lungs from eight animals were used for assessing the tumour progression to lungs. Lungs and serum collected from the remaining 8 animals were used to study the biochemical parameters. To assess the metastatic tumour progression, collected lungs were placed in a tissue culture dish containing 5ml 1x HBSS. Using forceps the lungs were thoroughly cleaned and transferred to another tissue culture dish. With curved scissors the lungs were minced into small pieces. The minced tissue was then transferred into a 15ml conical tube containing 2.5ml collagenase type IV cocktail. Tissue culture plates were again washed with 2.5ml 1xHBSS and transferred to the same 15ml conical tube to a final volume of 5ml. The samples were placed at 4^{0}C for 75 min on a shaker. After completion of enzyme digestion volume of the sample was made upto 10ml with 1xHBSS. Samples were filtered through a cell strainer to remove large chunks of undigested tissue. Samples were then centrifuged, supernatant was discarded and the pellet was resuspended in 10ml medium supplemented with 60µM 6-thioguanine. The resuspended cells were plated in a tissue culture dish and were evenly distributed by swirling the dish. The plates were then placed in 37^{0}C, 5% CO_2 incubator. When the cells were attached medium with unattached cells were removed. Cell density was assessed and photographed (Pulaski and Rosenberg, 2001).

6.2.2.2.1. Lung collagen hydroxyproline content

The lungs were homogenized and lung collagen hydroxyproline estimation was done by the chloramine-T method with hydroxyproline as standard (Bergman and Loxley, 1970).

6.2.2.2.2. Lung hexosamine content

Lyophilized tissue samples were hydrolysed and evaporated to dryness. Hexosamine was estimated in the presence of Ehrlich's reagent with glucosamine as standard (Elson and Morgan, 1933).

6.2.2.2.3. Lung uronic acid content

Uronic acid level was estimated in the presence of carbazole reagent with glucuronic acid lactone as standard (Bitter and Muir, 1962).

6.2.2.2.4. Serum sialic acid and γ-glutamyl transferase levels

Blood was collected by heart puncture, serum separated, and used to estimate serum sialic acid by thiobarbituric acid assay (Skoza and Mohos, 1976), with *N*-acetyl neuraminic acid as standard. γ-glutamyl transferase level in the serum was estimated by measuring the release of *p*-nitroaniline from γ-glutamyl *p*-nitroanilide in the presence of an acceptor (glycylglycine) using a *p*-nitroaniline standard (Szasz, 1976).

6.2.2.3 Metastatic tumour progression to liver

On the 32^{nd} day of tumour inoculation eight animals from each group was sacrificed. The liver was collected and placed in tissue culture dish containing 5ml 1x HBSS. Using forceps the liver was thoroughly cleaned and transferred to another tissue culture dish. Liver was minced into small pieces using curved scissors. The minced tissue was then transferred into a 15ml conical tube containing 2.5ml collagenase type I cocktail. Tissue culture plates were again washed with hyaluronidase cocktail and transferred again to the tube to a final volume of 5ml. Samples were placed at 37^0C for 20 to 30min on a shaker. After completion of enzyme digestion volume of the sample was made upto 10ml with 1xHBSS. Samples were filtered through a cell strainer to remove large chunks of undigested tissue. Samples were then centrifuged, supernatant was discarded and the pellet was resuspended in 10ml medium supplemented with 60µM 6-

thioguanine. Samples were plated in a tissue culture dish and were evenly distributed by swirling the dish. The plates were placed in a 37^0C, 5% CO_2 incubator. When the cells were attached medium with unattached cells were removed. Cell density was assessed and photographed (Pulaski and Rosenberg, 2001).

6.2.3. Determination of serum VEGF, IL-1β, TNF-α and GM-CSF levels

Mice were divided into two groups (8 animals/group). All animals were injected with a cell suspension of 0.1 ml, containing approximately 7×10^4 4T1 cells into the fat pad of the 4^{th} mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight. Blood was collected from the caudal vein of all the experimental animals on 7^{th} and 42^{nd} day of tumour induction. Serum was separated and used for the estimation of VEGF, IL-1β, TNF-α and GM-CSF using highly specific ELISA kits according to the manufacturer's instructions.

6.2.4. Expression of genes involved in metastasis

Total RNA was isolated from primary tumour of the punarnavine 40mg/kg body weight treated animals as well as control animals on the 32^{nd} day of tumour induction. cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase. Amplification was performed using specific primers of MMP-2, MMP-9, TIMP-1, TIMP-2 and VEGF. GAPDH was selected as a stable reference gene under the experimental conditions in this study.

6.3. Results

6.3.1. Effect of test materials on tumour volume and rate of survival

The effect of test materials on solid tumour reduction is presented in figure 6.1. Administration of punarnavine reduced the tumour volume in experimental animals compared to tumour control and tumour along with harmine and γ-hum treated groups. Mice carrying 4T1 cells alone (tumour controls) had a tumour

volume of 937±194mm^3 on day 42nd; it was reduced by the administration of punarnavine on the same day (408±139mm^3). When tumour-bearing mice were treated with harmine and γ-hum the tumour volume was reduced to 892±201 mm^3 and 886±143mm^3 on the same day. The data shows that there was significant reduction in tumour volume ($p<0.001$) in punarnavine treated group compared to the non significant reduction in tumour volume in harmine and γ-hum treated groups. The survival plot as shown in figure 6.2 also shows that the survival rate of animals treated with punarnavine was very high compared to the early death pattern shown by the tumour control, harmine and γ-hum treated groups.

6.3.2. Effect of test materials on metastatic tumour progression to lymphnode

Figure 6.3. gives a detailed picture of metastatic 4T1 cells present in the lymph node. The histopathology as well as the fold decrease in metastatic cell density clearly shows the reduction in the metastatic cells in the punarnavine treated group compared to tumour control, harmine and γ-hum treated groups.

6.3.3. Effect of test materials on metastatic tumour progression to lung

Figure 6.4. shows metastatic 4T1 cells present in the lung. The fold decrease in metastatic cell density in punarnavine treated group was very high compared to control and other two test materials treated groups. The histopathological analysis of the lung tissue was also confirmed the effect of punarnavine on the inhibition of metastatic progression compared to other groups.

6.3.4. Effect of test materials on metastatic tumour progression to liver

Liver metastasis also was significantly reduced by punarnavine treatment as shown in figure 6.5. Histopathological comparison of liver tissue and the metastatic cell density analysis in the four groups of animals also confirmed the effect of punarnavine in blocking the tumour progression in 4T1 induced animals compared to the other three groups of mice.

Figure 6.1. Solid tumour volume

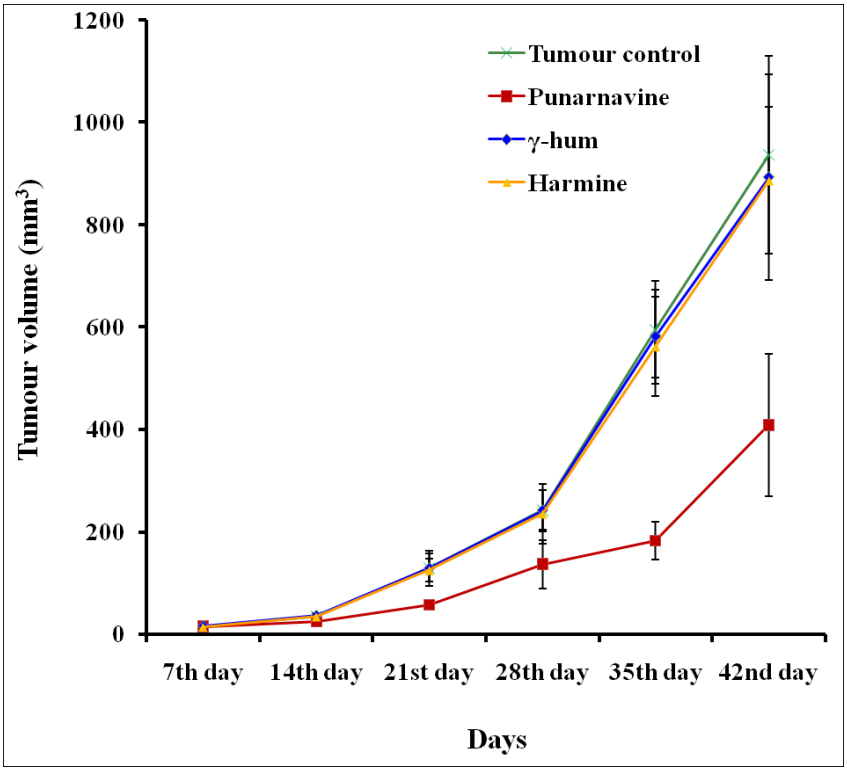


Figure 6.2. Rate of survival

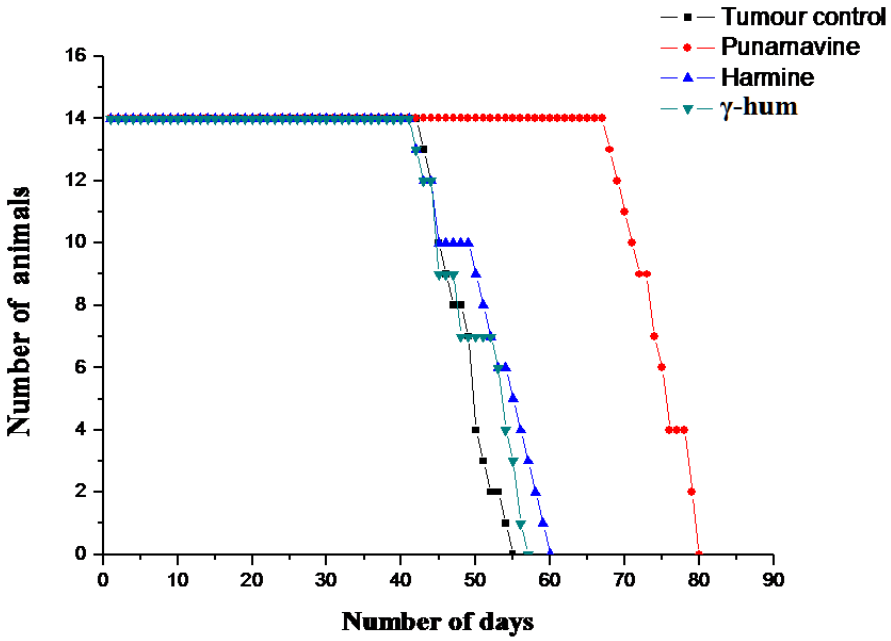
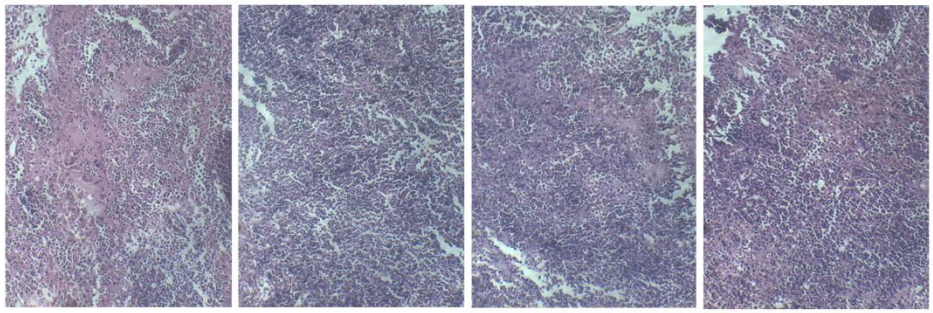


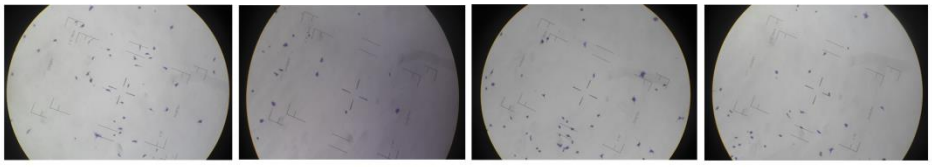
Figure 6.3. Metastatic progression to lymph node

