INTRODUCTION

Plants have a significant role in maintaining human health and improving the quality of human life for thousands of years and provided valuables in the form of medicines, beverages, cosmetics, and dyes. There exists a plethora of knowledge and information and benefits of herbal drugs in our ancient literature of Ayurvedic and Unani medicine. One of the earliest treatises of Indian medicine, the Charaka Samhita (1000 B.C.) mentions the use of over 2000 herbs for medicinal purposes. Exploration of the chemical constituents of the plants and pharmacological screening may provide us the basis for developing the leads for development of novel agents. In addition, herbs have provided us some of the very important life-saving drugs used in the armamentarium of modern medicine. However, among the estimated 250,000-400,000 plant species, only 6% have been studied for biological activity, and about 15% have been investigated phytochemically (Cragg et al., 1997). According to WHO survey 80% of the populations living in the developing countries rely almost exclusively on traditional medicine for their primary healthcare needs. The chemical constituents obtained from plants may be pharmacologically screened for developing novel agents (Balandrin et al., 1985; Cragg et al., 1997). Phytochemicals are compounds found in plants that are not required for normal functioning of the body, but have a beneficial effect on health or play an active role in amelioration of diseases. Phytonutrients have various health benefits, for example, they may have antimicrobial, anti-inflammatory, cancer preventive, antidiabetic, and antihypertensive effects to mention but a few (Ayoola et al., 2008).

Carcinogenesis is a multistage (initiation, promotion, and progression) process that encompasses multiple genetic and epigenetic events (Brickers et al., 1989; Brown and Balmain, 1995). Breast cancer is the most common cancer and cause of death in women and makes up one-tenth of all new cancer diagnoses worldwide (Bray et al., 2004; Ray et al., 2007). Breast cancer is one of the most serious problems in oncology. It is a leading cause of death among women in many countries. The American Cancer Society estimates that in 2005, approximately 2,11,240 women are newly diagnosed with this disease and 40,410 die annually. Despite significant advances in the treatment of breast cancer, this disease remains
the leading cause of death and the most commonly diagnosed cancers among women (Jemal et al., 2005, 2008). Development of mammary tumors requires aberrant accretion of cells caused by excessive proliferation, insufficient apoptosis or dysregulation of cellular differentiation (Hanahan and Weinberg, 2000; Kumaraguruparan et al., 2006). Epidemiological data from more than 250 case control and cohort studies shows an inverse relationship between the risk of certain types of cancer and consumption of dietary phytochemicals and fibers (Borek, 2004). Multiple mechanisms have been identified for the anti-neoplastic effects of plants, including antioxidant, anti-inflammatory and anti-proliferative activities and inhibition of bio-activating enzymes and induction of detoxifying enzymes (Le Marchand, 2002). Reactive oxygen species (ROS) are seemingly involved in a variety of important pathophysiological conditions including mutagenesis and carcinogenesis (Aruoma et al., 1994). Free radicals play an important role in tumor promotion by direct chemical reaction or alteration of cellular metabolic processes, and their scavengers (SOD and CAT) represents as inhibitors at different stages of carcinogenesis (Cerutti et al., 1985; Sun et al., 1990). The enzymes are found in cytosolic and mitochondrial functions mainly involved in the biotransformation and detoxification of carcinogens. The continuing severity and magnitude of the cancer problems make it imperative to develop chemopreventive strategies utilizing natural antioxidants to block the initiation, or arrest, or reverse the progression of pre-malignant cells. Antioxidants may protect against the toxicity of reactive oxygen species (ROS) by the prevention of ROS formation. The analysis of antioxidant levels is considered essential as they neutralize oxygen-free radicals. Development of life threatening diseases like cancer is linked to the availability of these antioxidants (Sen, 1995; Wilkinson and Clapper, 1997; Watterberg, 1997).

There has been an increasing interest in carcinogenic chemicals such as 7,12-dimethylbenz(a)anthracene (DMBA) to induce mammary carcinoma in rat models for the study of human breast cancers (Salami and Karami, 2003). The role of polycyclic aromatic hydrocarbons (PAH) is clearly implicated in the process of carcinogenesis especially 7,12-dimethylbenz[a]anthracene (DMBA), which is one of the most potent skin and breast carcinogens known (Lakshmi et al., 2009). DMBA induced experimental carcinogenesis is
preceded by a sequence of hyperplasia, dysplasia, and carcinoma (Shklar and Schwartz, 1996; Letchoumy et al., 2006). DMBA mediates carcinogenesis through formation of DNA adducts, DNA damage, generating excess reactive oxygen species and by producing chronic inflammation. Several studies suggested that DMBA mediated molecular, biochemical, genetic and histopathological changes were analogous to those observed in human cancers (Gimenez and Slaga, 1993; Miyata et al., 2001). DMBA-induced experimental carcinogenesis might therefore, be used as an ideal model to study the chemopreventive potential of medicinal plants and their active constituents. The evaluated results reported in DMBA induced carcinogenesis may assist the clinician in the diagnosis, prognosis and treatment monitoring of the cancer patients (Manoharan et al., 2009).

Angiogenesis, the formation of new blood vessels from the existing vascular network, is essential for continued tumor growth and metastasis (Folkman, 1992). Angiogenesis, the development of new blood vessels from pre-existing vasculature, has important roles in growth and development, wound healing and tumorigenesis. The vascular endothelial growth factor (VEGF) family of proteins has a pivotal role in regulating tumour angiogenesis (Shibuya and Claesson-Welsh, 2006). One of the most potent and specific angiogenic factors is VEGF, also known as vascular permeability factor and vasculotropin (Ferrara, 1996). Vascular endothelial growth factor (VEGF) is heparin-binding molecules with potent angiogenic properties both in vivo and in vitro. Vascular endothelial growth factor stimulates cell survival, migration and differentiation. It induces neovascularisation, and is required for the establishment of haematopoiesis; in malignant tumours, VEGF supports development of tumour vessels, which may lead to increased vascular permeability, and is shown to have a correlation with cancer prognosis and diagnosis (Folkman, 1995; Hormbrey et al., 2002). The growth of solid tumors is dependent on angiogenesis for the supply of nutrients and for the removal of metabolic waste products (Folkman, 1990, 1995). Higher VEGF levels have been found in the serum and urine of patients with different tumor types than in healthy individuals (Nguyen et al., 1994; Takei et al., 1994; Yamamoto et al., 1996; Heer et al., 2001) and in the serum of patients with metastatic disease than in those with localized disease (Kraft et al., 1999; Salven et al., 1999) Some studies in patients with breast cancer have investigated the potential value of
serum VEGF levels for diagnostic purposes (Takei et al., 1994; Yamamoto et al., 1996; Salven et al., 1997; Salven et al., 1999; Heer et al., 2001) or for monitoring the clinical course of disease (Pichon et al., 2000; Dirix et al., 1997; Sliutz et al., 1995; Findeisen et al., 2000).

Vascular endothelial growth factor (VEGF), a principal stimulant to endothelial cell growth and migration, is a 32- to 46-kDa homodimeric glycoprotein that promotes endothelial regeneration, stimulates the formation of collateral blood vessels, increases vascular permeability, and inhibits the function of antigen-presenting cells (Dvorak et al., 1999; Zachary, 1998). The VEGF family is composed of several isoforms (VEGF-A, B, C, and D) arising from tissue-specific, alternative splicing of transcripts from a single VEGF gene. VEGF is present in significant quantities in platelets, predominantly as the VEGF-A<sub>165</sub> isoform. Specific VEGF transmembrane tyrosine kinase receptors (Flt-1, KDR, Flt-4) are expressed almost exclusively on endothelial cells and mediate downstream intracellular second messenger pathways resulting in endothelial regeneration, stimulation of collateral blood vessel formation, increased vascular permeability, and lymphangiogenesis (Terman et al., 1992; De Vries et al., 1992). The VEGF family consists of VEGF-A, also called VEGF, which is produced in a number of splice variant isoforms, as well as VEGF-B, VEGF-C, and VEGF-D (Ferrara, 2002). VEGF-A is considered the most important of these with respect to tumor angiogenesis, especially the VEGF<sub>121</sub> and VEGF<sub>165</sub> splice variant isoforms (Ferrara, 2002). Many tumor types express abundant levels of VEGF, and the tumor cell population itself is usually the main source of this growth factor; infiltrating host stromal cells may also make a significant contribution in some cases (Ferrara, 2002). Both environmental and epigenetic factors, especially hypoxia (Shweiki et al., 1992) and genetic changes, including mutations that lead to activation of oncogenes or inactivation of tumor suppressor genes, can lead to VEGF induction or up-regulation in tumor cells (Rak et al., 1995). VEGF<sub>121</sub> and VEGF<sub>165</sub> can be secreted and bind to specific high-affinity VEGF receptor tyrosine kinases expressed by activated endothelial cells of newly formed blood vessels (Ferrara, 2002). VEGFR-2 appears the more functionally important of the two and can mediate signaling events involved in endothelial cell mitogenesis, migration, survival, and vascular permeability (Ferrara, 2002). In addition, VEGF can mobilize endothelial
precursor cells out of the bone marrow and into the peripheral circulation, where they can
differentiate and become incorporated into sites of ongoing angiogenesis (Ferrara, 2002; Asahara et al., 1999).

The p53 tumor suppressor protein is a critical regulator of cell cycle progression that
responds to DNA damage and certain other cellular stresses by arresting cell cycle
progression or by inducing apoptosis (Agarwal et al., 1998; Giaccia and Kastan, 1998; Prives, 1998). These responses are important for preserving the integrity of the genome of a
cell, thus preventing the transformation of a normal cell into a tumor cell. p53 normally is a
short-lived protein that is maintained at low levels in unstressed cells. After cells are
exposed to DNA damaging agents, nucleotide depletion, or hypoxia, the p53 protein is
transiently stabilized and accumulates in the nucleus. DNA damage also activates p53 as a
transcription factor, which in turn induces or represses the transcription of several that
regulate cell cycle progression. Both stabilization of the p53 protein and activation of its
sequence-specific DNA binding ability are widely believed to be mediated, at least in part,
by post-translational modifications to the p53 protein. p53 is phosphorylated on several sites
in its N-terminal transactivation domain as well as on several sites in the C-terminal
tetramerization/regulatory domain (Meek, 1998). While p53 seems to be dispensable for
normal development (Donehower et al., 1992), it plays an important role in regulating cell
fate in response to various stress conditions, either genotoxic (DNA alterations induced by
irradiation, UV, carcinogens, cytotoxic drugs) or not (hypoxia, nucleotide depletion,
oncogene activation, microtubule disruption, loss of normal cell contacts). The protein may
be viewed as a node for the stress signals, which are then transduced, mainly through the
ability of p53 to act as a transcription factor. p53 exerts its anti-proliferative action by
inducing reversible or irreversible (senescence) cell cycle arrest, or apoptosis. It may also
enhance DNA repair and inhibit angiogenesis. Many types of stresses may be encountered
during tumour development. The p53 function is often altered in cancer. It has been
suggested that p53 could have evolved in higher organisms specifically to prevent tumour
development (Vousden and Lu, 2002). It is believed that this specific action is exerted
mainly through the triggering of apoptosis (Haupt et al., 2003; Yu and Zhang, 2005).
Indeed, loss of p53 activity disrupts apoptosis and accelerates the appearance of tumours in
transgenic mice (Attardi and Jacks, 1999). In model rodent systems (e.g., DMBA-induced
mammary tumors in mice), p53 mutations in the pre-neoplastic lesions of the mammary glands are frequent (Jerry et al., 1993), though tumors seem to develop from the cells that retain their wild-type p53 status (Kito et al., 1996). In normal cells not exposed to stress, the level and activity of p53 are very low. Upon stress, p53 is activated through a series of post-translational modifications and becomes able to bind to specific DNA sequences. The p53 recognition sequence is very loose and has been found in several hundred genes that are differentially modulated (induced or repressed) depending on the cell type, the nature of stress and the extent of damage. At low cellular levels, p53 modulates only a subset of the genes regulated at higher levels. The kinetics of target gene modulation may also vary. In a study with a micro-array carrying 6000 capture sequences, 107 genes were found to be induced and 54 genes were repressed by p53 (Zhao et al., 2000).

The tumour suppressor gene p53 and its protein control critical cellular functions involved in apoptosis and in the control of the cell cycle (Lane, 1992; Harris, 1996). The encoded protein consists of 393 amino acids giving a molecular mass of 53, 000 Daltons. The N-terminal part of the protein is involved in transcription control, the middle portion is responsible for the DNA binding and the carboxy-terminal third of the protein facilitates the tetramarisation of the protein, which is claimed to be required for its function (Kirsch and Kastan, 1998). The p53 gene can be activated via the ataxia telangiectasia gene (ATM) by carcinogens, cytostatics, radiation, ultraviolet light, hypoxia or by an oncogene (Graeber et al., 1994; Harris, 1996; Serrano et al. 1997; Kirsch & Kastan, 1998; Bergh 1998). When cells acquire irreparable damage, the apoptotic machinery is activated, while in the case of repairable damage the cell cycle is retarded via p53-initiated downstream activation of the cyclin-dependent kinases. This results in inhibition of the cyclins, together with interaction with the retinoblastoma gene product aiming at controlling the cell cycle at specific check points. The normal p53 function can be inactivated by somatic and germ line mutations, binding to the oncogene murine double minute (MDM2) and binding to different viral oncoproteins (human papilloma virus protein E6, SV40 large T antigen, hepatitis B viral X protein, adenovirus protein E1A) (Harris, 1996). Somatic mutation of the p53 gene is, so far, the most common genetic abnormality described in human cancer. In pre-clinical model systems it was demonstrated that tumours with wild-type p53 status responded better to certain oncological therapeutic modalities than tumours with an altered p53 status (Clarke et
al., 1993; O’Connor et al., 1993; Lowe et al. 1993, 1994; Fan et al., 1994; Lim et al., 1994). Despite these straightforward findings it is also obvious that radiation and certain cytostatics may also induce apoptosis via a p53-independent pathway (Clarke et al., 1993; Beck and Dalton, 1997). However, the issue is complex, since mutant p53 has been claimed to interfere ‘with the p53-independent pathways of apoptosis’ (Li et al., 1998). p40, p51 and p73 have been demonstrated to have more or less sequence homology with p53 (Jost et al., 1997; Kaghad et al., 1997; Osada et al., 1998; Trink et al., 1998). These genes are now grouped together in the p53 family. How they are activated and may replace and function when p53 is mutated is not known. These aspects may be important both for p53-dependent and independent apoptosis.

