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., 2005a; Minn et al., 2005b). 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 breaching 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% O₂) to hypoxia (~1% O₂) is regulated by the hypoxia inducible factor -1 (HIF-1). This HIF-1 consists 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 cytoplasm 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 expressed 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 sesquiterpene 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
gas, 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_{13}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. Pharmacoglists 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\(^b\)), 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 (Jahanani 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, antimitagenic, antigenotoxic and hallucinogenic properties (Patel et al., 2012).