Histopathology



A B C D

Metastasized cells after 6-thioguanine treatment



A B C D

A.Tumour control
B. Punarnavine
C. Harmine
D. γ-hum

Metastasized cell density in fold change

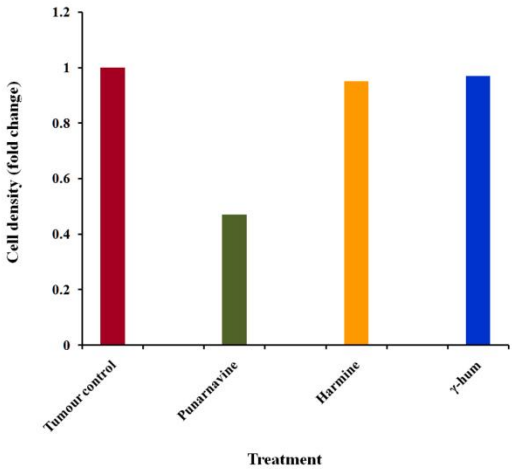
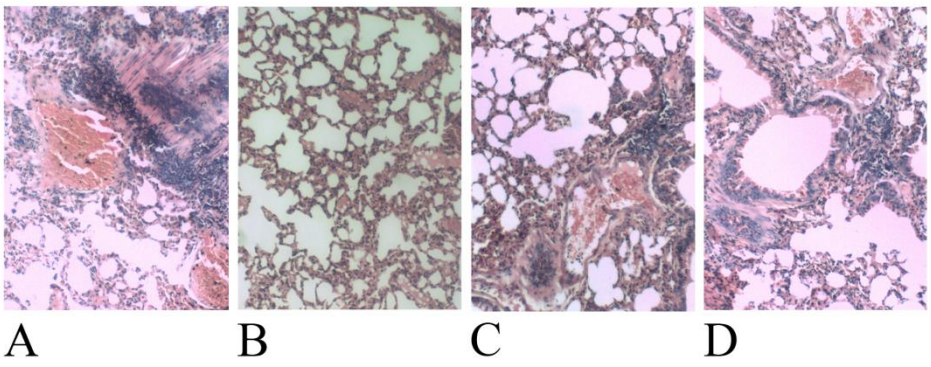
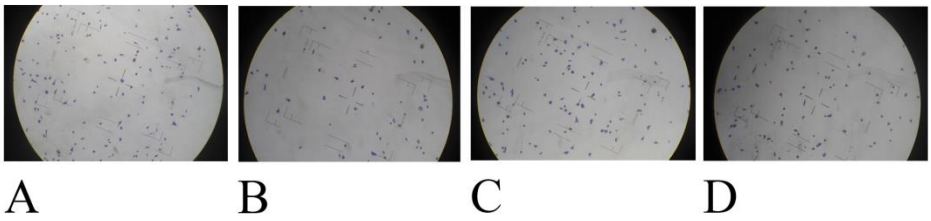


Figure 6.4. Metastatic progression to lung

Histopathology



Metastasized cells after 6-thioguanine treatment



A.Tumour control
B. Punarnavine
C. Harmine
D. γ-hum

Metastazised cell density in fold change

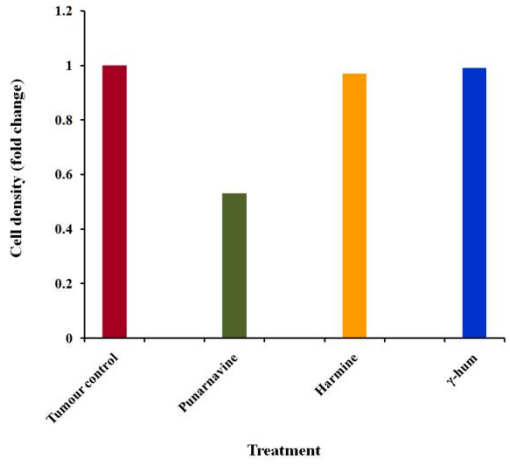
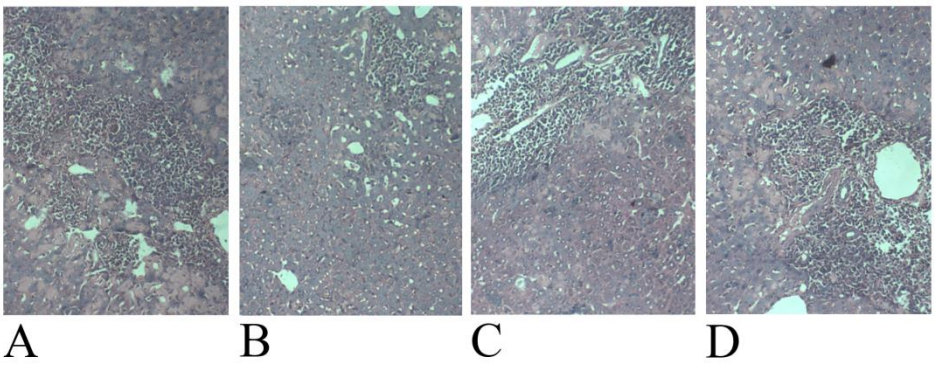
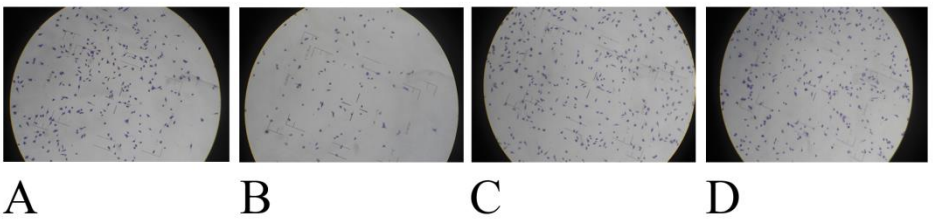


Figure 6.5. Metastatic progression to liver

Histopathology



Metastasized cells after 6-thioguanine treatment



A.Tumour control
B. Punarnavine
C. Harmine
D. γ-hum

Metastasized cell density in fold change

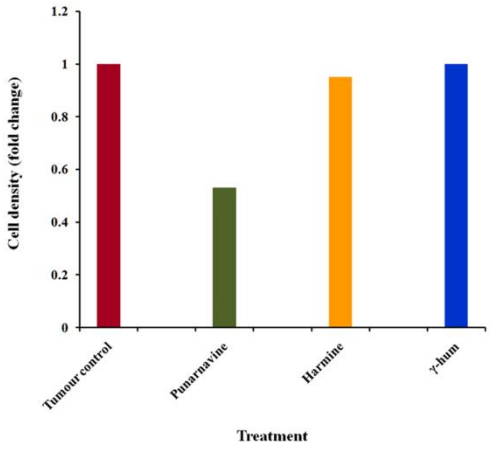
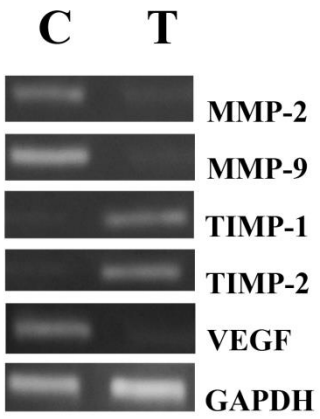


Figure 6.7. Effect of punarnavine on gene expression



C- Control; T-Punarnavine

6.3.5. Effect of test materials on biochemical parameters

Elevated levels of lung hydroxyproline, hexosamine and uronic acid were shown by 4T1 induced tumour bearing mice. By the administration of punarnavine this levels were significantly ($p<0.001$) reduced in punarnavine treated group. This reduction was not significant in the other two harmine and γ-hum treated groups. The serum sialic acid and GGT level also showed a similar pattern of reduction as above in the punarnavine ($p<0.001$) treated groups compared to the other three groups (table 6.1).

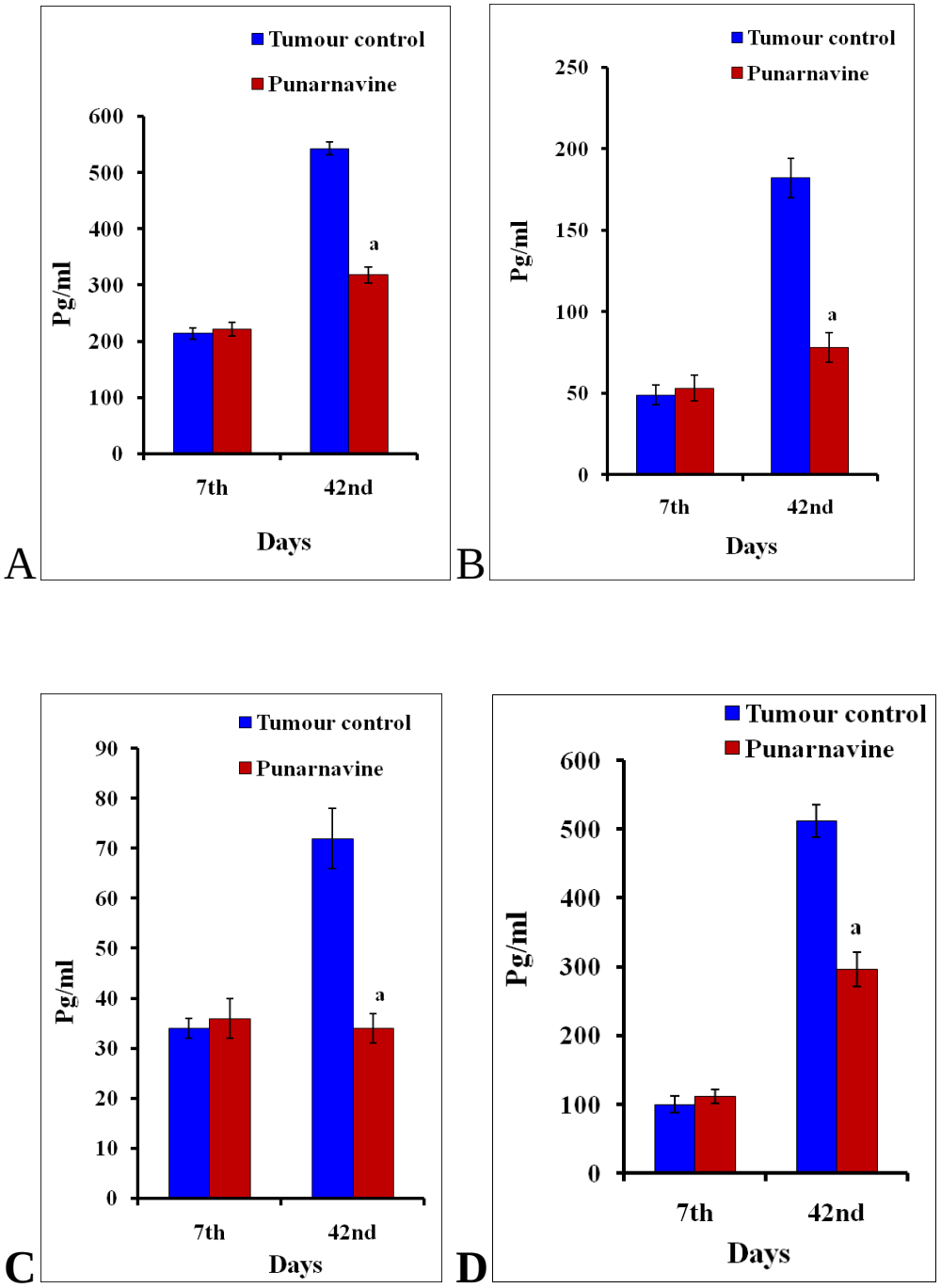
6.3.6. Effect of punarnavine on serum VEGF, IL-1β, TNF-α and GM-CSF levels

The effect punarnavine on the serum VEGF, IL-1β, TNF-α and GM-CSF levels confirmed its regulatory effect on these mediators. The VEGF level in the tumour bearing control group was 512±23pg/ml and GM-CSF level was 72±6pg/ml. IL-1β and TNF-α levels were 182±12pg/ml and 543±12pg/ml in the tumour control groups on the 42^{nd} day. These levels were significantly reduced in punarnavine treated groups VEGF (296±25pg/ml), GM-CSF (34±3pg/ml), IL-1β (78±9pg/ml) and TNF-α (318±14pg/ml) as shown in figure 6.6.

6.3.7. Expression of genes involved in metastasis

The mRNA expression of prometastatic genes such as MMP-2, MMP-9 and VEGF was found to be upregulated in the metastatic-tumour bearing control animals. The expression of these genes was down-regulated or inhibited in mice treated with punarnavine. It was interesting to note that mRNA expression of antimetastatic genes TIMP-1 and TIMP-2 were absent or down regulated in metastatic tumour bearing control animals and the punarnavine treatment could increase its expression in the treated group of animals (Figure 6.7).