First described in 1979, and initially believed to be an oncogene, p53 was the first tumour suppressor gene to be identified. p53 functions to eliminate and inhibit the proliferation of abnormal cells, thereby preventing neoplastic development. Abrogation of the negative growth regulatory functions of p53 occurs in many, perhaps all, human tumours. The p53 signalling pathway is in 'standby' mode under normal cellular conditions. Activation occurs in response to cellular stresses, and several independent pathways of p53 activation have been identified that appear to be dependent on distinct upstream regulatory kinases (Vogelstein et al., 2001). p53 remains the most commonly mutated gene in many common human cancers, with mutations estimated to occur in 50% of all cancers. Mutant proteins are almost always defective for sequence-specific DNA binding, and thus for transactivation of genes upregulated by the wild-type protein (Sigal and Rotter, 2000). Interestingly, the proportion of missense mutations in p53 is higher than that seen in other tumour suppressor genes, suggesting that expression of p53 mutants may confer selective advantage over and above loss of wild-type function (Hussain and Harris, 2000). Many human tumour-associated p53 mutants possess a number of properties absent from the wild-type protein (Sigal and Rotter, 2000). In a high proportion of cancers lacking mutations, p53 function is compromised by other recognised mechanisms (Vogelstein et al., 2001). In virus-associated cancers, this may occur via interaction with virally encoded proteins resulting in sequestration or enhanced degradation of p53. MDM2 binds to p53 and promotes the ubiquitination of the C-terminus of p53 and subsequent degradation. p14ARF
interacts with MDM2, preventing association of p53 and MDM2, and thereby stabilising p53. Degradation of p53 may therefore be inappropriately stimulated by overexpression of MDM2 or by deletion or epigenetic silencing of p14ARF. In undamaged cells, p53 levels are low because the protein is rapidly degraded through a ubiquitin-mediated pathway (Kubbutat et al., 1997; Haupt et al., 1997) that is mediated by the Mdm2, a protein that binds to the N terminus of p53 (Picksley et al., 1994; Kussie et al., 1996) and serves as an ubiquitin ligase (Honda and Yasuda, 1999). It was reported that incubating p53 with DNA-PK, a protein kinase activated by DNA strand breaks that phosphorylates human p53 in vitro on both Ser15 and Ser37 (Lees-Miller et al., 1992), inhibited the binding of p53 to Mdm2 (Honda and Yasuda, 1999; Pise-Masison et al., 1998). Thus, phosphorylation of these sites in vivo may stabilize p53 by preventing MDM2 binding. Loss of this protein has been reported in several common human cancers, particularly (but not exclusively) those in which the p53 gene is wild-type. Yet another mechanism of inactivation involves cytoplasmic sequestration of p53 protein, preventing nuclear localisation of the protein and thus inhibiting its activity. Intensive research efforts are presently being directed towards developing new methods to suppress the growth and progression of cancer cells in humans using small molecule therapeutics (Wang et al., 2003; Seemann et al., 2004). The qualitative and quantitative activity of p53 depends on its integrity (mutation status), its amount, and its specific posttranslational modifications induced by the activation of the different stress-induced signalling pathways. This leads to variable patterns of association between p53 and a number of other co-regulatory proteins, of which some may be tissue- or cell type-specific. Despite this complexity, p53 activity has been associated with prognosis and prediction of tumour response to various therapies and deserves further investigations with the perspective of developing more targeted treatments.

Cellular senescence is a stress response in which cell proliferation ceases after a finite number of cell divisions in normal somatic cells (Hayflick and Moorhead, 1961). Cellular senescence contributes to organisinal or tissue aging (Patil et al., 2005) as well as to cancer formation or development (Campisi, 2001). Diverse factors (i. e., telomere shortening, activation of oncogenes or tumor suppressor genes, oxidative stress, irradiation, cytotoxic chemicals, and inflammatory cytokines) induce cellular senescence (Collado et
Cellular senescence is also involved -directly or indirectly- in the pathophysiology of aging-associated diseases. In human tissues, especially skin (Dimri et al., 1995) and liver (Paradis et al., 2001), the number of senescent cells increases with age. It is well known that ultraviolet light from the sun is a key factor in inducing skin aging. UV irradiation causes cellular senescence in fibroblasts and keratinocytes, which, in turn, results in age-associated skin damages such as wrinkles and pigmentation (Makrantonaki and Zouboulis, 2007). Fibroblast senescence in skin ulcers also affects treatment efficacy and healing prognosis (Wall et al., 2008). Senescent cells are observed in many inflammatory tissues and tumor tissues such as rheumatoid arthritis (Schmid et al., 2004), osteoarthritis (Price et al., 2002; Dai et al., 2006), hepatitis (Paradis et al., 2001), chronic skin diseases (Harding et al., 2005), atherosclerosis (Minamino et al., 2003), benign prostate hyperplasia (Choi et al., 2000), and liver cancer (Paradis et al., 2001). Accumulation of senescent cells accelerates tissue damage due to the secretion of inflammatory cytokines and degrading enzymes and decreases the repair or regeneration capabilities of the tissue, contributing to the pathogenesis of aging-associated diseases (Campisi, 2005). Various plant extracts, including Persicaria hydropiper, Filipendula glaberrima, Nymphaea tetragona, Camellia japonica (Kim et al., 2007), horse chestnut (Fujimura et al., 2007), Malus doumeri (Leu et al., 2006), Paeoniae lactiflora (Lee et al., 2006) and Melothria heterophylla (Cho et al., 2006), have been reported to repress senescence phenotypes in fibroblasts. Hence, the presents study was conducted to determine p53 protein expression and phosphorylation using western blot on treatment with ethanolic extract of B. retusa to rats bearing mammary tumors.

Cancer cachexia is a common clinical problem that substantially impacts upon the quality of life and survival of cancer patients. The pathophysiology of this syndrome implicates tumour induced metabolic changes and immune response. Clinical manifestations include anorexia, chronic nausea and change in body image (Nayak, 2002). For all these reasons, it is important to develop a new strategy possessing anti-free radical scavenging and neoplastic properties. Hence, it is essential to investigate the phytotherapeutic origins in order to detect anticancer and free radical scavenging activities (Ioset et al., 2000). Moreover, the available semisynthetic anticancer drugs have more side effects and are
cytotoxic to human beings. Since modern medicine has no effective care for malignant cancers and tumors. Scientists are interested in finding a potent phytotherapeutic agent with non-cytotoxic properties (Wiart et al., 2000). Among several potential benefits of ayurvedic medicine, relief from cancer cachexia is the most valuable. Herbs used in cancer therapy provide not only total healing, but also reduces the side effects and cancer associated complications. It also avoids the need for supplemental therapy to manage cancer. Each herbal product contains multiple active principles that may operate synergistically, producing therapeutic benefits and lowering the risks of adverse effects. The anorexia or weight loss could be effectively managed by *Withania somnifera, Sida cordifolia, Asparagus racemosus, Vitis vinifera, Plumbago zeylenica, Tinospora cordifolia, Zingiber officinale, Coptidis rhizoma*, etc. These herbs have been shown to improve appetite, food intake, malnutrition, fatigue and sensation of well-being which could elicit body weight gain. These herbs may even stimulate the flow of digestive juices, thereby improving digestion and increasing the appetite. Thus, ayurvedic therapeutic regimen rejuvenates the body tissues, tones up the systems and act as a tonic to the body against cancer cachexia. This kind of orientation toward total healing and health promotion makes approach to cancer therapy promising. The ethanolic extract was more potent than aqueous extract in protecting against CCl₄ damage to enzyme activities and biochemical content, thus preventing hepatic and renal disorders in female albino mice (Cordeiro and Kaliwal, 2011). *B. retusa* possesses a potent protective effect and medicinal properties (Raja and Srilakshmi, 2010).

The bark of *B. retusa* has been reported to have antioxidant properties thus revealing protective effect of ethanolic extract against DMBA induced toxicity in female albino rats (Cordeiro and Kaliwal, 2011). The bark of *B. retusa* exhibited variations in their contents of phytoconstituents depending upon the geographical location from where they were collected which was evident and supported from their extractive values and total polyphenol content. The methanolic extract of the plant collected from Maharashtra and Andhra Pradesh regions and the plant collected from the region of Maharashtra was found to be superior with respect to extraction yield and radical scavenging activity. These differences may possibly be related to the natural climatic differences which occur over a particular geographical area to be influenced by several climatic factors (Banerjee and Bonde, 2011). A number of factors
influence the concentration of the active constituent’s particularly phenolic compounds present in the herbals. Some of the notable factors are time and period of collection, geographical origin and climatic conditions. Sometimes, the influence of these factors may lead to even absence of active constituents in the same plant collected from different regions, as evidenced by several research reports (Houghton., 1998; Bilia, 2002; Marcus et al., 2002; Banerjee and Bonde, 2011). In the first chapters, the phytochemical analysis has revealed the presence of bitter principles, flavonoids, saponins, coumarins, reducing sugars, proteins, tannins, quinones and glycosides which may be responsible for the various biological activity. The antimicrobial activity of the ethanolic extract was highest against broad range of microbes in comparison to other extract along with anticancer, hepatoprotective, nephroprotective and in vivo antioxidant activity was highest for the ethanolic extract was noted in earlier chapters. Hence, ethanolic extract of the stem bark material of Bridelia retusa was used in the present investigation to study in vivo anticancer activity in DMBA induced mammary carcinogenesis in Sprague Dawley rats.
MATERIALS AND METHODS

Animals

Sprague Dawley virgin female rats procured from private breeder aged 45-55 days weighing between 80-100 gm were used in the experiments. The animal experiments were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (493/01/a/CPCSEA). The Institutional Animal Ethics Committee approved experimental design performed in this study for the use of Sprague Dawley rats as an animal model for anticancer activity. The rats were maintained in P.G. Department of Pharmacology and toxicology, KVAFSU, Veterinary College, Bangalore. They were housed in separate polypropylene cages containing sterile paddy husk as bedding material. Standard pellet diet “Gold Mohar” (Hindustan Lever company, Mumbai) was provided along with tap water ad libitum. The rats were maintained under normal day/night schedule (12L:12D) at room temperature 25±2°C.

Chemicals and Treatment

DMBA 7,12-dimethylbenz(a)anthracene (Sigma-Aldrich) toxicity was induced by intra-mammary (subcutaneous) injections of 20 mg diluted in sesame oil (0.5 mg in 0.5 ml sesame oil prepared just before administration) given once per week for four weeks. Cyclophosphamide (Kidwai Cancer Hospital, Bangalore) was used at 1 mg/kg in 0.4 % carboxymethyl cellulose as standard anticancer drug. Bridelia retusa S. bark extracts were administered at doses below acute LD_{50} level of intoxication according to the body weight of the rats. Oral suspensions containing 50, 100 and 150 mg/kg b. wt. of ethanolic extract were prepared in (CMC) carboxymethyl cellulose (0.4%).

Experimental Schedule

Mammary tumors were induced by DMBA induced in rat model (Samy et al., 2006). Rats were divided into six groups of six rats each as follows. Carboxymethyl cellulose (CMC) suspension (0.5 ml in distilled water) was gavaged orally to group I with normal food and water (negative control) and DMBA treated rats were used for mammary cancer
studies (positive control). The group III received 1 mg/kg cyclophosphamide (CYC) and the remaining were given three doses 50, 100 and 150mg/kg of ethanolic extract to mammary cancer induced rats for 20 days after tumors were visible. The rats were monitored for change in body weight and tumors were palpated weekly during the experiment. The mammary glands were evaluated for the presence of grossly detectable mammary tumors and the dissected animals with tumors were photographed to provide identification record on the location and gross morphology of lesions. Blood was collected for estimation of blood and serum parameters. The tumor size was measured by vernier calipers after DMBA exposure. All the rats were necropsied by mild ether anesthesia and vital organs were dissected out. The oxidative stress parameters of superoxide dismutase, catalase and lipid peroxidation were assayed in the mammary tissue, liver, kidney and blood. Tissue portion was used for histological studies.

**Histological studies**

Freshly removed tissue were cleared off from the adnexal tissues using saline, placed in blotting paper and gently pressed in blotting paper to remove excess saline adhering to the organs. The representative tissues were collected in neutral buffered formalin (NBF) for histological study. The tissues were embedded in paraffin followed by sectioning at 5 μm using microtome and stained with haematoxylin and eosin (Luna, 1968).

**Serum parameters**

Blood was withdrawn from the retro-orbital plexus puncture technique using microhaematocrit capillary tubes under ketamine (40 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia. Blood samples (without anticoagulant) were collected and allowed to coagulate followed by centrifugation to obtain serum for estimation.

**Vascular Endothelial growth Factor (VEGF)**

VEGF levels were quantified using indirect enzyme immunoassorbent assay (ELISA) technique. The sample (100μl) was coated to the centre of the 96-well microtiter ELISA plates in 50 mM coating buffer (477 mg Na₂CO₃ and 879 mg NaHCO₃ in 300 ml distilled water, pH 9.6). The plates were covered with foil and incubated for 16 hrs or overnight at 4°C.
4°C. The plates were washed thrice with (0.1 % (v/v) Tween 20 in PBS) washing buffer (5 min each) followed by blocking with blocking buffer (3% Bovine Serum Albumin (BSA) in PBS) at room temperature. The plates were again washed thrice with washing buffer (5 min each). The immunoaffinity purified anti-VEGF antibody (primary antibody: rabbit anti-VEGF) were diluted in incubation buffer (1:1,000 in 0.05 M PBS (1.16 g Na₂HCO₃, 0.1 g KCl, 0.1 g K₃PO₄ and 4 g NaCl in 500 ml distilled water. pH 7.4), pH 7.4) of which100µl was added and incubated at 37°C for 2 hrs. The plates were washed thrice with washing buffer (5 min each) followed by incubation with 100µl of (secondary antibody: goat anti-rabbit ALP) secondary antibody (1:5,000) conjugated to ALP and incubated for 2 hrs at 37°C. The plates were again washed thrice with washing buffer (5 min each) and incubated with 100µl of pNPP substrate solution (Freshly prepared 5 mg p-nitro phenyl phosphate in 5ml substrate buffer (1 mM Diethanolamine buffer: 97 ml diethanolamine and 100 mg MgCl₂.6H₂O, adjust pH to 9.8 with concentrated HCl and make volume to 1000 ml)). After sufficient color development (if it is necessary) followed by addition of 50µl of (0.1 n NaOH) stop solution to the wells. The VEGF standards (10 pg/ml to 100 ng/ml) were prepared and run along with the samples as reference. Absorbance was read at 405 nm using Medispec ELISA reader.

**Serum Glutamic Oxaloacetic Transaminase (SGOT)**

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood. Elevated levels are found in myocardial infarction, cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases, primary muscle diseases. Decreased levels may be found in pregnancy, Beri Beri and Diabetic ketoacidosis. SGOT (ASAT) catalyzes the transfer of amino group between L-Aspartate and a Ketoglutarate to form Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with NADH in the presence of Malate Dehydrogenase to form NAD.

**Serum Glutamic Pyruvic Transaminase (SGPT)**

Alanine aminotransferase (ALAT) also known as glutamate pyruvate transaminase (GPT) is a transaminase. The highest levels are found in the liver and kidneys, and in
smaller amounts in heart and skeletal muscle. SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction. SGPT (ALAT) catalyzes the transfer of amino group between L-Alanine and a Ketoglutarate to form Pyruvate and Glutamate. The Pyruvate formed reacts with NADH in the presence of Lactate Dehydrogenase to form NAD.

The estimation of SGPT and SGOT was performed using clinical chemistry analyzer, Microlab 300 (Vitalab Scientific, Netherlands) and commercially available diagnostic kits from Merck (Merck specialities Ltd., Ambernath) and following the instructions furnished in the leaflets supplied along with kits. The estimation of SGOT and SGPT were performed by taking 1ml of the reagent present in the kits into a test tube containing 100 μl of serum. The solution was mixed thoroughly and incubated at 37°C for 5 minute. Later the solution was processed by placing the test tube in semiautomatic biochemistry analyzer. One international unit (IU) of SGOT and SGPT (IU/L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

**Oxidative stress parameters in tissues**

**Preparation of tissue homogenate**

Immediately after sacrificing the animals, the tissues were processed for the estimation of antioxidant enzymes (Bruce and Baudry, 1998). The tissue was washed in ice cold normal saline and blotted dry followed by storage at -20°C for further analysis. The tissue was crushed in ice cold 0.05 M phosphate buffer (pH 7.4) and 10% homogenate (w/v) was prepared. The homogenate was centrifuged at 1,500 g for 1 hr at 4°C. The supernatant was used for estimation. The estimation of protein was carried out by method of Lowry et al. (1951) as described in chapter III.

**Estimation of lipid peroxidation (LPO)**

The level of TBARS (thiobarbituric acid reactive substances) in the tissue homogenate was estimated (Ramamarayan et al., 2000). The reaction of malonaldehyde
(MDA), breakdown product of polyunsaturated acids with thiobarbituric acid (TBA) to give a brilliant pink color product that can be estimated by measuring absorbance at 532 nm.

The homogenate was suspended in 0.1 M Tris HCl (pH 7.4) and peroxidation was terminated with ice cold, 76% trichloroacetic acid (TCA) in 2.3 N HCl followed by addition of freshly prepared TBA reaction mixture (water: 67 μM BHT in ethanol: 1.5% TBA in 0.2 M tris HCl , pH 7.0:: 1:1:5). The mixture was vortexed and incubated at 80°C for 30 min. The TBA test was stopped by plunging into ice bath and adding ice cold 91% TCA followed by chloroform and centrifuged at 2000 g for 15 min at 4°C. The chloroform phase was separated and absorbance measured at 532 nm. The total TBARS was expressed in terms of nmol MDA/ min/mg proteins.

**Estimation of superoxide dismutase (SOD)**

Superoxide dismutase (EC 1.15.1.1) was determined as per method described by Marklund and Marklund (1974). Superoxide is an ion produced as a product of auto-oxygenation of pyrogallol at pH 8.2. The ability of this enzyme to inhibit auto-oxygenation of pyrogallol provides basis for enzyme activity.

The tissue homogenate is treated with ethanol and chloroform followed by centrifugation at 13,000 g for 15 min at 4°C. The supernatant in 0.2 M tris HCl was treated with pyrogallol and the absorbance measured for 3 min at 420 nm. As reference pyrogallol in distilled water was used. The enzyme activity was expressed as units/mg protein. One unit of enzyme corresponds to the amount of enzyme that inhibits pyrogallol auto-oxygenation by 50%.

**Estimation of catalase (CAT)**

Catalase (EC 1.11.1.6) was determined as per method described by Caliborne (1985). The enzyme activity was determined by monitoring the decrease in absorbance at 240 nm due to H₂O₂ decomposition. The difference in extinction coefficient per unit time was used as measure of catalase activity.
The homogenate was treated with 30 mM H₂O₂ and the absorbance measured at intervals of 1 min at 240 nm. Blank of distilled water was used. The enzyme activity was expressed as μmol of H₂O₂ decomposed per min per mg protein.

**Oxidative stress parameters in blood**

Blood was withdrawn from the retro-orbital plexus puncture technique using microhaematocrit capillary tubes under ketamine (40 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia. Blood samples using calcium disodium EDTA as anticoagulant were collected. RBCs were collected after centrifugation at 2000 g for 10 min at 4°C. It was then suspended in phosphate buffered saline (PBS) for estimation of antioxidant enzymes.

**Estimation of lipid peroxidation (LPO)**

The level of TBARS (thiobarbituric acid reactive substances) in the tissue homogenate was estimated. The reaction of malonolaldehyde (MDA), breakdown product of polyunsaturated acids with thiobarbituric acid (TBA) to give a brilliant pink color product that can be estimated by measuring absorbance at 532 nm (Stocks, 1971).

Trichloroacetic acid (TCA), 28% was added to the RBC suspension in PBS and centrifuged. To the supernatant 1% thiobarbituric acid was added, heated in boiling water for 60 min and cooled immediately. The absorbance was measured spectrophotometrically at 532 nm.

**Estimation of superoxide dismutase (SOD)**

Superoxide dismutase (EC 1.15.1.1) was determined as per method described by McCord (1969). Superoxide is an ion produced as a product of auto-oxidation of pyrogallol at pH 8.2. The ability of this enzyme to inhibit auto-oxidation of pyrogallol provides basis for enzyme activity.

It was estimated in the erythrocyte lysate prepared from the RBC suspension. To the lysate, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 min by spectrophotometer.
**Estimation of catalase (CAT)**

The enzyme activity was determined by monitoring the decrease in absorbance at 240 nm due to H$_2$O$_2$ decomposition. The difference in extinction coefficient per unit time was used as measure of catalase activity.

Catalase (EC 1.11.1.6) activity was determined in erythrocyte lysate using Aebi’s method with some modifications. The lysate was added to a cuvette containing 2 ml of phosphate buffer (pH 7.0) and 30 mM H$_2$O$_2$. Catalase activity was measured at 240 nm for 1 min using spectrophotometer. The molar extinction coefficient of H$_2$O$_2$, 43.6 M cm$^{-1}$ was used to determine the catalase activity.

**Expression and phosphorylation of p53 protein**

Total protein was isolated from the given tissue samples in the presence of protease and phosphatase inhibitors (1.5 ml of 1X PBS). The quantification of the protein extracted was done using Bradford method and the protein concentration was found to vary from 0.5 mg/ml to 1 mg/ml. 5 µl of p53- polyclonal antibody (Igenex, San Diego) was added to the above protein extract to capture p53 antigen, this protein and p53-Ab complex was captured with protein A resin beads. After incubation, the beads were collected, washed and loaded on 12% SDS-PAGE in three sets. One set was stained with Coomassie blue stain and the other gels were subjected to electrotransfer onto Nitrocellulose membrane used for western blotting experiments. After blotting, the membrane was blocked with 3% BSA for overnight at 4°C. The two membranes were then probed separately with p53-polyclonal antibody (Igenex, San Diego) and $\alpha$-phosphotyrosine antibody. The blots were then developed and analyzed.

**Statistical analysis**

The statistical significance between control and experimental data was subjected to analysis of variance (ANOVA) together with Dunnett’s test (p<0.05) as described in chapter III.
OBSERVATIONS

a. Effect of ethanolic extract of stem bark of Bridelia retusa (EESBB) on body, organs weight and tumor size of DMBA treated rats (Table 5.1; Graph 5.1).

Body weight

In control rats, body weight gain was 46.17 g which on exposure to DMBA was 33.83 g. The body weight gain after treatment with cyclophosphamide (standard drug), 50, 100 and 150 mg/kg of ethanol extract to DMBA induced mammary cancer in rats was 38.84, 34.34, 35.84 and 38.17 g, respectively.

The toxic effect of DMBA was observed by significant decrease in the body weight of rats when compared with that of normal control. The protective activity of the ethanolic extract was justified by the significant increase in the weights when compared with that of DMBA treated group. Weight loss observed in DMBA treated group was rectified best in 150 mg/kg extract treated group which was almost that of standard drug.

Tumor Size

The control rats showed no tumors but the tumor size observed on exposure to DMBA was 24.42 mm while the tumor size obtained after treatment with cyclophosphamide (standard drug), 50, 100 and 150 mg/kg of ethanol extract to DMBA induced cancer in rats was 6.40, 7.78 6.96 and 6.63 mm, respectively. The carcinogenic effect of DMBA was observed by significant increase in the tumor size of rats when compared with that of normal control. The protective activity of all doses of ethanolic extract was justified by the significant decrease in the tumor size in extract and standard drug treated groups when compared with that of DMBA treated group. The decrease in tumor size observed in extract treated group was maximum in 150 mg/kg which approached that of standard drug.

Histologic observation of the mammary tissue of control rats revealed that the branch duct system is surrounded by relatively dense fibrous interlobular tissue. The duct system is lined by cuboidal or columnar epithelial cells with oval nuclei (Fig. 1). Histologic examination of the mammary tissue of rats treated with DMBA showed invasion of tissue
by ductal breast carcinoma along with profound dysplasia expanding into stroma. In the ductal carcinoma, necrosis was also detected which was not only restricted to the ducts (Fig. 2). Histologic study of mammary tissue of rats treated with DMBA and cyclophosphamide showed overall appearance of the normal mammary tissue with minimum ductal carcinoma restricted to ducts (Fig. 3). Histologic observation of the mammary tissue of rats treated with DMBA along with 50 and 100 mg ethanol extract showed decreased invasion and dysplasia with appearance of glandular spaces (Fig. 4 and 5). Histologic observations of the mammary tissue of rats treated with DMBA and 150 mg extract revealed the overall appearance approaching the normal mammary tissue with minimum carcinoma (Fig. 6). Thus, the ethanolic extract showed decrease in dysplasia and ductular carcinoma in the mammary tissue when administered to DMBA treated rats, thus providing gradual protection against the toxic effects with best effects at 150 mg which was comparable to standard drug.

**Organs weight**

**Liver**

In control rats, the weight of liver was 2.88 g which on exposure to DMBA was 2.23 g. The change in body weight after treatment with cyclophosphamide (standard drug), 50, 100 and 150 mg/kg of ethanol extract to DMBA induced cancer in rats was 2.82, 2.47, 2.64 and 2.70 g, respectively. The toxic effect of DMBA was observed by significant decrease in the weight of the liver in rats when compared with that of normal control. The ethanolic extract caused significant increase in the weights when compared with that of DMBA treated group, though cyclophosamid caused more significant increase. The decrease in weight observed in DMBA treated group was rectified best by standard drug while the extract groups were less effective.

The histologic observations of the liver in control rats revealed normal structure of the liver lobules. The hepatic lobules possesses the rows of cuboidal or polyhedral hepatic cells which forms hepatic cords from the center to periphery of the lobule. Hepatic cords were arranged radially around the central vein (Fig.1). In the rats treated with DMBA, histologic study of the liver revealed dilation of central vein and infiltration around portal triad (Fig. 2). Histologic study of liver of rats treated with DMBA and cyclophosphamide
showed overall appearance of the normal tissue with minimum damage (Fig. 3). In the DMBA treated rats with 50, 100 and 150 mg/kg extract, histologic study of the liver exhibited almost normal structure of liver lobules showing hepatic cords with cuboidal or polyhedral hepatic cells with sinusoids (Fig. 4, 5, 6). The histological changes caused due to DMBA in liver on treatment with ethanolic extract showed a reversal to normal appearance wherein 150 mg gave the best results which was comparable to standard drug.

**Kidney**

In control rats and DMBA treated rats, the weight of kidney was 0.65 and 0.56 g, respectively. The change in body weight after treatment with cyclophosphamid (standard drug), 50, 100 and 150 mg/kg of ethanol extract to DMBA induced cancer in rats was 0.61, 0.58, 0.59 and 0.61 g, respectively. The toxic effect of DMBA was observed by significant decrease in the weight of kidney in cancer bearing rats when compared with that of normal control. The ethanolic extract showed a non significant increase in the weights when compared with that of DMBA treated group. Neither the standard drug nor the extract showed a significant protection against kidney weight loss observed in DMBA treated rats.

Histologic observations of the kidney of control rats revealed that the cortex consists of numerous Bowman’s capsule which are double layered cup like structure inside highly anatomizing network of afferent and efferent arterioles called glomerulus. The cortical tubules were well organized with connective tissue and inter tubular spaces. Tubular walls were made up of thick epithelial cells (Fig. 1). Histologic examination of the kidney of rats treated with DMBA showed glomerular which were loosely attached to Bowman’s capsule (Fig 2). Histologic study of kidney of rats treated with DMBA and standard drug displays tubular cells with almost normal glomerulus (Fig. 3). Histologic observation of the kidney of rats treated with DMBA and 50 mg ethanol extract showed decreased glomerular and tubular epithelial damage (Fig. 4). Histologic observations of the kidney of DMBA treated rats with 100 and 150 mg ethanol extract revealed lesser glomerular and tubular epithelial damage with mostly normal appearance (Fig. 5 and 6). The DMBA histological changes in kidney on treatment with ethanolic extract showed a reversal to normal appearance wherein 150 mg gave the best results which was comparable to standard drug.
b. Effect of ethanolic extract of stem bark of *Bridelia retusa* (EESBB) on serum parameters of DMBA treated rats. (Table 5.2; Graph 5.2)

**vascular Endothelial Growth Factor (VEGF)**

Serum VEGF levels in control and DMBA treated rats were 480 pg and 280 ng, respectively. The serum VEGF levels after treatment with cyclophosphamide (standard drug), 100 and 150 mg/kg of ethanol extract to DMBA induced cancer in rats was 800 pg, 6 and 3.2 ng, respectively.