Figure 6.6. Effect of punarnavine on cytokine levels



A. TNF-α; B. IL-1β; C. GMCSF; D. VEGF

$^{a}p<0.001$

Table 6.1. Biochemical parameters

Treatment	Hydroxyproline (µg/mg protein)	Uronic acid (µg/100mg tissue wet weight)	Hexosamine (µg/100mg tissue dry weight)
Tumour Control	24.55±3.04	339.1±37.24	4.6±0.86
Punarnavine	9.9±1.21***	109±13.6***	1.7±0.42***
Harmine	25.2±2.8	347.11±36.81	5.1±1.2
γ-hum	25.5±2.9	348.2±37.5	5.05±1.1

Treatment	Sialic acid (µg/ml serum)	GGT (nmol/ml serum)
Tumour Control	128.67±14.13	121.8±11.9
Punarnavine	56.6±11.8***	51.9±6.7***
Harmine	128.4±15.4	125.5±8.4
γ-hum	129.2±16.5	122.3±9.5

Values denote mean ± SD, ***p< 0.001

6.4. Discussion

In the present study we analysed the effect of three potent plant isolated test materials in a drastic metastatic condition established by the inoculation of 4T1 breast mammary carcinoma cells in mice. Initially the results on solid tumour reduction and rate of survival showed the potent effect of punarnavine on primary breast tumour growth compared to harmine and γ-hum. These results were further confirmed by monitoring the organ specific metastatic progression in the tumour bearing animals. The metastasized 6-thioguanine resistant cells of lympnode, lung and liver collected on different days after sacrificing the animals when allowed to grow in culture the punarnavine treated group showed the maximum lower cell density. The histopathology of these organs was also in favour for the efficacy of punarnavine in combating metastatic progression in comparison to other test materials.

The assessment of biochemical parameters like hydroxyproline, hexosamine uronic acid, sialic acid and GGT were also undoubtably proved the dominating inhibitory effect of punarnavine on breast tumour metastasis. Hydroxy proline is posttranslationally produced from proline and forms a major component of collagen the main cellular matrix protein. The degradation of collagen in the metastatic tumour tissues will result in an elevated level of hydroxyproline (Phang et al., 2008). The acidic and basic modifications of monosaccharides found in the extracellular matrix yield uronic acids (glucuronic acid) and amino sugars (hexosamines) and they form a vital part in many structural polysaccharides and glycosaminoglycans (GAG) found in the ECM. Hexosamine found in the ECM serves as ground substratum for collagen synthesis (West et al., 1985). Elevated expression of sialoglycans in the circulation of tumour bearing animals correlate with tumour aggressiveness and ability to metastasize and invade surrounding tissues. Aberrant sialylation contribute to therapy resistance of cancer. Sialic acids promote tumorigenesis by facilitating escape from apoptosis, formation of metastasis, and resistance to therapy. Selective approaches interfering with sialic acid expression would therefore have great potential to counteract tumour growth and metastatic formation (Bull et al., 2014). Higher serum levels of GGT are associated with an

increased cancer risk. GGT is disregulated in malignant cells and this will produce reactive oxygen species resulting in more aggressive tumour formation (Fentiman, 2012).

Numerous studies have indicated that tumor cells exhibit an elevation in the constitutive production of proinflammatory cytokines such as TNF-α, IL-1β, and GM-CSF. TNF-α can act as endogenous tumour promoter involved in the tumour progression (Balkwill, 2006). IL-1 expression increases the tumour invasiveness and metastasis by enhancing the expression of adhesion molecules on endothelial and malignant cells into the circulation and their dissemination to remote tissues (Guo et al., 2012). Elevated serum levels of tumour promoting factor GM-CSF in cancer patients is considered as marker with high diagnostic sensitivity. GM-CSF will enhance invasive capacity of cancer cells via increased expression of matrix degrading proteins. It is also involved in enhancing the expression of MMPs and this was reversible by GM-CSF blocking (Gutschalk et al., 2013). Lowering of the serum levels of these cytokines indicate the potent effect of punarnavine on the inhibition of cytokine mediated signaling that play an important role in tumorigenesis.

MMPs are a family of zinc dependent endopeptidases and MMP mediated ECM degradation leads to cancer cell invasion and metastasis. MMP-9 is critical for the formation of metastatic niche due to its ability to liberate VEGF and thereby support angiogenesis. MMP-2 and MMP-9 enhance tumour cell migration and contribute to the establishment of metastasis–prone sites at tumour distant organs (Kessenbrock et al., 2010). MMPs are produced in the zymogen form and their activation and activities are regulated by TIMPs. The disruption of MMP-TIMP balance will result in tumour invasion and metastasis (Chirco et al., 2006). In the present study, punarnavine treatment downregulated the expression of MMPs at the same time upregulated the expression of TIMPs, indicating its regulatory effect on the inhibition of MMP. When MMPs are lacking or TIMPs are over produced, formation of new tumours decreases as evident from these results.

Autocrine VEGF signaling crucial in highly aggressive cancers is mediated by growth, survival, migration and invasion of cancer cells (Kohno and Pouyssegur, 2006). In this study, the expression of VEGF has been downregulated and as supporting evidence the serum VEGF level was also reduced by the treatment. These results show that punarnavine could inhibit all the pathways that link the MMPs and VEGF to tumour survival, proliferation, and invasion.

Chapter 7

Punarnavine: Evaluation of the possible therapeutic application in conventional radiotherapy

7.1. Introduction

Cells capable of remerging and establishing a metastatic tumour condition from its quiescent state after tolerating an episode of cancer therapeutic approaches is often called cancer stem cell or cancer initiating cell. Hypoxic condition with insufficient oxygenation and the presence of other favourable mediators like hypoxia inducible factor, vascular endothelial growth factor and cytokines will accelerate this type of cell selection within the tumour mass (Collet et al., 2015). Exposure with ionizing radiation will positively modulate existing conditions in favour of the survival and expansion of cancer stem cells in a hypoxic microenvironment. The phenomenon of radio resistance is well exhibited by the hypoxic cells than the non hypoxic ones. Major repair pathways are also negatively regulated by hypoxia (Liu et al., 2015). In solid tumours abnormal blood vessel formation and perfusion, increase in cancer cell proliferation with high oxygen consumption all these can contribute to low oxygen concentrations and the resultant hypoxic progression (Justus et al., 2015). The median value of partial oxygen pressure (pO_2) is 10mm Hg in breast cancers compared to the value of 65mm Hg in normal breast tissue.

Radiotherapy is one of the prime treatment modality for breast cancer. Heterogenous hypoxic areas present in locally advanced breast cancers contribute to therapeutic resistance, metastases and poor patient survival. Meagre response to radiotherapy in about 50% of locally advanced breast cancers is due to the presence of these hypoxic regimes and the inability of ionizing radiation to produce DNA damage in the absence of oxygen (Aravindan et al., 2013). Hypoxic tumour cells are capable of active proliferation, invasion, metastasis and neovascularisation (Ruan et al., 2009). Specific targeting of hypoxic process will be the future arena of novel cancer therapies. The plant derived compounds are a tremendous source of active therapeutic agents with fewer side effects that can be used alone or in combination with other therapeutic methods. In the present study we investigated the effect of punarnavine during X-irradiation in mouse 4T1 breast tumour model.

7.2. Materials and Methods

Plant material: - Punarnavine an alkaloid from *Boerhaavia diffusa*

Animals: - Female BALB/c mice (8 weeks old)

Dosage: - Punarnavine - 40mg/kg body weight
Administration - Intraperitoneal (i.p) for ten consecutive days.

Cell line: - 4T1 mouse breast carcinoma cell line
Site of injection subcutaneous to the abdominal mammary fat pad

7.2.1. Monitoring *in vivo* tumour growth in X-irradiated mice

Female BALB/c mice were divided into eight groups (8 animals/group). All animals were injected with a cell suspension of 0.1 ml, containing 4×10^5 4T1 cells into the fat pad of the 4^{th} mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight intraperitoneally for 10 days starting from 24 hour after day 14 of tumour induction. Groups III, IV, and V animals were exposed to a single dose of 6, 4, and 2Gy radiation respectively on day 14, and kept as a radiation control. Groups VI, VII, and VIII animals were exposed to 6, 4, and 2Gy radiations respectively on day 14 and all animals were treated with punarnavine 40mg/kg body weight intraperitoneally for 10 days starting from 24 hour after X-irradiation. The tumour volume was measured on day 15, day 21 and on day 28 after radiation exposure. Solid tumour development was measured with vernier calipers and calculated using the formula $V = LW^2/2$ (V, tumour volume; L, Length; W, Width).

7.2.1.1. Irradiation procedure

Experimental animals were exposed to a single dose of X-radiation (6, 4 and 2Gy) using 6MV Linear accelerator (LINAC) (Varian Medical systems, USA). The animals were kept immobilized in a specially designed, well-ventilated cage

without any anaesthesia and exposed to whole-body irradiation at a rate of 1Gy/min. The radiation field size was 25 × 25 cm^2, at a distance 100cm from the source.

7.2.2. Determination of serum vascular endothelial growth factor (VEGF) level

Blood was collected from the above groups of animals by caudal vein bleeding on day 21 and on day 28 after radiation exposure. Serum was separated and used for the estimation of VEGF level using enzyme linked immunosorbent assay kit (ELISA) according to the manufacturer's instructions.

7.2.3. Immunohistochemical analysis

Immunohistochemical staining of the excised tissues were performed by means of a double antibody labelling method. Briefly resected primary tumours on day 28^{th} were fixed in neutral buffer containing 3.7% formalin for 24 hours, processed, and embedded in paraffin. The paraffin tissue blocks were cut into 5 micrometer thick sections, placed on glass slides, and deparaffinized in sequential baths of xylene. Rehydration was done by graded alcohol series and distilled water. The endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide for 10 min followed by incubation with anti-CD-31 (diluted at 1:100) antibody overnight at 4^0C. After incubation, the unbound primary antibody was washed off with PBS-T (Phosphate Buffered Saline with 0.1% Tween-20). The sections were then covered with HRP conjugated rabbit anti–mouse IgG (diluted at 1:200) secondary antibody, incubated for 45-60 min at room temperature, and washed with PBS-T. Visualisation was done using diaminobenzidine chromogen that yields a brown coloured deposit. These sections were then counterstained with Methyl green and photographed.

7.2.4. Gene expression of VEGF and HIF-1α

4T1 primary tumour tissue was excised and total RNA was isolated, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase. Amplification was performed using specific primers of VEGF and HIF-1α.

7.2.5. Proliferation assay

4T1 cells (5×10^3 cells/well) were cultured on several 96-well culture plates for 24 hours and the plates were then exposed to different doses of X-radiation (0.5, 1, 2, 3, 4, and 5Gy) in the presence or absence of punarnavine (5μg/ml). After 48 hours of incubation ^{3}H thymidine was added to each well (1 μCi /well) and further incubated for an additional 18 hours. After incubation, DNA was precipitated using 10% ice-cold perchloric acid, and pellets were dissolved in NaOH and transferred to 5ml scintillation fluid. Radioactivity was measured using Rack Beta fluid scintillation counter.

7.2.6. Determination of *in vitro* VEGF level

4T1 cells were grown in complete DMEM on 24-well culture plates, 24 hour after incubation culture medium was replaced with serum-free DMEM. One group of cells were incubated with or without punarnavine (5μg/ml) and another group of cells were irradiated with 1Gy of radiation and further incubated with or without punarnavine (5μg/ml). Both groups were incubated for 72 hours. Conditioned medium was then collected on 24, 48, and 72 hours after irradiation, centrifuged, and stored for conducting invasion assays, gelatin zymographic analysis and gene expression studies that to be done after an estimation of VEGF content. VEGF was measured using ELISA kit according to the manufacturer's instructions.

7.2.7. Invasion assay

The invasion assay was carried out in modified Boyden chambers. The lower compartment of the chamber was filled with conditioned medium containing

maximum VEGF content from the above experiment (section 7.2.6.) and a polycarbonate filter coated with 25µg of type I collagen was placed on top. 4T1 cells were irradiated with 1Gy of X-radiation and then seeded (10^5 cells/150 µl DMEM) onto the upper chamber in the presence or absence of punarnavine (5µg/ml) and incubated at 37°C in 5% CO_2 atmosphere for 10 hours. After incubation, the filters were removed, fixed with methanol, and stained with crystal violet. Cells migrating to the lower surface of the polycarbonate filters were counted under a microscope. The results are expressed as the percentage inhibition of invasion**.**

% inhibition of invasion = 100 - (mean number of migratory cells in test/ mean number of migratory cells in control) x 100

7.2.8. Gelatin zymography

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed with 0.1% gelatin incorporated in the separating gel (Billings et al., 1991). Conditioned medium with maximum VEGF level from the above mentioned experiment (section 7.2.6.) was used for zymographic analysis. Samples equivalent to 100µg protein was activated with 5µl trypsin solution (75 µg/ml) in the presence and absence of punarnavine (5µg/ml) in 0.1 M Tris–HCl, 10mM $CaCl_2$ buffer (pH8) and incubated for 1 hour at room temperature. Sample loading and electrophoresis was carried out in a same manner as mentioned in section 5.2.9.

7.2.9. Expression of VEGF, HIF-1α, and Flk-1 genes under hypoxic condition

4T1 cells and HUVECs (2×10^4 cells) were seeded in 96 well plates containing conditioned medium (with maximum VEGF) from the above experiment (section 7.2.6.) and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. These cells were then exposed to hypoxia (1% O_2, 5% CO_2 and 94% N_2) in a modular incubator chamber in the presence or absence of punarnavine (5µg/ml) for 4 hours at 37°C. cDNA was synthesized using Moloney murine leukemia

virus reverse transcriptase. Amplification was performed using specific primers of VEGF and HIF-1α for 4T1 cells and Flk-1 for HUVECs.