The serum VEGF levels significantly increased in tumor bearing rats in comparison to the normal control rats which was reduced significantly on treatment with cyclophosphamide and ethanolic extract indicating their anti-angiogenic potentials. The lowering in serum VEGF levels due to cyclophosphamide was better than the highest concentration of 150 mg of ethanolic extract.

**Aspartate Transaminase (ASAT) / Serum Glutamic Oxaloacetic transaminase (SGOT)**

In the control rats, activity of SGOT was 140.40 IU which on exposure to DMBA was 185.61 IU. The SGOT activity after treatment with cyclophosphamide (standard drug) was 146.62 IU but with 50, 100 and 150 mg/kg of ethanol extract to DMBA rats was 168.70, 162.54 and 155.75 IU respectively.

The effect of DMBA was observed to be toxic due to the significant increase in the activity of SGOT when compared with that of normal control. The protective effect was observed by significant decrease in the activity of SGOT of extract treated group when compared with that of DMBA control. The activity of standard drug showed significant decrease which was better than highest concentration of extract treated group, which gave best results in comparison to other extract treated groups.
Alanine Transaminase (ALAT) / Serum Glutamic Pyruvic transaminase (SGPT)

In the control rats and DMBA treated rats, activity of SGPT was 50.44 and 71.32 IU, respectively. The SGPT activity after treatment with cyclophosphamide (standard drug) 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 57.54, 69.49, 63.71 and 60.65 IU, respectively.

The activity of SGPT significantly increased in the DMBA treated group when compared with that of normal control. The extract showed significant decrease in the activity of SGPT when compared with that of DMBA treated control rats. Among the extract treated group, 150 mg/kg showed significant decrease and similar activity of SGPT to that of standard drug.

c. Effect of ethanolic extract of stem bark of *Bridelia retusa* (EESBB) on oxidative parameters (lipid peroxidation, SOD and CAT) in mammary tissue, liver, kidney and blood of DMBA treated rats. (Tables 5.3, 5.4, 5.5, 5.6 and 5.7; Graphs 5.3, 5.4, 5.5, 5.6 and 5.7)

**Lipid peroxidation (LPO)**

**Serum**

In the control rats, lipid peroxidation was 1.74 nmole which on exposure to DMBA was 1.94 nmole. The lipid peroxidation after treatment with cyclophosphamide (standard drug) was 1.86 nmole but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 1.97, 1.92 and 1.91 nmole, respectively.

The toxic effect of DMBA was justified by the significant increase in lipid peroxidation when compared with that of normal control. The antioxidant effect of extract was observed by significant decrease in lipid peroxidation when compared with that of DMBA treated control rats. The 150 mg/kg extract treated group showed similar and significant decrease in LPO to that of standard drug, which gave best results in comparison to other extract treated groups.
**Erythrocytes**

In the control rats, lipid peroxidation was 3.41 nmole which on exposure to DMBA was 4.22 nmole. The lipid peroxidation after treatment with cyclophosphamide (standard drug) was 3.51 nmole but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 3.80, 3.62 and 3.59 nmole, respectively.

The toxic effect of DMBA was justified by the significant increase in lipid peroxidation when compared with that of normal control. The antioxidant effect of extract was observed by significant decrease in lipid peroxidation when compared with that of DMBA positive control. The 150 mg/kg extract treated group showed significant decrease in LPO and hence similar antioxidant activity to standard drug, which gave best results in comparison to other extract treated groups.

**Mammary tissue**

In the mammary tissue of the control rats, lipid peroxidation was 0.70 nmole which on exposure to DMBA was 1.86 nmole. The lipid peroxidation after treatment with cyclophosphamide (standard drug) was 1.26 nmole but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 1.74, 1.59 and 1.30 nmole, respectively.

The toxic effect of DMBA was justified by the significant increase in lipid peroxidation in the mammary tissue when compared with that of rats of normal control. The antioxidant effect of extract was observed by significant decrease in lipid peroxidation in the mammary tissue when compared with that of DMBA treated control rats. The 150 mg/kg extract treated group showed significant decrease in LPO and similar antioxidant activity to that of standard drug, which gave best result in comparison to other extract treated groups.

**Liver**

In the liver of the control rats, the lipid peroxidation was 0.55 nmole which on exposure to DMBA was 1.85 nmole. The lipid peroxidation after treatment with cyclophosphamide (standard drug) was 0.96 nmole but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 0.94, 0.93 and 0.92 nmole, respectively.
The lipid peroxidation significantly increased in the liver of DMBA treated group when compared with that of normal control. The extract showed antioxidant effect by significant decrease in lipid peroxidation in the liver when compared with that of DMBA treated control. Among the extract treated group, 150 mg/kg showed similar and significant decrease in lipid peroxidation to that of standard drug.

**Kidney**

In the control rats, lipid peroxidation in the kidney was 0.75 nmole while that on exposure to DMBA was 1.95 nmole. The lipid peroxidation after treatment with cyclophosphamide (standard drug), 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 1.04, 0.98, 0.95 and 0.94 mg, respectively.

The effect of DMBA was observed to be toxic due to the significant increase in lipid peroxidation in the kidney when compared with that of normal control. The antioxidant effect was observed by significant decrease in lipid peroxidation in the kidney of extract treated group when compared with that of DMBA control. The antioxidant activity of standard drug was similar and significant decrease in LPO to 150 mg/kg extract treated group, which gave best results in comparison to other extract treated groups.

**Superoxide dismutase (SOD)**

**Serum**

In control rats, activity of SOD was 3.24 μmoles and that of DMBA control rats was 2.44 μmoles. The SOD activity after treatment with cyclophosphamide (standard drug) was 3.15 μmoles but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 3.06, 3.08 and 3.16 μmoles, respectively.

The effect of DMBA was observed to be toxic due to the significant decrease in the activity of SOD when compared with that of normal control. The antioxidant effect was observed by significant increase in the activity of SOD in the extract treated group when compared with that of DMBA control. The antioxidant activity of group treated with
standard drug was significant increase in SOD activity and similar to 150 mg/kg extract treated group, which gave best results in comparison to other extract treated groups.

**Erythrocytes**

In the control rats, activity of SOD was 3.30 Units which on exposure to DMBA was 2.60 Units. SOD activities after treatment with cyclophosphamide (standard drug) was 4.14 Units but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 3.06, 3.56 and 3.84 Units, respectively.

The activity of SOD significantly increased in the DMBA treated group when compared with that of normal control. The extract showed antioxidant effect by significant increase in the activity of SOD when compared with that of DMBA treated control group. The activity of SOD was brought to that of normal by 150 mg/kg of extract treated group which was significantly less than cyclophosphamide treated group.

**Mammary tissue**

In the mammary tissue of the control rats, activity of SOD was 3.47 Units which on exposure to DMBA was 1.50 Units. The SOD activity after treatment with cyclophosphamide (standard drug) was 3.56 Units but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 3.56, 2.61, 2.84 and 3.04 Units, respectively.

The toxic effect of DMBA was justified by the significant decrease in the activity of SOD in the mammary tissue when compared with that of normal control. The antioxidant effect of extract was observed by significant increase in the activity of SOD in the mammary tissue when compared with that of DMBA cancer control. The 150 mg/kg extract treated group showed significant decrease in LPO and similar antioxidant activity to that of standard drug, which gave best result in comparison to other extract treated groups.

**Liver**

In the liver of the control rats, activity of SOD was 6.14 Units which on exposure to DMBA was 5.07 Units. The SOD activity after treatment with cyclophosphamide (standard
drug) was 5.96 Units but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 5.57, 5.84 and 6.03 Units, respectively.

The activity of SOD significantly increased in the liver of DMBA treated group when compared with that of normal control. The extract showed antioxidant effect by significant increase in the activity of SOD in the liver when compared with that of DMBA treated control rats. The activity of SOD was brought to that of normal by 150 mg/kg of extract treated group which was significantly more than cyclophosphamide treated group.

Kidney

In the kidney of the control rats, activity of SOD was 4.24 Units and that of DMBA control rats was 2.07 Units. The SOD activity after treatment with cyclophosphamide (standard drug) was 4.06 Units but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 3.03, 3.07 and 3.15 Units, respectively.

The effect of DMBA was observed to be toxic due to the significant decrease in the activity of SOD in the kidney when compared with that of normal control. The antioxidant effect was observed by significant increase in the activity of SOD in the kidney of extract treated group when compared with that of DMBA treated control rats. The antioxidant activity of rats treated with standard drug was significant increase in the SOD activity and similar to 150 mg/kg extract treated group, which gave best results in comparison to other extract treated groups.

Catalase (CAT)

Serum

The activity of CAT was 1.24 μmoles in control rats, which on exposure to DMBA was 1.04 μmoles. The CAT activity after treatment with cyclophosphamide (standard drug) was 1.18 μmoles but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 1.12, 1.15 and 1.16 μmoles, respectively.
The effect of DMBA was observed to be toxic due to the significant decrease in the activity of CAT when compared with that of normal control. The antioxidant effect was observed by significant increase in the activity of CAT in the extract treated group when compared with that of DMBA control. The increase in the activity due to standard drug was significant and similar to 150 mg/kg extract treated group, which gave best results in comparison to other extract treated groups.

**Erythrocytes**

In the control rats, activity of CAT was 166.62 μmoles which on exposure to DMBA was 130.50 μmoles. The CAT activity after treatment with cyclophosphamide (standard drug) 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 144.37, 135.44, 137.72 and 140.81 μmoles, respectively.

The activity of CAT significantly decreased in the DMBA treated group when compared with that of normal control. The extract showed antioxidant effect by significant increase in the activity of CAT when compared with that of DMBA treated control. Among the extract treated group, 150 mg/kg showed significant increase in activity of CAT and similar to that of standard drug.

**Mammary tissue**

In the mammary tissue of the control rats, activity of CAT was 48.41 μmoles which on exposure to DMBA was 12.49 μmoles. The activity of CAT after treatment with cyclophosphamide (standard drug) was 34.38 μmoles but with 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 27.55, 30.49 and 34.96 μmoles, respectively.

The toxic effect of DMBA was justified by the significant decrease in the activity of CAT in the mammary tissue when compared with that of normal control. The 150 mg/kg extract treated group showed significant increase in CAT activity and similar when compared to that of standard drug, which gave best results in comparison to other extract treated groups.
Liver

In the liver of the control rats, activity of CAT was 51.40 μmoles which on exposure to DMBA was 28.16 μmoles. The CAT activity after treatment with cyclophosphamide (standard drug) 50, 100 and 150 mg/kg of ethanol extract to DMBA treated rats was 45.37, 36.44, 36.95 and 37.56 μmoles, respectively.

The activity of CAT significantly decreased in the liver of DMBA treated group when compared with that of normal control. The extract showed antioxidant effect by significant increase in the activity of CAT in the liver when compared with that of DMBA treated control. Among the extract treated group, 150 mg/kg showed significant increase in activity of CAT and similar to that of standard drug.

Kidney

In the kidney of the control rats, activity of CAT was 46.64 μmoles which on exposure to DMBA was 30.54 μmoles. The CAT activity after treatment with cyclophosphamide (standard drug) was 44.38 μmoles but with 50, 100 and 150 mg/kg of ethanol extract to DMBA cancer rats was 34.43, 35.74 and 36.86 μmoles, respectively.

The effect of DMBA was observed to be toxic due to the significant decrease in the activity of CAT when compared with that of normal control. The antioxidant effect was observed by significant increase in the activity of CAT in the kidney of extract treated group when compared with that of DMBA treated control group. The CAT activity in group treated with standard drug was significantly increased and similar to that of 150 mg/kg extract treated group, which gave best results in comparison to other extract treated groups.

d. Effect of ethanolic extract of stem bark of Bridelia retusa (EESBB) on expression and phosphorylation of p53 protein in mammary tissue of DMBA treated rats.

SDS-PAGE analysis of whole protein extract from the tissue samples.

The present investigation was attempted to elucidate the protein profiling of the whole protein extract obtained from the mammary tissue isolated from the rats of all six
groups. The protein expression was observed at 66, 30 and 18 kDa for control group. In cancer bearing rats induced by DMBA and cancerous rats treated with standard drug, the protein expression of 66 kDa was obtained. The expression of protein of 66 and 51 kDa was observed for cancer rats treated with 50 mg ethanolic extract. Groups of cancer rats treated with 100 and 150 mg ethanolic extract showed protein expression of 66, 51, 30 and 18 kDa (Fig. 1).

**SDS-PAGE of the immunoprecipitated proteins before transfer to nitrocellulose membrane**

The present investigation was attempted to elucidate the protein profiling of the immunoprecipitated protein using antibodies against p53. The p53 proteins and antibody complex precipitated were recovered with protein A beads in the process to obtain p53 proteins. The protein expression was observed at 51 kDa for all control, DMBA intoxicated rats, rats after treatment with cyclophosphamide (standard drug), 50 and 100 mg/kg of ethanol extract to DMBA induced mammary cancer groups and 150 mg/kg of ethanol extract to DMBA induced mammary cancer groups at 51, 30 and 25 kDa (Fig. 2).

**Western blotting analysis with anti-p53 antibody and anti-alpha phosphotyrosine antibody.**

The western blotting analysis for detection of p53 protein expression was observed at 50 kDa for all groups (Fig. 3). p53-Phosphorylation was observed in samples for all groups below 66 kDa with bands of different intensity (Fig. 4).
DISCUSSION

Polycyclic aromatic hydrocarbons (PAH) is a class of organic pollutants that are released into the environment in large quantities, mainly due to human activities. PAH are components of crude and refined petroleum, and coal. Many PAH are quite persistent and some are potent carcinogenic agents. Most PAH in the environment are found during incomplete combustion of organic matter at high temperatures. In addition, many domestic and industrial activities involve pyrosynthesis of PAH. The resulting PAH may be released to the environment as airborne particulates, or as solid or liquid by-products of the pyrolytic process (Neef, 1985). 7,12-Dimethylbenz (a) anthracene (DMBA) is one of polycyclic aromatic hydrocarbons chemical group. DMBA is well known as cytotoxic, carcinogenic, mutagenic and immunosuppressive agent (Smith et al., 1999; Spitsbergen et al., 2000; Miyata et al., 2001; Wijnhoven et al., 2001; Lindhe et al., 2002; Buters et al., 2003). DMBA is highly carcinogenic in experimental animals. Large single and multiple doses produce tumors of the skin, breast, and stomach or leukemias regardless of route of administration. Skin of mice is particularly sensitive to low, topically applied doses (NIH, 2009). This carcinogen reacts with rapidly proliferating cells in the terminal end buds of mammary glands forming DNA adducts which transform normal terminal end buds to malignant pathways (Russo et al., 1982, 1983).

Sprague Dawley DMBA-induced mammary gland tumors, has been largely used due to the chemical induction efficiency and the specific susceptibility of this laboratory animal to breast neoplastic lesions which is consistent with results from the DMBA induction protocol followed (Russo and Russo, 1996). Mammary carcinomas appeared in a higher proportion and earlier in Sprague Dawley than in Wistar rats exposed to different chemical carcinogen (Gruenstein et al., 1966). The induced breast cancer models also allow the observation of all the stages during the carcinogenesis process which can be especially useful to evaluate the effect of external conditions like diet, nutrients and therapeutic agents. The susceptibility of Sprague Dawley rats to DMBA is maximal at 55 to 60 days of age and is abolished by ovarietcomy, suggesting the inducible action of carcinogen depends on ovarian secretions. The estrogenic properties of DMBA molecule may be due to long lasting effects that the carcinogen exerts on the plasma membrane of estrogen sensitive neurons.
DMBA can interact with estrogen receptor and partially mimic both positive and negative feedback of estradiol (Dao, 1962; Daneil and Jovco, 1983; Lenoir et al., 2005). DMBA induced experimental carcinogenesis is preceded by a sequence of hyperplasia, dysplasia, and carcinoma (Shklar and Schwartz, 1996; Letchoumy et al., 2006).

Occurrence of cancer is associated with the natural loss of the cells in any tissue or organ and thus undergoing unregulated growth. DMBA mediates carcinogenesis through formation of DNA adducts (Russo et al., 1982, 1983), DNA damage, generating excess reactive oxygen species and by producing chronic inflammation. Several studies suggested that DMBA mediated molecular, biochemical, genetic and histopathological changes were analogous to those observed in human cancers. DMBA-induced experimental carcinogenesis might therefore, be used as an ideal model to study the therapeutic potential of medicinal plants and their active constituents. The evaluated results reported in DMBA induced carcinogenesis may assist the clinician in the diagnosis, prognosis and treatment monitoring of the cancer patients (Gimenez-Conti and Slaga, 1993; Miyata et al., 2001). DMBA, a member of the polycyclic aromatic hydrocarbons, is present in the environment as a product of incomplete combustion of complex hydrocarbons. Being an indirect carcinogen, DMBA requires metabolic activation to become a carcinogen. DMBA is metabolized by cytochrome P4501A1 in liver microsomes and by cytochrome P4501B1 in primary bone marrow stromal cells to form diol epoxides and other toxic ROS. The toxic metabolites of DMBA, including diol epoxides, are capable of binding to adenine residues of DNA causing chromosomal damage (Guerin, 1978). Accumulation of reactive oxygen species in cells damages the defence mechanisms of the body including the DNA structure and enzymatic balance triggering the process of cancer pathogenesis (Mates and Sanchez-Jimenez, 2000).

Covalent DNA adducts derived from DMBA that were analyzed in previous studies were anti-diolepoxide- deoxyguanosine (anti-dG), syn-diol-epoxide deoxyadenosine (syn-dA), and anti-diol-epoxide deoxyadenosine (anti-dA) (Singletary et al., 1990). They occurred in both the target (mammary epithelial tissue) and the non-target organ (liver). Daniel and Joyce (1983) and Singletary et al. (1990) have shown that, following DMBA
administration, anti-dG is the major adduct formed in rat mammary tissue in vivo. Maximum DNA binding in the mammary tissue of Sprague–Dawley rats was detected 24–48 h after DMBA administration. Phytoconstituents in the extract in the present study may be responsible for anticancer activity. Ellagic acid and several other dietary phenolics have been considered blocking agents (Wattenberg, 1985; Ip and Ganther, 1991; Kashiwada et al., 1994), because of their frequently observed anti-carcinogenic activity when administered during the initiation phase in rodent models. The studies presented previously showed that ellagic acid also can act as an inhibitor of DMBA initiated stomach and swim-bladder carcinogenesis in the rainbow trout model wherein it was relatively ineffective as a blocking agent, but provided substantial protection in the post-initiation (promotion/progression) phase of carcinogenesis in stomach (Hartigg et al., 1996). These studies support recently published evidence from rats (Akagi et al., 1995; Siglin et al., 1995) that dietary ellagic acid can suppress tumor development, in this case in a complete carcinogenesis protocol without exogenous promoter. It has shown anti-oxidant and anti-promoting activity against TPA in DMBA-initiated mouse skin when given topically (Gali et al., 1992) and caused a sustained decrease of cellular proliferation in tongue epithelium after 4-nitroquinoline-1-oxide initiation in rats (Haslam, 1989). Recent reports demonstrate inhibitory activity of ellagitannins on protein kinases including protein kinase C (Kashiwada et al., 1994; Dow et al., 1994) and the inhibition of DNA-topoisomerases (Constantinou et al., 1995) as also reported in green tea polyphenols (Lee and Lin, 1997; Chen et al., 1999). The influence on such signal transduction/cell proliferation enzymes could be the basis of its anti-proliferative and tumor suppressing activity. Tea polyphenols might suppress mitogenic signal transduction and other unknown proliferative signals. These effects might have an inhibitory effect on proliferation and cell cycle progression, even on apoptosis. Another report indicated that treatment of rats with green and black tea for 4 or 6 weeks caused significant induction of cytochrome P450 enzymes, such as CYP1A2, CYP1A1, CYP2B, and CYP4A1 (Sohn et al., 1994; Bu-Abbas et al., 1994). Induced phase I enzymes might be metabolic activators of carcinogens, for example, CYP1A2 may play an important role in the activation of aromatic and heterocyclic amine procarcinogens.
The exploration of oral administration of anticancer drugs that have been available for many years (i.e., etoposide, cyclophosphamide, and idarubicin) and novel strategies for oral use of anticancer drugs traditionally administered (paclitaxel plus cyclosporin, 5-fluorouracil plus eniluracil), leading to a new era in the administration of cancer chemotherapy (DeMario et al., 1998). Oral cyclophosphamide has been an important component of adjuvant therapy for breast cancer for over a decade, permitting self administration in a convenient setting and allowing patients to have a greater role in their therapy. With the development of oral anthracyclines (such as idarubicin) and less variable approaches for oral 5-fluorouracil administration (such as coadministration with eniluracil), treatment regimens with only oral chemotherapy are now under clinical evaluation for adjuvant breast cancer (McMenemy and Clark, 1997). Patient compliance with prescribed drug therapy regimens is also an important variable that is of greatest concern when medications are self-administered, as is typically the case with oral chemotherapy. Inability of patients to successfully comply with a treatment regimen is thought to be a major source of therapy failure for many diseases. A noncompliance rate of 43% was observed in breast cancer patients treated with an outpatient, oral cyclophosphamide regimen (Leovits et al., 1990) hence search for better drug is still going on.

Comprehensive reviews provide strong evidence that high intake of vegetables and fruits are associated with reduced cancer incidence (Block et al., 1992). It is known that balance between Phase I and Phase II enzymes can afford protection against numerous chemical carcinogens, and the induction of antioxidant enzyme facilitates their degradation from the body (Miller, 1998). It undergoes oxidation to 1,2-diolepoxide, which binds to and damages DNA (Sims and Grover, 1991). Phase I enzymes are known to induce tumour formation by activating procarcinogens which modify genomic DNA and black tea polyphenols probably inhibit cytochrome P450 dependent bioactivation of the carcinogen (Catteral et al., 1998). DMBA is known to produce toxic and highly diffusible reactive oxygen species capable of producing deleterious effects at sites far from the target tissue (Chou et al., 1998). In particular, the erythrocytes are prone to oxidative damage due to high content of iron and polyunsaturated fatty acids and their role as O₂ transporters (Hebbel, 1986). Thus, enhanced concentration of hepatic and blood lipid peroxides in DMBA-painted
animals can be ascribed to the excessive generation of oxygen free radicals (OFR) by DMBA.

**a. Effect of ethanolic stem bark extract of *Bridelia retusa* (ESBEB) on body weight, tumor size and organs weight of DMBA treated rats**

**Body weight**

In the present study of the toxic effects of DMBA was observed due to a significant decrease in the body weights of rats. Weight loss observed in DMBA treated group was rectified best in 150 mg/kg extract treated group which was similar to that of cyclophosphamide (standard drug). There was a severe body weight loss observed at the end of the experiment in cancer bearing rats versus the control rats in previous study as also observed in present findings. On treatment with Kalpaamruthaa (a siddha formulation contain *Semecarpus anacardium* Linn., *Emblica officinalis* and honey), the gradual increase in body weight in earlier studies reported the counteractive property towards DMBA (Krishnamurthy *et al*., 2006) which was shown by ethanolic extract of the present study. The gradual enhanced weight observed due to ethanolic extract in the present study was best with 150 mg/kg and comparable to that of standard drug. Similar increase in loss of body weight by berberin (berberine is an isoquinoline alkaloid isolated from the roots and bark of several herbs including *Berberis vulgaris*, *Coptis chi-nensis*, *Hydrastis Canadensis* and *Berberis aquifolium*) from earlier studies have been reported (Shanmugam *et al*., 2010). The weight gain due to olive oil in DMBA treated animals was higher with higher weight index homogeneity (Pereira *et al*., 2009). Mean weight loss in the 1500 mg/kg MTBE treated group was 6% over the first two weeks, but several animals experienced much greater weight reduction, after which MTBE (methyl tertiary-butyl ether) doses were reduced to allow MTBE-treated animals to recover from an initial body weight decline was observed in previous findings. Body weight in the 800 mg/kg group began to decline dramatically only after two weeks of treatment, and only toward the end of the experiment in the 400 mg/kg dose group as reported by De Peyster *et al*. (2003).

In mammary carcinoma bearing animals, there was a sharp drop in their body weight which may be due to the cancer cachexia. Cancer cachexia results in progressive loss of
body weight, which is mainly accounted by wasting of host body compartments such as skeletal muscle and adipose tissue (Krishnamurthy et al., 2006) which may have also been the case in the present study. The protective effect due to ethanolic extract especially at 150 mg was comparable to standard drug in the present study which may be attributed to its phytoconstituents.

**Tumor size**

In the present study of the toxic effects of DMBA was observed due to a significant increase in tumor size of rats. The tumor size observed in DMBA treated group was rectified best in 150 mg/kg extract treated group which was similar to that of cyclophosphamamide (standard drug). Similar inhibited tumor growth by methanolic extract of *Ganoderma lucidum* was observed in previous studies (Lakshmi Krishnamurthy et al., 2009). The total tumor diameter in the *mekabu* treated group was significantly smaller than in the control group in earlier studies (Funahasi et al., 2001). The female rats treated with DMBA and consuming standard food presented a much higher percentage of large tumors in comparison to the ones that had a diet based on olive oil (Pereira et al., 2009).

The histologic examination of the mammary tissue of rats treated with DMBA in the present study, showed invasion of tissue by ductal breast carcinoma along with profound dysplasia expanding into stroma. Along with ductal carcinoma, necrosis was also detected which was not only restricted to the ducts. The ductular appearance with sharply defined granular spaces was lost while rats treated with DMBA along 50 and 100 mg ethanol extract showed decreased invasion and dysplasia with appearance of glandular spaces. Histologic observations of the mammary tissue of DMBA treated rats with standard drug and 150 mg extract revealed the overall appearance approaching the normal mammary tissue with minimum carcinoma. Similar overall appearance of the breast much like that of normal control rats after treatment with spirulina was observed in previous study. Invasive breast cancer with mitotic figures, necrosis, fibrotic stroma and malignant epithelial cells from small ductal structures were observed in cancer controls (Bin-Meferij, 2008). Histologically, the mammary tumors were cystic adenocarcinoma, and tumors in the *mekabu* treated group of previous findings showed a decreased density of epithelial cells.
and fibrosis (Funahashi et al., 2001). Several studies indicated that the site of origin of human mammary carcinoma may be in lobules and terminal ductules of the mammary gland (McCarty, 1913; Cheatle, 1926; Muir, 1941; Wellings et al., 1975; Wellings and Jensen, 1973) which may be so in present study due to occurrence of ductal carcinoma. When 7,12-dimethylbenz(a)anthracene (DMBA) was administered to the rat, numerous mammary gland dysplasias and mammary carcinomas occur (Huggins et al., 1961; Dao et al., 1964; Beuving et al., 1967) as also seen in the present study. It has been reported that approximately 80% of the tumors arising are ovarian hormone dependent (Huggins et al., 1959; Forest, 1972). Rat mammary carcinomas are claimed to arise in small mammary ducts (Middleton, 1965; Sinha and Dao, 1975; Russo et al., 1975) or from hyperplastic alveolar nodules (Beuving, 1968; Beuving and Bern, 1972).