7.3. Results

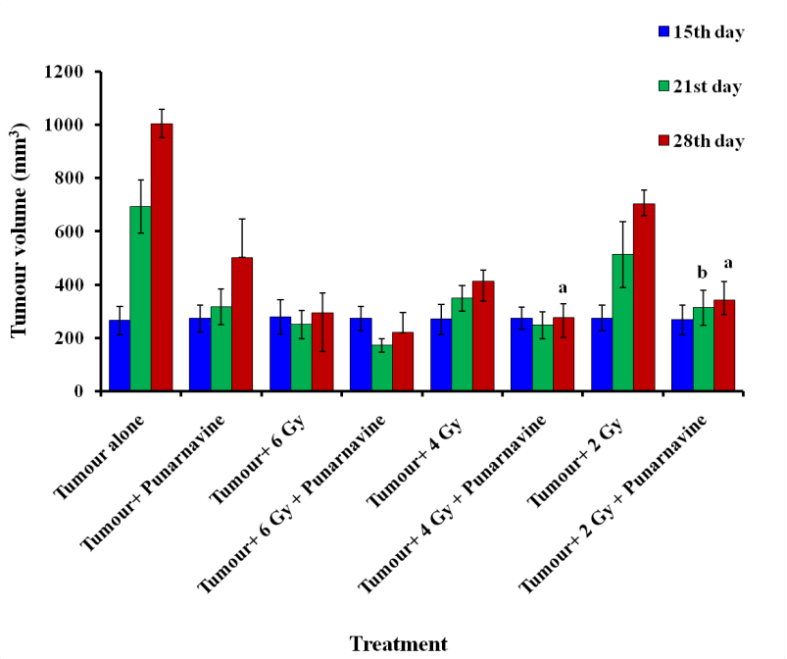
7.3.1. Effect of punarnavine on *in vivo* tumour growth in X-irradiated mice

The effect of punarnavine on solid tumour reduction is presented in figure 7.1. Administration of punarnavine reduced the tumour volume in all groups compared to control (radiation alone) groups. Mice carrying 4T1 cells alone (tumour controls) had a tumour volume of 1003±144mm^3 on day 28; it was reduced by the administration of punarnavine on the same day (500±74mm^3). Radiation treatment significantly decreased the tumour volume in the treated groups of animals. When tumour-bearing mice were exposed to 6Gy radiation the tumour volume was reduced to 293±74mm^3 on day 28. There was a non significant reduction in tumour volume by the administration of punarnavine in 6Gy group 219±43mm^3. 4Gy radiation treated group of animals showed a reduction in tumour volume of 410±53mm^3, when punarnavine was administered this reduction was further lowered significantly ($p<0.001$) and had a decreased tumour volume of 275±70mm^3 on day 28. The 2Gy radiation reduced tumour volume to about 701±147mm^3 at the same time on 28th day. There was a significant ($p<0.001$) reduction in tumour volume in punarnavine along with 2Gy treated group 341±74mm^3. This reduction in tumour volume in 2Gy radiation along with punarnavine is more effective than 4Gy alone treated control group.

7.3.2. Effect of punarnavine on serum VEGF level

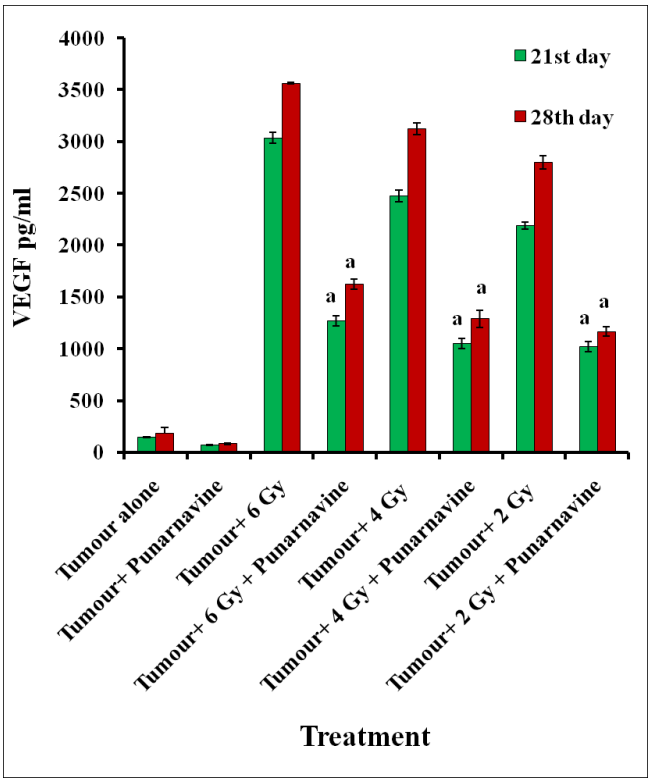
The serum VEGF level during tumour progression in radiation alone and radiation along with punarnavine treated groups of animals were shown in figure 7.2. Serum VEGF level was elevated during tumour progression. Administration of punarnavine significantly ($p<0.001$) reduced serum VEGF compared with untreated tumour controls. Radiation exposure drastically enhanced the serum VEGF level. Tumour bearing animals exposed to 6Gy radiation showed a

Figure 7.1. Effect of punarnavine on *in vivo* tumour growth in X-irradiated mice



[a]p<0.001, [b]p<0.01

Figure 7.2. Effect of punarnavine on *in vivo* VEGF level



[a]p < 0.001

maximum VEGF level on day 28. The 4Gy and 2Gy radiation treated animals also showed a significant increase in VEGF level. This increase in all the three doses of radiation treated groups were significantly ($p<0.001$) reduced by radiation along with punarnavine administration.

7.3.3. Immunohistochemical analysis

Figure 7.3 shows the immunohistochemistry of tumour tissue for CD31 to analyze the vascular mass. Immunohistochemistry studies revealed an increase in the CD31 endothelial cell marker, suggesting a hypoxia mediated activation of the neovascularizing pathways. By Punarnavine treatment this vascular mass was markedly reduced in different doses of radiation treated groups showing its effect on neovascularizing process.

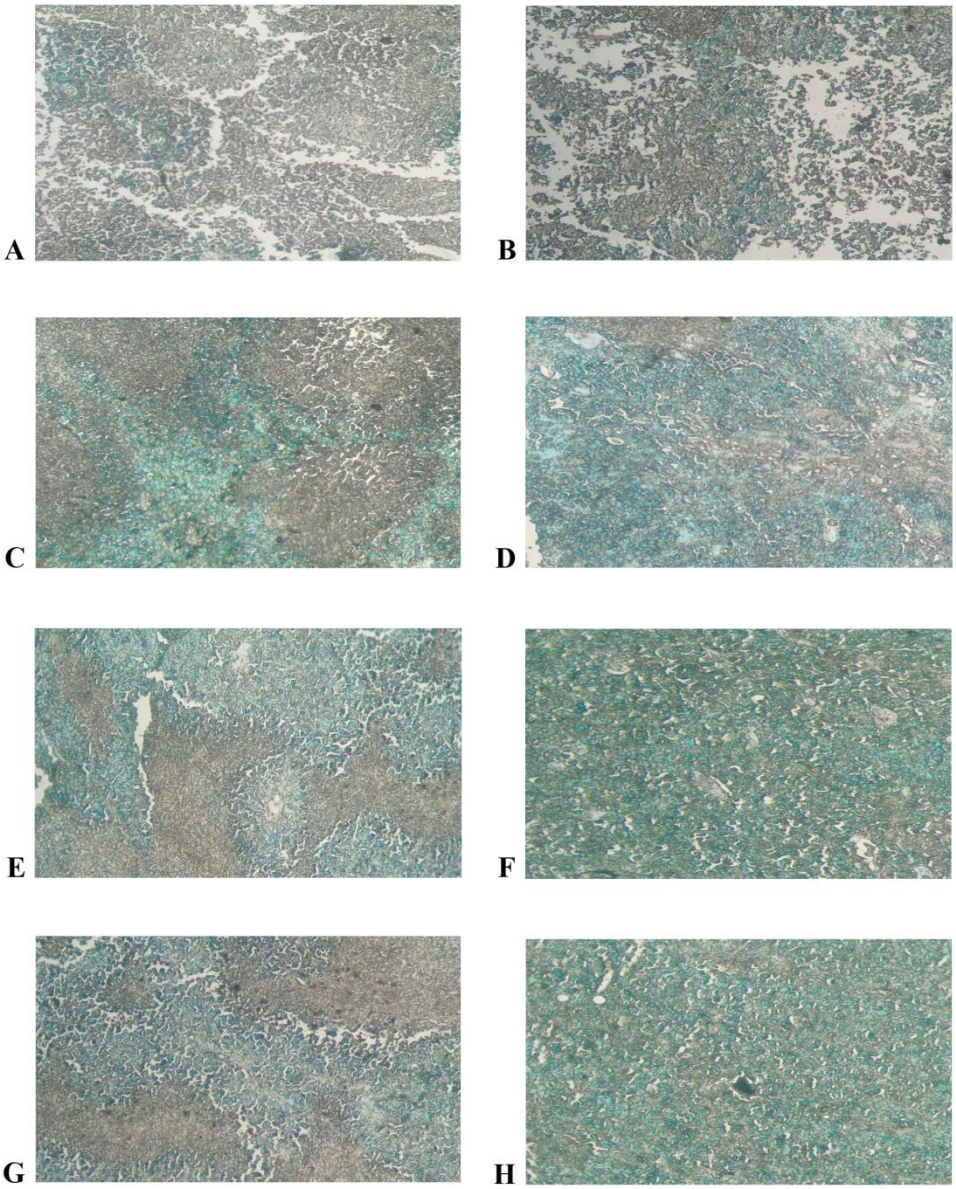
7.3.4. Gene expression of VEGF and HIF-1α

Gene expression of VEGF and HIF-1α is given in figure 7.4. We observed upregulated expression of these genes in the untreated tumour-bearing mice exposed to different doses of radiation. Punarnavine administration down regulated the expression of VEGF and HIF-1α in the tumour bearing mice exposed to different doses of radiation.

7.3.5. Effect of punarnavine on cell proliferation and *in vitro* VEGF level

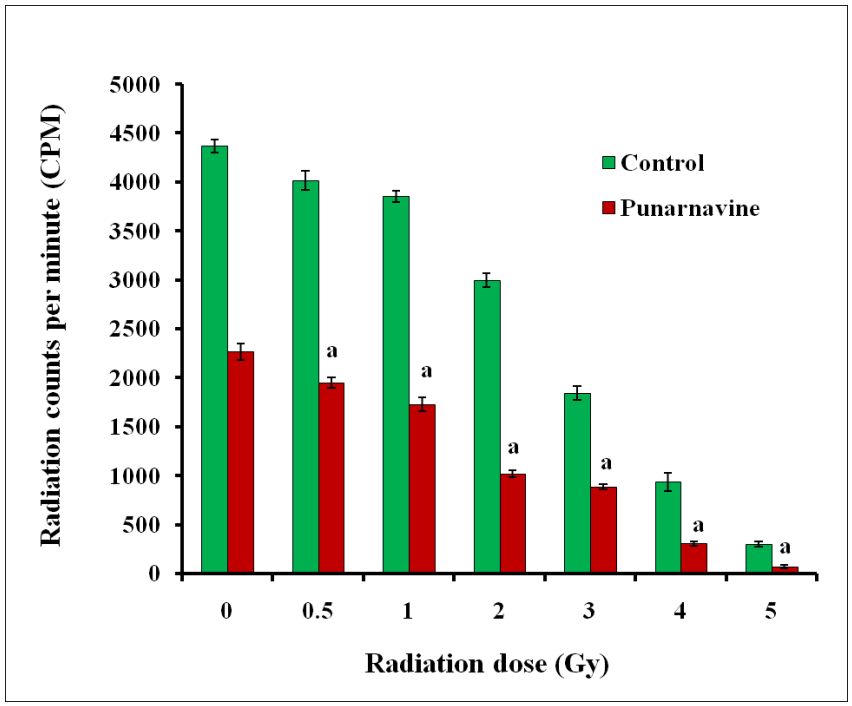
Figure 7.5 shows the effect of radiation and punarnavine on the inhibition of 4T1 cell proliferation. Cell proliferation was analyzed by the ^{3}H-thymidine incorporation assay. Cell proliferation was markedly decreased by the increase of radiation dose. Proliferation was further decreased when the cells were treated with punarnavine. Figure 7.6 shows the effect of radiation on *in vitro* VEGF level. Our data indicates that radiation elevated the VEGF level. After 48 hours by radiation treatment, 4T1 cells showed a maximum VEGF level (1415±66pg/ml) that was significantly ($p<0.001$) decreased by treatment with punarnavine (861±21 pg/ml).