Mirunalini et al. (2010) have also reported highly invasive tumor cells in mammary tissue of DMBA treated rats which was reduced on treatment with melotinin. Though treatment with the mushroom extract could ameliorate the histopathological alterations to a significant extent, animals treated with DMBA alone reported (Lakshmi et al., 2009) to show typical hyperplasia and invasive ductal carcinoma while the extract treatment was found to inhibit the necrosis of epithelial cells. The vast majority of the lesions that developed in the rat mammary glands treated with DMBA were mostly carcinomas along with necrosis which were reduced on administration of luteolin and cyclophosphamide (Samy et al., 2006) which occurred in the present study as well. As it is known that garlic contains many organosulphur compounds which react with sulphhydryl groups of tumor cell proteins (Willis, 1956; Weisberger and Pensky, 1958), suggesting that the tumor cells are probably attenuated by the similar action of garlic extract. Working with a transitional cell carcinoma (MBT-2), Lau et al. (1986) have shown that garlic extract exhibited significant effectiveness in inhibiting tumor growth in mice through elicitation of activated macrophages and lymphocytes. It is, therefore, not improbable that garlic extract played a dual role, inactivating sulphhydryl compounds of tumor cells (Fujiwara and Natata, 1967) and cytotoxic destruction of tumor cells by activated macrophages and lymphocytes leading to inhibition of tumor growth (Lau et al., 1986). Thus the active compounds of the ethanolic extract may be considered to play an important role in cancer therapy. The ethanolic extract
at 150 mg in the present study showed protective effect comparable to standard drug, thus indicating its potential as anticancer drug. Further research needs to be proceeded to understand the actual mechanism which may be due to individual or combinational effect of various phytoconstituents.

**Organs weight**

In the present study the standard drug showed protective effect against DMBA treated rats in elevating the weight loss of liver but no significant change on kidney was observed while the protective effect of extract was negligible. This was also observed in earlier studies that the significant decrease in kidney weight noticed in animals exposed to lead and the no significant decrease in kidney weight noticed in animals administered tomato paste alongside lead exposure (Emmanuel et al., 2009). Both absolute liver weight and relative mean liver to the body weight ratios (De Peyster et al., 2003) were similar in all groups at the end of 28 days of different dosing of MTBE (methyl tertiary-butyl ether). Shivanandappa and Krishnakumari (1981) have reported that the liver and kidney weights decreased in rats treated with BHC.

In the rats treated with DMBA of the present study, histologic study of the liver revealed dilation of central vein and infiltration around portal triad. Histologic study of liver of the rats treated with DMBA on administration of cyclophosphamide showed overall appearance of the normal tissue with minimum damage best at 150 mg of the extract. Similar results were (Mirunalini et al., 2010) reported with mononuclear inflammatory infiltrate and damage around portal triad in liver of DMBA treated rats. In the DMBA treated rats with 50, 100 and 150 mg/kg extract, histologic study of the liver exhibited almost normal structure of liver lobules showing hepatic cords with cuboidal or polyhedral hepatic cells with sinusoids. Histologic examination of the kidney of rats treated with DMBA showed glomerular and tubular epithelial damage. Histologic observation of the kidney of DMBA treated rats with 50 and 100 mg ethanol extract showed decreased glomerular and tubular epithelial damage. Histologic observations of the kidney of rats treated with DMBA along with standard drug and 150 mg ethanol extract revealed lesser glomerular and tubular epithelial damage with mostly normal appearance. Earlier reports
showed glomeruli with cellular proliferation and tubular epithelial damage in the kidney in DMBA rats wherein the histological changes were reduced on administration of melotinin to DMBA treated rats (Mirunalini et al., 2010).

The weight loss in liver and kidney due to DMBA in the present study was rectified by ethanolic extract best at 150 mg which was comparable to standard drug. The histophaological findings also supported the toxic effects of DMBA and protective effects of B. retusa ethanolic extract. Meng et al. (2010) have reported that the treatment with 17β-estradiol in overiectomiced mice caused significant decrease in the weight of liver and kidney. Contrary to present study, the body weight, liver weight, and relative liver weight (liver weight/body weight ratio) were not significantly influenced by the carcinogen treatment or garlic supplementation in previous investigations (Kyung-Ae Park et al., 2002). Janardhan and Sisodia (1990) reported that rats treated with monocrotrophos exposed for 30 days caused decrease in liver and kidney weights, with an evident histopathological symptoms in liver and kidney as seen in present study. No histological differences were seen in liver and kidney tissues (Hallowes, 1971) unlike present study which showed significant negative effects of DMBA on liver and kidney weights which was rectified in dose dependent manner by ethanolic extracts comparable to standard drug.

In conclusion, the significant decrease in the body weight as well as the relative weights of liver and kidney in the present study may be due to pathophysiological and histological alterations or functional impairment. Decrease in body, liver and kidney weights was observed in pesticides like mancozeb, monocrotrophos, dimethoate, methomyl, etc treated rats and mice (Adilaxmamma et al., 1994; Ratnasoorya et al., 1995; Kacker et al., 1999; Baliger and Kaliwal, 2000, 2002, 2003, 2004; Radhika and Kaliwal, 2002; Mahadeswami and Kaliwal, 2002; Shreelakshmi and Kaliwal, 2007; Kshreesagar and Kaliwal, 2008; Manwadi and Kaliwal, 2010). Rats treated with monocrotrophos and BHC showed decrease in liver and kidney weights (Sisodia, 1990; Shivanandappa and Krishnakumari, 1981). The body weight increased and tumor size decreased due to treatment with luteolin (hydroxylated flavone derivative) and cyclophosphamidue in DMBA-induced mammary tumors in rats (Samy et al., 2006) as also seen in the present study.
Semanogh et al. (2001) have reported a significant change in absolute organ and body weight gain, but there was a decrease in liver/body weight ratio of rats exposed to carbachol at higher doses. The findings of earlier studies indicated that dietary garlic could inhibit DEN (Diethlynitrosamine) initiated hepatocarcinogenesis, but had no effect on body weight gain and dietary intake (Kyung-Ae Park et al., 2002). But in the present study the ethanolic extract had positive effect on body weight gain and decrease in tumor size. The tumor bearing rats of earlier study consumed less food, and the differences in weight between the control and tumor-bearing rats were more pronounced which was also observed in the present study. With time, the number of tumors arising per rat increased gradually along with the average tumor diameter increased (Barclay et al., 1967). Resveratrol and EGCG (epigallocatechin-3-gallate) from grapes protection against cancer was observed by loss of weight or differences in food and drink consumption of previous investigation (Whitsett et al., 2006). The lack of alteration of body weights was also observed by Juan et al. (2002) showed that daily administration of resveratrol (20 mg/kg) had no effect on final body weights or on the tissue weights of the lungs, heart, liver, kidney, or adrenal glands. Likewise, Hirose et al. (1994) have reported no differences in body, liver, or kidney weights after treatment with green tea catechins in the diet or in the water, thus indicating protective role as seen with ethanolic extract at 150 mg/kg and standard drug which reversed the loss in weights caused my DMBA induced stress. The most highly malignant tumors in rats have some common features with intraductal and infiltrating ductal carcinomas in humans, however they are a minority of tumors induced by the commonly used regimes. The abnormal epithelium usually remains rigidly confined by the adjacent stroma, shows no clear evidence of invasion and varies considerably within and between tumors. All of these features complicate the distinction between benign and malignant lesions (Russo et al., 1990). Both the MNU and the DMBA models include reliability of tumour induction, organ site specificity, tumours of ductal origin and predominantly carcinomatous histopathological characterisation, tumours of varying hormone responsiveness, and the potential to examine tumour initiation and promotion processes (Thompson and Adlaka, 1991). The proportion of benign tumours induced by MNU is lower than that induced by DMBA (Gullino et al., 1975). Mc Cormick et al. (1981) concluded that one of the major disadvantages of the DMBA model is the lack of tumor metastases. Hence, in the present
study also no metastases was obtained using DMBA as carcinogen. Although, the carcinomas (ductal) retained normal architecture of the mammary gland, there was invasion of the surrounding tissues and the tissue invasion was mostly local. Though no metastasis was observed in the present study, vital organs like liver and kidney showed toxicity signs due to DMBA (Russo et al., 1990). No metastases in distant sites were observed due to methyl nitrosourea (MNU) in earlier histological studies (Liska et al., 2000). Thus, in the present study the carcinogenesis was localized to mammary glands, which was confirmed histologically though toxicity was observed in other vital organs. Further investigation and long term exposure will be needed to understand the protective role of ethanolic extract of *B. retusa* in DMBA treated animals and its effect on other organs.

b. Effect of ethanolic stem bark extract of *Bridelia retusa* (ESBEB) on serum parameters in DMBA treated rats

Transaminases are intracellular enzymes, which exist in only small amounts in serum; therefore, damage to liver cells may result in leakage of the enzymes into plasma due to a large concentration gradient (Wroblewski and La Due, 1955). Aspartate Transaminase (ASAT) / Serum GOT (Glutamic Oxaloacetic Transaminase) catalyses the reversible interconversions between glutamate and aspartate and their 2-oxo analogues (Sadasivam and Manickam, 2008). The effect of DMBA in the present findings showed an increase in the activity of SGOT and SGPT in the serum. The protective effect was observed by decrease in their activities in the serum of extract treated group. In the CCl₄ intoxicated control group of earlier study, the SGOT and SGPT activities increased significantly when compared with the normal group. In contrast, the group treated with fucosterol (Pelvetia siliquosa) decreased significantly the elevated transaminase activities (Sanghyun Lee et al., 2003). The antioxidant activity of standard drug was significant and similar to 150 mg/kg extract treated group, which gave best result in comparison to other extract treated groups. Increase in serum transaminase in presence of trichloroacetic acid (TCA) was reported by Demir and Celik (2006). TCA, as a toxicological agent like other pesticides, might interact primarily with liver tissue cell membranes, resulting in structural damage and changes in metabolism of the constituents. In the Wistar rat model of CCl₄ induced hepatocyte injury, elevation in the serum transaminase levels was observed in earlier studies. At higher concentration (4
mg/ml) of Essiac (a blend of the four herbs *Arctium Lappa*, *Rheum palmatum*, *Rumex acetosella* and *Ulmus rubra*) decreased the elevation due to CCl₄ challenge over the vehicle control alone which coincided with present study that was possible at 150 mg of ethanolic extract. Within the parameters studied, there appeared to be some indication of a dose response trend in much of the data as also seen in earlier findings (Leonard *et al.*, 2006). Similarly effect of anthocyanins to cadmium chloride induced toxicity significantly decreased transaminase activity in sera. SGOT levels were acceptable indicators that showed the addition of standard drug and ethanolic extract relieved the adverse effect of DMBA induced stress similar to the effects of vitamin C and propolis extract on stress originating from lead (Seven *et al.*, 2010). The anthocyanins used in the investigations of previous studies demonstrated protective action against the damages of hepatocytes by cadmium since they normalized the activities of enzymes (Kowalczyk *et al.*, 2003). The previous investigation revealed that the given repeated doses of profenofos produced damage in liver confirmed by significant elevation in serum transaminase levels which are markers of impaired liver function (Mohamed *et al.*, 2010) which may be also seen in present study by multiple doses of DMBA.

Thus, the increase in transaminase activity due to DMBA was reduced on treatment with *B. retusa* extract in the present study in dose dependent manner which was comparable with that of standard drug. Increase in transaminases in circulation in the present study was supported by increase activity due to lead acetate in drinking water of experimental animals (Madiha *et al.*, 2011). The toxicant induces a disturbance in the physiological state, which affects the enzyme activity. It then causes distortions in the cell organelles, which may cause the elevation in the activity of various enzymes (Sharma *et al.*, 2010). Liver, being one of the major organs involved in the storage, biotransformation and detoxification of toxic substances, is of interest in heavy metal and pesticide poisoning. The activities of liver enzymes of rats given lead acetate in their drinking water were increased in plasma. This was due to lyses of cells and the release of these enzymes into the circulation which may be so in the present study. Several studies have demonstrated changes in aminotransferase levels in rats exposed to lead (Madiha *et al.*, 2011). Amino transaminases are two of the most reliable markers of hepatocellular injury or necrosis. Their levels can be elevated in a
variety of hepatic disorders. They are specific for hepatic injury because they are present mainly in the cytosol of the liver and in low concentrations elsewhere (Giboney, 2005). The present study showed protective effect at higher concentration which was contrary to the study of Ahmed et al. (2010) that suggested the reduction in blood levels was at lower doses but not at higher doses. It may be following the theory of increased potency on increased dilution dictated that the more times the remedy was diluted the more powerful it became, however it was invalidated by pharmaceutical dose-response studies which show that increasing dosage increases the effect of a drug (Jackson, 2006) as observed in the present study. Elevated serum GOT has been associated with several pathologic conditions in addition to damaged heart muscle and liver (Agress, 1959). Since this enzyme was present in other tissues such as skeletal muscle, brain, kidney, testes, lung, and spleen (Udenfreind et al., 1960), its concentration in serum could conceivably be increased as a result of damage to these tissues. Although all the tumors studied earlier and presently were associated with some elevation of serum GOT, the serum GOT increased with increased amounts of tumor tissue present (Dyer et al., 1961). Thus increase in transaminases in the present study coincided with that of previous findings indicating the extent of damage to the tissue caused by leakage of these enzymes into the serum. The ethanolic extract may provide protection by decreasing damage to cells of the specific tissue producing these enzymes achieved best at 150 mg comparable to standard drug.

Malignant tissues produce multiple growth factors and cytokines to induce angiogenesis, which is essential for tumor growth, invasion and metastasis (Cao, 2004; Cao, 2009). Among tumor-derived angiogenic factors, vascular endothelial growth factor (VEGF) is probably one of the best-characterized molecules. VEGF displays multiple physiological and pathological functions by targeting both vascular and non-vascular systems (Xue et al., 2008; Xue et al., 2009; Crawford and Ferrara, 2009). It is well known that to grow beyond the size of 2–3 mm, it is essential for a tumor to acquire new blood vessels. This process of neovascularization is known as angiogenesis (Gimbrone et al., 1972). VEGF3 is one of the most potent angiogenic cytokines. It causes mitosis of endothelial cells and increases blood vessel permeability. This increased permeability results in the extravasation of macromolecules such as fibrinogen, which provide an
extraluminal meshwork over which endothelial cells can organize and tumor cells can migrate (Dvorak, 1986). Unlike other cytokines, VEGF is a selective cytokine, acting exclusively on vascular endothelial cells (Keck et al., 1989). Indeed, it has been shown that other mediators of neovascularization including interleukins, oncogenes, and some growth factors may produce their effects by altering the expression of VEGF, suggesting that VEGF may be the final common pathway for all pathological in vivo angiogenesis (Ferrara, 1996).

VEGF may exist in one of four forms, namely, VEGF165 (the most abundant form, soluble, and tissue bound), VEGF121 (soluble), VEGF189 (bound), and VEGF206 (extremely rare, bound form). Each of these forms has similar biological activity. The ELISA kit used to detect VEGF in the serum in this study is specific for VEGF165 but will also detect VEGF121. Thus, the detected levels are indicative of the total circulating level of VEGF (Yamamoto et al., 1996). Cellular sources of VEGF, other than tumor cells, are multiple. Most of these sources are involved in normal adult tissue repair and remodeling processes. Mast cells and muscle cells are an important source of VEGF (Gavin et al., 2000; Richardson et al., 1999). Fibroblasts, PMN cells, and monocytes (MOs) are sources of VEGF in the wound-healing process (Steinbrech et al., 1999; Edelman et al., 1999). Multiple factors, including hypoxia, nitric oxide, and inflammatory cytokines such as MCP-1, (Goede et al., 1999) interleukin 1, and interleukin 6, are involved in controlling VEGF expression in normal tissue repair and remodeling events (Frank et al., 1999; Levitas et al., 2000; El-Awad et al., 2000). The raised serum VEGF levels in tumor bearing rats were lowered due to treatment with cyclophosphamide (standard drug) which was better than that of ethanolic extract. The serum VEGF levels of the cancer patients group were reported to be significantly elevated compared with those of the controls (Heer et al., 2001) as also seen in the present study. Serum VEGF was reported to be significantly elevated in patients with metastatic differentiated thyroid cancer but not in patients with poorly differentiated thyroid cancer metastases (Tuttle et al., 2001) indicating metastases in the present study. Serum VEGF levels were significantly raised in ductal but not in lobular carcinoma (Heer et al., 2001) as also seen in the present study wherein all rats showed ductal carcinoma and hence raised levels of VEGF. This finding agrees with that of Dvorak et al. (1995).
Neoangiogenesis plays a key role in tumor progression of solid neoplasm (Folkman, 1995; Kern and Lippman, 1996; Leek et al., 1996; Weidner et al., 1996). Tumor cells may induce angiogenesis via the release of numerous growth factors, prostaglandin, etc., and by their attraction of inflammatory cells which in turn release multiple angiogenic stimuli. A number of antiangiogenic agents have been recently discovered, and some are under early clinical evaluation (Sledge, 1996; Harris et al., 1996; Dickson et al., 1996). In animal models, treatment with angiogenesis inhibitors has a proven antitumor effect in vivo, and can both reduce metastases and lead to regression of primary growth by necrosis following capillary retraction (Klauber et al., 1997; Folkman, 1996). There is evidence that several common anticancer agents including cyclophosphamide (CTX), doxorubicin (Adriamycin) and paclitaxel (Taxol) have antiangiogenic activity in the animal model (Klauber et al., 1997; Hirat et al., 1989; Steiner et al., 1992) as also seen in the present study, cyclophosphamide showed decrease in the levels of VEGF.

A marked increase in VEGF levels has been observed in various types of cancer including anal carcinoma (Kusumanto et al., 2003), lymphoma (Salven et al., 1999), lung cancer (Salgado et al., 1999; Yanagawa et al., 1999; Matsuyama et al., 2000; Kishiro et al., 2002), gastric carcinoma (Hyodo et al., 1998; Kraft et al., 1999; Yoshikawa et al., 2000), ovarian cancer (Hyodo et al., 1998; Tempfer et al., 1998; Kraft et al., 1999; Yoshikawa et al., 2000), renal cell carcinoma (Dosquet et al., 1997; Sato et al., 1999; Jacobsen et al., 2002; Ljungberg et al., 2003), brain tumour (Stockhammer et al., 2000), hepatocellular carcinoma (Poon et al., 2001), breast cancer (Bando et al., 2005; O'Riain et al., 2005), prostate cancer (Kaushal et al., 2005; Li et al., 2005) and colorectal cancer (Haraguchi et al., 2002; Karayiannakis et al., 2002; Werther et al., 2003) as also seen in the present study. Of these, the last three are most extensively studied. Numerous studies have documented abnormal VEGF expression in malignant cells and elevated serum VEGF levels present in patients with a wide variety of malignancies. Serum VEGF levels often correlate with stage of disease and with poor prognostic clinical features (Adams et al., 2000; Boss et al., 2001; Braybrooke et al., 2000; Broll et al., 2001; Byrne and Bundred, 2000; Chin et al., 2000; Dietz et al., 2000; Feldman et al., 2000; George et al., 2000; Haggstrom et al., 2000; Kuniyasu et al., 2000; Jones et al., 2000; Li et al., 1999; Lissoni et al., 2000; Matsuyama et
al., 2000; Moon et al., 2000; Poon et al., 2001; Riedel et al., 2000; Salven et al., 2000; Sezer et al., 2001; Ugurel et al., 2001). Both breast and prostate cancer are commonly diagnosed malignancies, and are among the top leading causes of death responsible for 15 and 10% of cancer deaths in women and men, respectively in 2005 (Uzzan et al., 2004; Jemal et al., 2005) while colorectal cancer is responsible for 10% of all cancer deaths in 2005 (Jemal et al., 2005). For these cancer types, significant correlations between VEGF and the extent of tumour vascularisation, tumour stages and metastasis have been reported (Duque et al., 1999; Gasparini, 2000; Karayiannakis et al., 2002). VEGF has been considered a significant indicator of cancer, and blood VEGF levels are often used to estimate the degree of tumour development. However, the debate is still on as to the origin and location of VEGF. Serum, plasma and whole blood have been commonly used to determine VEGF levels in the body, but it is not clear which measurement can provide the best prognostic information. Plasma is the free circulating, liquid component of blood, in which blood-formed elements are suspended. Serum is plasma with all coagulation factors removed, and is obtained by clotting the blood before centrifugation. Because coagulation results in the release of VEGF from platelets, serum VEGF concentration counts both plasma VEGF and platelet-held VEGF.

Capsaicin was reported to block the downstream event of VEGF-induced KDR / FIK-1 signaling such as, the activation of p38 mitogen-activated protein kinase and p125 FAK tyrosine phosphorylation that are required for the mitogenic activity of VEGF in endothelial cells (Mifflin et al., 2002; Yang et al., 2002; Catley et al., 2003). Cancer cells produce numerous blood vessels through which nutrients are siphoned from individuals carrying one form of cancer or the other. The activity of VEGF is needed to achieve this fit and is therefore dependent on its phosphorylation, before it becomes active. Capsaicin is known to block the phosphorylation of VEGF. Abnormal or improper activation of downstream transcription factor can result in uncontrolled cell growth, leading to malignant transformation, abnormal cell proliferation and growth. Thus the active constituents in the extract of the present study may be responsible for the decrease in levels of sera VEGF. Curcumin inhibits angiogenesis directly and via regulation of angiogenic growth factors like vascular endothelial growth factor, basic fibroblast growth factor and epidermal growth
factor, as well as the genes like angiopoietin 1 and 2, hypoxia-inducible factor-1, heme oxygenase-1, and the transcriptional factors like NF-κB (Strimpakos and Sharma, 2008). Inhibition of angiogenic growth factor production and metalloproteinase generation, both integral to the formation of new vasculature, has also been influenced by curcumin in non-malignant and malignant cells growth (Mohan et al., 2000; Choi et al., 2006).

c. Effect of ethanolic stem bark extract of Bridelia retusa (ESBEB) on oxidative parameters (Lipid peroxidation, SOD and CAT) in blood, mammary tissue, liver and kidney of DMBA treated rats

Lipid peroxidation (LPO)

Lipid peroxidation, oxidative deterioration of lipid bilayer, has been measured as an index of production of excess ROS. The formation of malondialdehyde is considered as an index of lipid peroxidation that causes cell injury (Mishra et al., 2009; Vennila et al., 2010). Elevation of Lipid Peroxides, as indicated by increased MDA was observed in breast cancer bearing animals. Significant increase in LPO in carcinogenic process may be due to abnormal levels of reactive oxygen species (ROS). ROS production in excess of cellular antioxidant capacity may result in damage to lipid, protein, RNA and DNA or other effects (Vennila et al., 2010). A significant increase in lipid peroxidation was observed due to DMBA in the present study in the mammary tissue, liver and kidney. The standard drug and 150 mg/kg ethanolic extract was considered to decrease LPO in a similar manner. Significant increase in LPO associated with various forms of carcinogenesis has been documented widely and the scavengers are known to play an important role in cancer prevention. The enhanced LPO in mitochondria may decrease mitochondrial membrane fluidity, increase the negative surface charge distribution and alter membrane ionic permeability including proton permeability, which uncouples oxidative phosphorylation (Hennipman et al., 1998). Oral administration of green or black tea leaf powder inhibited the lipid peroxidation of liver induced by tert-butyl hydroperoxide in rats (Sano et al., 1995) while in the kidney, the antioxidant effect was observed only for the green tea-fed group.

Kalpaamruthaa (includes Semecarpus anacardium Linn nut milk extract and dried powder of Phyllanthus emblica fruit and honey) treated animals of previous studies showed
reduction towards the normal levels demonstrating the protective effect. This could possibly due to modulation of the antioxidant system that could decompose the peroxides and thereby offering a protection against lipid peroxidation (Arulkumaran et al., 2007) which may have been the reason in the present study. The drug (fungal taxol) treated and commercial drug treated (paclitaxel) rats in earlier studies showed decreased LPO levels when compared to DMBA administrated rats. While lipid peroxides level were higher in cancer bearing animals but reduced in taxol treated animals (Vennila et al., 2010). In a previous study related to propolis supplementation conducted to determine the treatment effect of propolis in cancer bearing female rats (Padmavathi et al., 2006), it was determined that porpolis addition significantly decreased the MDA levels as lipid peroxidation indicators in the breast and liver tissues of cancer created rats which was also seen in present study. Ethanolic extract of *Etlingera elatior* showed reduced lipid peroxidation in liver and sera of rats on exposure to lead (Haleagrahara et al., 2010; Jackie et al., 2011). LPO was found to be increased significantly in the plasma and RBCs of DMH-treated rats when compared to control rats (Thiyagarajan Devasena et al., 2006). DMH (dimethylhydrazine), a procarcinogen undergoes oxidative metabolism in the liver which results in the production of active carcinogenic electrophile (diazonium ion) that is released into the circulation eventually leading to LPO in plasma and RBCs (Bobek et al., 2000).

The contents of TBARS, acts as an index of lipid peroxidation, were unchanged by garlic and carcinogen (Kyung-Ae Park et al., 2002), contradictory to the reported inhibition of peroxidation by garlic extracts in the carbon tetrachloride-induced liver injury (Kagawa et al., 1986), and that garlic powder diet suppressed the contents of TBARS in the promotional stage of hepatocarcinogenesis (Park and Choi, 1997). Co-administration of extract of *M. laetum* leaves to profenofos intoxicated rats effectively inhibited membrane lipid peroxidation in both liver and kidney compared with profenofos intoxicated rats, indicating its potential antioxidant beneficial action. So the antioxidant ability of the extract may be responsible for protecting the liver and kidney tissues from the oxidative tissue damage in response to profenofos toxicity as seen in present study against DMBA intoxication. This beneficial effect of this extract may be attributed to its capability to increase the endogenous antioxidant defensive capacity of the liver and kidney to combat oxidative stress induced by
profenofos. This emphasizes the fact that herbal medicines often contain multiple active substances which have antioxidant activities (Mohamed et al., 2010). Lipid peroxidation and toxicity that is associated with oxygen radicals have been suggested as major causes of cancer and oxidative stress-related diseases (Park and Choi, 1997; Park et al., 2001) also lipid peroxidation decreases the membrane stability (Rowe and Wills, 1976). The above mentioned earlier data revealed significant increase in MDA due to stress which was similar to present study in rats treated with DMBA (the biomarker of lipid peroxidation). Hence, in the present study the ethanolic extract reduced LPO by protection against ROS responsible for the overall damage which may be attributed to single or multiple active constituents that needs to be investigated.

**Superoxide dismutase (SOD)**

SOD converts superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) and is a major defense system for aerobic cells in combating the toxic effects of superoxide radical (Mishra et al., 2009; Vennila et al., 2010). SOD acts as an anti-carcinogen inhibitor during initiation and promotion/transformation stages of carcinogenesis (Samy et al., 2006). SOD is the first and the most important line of antioxidant enzyme defense against ROS and particularly oxygen radical. SOD demolishes superoxide by converting it into peroxide that can in turn be destroyed by CAT (Fridovich, 1995). DMBA in the present study showed a significant decrease in the activity of SOD in the mammary tissue, liver kidney and blood which was rectified by ethanolic extract in dose dependent manner with 150 mg/kg showing best effect which was similar to standard drug. Vennila et al. (2010) reported that the activities of SOD in mammary tissue were significantly reduced while the activities of SOD were significantly increased on treatment with taxol when compared to cancer bearing animals as also seen in present study. The previous findings revealed significant decrease in the activities of SOD in liver mitochondria of carcinoma bearing groups that have been reported to increase on administration of Kalpaamruthaa preparation increased the activities SOD to near normal levels. Increase in SOD activity should accelerate the removal of the reactive oxygen species (Arulkumaran et al., 2007) which may have been the reason for the increase in SOD activity in the present study as well. Increase in the SOD activity in the kidney due to Moringa oleifera extract accelerates the removal of ROS (Paliwal et al., 2011) which may
have been the case in present study. The decrease in SOD activity on treatment with DMBA was consistent with previous results which showed that the level of SOD decreased in erythrocytes of exercise group of rats by Hamid *et al.* (2011).

Superoxide dismutase plays an important role in catalyzing the dismutation of superoxide radicals. SOD enzymes work in conjunction with H$_2$O$_2$ removing enzymes, such as catalase and glutathione peroxidase. These antioxidant enzymes depend on various essential trace elements and prosthetic groups for proper molecular organization and enzymatic action. Increase in SOD activity should accelerate the removal of the reactive oxygen species. Catalase, whose activity has also been augmented by extract, helps in removing the hydrogen peroxide produced by the action of SOD, 150 mg when compared with other doses. Induced SOD activity along with that of catalase explains the decrease in lipid peroxidation, which is an indicator of oxidative stress that persists in the cell. The decreased lipid peroxidation in the present study is in correlation with the induction of antioxidant enzymes by the *Moringa oleifera* extract (Paliwal *et al.*, 2011). Although the possible mechanism(s) of protection against DMBA induced hepatotoxicity and nephrotoxicity was studied in the current study, it is possible that the protective effect of the extract is mediated through antioxidant and/or free radical scavenging activities. Literature has shown medicinal plants with hepatoprotective and nephroprotective properties to mediate their protection via antioxidant and/or free radical scavenging activities due to the high concentration of flavonoids and alkaloids they contain (Olagunju *et al.*, 2009). In this respect, *Moringa* pods contain important bioactive compounds including glucosinolates, isothiocyanates, thiocarbamates, and flavonoids (Bharali *et al.*, 2003). These compounds quench ROS, chelate metal ions and regenerate membrane-bound antioxidants. Then present finding was consistent with previous studies, which demonstrated the antioxidant activity of *Moringa* extract (Kumar and Pari, 2003; Arabshahi *et al.*, 2007).