Figure 7.3. Immunohistochemical analysis



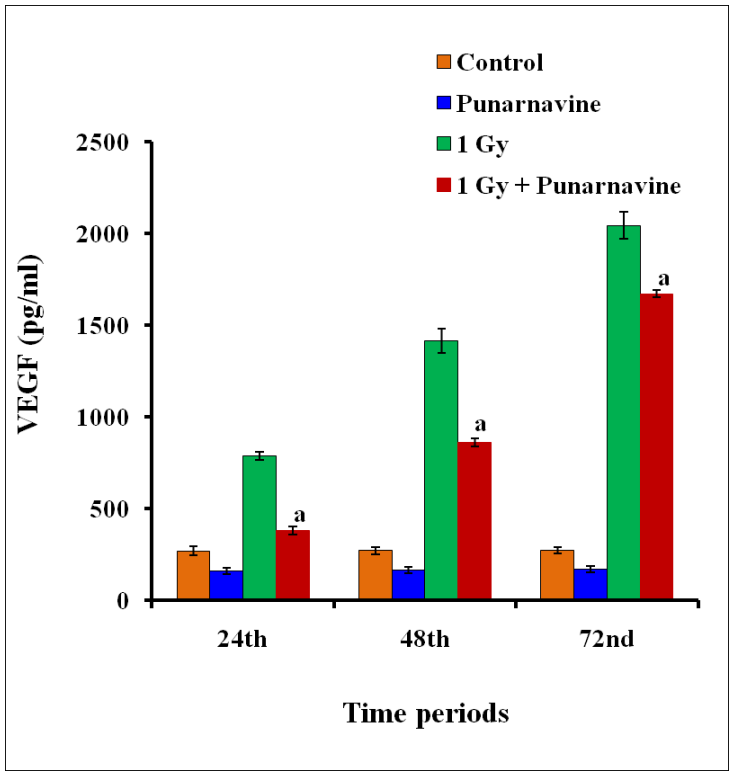
A. Tumour alone
B. Tumour + punarnavine
C. Tumour + radiation (6 Gy)
D. Tumour + radiation (6 Gy) + punarnavine
E. Tumour + radiation (4 Gy)
F. Tumour + radiation (4 Gy) + punarnavine
G. Tumour + radiation (2 Gy)
H. Tumour + radiation (2 Gy) + punarnavine

Figure 7.5. Proliferation assay



[a]p < 0.001

Figure 7.6. Effect of punarnavine on *in vitro* VEGF level



[a]p < 0.001

7.3.6 Collagen Matrix Invasion Assay

The effect of punarnavine on the invasion of irradiated 4T1 cells is shown in figure 7.7. Irradiated control cells showed the maximum invasion through the polycarbonate filter membrane. punarnavine treatment significantly inhibited cell invasion by 86%.

7.3.7 Gelatin zymography

Punarnavine inhibited the activation of MMPs produced by irradiated 4T1 cells, as shown in figure 7.8. Conditioned medium after trypsin activation showed digested clear areas at 92 and 72 kD, which was identical to MMP-9 and MMP-2 activity (Gel no.2) Gels loaded with conditioned medium without trypsin activation did not show any clear areas, indicating the inactive form of the enzyme (Gel no.1). After incubation with 10mM EDTA, trypsin activated conditioned medium loaded gels did not show clear areas, which indicate that the enzyme responsible for degradation is metalloproteinase (Gel no.3). When conditioned medium was treated with punarnavine during trypsin activation, no clear bands were observed (Gel no.4), indicating that punarnavine inhibited the activation of proenzyme to active enzyme.

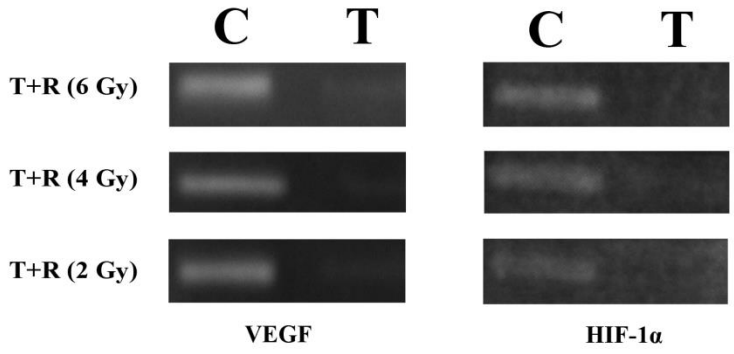
7.3.8 Expression of VEGF, HIF-1α, and Flk-1 genes

VEGF, HIF-1α, and Flk-1 genes were clearly expressed in hypoxia-exposed untreated cells (figure 7.9). The expression of all of these genes was down-regulated or suppressed by treatment with punarnavine showing its inhibitory effect on these marker genes involved in hypoxic tumour progression.

7.4. Discussion

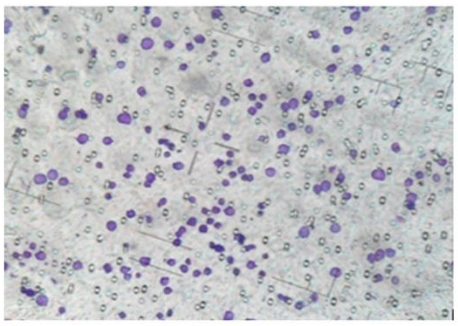
Many studies have demonstrated that the growth of solid tumours and their metastases are dependent on angiogenesis, which is regarded as a critical event in tumour development (Bergers et al., 2003). HIF-1α contributes to tumour aggressiveness, invasiveness, and resistance to radiotherapy and chemotherapy

Figure 7.4. Gene expression of VEGF and HIF-1α

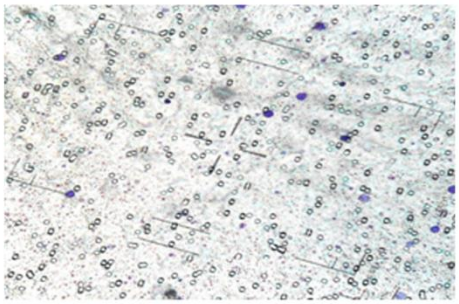


C-Tumour control; T-punarnavine treated

Figure 7.7. Collagen matrix invasion assay



Untreated irradiated control

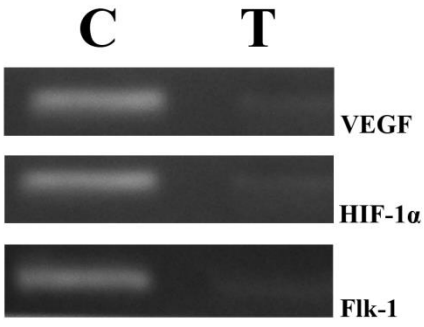


Irradiated punarnavine treated

Figure 7.8. Gelatin zymography

1. Conditioned medium from untreated, irradiated 4T1 cells without trypsin activation
2. Conditioned medium from untreated, irradiated 4T1 cells after trypsin activation
3. Conditioned medium from untreated, irradiated 4T1 cells after trypsin activation+EDTA
4. Conditioned medium from irradiated 4T1 cells (5 microgram/ml punarnavine) after trypsin activation

Figure 7.9. Gene expression of VEGF, HIF-1α, and Flk-1



C-Tumour control; T-punarnavine treated

(Diaz et al., 2005). Over expression of HIF-1α is correlated with vascular density in tumours, indicating that HIF-1α is a key initiator of angiogenic activity (Giatromanolaki et al., 2001; Sivridis et al., 2002). Punarnavine efficiently down regulated the expression of HIF-1α and potentially inhibited vascular endothelial growth factor levels. Although punarnavine is known to inhibit VEGF expression via multiple pathways its effect on the VEGF expression in a hypoxic condition is not well understood. In this study we have demonstrated that VEGF, the hypoxia induced potent vascularisation factor and HIF-1α the transcription factor required for its activation can be regulated by punarnavine in 4T1 mouse breast cancer cells. Punarnavine may play a direct role in degradation of HIF-1α and succeeding VEGF signalling in 4T1 cells leading to inhibition of hypoxia induced tumour angiogenesis which is consistent with previous studies that punarnavine process antiangiogenic and antimetastatic activity.

Resistance to radiation therapy is often associated with an increased cellular invasion and metastatic potential (Postovit et al., 2004). Hypoxic cells are 2 to 3 fold more resistant to radiation than well-oxygenated cells because the biological effect of radiation is greatly influenced by the presence or absence of molecular oxygen at the time of irradiation (Hall, 1994; Gray et al.,1953). In the present study, the administration of punarnavine along with radiation significantly reduced tumour volumes compared with untreated radiation controls in 4T1 tumour-bearing mice. The treatment along with the radiation also decreased the cell proliferation and survival fraction of 4T1 cells. VEGF is produced by tumour cells, and its binding with the VEGF receptor Flk-1 (which is expressed on vascular endothelial cells) leads to the proliferation and migration of endothelial cells (Ferrara, 2004; Liu et al., 2005). In our study, the serum VEGF level was elevated during tumour progression, and punarnavine significantly reduced this level in tumour-bearing animals. Ionizing radiation also drastically enhanced the serum VEGF level, which was found to be decreased by the administration of punarnavine. Gene expressions of VEGF, HIF-1α, and Flk-1 were found to be down-regulated by treatment with Punarnavine.

CD31 is a transmembrane glycoprotein that is highly expressed in endothelium. Its localization at the endothelial cell junctions suggests an important role in transendothelial cellular migration (Zocchi et al., 1996). Immunohistochemical analysis showed comparatively less tumour vasculature in punarnavine treated tumour bearing animals. MMPs are zinc-dependent proteolytic endo-peptidases (Matrisian, 1992) involved in cancer progression. They stimulate cancer cell growth, migration, invasion, and metastasis. Our study clearly demonstrated that punarnavine treatment significantly inhibited the activation of MMP-2 and MMP-9 in 4T1 cells, and decreased the invasion of 4T1 cells through a collagen coated polycarbonate filter membrane, which supports the above observations. In nutshell the results indicate that punarnavine administration significantly reduced tumour volumes in irradiated experimental mice, inhibited tumour cell invasion, MMP activation, VEGF level, and gene expressions of HIF-1α and VEGF in a condition of ionizing radiation exposure. All these strongly suggest that punarnavine inhibited the invasion of irradiated tumour cells by regulating HIF-1α, MMP-2, MMP-9, and VEGF.

Chapter 8

Emilia sonchifolia (L.) DC: Evaluation of the possible therapeutic application in conventional chemotherapy

8.1. Introduction

There is a need to develop protective agents to combat major side effects of chemotherapy, without a negative impact on the immune system while not negating the expected therapeutic outcome. This study is a pioneer attempt to find out the ameliorating effect of an active fraction from the medicinal plant *Emilia sonchifolia*, enriched with the major sesqueterpene γ-humulene (γ-hum). The major urological side effects of oxazaphosphorine cytostatic drug cyclophosphamide (CP) include urinary bladder oedema, urothelial damage, haemorrhage and disturbances in voiding (Corrow and Vizzard, 2009). Hepatic cytochrome p450 oxidase converts CP to 4-hydroxy cyclophosphamide (Huttunen et al., 2011), and these renally excreted 4-hydroxyl metabolites is responsible for its toxicity. In addition induction of oxidative stress by acrolein, one of the major metabolite of CP has deleterious effect on the bladder epithelium paving way to hemorrhagic cystitis (Emadi et al., 2009). An effective uroprotector either stabilize these toxic metabolites and thereby prevent its release or detoxify the acrolein being generated.

Lot of substances were screened for their ability to act as regional antidots and among them the compound sodium 2-mercaptoethanesulfonate (MESNA) found to be most promising (Brock, 1989). Administration of MESNA to alleviate the urotoxic effects of CP is not completely free of inauspicious events (Berlin and Mu Hugh, 1999). Although as a known uroprotector available today, MESNA was used as standard in the present study. The system is said to be under oxidative stress when the levels of antioxidants are low or the levels of oxidants are high in such a situation the oxygen free radicals will be higher than their normal levels. Antioxidant compounds can be used in chemotherapy to protect against these harmful free radicals. Considering this aspect, we have done a thorough investigation of the effect of our test material on the renal antioxidant system.

8.2. Materials and Methods

Plant material: - An active fraction from *Emilia sonchifolia*, enriched with γ-humulene (71%) (γ-hum).

Dosage: - γ-hum 5mg/kg body weight was intraperitoneally (i.p) administered for five consecutive days.

Animals: - Swiss Albino mice (6-8 weeks old)

Experimental design

Swiss Albino mice divided into 4 groups with a number of 24 animals/group. The mice in Group I was kept as normal without any treatment. Remaining 3 groups were treated with CP (1.5mmol/kg body weight, single acute dose). Group III was administered with γ-hum and Group IV with MESNA (150 mg/kg body weight, single dose) before CP administration. Group II kept as control with CP alone. From each group 8 mice were sacrificed at 4th hour, 24th hour and 48th hour after CP administration. Urine was collected prior to sacrifice (Wood et al., 2001). Blood was collected after sacrifice by heart puncture, and serum was separated and used for further analysis.

8.2.1. Biochemical investigations

The excised kidney and urinary bladder of the sacrificed animals were rinsed in ice cold normal saline. Tissue homogenate of these organs were prepared in chilled Phosphate buffered saline (PBS) in order to perform following biochemical parameters.

8.2.1.1. Reduced glutathione (GSH)

Briefly 125µl of 25% TCA was added to 0.5ml of the homogenate and kept cooled on ice for five minutes. Ellman's reaction of GSH with Ellman's reagent

dithionitrobenzene was used for estimation of reduced GSH according to the method of Moron et al. (1979).

8.2.1.2. Lipid peroxidation, total protein, serum creatinine and urea nitrogen

The levels of malondialdehyde (MDA) was estimated by extracting MDA-thiobarbituric acid (TBA) adduct using TBA and its subsequent measurement at 532nm (Ohkawa et al., 1979). Total protein, serum creatinine and urea nitrogen content was estimated using standard kits according to the manufacturer's instructions.

8.2.1.3. Antioxidant enzyme measurements

The supernatant obtained after centrifugation (5,000g for 10 min at 4^0C) of the kidney homogenate was assayed for superoxide dismutase (SOD) using nitroblue tetrazolium (NBT) reduction method of Mc Cord and Fridovich, 1969. Glutathione peroxidise (GPx) by the method of Hafeman et al., 1974 and catalase (CAT) by Aebi, 1984.

8.2.2. Serum cytokine levels

Serum collected from the sacrificed animals were quantified for various murine cytokines such as IL-2, IFN-γ and TNF-α on the same day using highly specific quantitative sandwich enzyme-linked immunosorbent assay kits according to the instructions of the manufacturer.

8.2.3. Bladder morphology and histopathology

Urinary bladder removed immediately after sacrificing the animals was examined on three different time intervals 4, 24 and 48 hours respectively. The morphological changes like colour, inflammation etc. Were observed and were confirmed by three different persons (Davis and Kuttan, 2000). Histopathology was done by fixing the bladder in 10% formaldehyde. Different concentrations

of alcohol were used for further treatments, and then the tissue was fixed in paraffin wax. Staining of sections with 4μm thickness was done using eosin and hematoxylin and thereafter subjected to microscopic analysis.

8.3. Results

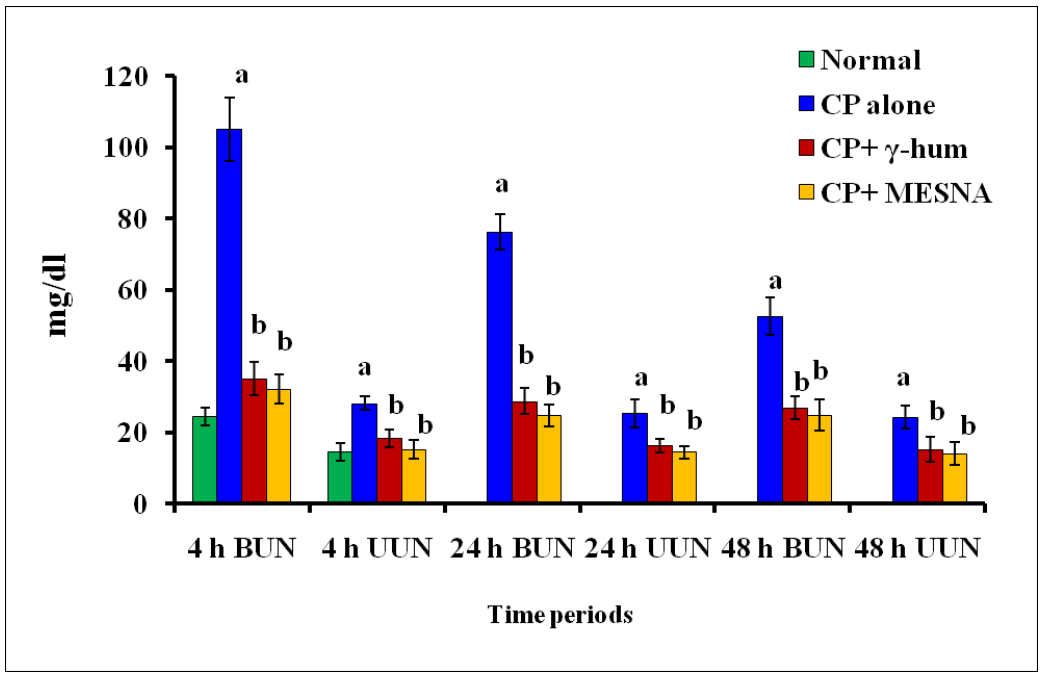
8.3.1. Effect of γ-hum on BUN and UUN

There was an elevation in the blood and urine urea nitrogen level in animals by CP treatment when compared to normal animals (BUN: 24.48±2.58mg/dl; UUN: 14.48±1.3mg/dl). In the CP treated animals the BUN levels were 105.08±8.91mg/dl (4h), 76.26±4.94mg/dl (24h), 52.51±5.31mg/dl (48h) and UUN levels were 28.13±1.93mg/dl (4h), 25.37±3.95mg/dl (24h), 24.27±3.3mg/dl (48h) indicating renal damage. γ-hum treatment significantly reduced ($P<0.001$) these elevated levels to (BUN 35.12±4.67mg/dl) (4h), 28.7±3.64mg/dl (24h), 26.81±3.18mg/dl (48h)) and (UUN 18.35±2.55mg/dl (4h), 16.22±1.84mg/dl (24h), 15.25±3.47mg/dl (48h)) as shown in figure 8.1. These results were almost similar to the established uroprotector MESNA indicating their protective effect from the CP induced toxicity.

8.3.2. Effect of γ-hum on the total protein and serum creatinine levels

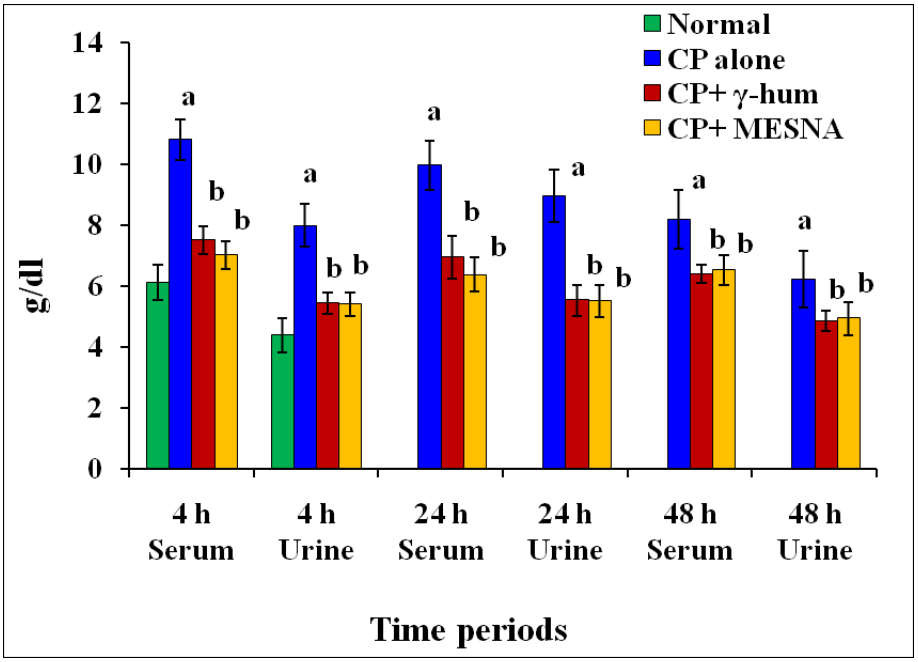
The CP administration drastically enhanced the total protein levels in serum to 10.81±0.67g/dl at 4h, 9.95±0.8g/dl at 24h and 8.19±0.96g/dl at 48h compared to the normal serum protein level 6.92± 0.57g/dl. Similarly the urine protein levels were elevated to 7.98±0.7g/dl at 4h, 8.95±0.85g/dl at 24h and 6.21±0.94g/dl at 48h compared to the normal urine protein level 4.38±0.53g/dl. The γ-hum treatment significantly diminished ($P<0.001$) these serum protein levels to 7.5±0.45g/dl at 4h, 6.94±0.71g/dl at 24h and 6.39±0.31g/dl at 48h and the protein in the urine to 5.44±0.35g/dl at 4h, 5.53±0.51g/dl at 24h and 4.85±0.32 g/dl at 48h and these results were almost comparable to the uroprotector MESNA. Similarly there was a hike in creatinine level in the serum by CP treatment (0.93±0.14mg/dl), (0.79±0.08mg/dl) (0.65±0.07mg/dl) at 4, 24 and 48h respectively. By γ-hum treatment this increased creatinine content was

Figure 8.1. Blood and urine urea nitrogen level



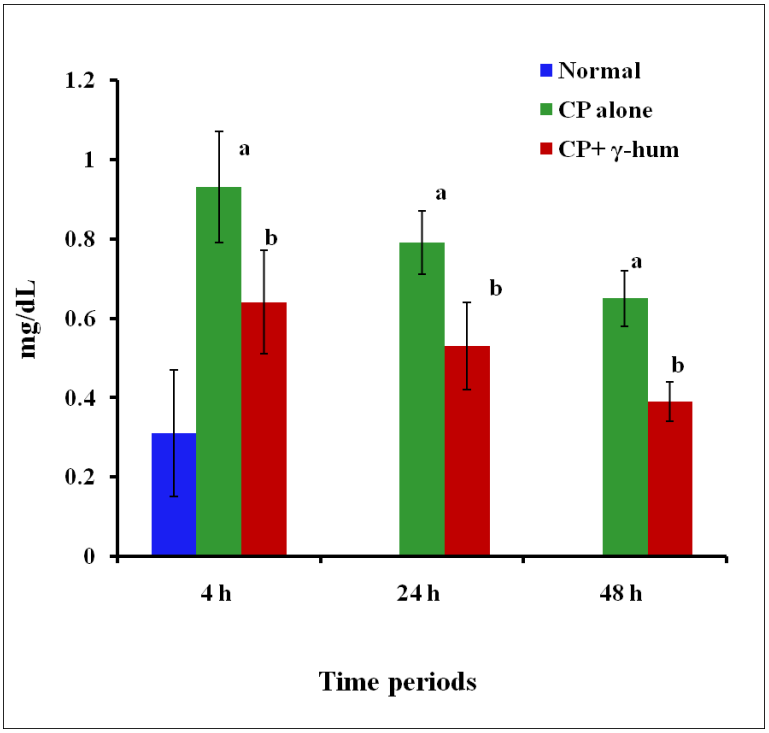
$^{a}p < 0.001$, $^{b}p < 0.01$

Figure 8.2. A. Total protein



$^{a}p < 0.001$, $^{b}p < 0.01$

B. Serum creatinine



$^{a}p < 0.001$, $^{b}p < 0.01$

reduced in a time dependent manner (0.64±0.13mg/dl), (0.53±0.11mg/dl) in 4h 24h and by 48thh attained a near normal value (0.39±0.05mg/dl), demonstrating its promising regulatory effect (figure 8.2).

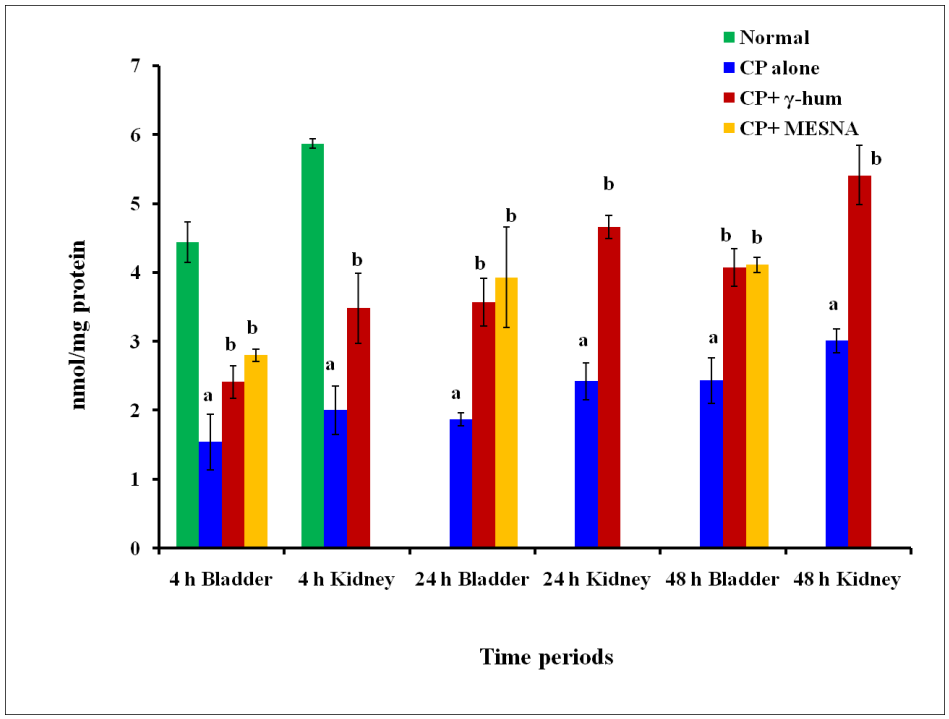
8.3.3. Effect of γ-hum on GSH levels

CP treatment reduced the bladder GSH levels (1.54±0.4nmol/mg protein (4h), 1.87±0.09nmol/mg protein (24h), 2.43±0.33nmol/mg protein (48h)) and renal GSH levels 2±0.35nmol/mg protein, 2.42±0.27nmol/mg protein, 3.01±0.17nmol/mg protein at 4, 24 and 48 hour respectively compared with normal levels of GSH in bladder 4.44±0.29nmol/mg protein and kidney 5.87±0.07nmol/mg protein. The CP along with γ-hum significantly increased the bladder glutathione levels to 2.41±0.24nmol/mg protein (4h), 3.57±0.35 nmol/mg protein (24h), 4.07±0.27nmol/mg protein (48h) similar to the results obtained by MESNA treatment. The renal glutathione levels were also increased by γ-hum treatment to 3.48±0.51nmol/mg protein (4h), 4.66±0.17nmol/mg protein (24h), 5.41±0.43nmol/mg protein (48h) as shown in figure 8.3.

8.3.4. Effect of γ-hum on lipid peroxidation and renal antioxidant system

The renal antioxidant levels of SOD, CAT and GPx in 3 different time intervals were depicted in figure 8.4. The renal antioxidant status was very much lowered in the CP treated animals. The γ-hum treatment enhanced this lowered levels showing its potent antioxidant and free radical scavenging effect. CP elevated the lipid peroxidation 1.16±0.32nmoles/mg protein, 2.5±0.47nmoles/mg protein, 2.25±0.34nmoles/mg protein, 2.09±0.27nmoles/mg protein at time intervals of 4, 24 and 48h. γ-hum significantly ($P<0.001$) reduced this levels to 1.75±0.12nmoles/mg protein, 1.26±0.21nmoles/mg protein, 1.13±0.33 nmoles/mg protein in the respective time intervals pointing out towards a cell level protection (Figure 8.4).

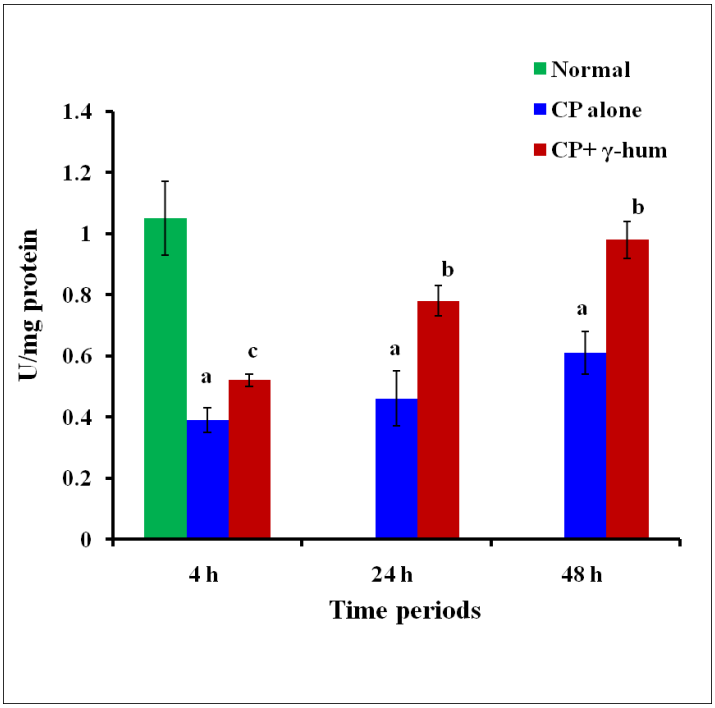
Figure 8.3. Bladder and kidney GSH level



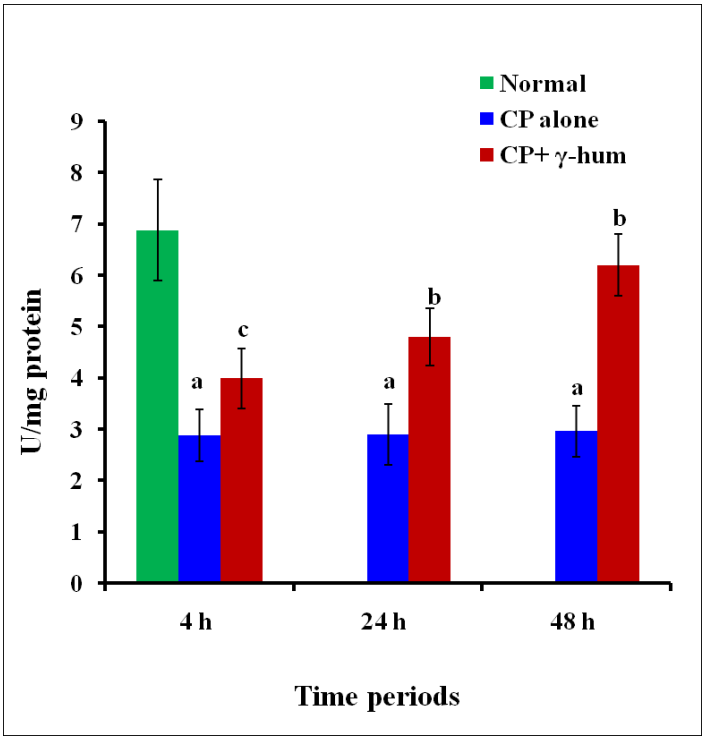
$^{a}p<0.001$, $^{b}p<0.01$

Figure 8.4. Renal antioxidant levels

A. SOD

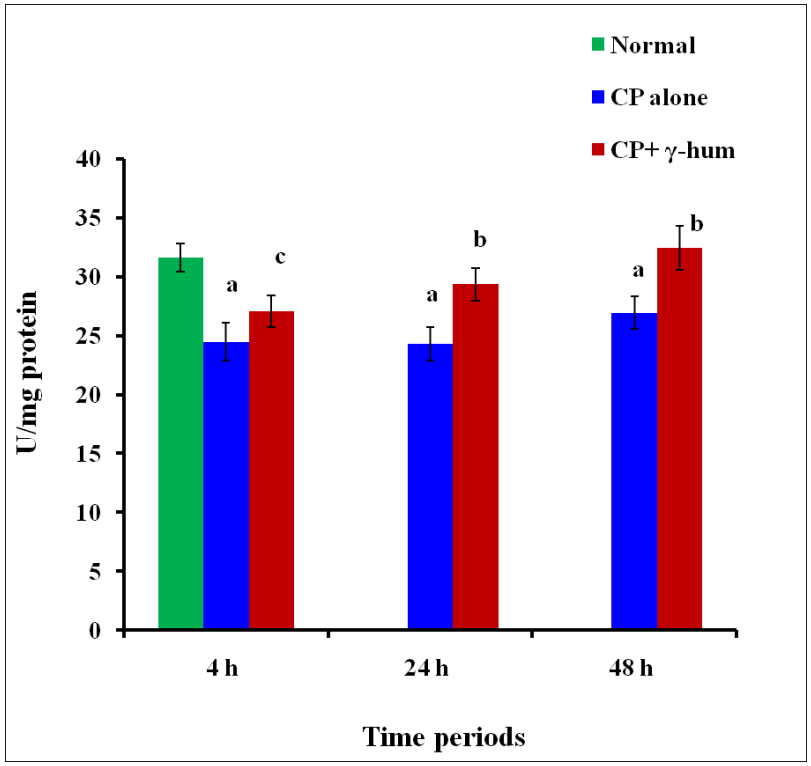


B. Catalase

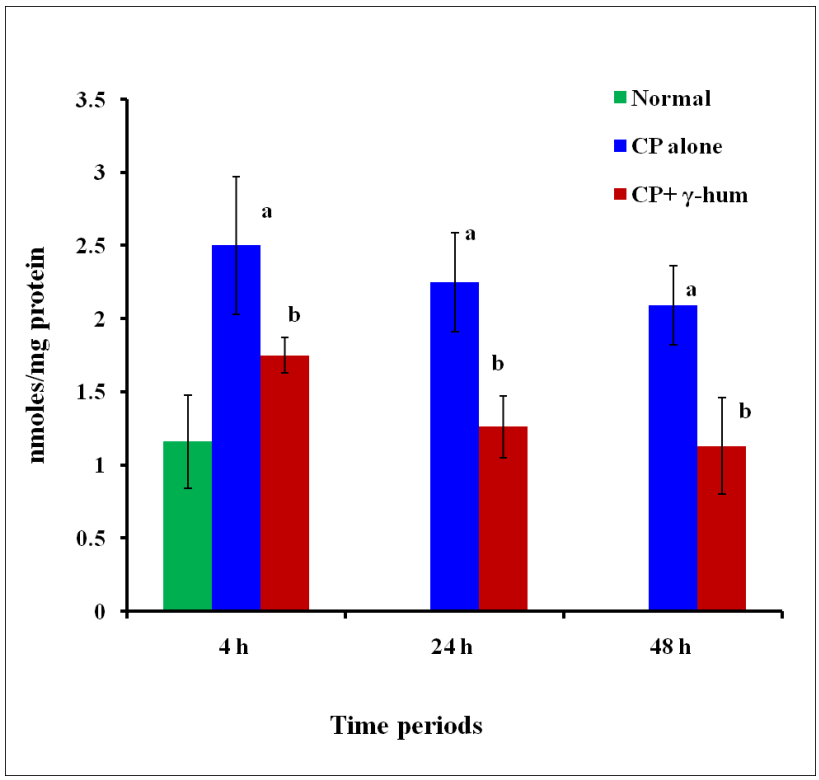


[a]p<0.001, [b]p<0.01, [c]p<0.05

C. GPx



D. Lipid peroxidation



[a]p<0.001, [b]p<0.01, [c]p<0.05

8.3.5. Effect of γ-hum on cytokine markers and histopathology

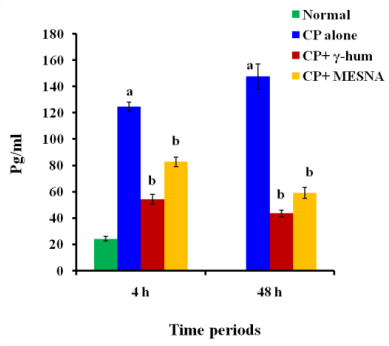
The three serum cytokine markers were positively regulated by γ-hum treatment. IFN-γ in CP+γ-hum and in CP+MESNA showed a time dependent increase that finally normalized in about 48 hours. IL-2 was also performed a similar strategy both compared to the decreased level in CP alone treatment. The effect was reversed in the case of TNF-α with a diminished level in the γ-hum and MESNA treated groups (figure 8.5). Apparent differences in the morphology of urinary bladder between control and treated groups were evident at different time intervals of sacrifice. There was an inflamed outlook with dark colouration at 4 h of CP treatment; in CP+γ-hum slight inflammation with usual colour was observed. Severe haemorrhage was shown at 24h of treatment in control compared to γ-hum+CP treated bladder which showed a tendency toward normalisation with slight haemorrahage. At 48h the condition became worse in CP group in comparison to γ-hum+CP that looked like normal. Use of the uroprotective agent MESNA produced appreciable results as well in the bladders. Bladder pathology was almost normalised by γ-hum treatment along with CP administration as shown in the histopathological sections at different time intervals (figure 8.6). In combination with γ-hum the necrosis of cells were reduced and the appearance was like normal epithelium with numerous folds and rugae.

8.4. Discussion

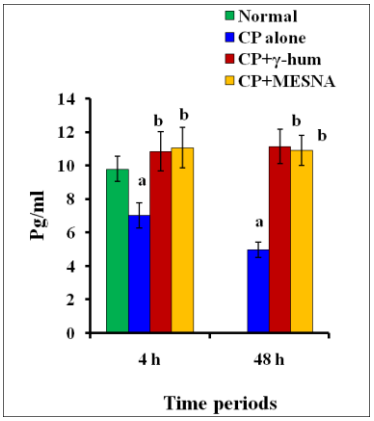
The toxic side effects of CP on the hepatic, urinary (Morandi et al., 2005), renal (Amudha et al., 2007) and haematopoietic (Papaldo et al., 2005; Schwartz et al., 2005) systems of the body often masks its beneficial therapeutic effects**.** Results obtained in the current study were found to be very promising for the usage of γ-hum, as an ameliorating agent against CP induced damages. γ-hum is found to be equal to or may be greater promising in comparison to MESNA because of its null side effects. The protective effects of γ-hum can be attributed to the already known free radical scavenging and anti-inflammatory effects of *E. sonchifolia*. The severely impaired bladder morphology along with bladder inflammation and oedema due to CP administration was markedly revamped by

Figure 8.5. Serum cytokine levels

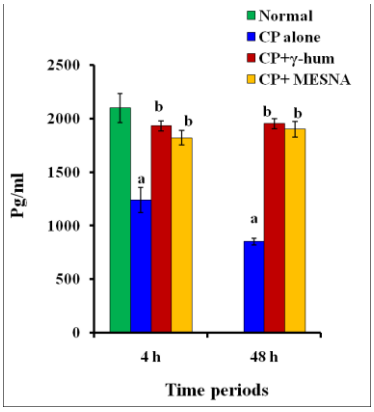
A. TNF-α



B. IL-2

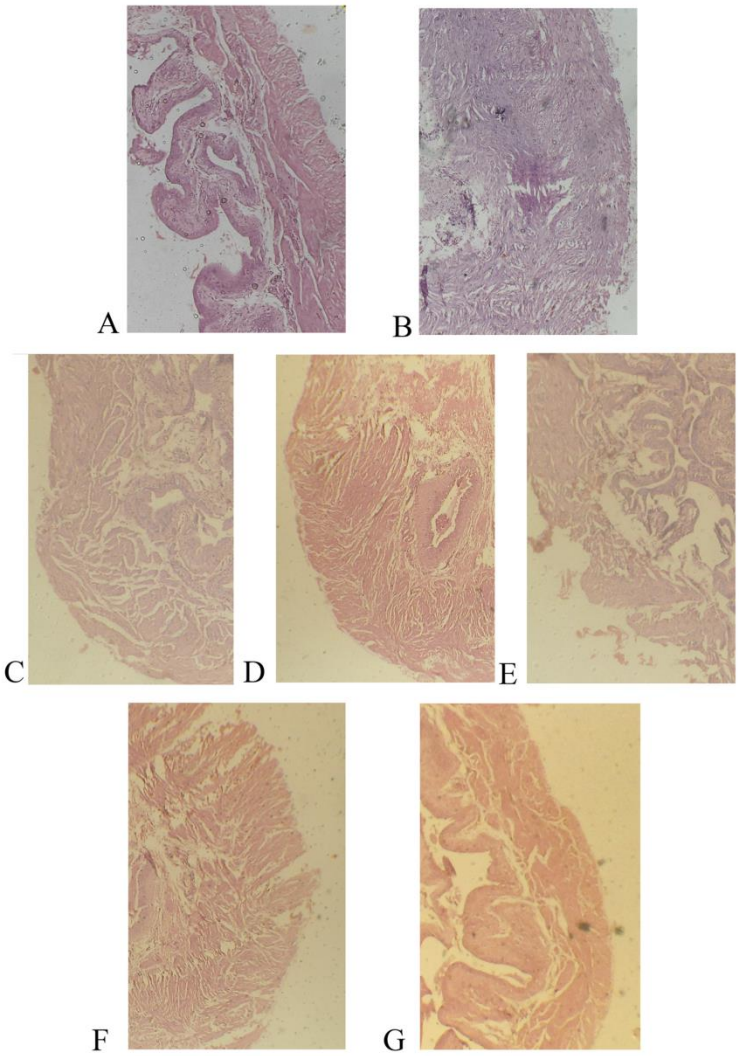


C. IFN-γ



$^{a}p<0.001$, $^{b}p<0.01$

Figure 8.6. Histopathological analysis of urinary bladder in cross section after different time points



A. Normal
B. 4h after CP administration
D. 24h after CP administration
F. 48h after CP administration
C. 4h after CP+γ-hum administration
E. 24h after CP+γ-hum administration
G. 48h after CP+γ-hum administration

γ-hum treatment as evident from the histopathology. Histology revealed about normal structural organization along with functional recovery of the bladder tissues.

The inflammation and shifts in overall redox cycling is due to the pro oxidative metabolites of CP and also by peroxidation and additional engendering of free radicals (Kehrer and Biswal, 2000). GSH maintains cell integrity by protecting cellular constituents from oxidising and alkylating agents or by its involvement in detoxification mechanisms (Masella et al., 2004) and its reduction make mitochondria susceptible to free radicals prodded by itself leading to severe after effects (Hatono et al., 1996). Interactions of acrolein with GSH and one of the constituent aminoacid of GSH will lead to the reduction in GSH level (Manesh and Kuttan, 2005) and the resultant death or apoptosis of the cells lining the bladder. This hike in lipid peroxidation and reduction in GSH in CP administered animals can be reversed remarkably by γ-hum administration as evident from the graphical depiction of the results.

Apart from this the antioxidant levels were significantly increased suggesting the role of the antioxidant system in alleviating the CP induced toxic effects. When the levels of antioxidant enzymes are elevated the vulnerability to cell injury will subsequently diminish (Werts and Gould, 1986). Cellular enzymatic antioxidants SOD, CAT and GPx offer significant protective effects (Cerutti et al., 1994). These antioxidants also regulate intra cellular LPO hike and reduced antioxidant enzyme content well exhibited while in CP administration. Impediment in peroxidase and catalase enzyme levels along with a hike in malondialdehyde (MDA) denotes the harm caused by the ROS produced and the resulting severe oxidative stress on the urinary bladder (Sulkowska et al., 1998). γ-hum significantly negate toxic effects as confirmed from the results of the renal antioxidant status after treatment with this test material in CP induced animals. This regulatory effect γ-hum on the antioxidant system may be attributed to its free radical scavenging activity or by inhibiting seepage of lipid peroxides that well complements the previous studies reporting the antioxidant effects of the plant *E. sonchifolia*.

In chronic bladder inflammatory conditions cytokines play a major role in enhancing or diminishing the severity by acting as targets of therapy or as markers to denote up to date status (Erickson et al., 2005). TNF- α is such a candidate that contribute to the pathogenic complications in various renal dysfunctions (Vielhauer and Mayadas, 2007). This situation of TNF-α elevation indicating toxicity of CP is found in the control animals. Our studies have demonstrated that γ-hum could reduce this elevated level denoting its protective action. The excessive release of IFN-γ is also a marker in severe inflammation and autoimmune conditions and its elevated level can check the length of inflammatory process (Muhl and Pfeilschifter, 2003). IL-2 inhibit IL-17 dependent inflammatory process by suppressing the formation of inflammatory T helper 17 (Th17) with modification of sites in the genes involved in Th 17 differentiation with STAT 5 substitution, in place of STAT 3 transcription factor and also by down regulating the expression of IL-6 receptor, thereby checking its further signalling processes. IL-2 also involved in the Tregs (Regulatoy T cells) development and maintenance (Laurence et al., 2007; Yang et al., 2011). Thus IL-2 limits overall inflammation by opposing actions on Tregs and the Th 17 cells. Studies revealed small amount of 1L-2 may reduce inflammation and alleviate the ailment (Koreth et al., 2011; Saadoun et al., 2011). The levels of these two cytokines were significantly increased by γ-hum as well as MESNA treatment revealing the significant ameliorating effects of γ-hum my modulating the immune response complimenting the results obtained in our recently published immunomodulatory study on the plant (George and Kuttan, 2015).

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Summary

In the present study we evaluated the inhibitory effect of selected plant natural products on the breast cancer development and progression along with an analysis of the effect of these products on other allied aspects of tumour development. For the study on metastasis we selected the syngenic mouse 4T1 breast tumour model and the plant isolates used were punarnavine, harmine and an active fraction from the plant *Emilia sonchifolia* (γ-hum) enriched with the major sesqueterpene γ-humulene (γ-hum). From the results of the study it was clearly evident that punarnavine the quinolizidine alkaloid, isolated from the plant *Boerhaavia diffusa* significantly inhibited the primary tumour growth in the orthotropic site of induction and increased the life span of animals in the treated groups. The organ specific metastatic progression of tumour from the primary site to the distant organs via the lymphatic system was efficiently reduced by the punarnavine administration. The assessment of biochemical parameters and the analysis of inflammatory cytokines along with a gene expression study of the major genes involved in the metastatic progression undeniably proved the potential of punarnavine in combating breast tumour development and progression compared to the tumour control and the other two plant isolates used in the study.

When we tried to analyse whether this potent alkaloid punarnavine can be used in conventional radiotherapy the results obtained were quite promising. Here we find out the efficacy of punarnavine in down regulating the expression of the hypoxia inducible factor α that contribute immensely to the survival of hypoxic tumour cells and their retrieved growth and tumour development even after an episode of radiation therapy. The hypoxia induced vascularisation via the vascular endothelial growth factor was also significantly inhibited by the punarnavine administration along with reduced tumour vasculature as evident from the immunohistochemical analysis. The effect of punarnavine administration on the tumour development in X irradiated animals resulted with a high level of reduction in subsequent tumour development in the primary site of tumour induction after different doses of radiation exposure. Further the treatment efficiently inhibited the invasion of irradiated cells with negatively influencing the activation of matrix metalloproteinases and thereby inhibiting its effects on influencing the inavasive capacity of surviving tumour cells. These

experiments for evaluating the potential of the usage of punarnavine in conventional radiotherapy using X irradiation in 4T1 tumour bearing mice once again proved the efficacy of this potent compound in the combinational therapeutic applications in conventional treatments.

The studies on the effect of the plant natural products on the other allied aspects of tumour growth and development revealed the effects of these isolates on the immune, inflammatory and angiogenic systems. The plant *E. sonchifolia* found to enhance the immune parameters, trigger stem cell proliferation as well as differentiation, and heighten antibody responses in a well-regulated way, which may be specifically mediated through various cytokine molecules. This immunomodulatory activity of the plant involved the combined action of humoral and cell-mediated immune responses as proven by the proliferation assay. Besides, the extract delivers enhanced CTL activity in tumour-bearing animals, which again signifies the augmented involvement of a cell-mediated immune system to defend against the tumour. Hence, it can be stated that the plant could definitely act as a nontoxic immunomodulator. As an immunomodulator, *E. sonchifolia* does not tend to overboost immunity but rather provides a stimulated and optimized immune response compared to the untreated group of animals. These results may be considered as a solid scientific evidence for its conventional and traditional medicinal uses for a number of ailments by regulating the body's defense system against pathological manifestations.

The active fraction from *E. sonchifolia* containing the major sesqueterpene γ-humulene found to be quite promising with its anti-inflammatory, antiangiogenic and applications in conventional chemotherapy. The anti-inflammatory effects of γ-hum was evident with a reduction in the paw oedema induced by acute and chronic inflammatory agents. The lipopolysaccharide induced inflammatory response was also significantly reduced by the treatment as evident from the levels of proinflammatory cytokine, C-reactive protein and nitric oxide levels. Gene expression analysis of the cycloxygenase (COX-2) and inducible nitric oxide (iNOS) also showed the effect of γ-hum in efficiently down regulating the genes involved in the process.

Antiangiogenic therapy that targets vascular growth within the tumor is now widely accepted to treat various tumours, because the agents used in this treatment modality have fewer side effects due to the quiescent nature of the blood vessels in adults. The present study clearly proved the inhibitory effect of γ-hum on tumor angiogenesis by efficiently decreasing MMPs, VEGF, and proinflammatory cytokines while at the same time increasing the level of TIMP. The retarding effect of γ-hum was clear-cut from the decreased capillary formation and prevention of microvessel outgrowth from the aorta. Additional evidence for the inhibitory effect of γ-hum is the impediment on endothelial cell proliferation, invasion, migration, and also hampering of the activation of proenzyme to active enzyme as evident by the gelatin zymographic analysis.

Harmful metabolites will be accumulated in the bladder than in any other areas and that makes this storage organ the most susceptible for the toxic effects of therapeutics. In this scenario we analysed the effect of γ-hum on the conventional chemotherapy using cyclophosphamide, the oxazaphosphorine cytostatic drug. The results obtained altogether proved the safeguarding effect of γ-hum in an *in vivo* experimental mice model. Current study revealed the protective effects of γ-hum by implicating reduced levels of urea nitrogen, total protein, creatinine and lipid peroxidation to almost normal. Revamping of GSH level, cellular antioxidants and marker cytokine levels towards positive amelioration. These results points towards the usage of γ-hum in combination for allevating the toxic side effects of conventional chemotherapy.

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