Jackie *et al.* (2011) have reported increased SOD activity in the sera of rats caused on exposure to lead but the administration of ethanolic extract of *Etlingera elatior* caused increase in antioxidant enzymes. Restoration of SOD activities in the circulation of DMH + curcumin analog administered rats showed that curcumin analog normalizes the circulatory
antioxidant status and ameliorates DMH-induced oxidative stress was reported by Devasena et al. (2006). Daily oral supplementation with α-tocopherol increased the level of SOD in RBCs decreased due to streptozotocin induced stress in rats, thus providing protection against oxidative damage similar to the present study (Musalmah et al., 2002). Wei et al. (1993 and 1995) have observed that genistein could suppress H$_2$O$_2$ production and inhibits superoxide formation, thus genistein may diminish superoxide anion formed during exercise. Thus decrease in SOD activity in various tissues, RBCs and serum showed decreased activity indicating stress induced by DMBA which was normalized by the ethanolic extract of B. retusa. This may be due to protection against production of radicals which may have inhibited activity of the enzymes.

**Catalase (CAT)**

Catalase decomposes hydrogen peroxide (H$_2$O$_2$) into H$_2$O and O$_2$ and protects the tissue from highly reactive hydroxyl radicals (Mishra et al., 2009; Vennila et al., 2010). In the present study, DMBA showed a significant decrease in the activity of CAT in the mammary tissue, liver and kidney. The ethanolic extract improved the activity in dose dependent manner with 150 mg/kg showing best effect which was similar to standard drug. The activities of CAT in mammary tissue were significantly reduced in previous study of taxol and the activities of CAT were significantly increased when compared to cancer bearing animals (Vennila et al., 2010). Arulkumaran et al. (2007) have observed a significant decrease in the activity of CAT in liver mitochondria of carcinoma bearing groups. Administration of Kalpaamruthaa preparation, increased the activity of CAT to near normal levels. Catalase, whose activity was also augmented by *Moringa oleifera* extract in previous study in kidney of mice, which helps in removing the hydrogen peroxide produced by the action of SOD. Induced SOD activity along with that of catalase, which is an indicator of oxidative stress that persists in the cell (Paliwal et al., 2011). The earlier data revealed significant decrease in the antioxidant enzyme, CAT, but the co-administration of extract of *M. laetum* leaves to profenofos intoxicated rats effectively rectified antioxidant enzyme in both liver and kidney compared with profenofos intoxicated rats, indicating its potential antioxidant beneficial action. The beneficial effect of this extract may be attributed to its capability to increase the endogenous antioxidant defensive capacity of the liver and
kidney to combat oxidative stress induced by profenofos, thus emphasizing the fact that herbal medicines often contain multiple active substances which have antioxidant activities which may have been the case in present study (Mohamed et al., 2010).

Hamid et al. (2011) reported decreased activity of CAT on exercise which coincided with the present result when DMBA stress was induced. Szatrowski and Nathan (1991) have suggested that tumor cells produce substantial amount of H₂O₂ that is released into the circulation. Thus, the increased susceptibility of plasma and RBCs of DMH-treated rats could be due to the production of H₂O₂ by the tumor cells. Hence, a reduction in the activity of CAT was observed as a result of DMH (Devasena et al., 2006) that coincides with DMBA effect in the present study. Similar to present findings of the B. retusa extracts, Devasena et al. reported that the activity was increased on treatment with curcumin analog used against cancerous rats. On the contrary to the present study cisplatin caused an increase in serum CAT and the protection was observed by lowering of the activity (Azu Mohamed et al., 2010). This study wherein DMBA induced stress agrees with Somani et al. (1995) that erythrocyte catalase activities were significantly decreased by one session of acute exhausting exercise. Thus above mentioned earlier findings coincide with that of the present study that showed decrease in CAT due to DMBA was rectified by ethanol extract.

Thus, conclusively the stress caused due to DMBA in the present study showed a significant decrease in the activity of SOD and CAT while significant increase in lipid peroxidation in the mammary tissue, liver and kidney when compared to negative control. The antioxidant effect of extract was observed by significant increase in the activity of SOD and CAT while significant decrease in lipid peroxidation when compared to DMBA positive control. Reduced activities of SOD and CAT can cause accumulation of superoxide anion and H₂O₂ with deleterious consequences including DNA strand breaks and conformational changes in proteins (Bize et al., 1980; Scott et al., 1991). The naturally occurring free radical scavenger of the ethanolic extract in the present study lowered the MDA level suggesting reduced LPO. Due to the free radical scavenging property, which helps to improve the antioxidants defense system and prevent the damage induced by free radicals. In earlier studies, CAT and SOD activity of liver and kidney was lower in animals with
breast cancer, higher levels of CAT and SOD activity were recorded on treatment. The lipid peroxide levels were increased more in the breast cancer bearing animals; whereas, little reduction was found in the rats treated with luteolin as well as the cyclophosphamide individually treated groups of earlier studies (Samy et al., 2006) which was also seen in the present study with ethanolic extract and cyclophosphamide as standard drug. Similar effects have been reported by ethanolic extract of *Symplocos racemosa* on SOD, CAT and LPO was observed in acute experimental liver injury induced by administration of DMBA (Vijayabaskaran et al., 2010).

It has been discovered that DMBA toxicity leads to free radical damage via two different pathways: (1) the production of reactive oxygen species (ROS), including hydroperoxides, singlet oxygen, and hydrogen peroxide, and (2) the direct reduction of antioxidant reserves (Sharma *et al.*, 2010). The balance between the production of oxidants and the scavenging of those oxidants by antioxidants determines the extent of lipid peroxidation. DMBA in previous investigation at a dose of 15 mg/ kg body weight caused significant increase in the LPO level and significant decrease in CAT and insignificant decrease in SOD activity, in comparison to control group which was achieved at 20 mg/kg in the present study given as multiple doses. Intake of hydro-ethanolic extract of *Moringa oleifera* at dose of 200 and 400 mg/kg and BHA (0.5%) along with DMBA significantly improved the LPO level, SOD and CAT activity as seen at 50, 100 and 150 mg of ethanol extract with 150 mg giving the best result in the present study. In the present study, the activities of SOD and CAT antioxidants were reduced by DMBA, thus rendering the tissues to the peroxidative damage which was also shown in previous observation (Paliwal *et al.*, 2011).

The impaired liver and kidney functions in response to ingestion of repeated doses of the insecticide presented in the previous investigation may be due to the oxidative tissue damage caused by the massive production of reactive oxygen species (ROS) and disturbance in the protective physiological moieties (as antioxidant defense mechanism systems) causing lipid peroxidation, a process leading to damage to the macromolecules in vital biomembranes (Bergamini *et al.*, 2004) which may also be the reason in the present study.
This might be due to decrease in the free radical generation due to the presence of phenolic compounds. Antioxidant activity of phenolic compounds mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules though phenolic compounds were not detected in the present study, hence other phytoconstituents may be needed to be considered. Numerous studies have explained that DMBA induces substantial oxidative effects in liver and an oxidant closely associated with tumour promotion, correlated with our study (Izzoti et al., 1999).

More than two-fold increase in lipid peroxidation in the liver of DMBA induced group may be due to increased peroxidation of the primary substrates and polyunsaturated fatty acids (Szewezyk and Wojtczak, 2002). *Etlingera elatior* ethanolic extract from the inflorescence caused reversal of lead induced changes in the oxidative serum biomarkers which was best at higher concentration of 200 mg/kg. This was attributed to polyphenol, flavanoids and flavones present in the extract responsible for free radical scavenging and antioxidant properties (Jackie et al., 2011). This was achieved at 150 mg/kg of ethanolic extract in the present study which may also be attributed to flavonoids or flavones that was detected in the extract, hence further investigation becomes necessary to determine the active constituent(s). As mentioned earlier (Szatrowski and Nathan, 1991), tumor cells produce substantial amount of H₂O₂ which is released into the circulation. Accumulation of H₂O₂ results in formation of hydroxyl radical (OH•) and/or a highly toxic hypochlorous acid formed as a result of metabolism of H₂O₂ by circulatory neutrophil-derived myeloperoxidase (Weiss et al., 1982). Thus, H₂O₂ produced by tumor cells elicits an oxidative stress in RBCs as also seen in present study. SOD and CAT, the primary antioxidant enzymes reported to be more sensitive to oxidative stress (Moghadasian et al., 1996; Taniguchi et al., 1999). Therefore, the decreased activities of SOD and CAT in the rats of present study may be due to this oxidative stress. Erythrocytes are the first to react to increased activity of free radical oxidation and to exhaust their compensatory potential. Previous studies on erythrocyte antioxidant capacity and human disease relation showed that some changes in activities of the antioxidant enzymes in the cell may occur (Karatas et al., 2003).
Plasma viscosity remains stable in serum oxidation of different degree, and therefore, erythrocytes are responsible for changes in blood rheology during intensification of free radical oxidation. It was proposed that a superoxide anion channel allows the transport of superoxide and other free radicals into the red blood cell, where they are deactivated by the erythrocyte antioxidant system which effectively prevents extensive oxidative damage to tissues (Roitman et al., 2001). In addition to its function of gas exchange, the erythron provides a mechanism for the inactivation of reactive oxygen and oxide radicals in vivo. In carrying out this function, individual erythrocytes undergo changes in biochemical and structural properties, which are reflected by shape and functional alterations (Richards et al., 1998). The oxidative damage increases the antioxidant system of the erythrocytes which may have been the reason in the present study as well. The level of antioxidants TBARS was increased and activities of SOD and CAT was reduced which was significantly increased on treatment with aqueous extract *Terminalia arjuna* bark (Dhanarasu et al., 2010) as also seen in the present study. Enhanced lipid peroxidation associated with antioxidant depletion in circulation is a characteristic finding in malignant transformation. Free radicals, which are highly toxic to the traverse membranes and cause deleterious effects at sites far from the tumor (Dreher and Junod, 1996) which may have been the reason for toxicity visible other than in mammary tissue in the present study. Erythrocytes are constantly exposed to oxidative stress, and the susceptibility of erythrocytes to oxidative stress has been reported in several pathological conditions, including oral cancer (Szatrowski and Nathan, 1991). Elevated lipid peroxidation in cancer patients may also be correlated to their poor antioxidant system (Sabitha and Shyamaladevi, 1999). Thus, the observed increase in plasma lipid peroxides in DMBA-treated animals in the present study may be due to overproduction and diffusion from the damaged erythrocytes and erythrocyte membranes. A decrease in the activities of SOD and CAT, the major cellular detoxifying enzyme systems, has been reported in malignancies (Corrocher et al., 1986; Arivazhagan et al., 1997), our results are in line with these findings.

Nevertheless, the fact that mature erythrocytes lack a nucleus, and given that the life cycle of erythrocytes is about 120 days, argues against a profound effect of vitamin E in a 2–6 week period either on bone marrow or directly on the blood which may have also been
the case in present study (Huang et al., 1999; Liu et al., 2003). A dose dependent free radical scavenging effect of vitamin E has been shown in previous studies as a way in which vitamin E causes a time-limited defense against lipid peroxidation, which would then be terminated after total consumption of vitamin E (Niki et al., 1984). By scavenging free ROS, vitamin E increases the total antioxidant capacity, and can theoretically up-regulate the activity of SOD and other antioxidant enzymes, as ROS are potent inhibitors of these antioxidant enzymes (Bray et al., 1974; Meier et al., 1994; Fujii and Taniguchi, 1999). So it is theoretically possible that vitamin E exerts a dose-dependent effect on antioxidant enzymes which was also seen in present study with the crude extract. Oral administration of the aqueous extract *Terminalia arjuna* bark at 500 mg/kg not only prevented the tumor formation but also significantly improved the status of lipid peroxidation and antioxidants in DMBA-painted animals, which clearly indicates its potent antilipidperoxidative, and antioxidant potential in DMBA-induced hamster buccal pouch carcinogenesis (Dhanarasu et al., 2010) which was also seen in the present study against DMBA induced mammary carcinogenesis best at 150 mg/kg of Ethanolic extract of *B. retusa*. The modulation of circulatory antioxidants effect of the extract is probably due to the presence of several bioactive principles and their synergistic effects. Since the crude extract was taken for investigation along with phytoconstituents even vitamins may be responsible for the protective effects.

Anbuselvam et al. (2007) have demonstrated the protective effect of *Operculina turpethum* against DMBA-induced oxidative stress with reference to breast cancer in experimental rats. They suggested that the antioxidant activity of *Operculina turpethum* played a protective role against DMBA induced breast cancer. Kumaraguruparan et al. (2007) have reported that the black tea polyphenols in DMBA induced mammary carcinogenesis showed modulating effect on xenobiotics metabolizing enzymes, oxidative stress, cell proliferation, apoptosis and angiogenesis. Although the exact mechanism of action of the ethanolic extract is unclear in the present study, its anti-lipid-peroxidative, antioxidant and modulating effect on detoxification cascade could play a possible role. The DMBA toxicity stimulates the oxidative stress and the antioxidant enzymes, induced as a defense mechanism. It has been reported that DMBA toxicity leads to free radical damage
via two different pathways: (a) the production of reactive oxygen species (ROS), including hydroperoxides, singlet oxygen, and hydrogen peroxide, and (b) the direct reduction of antioxidant reserves (Sharma et al., 2010). Since oxidative stress plays a major role in DMBA toxicity, thus the external antioxidant system provided by the extract in the present study may be critical in providing protection.

It is known that various initiators and promoters of carcinogenesis act via generation of activated forms of oxygen and associated lipid peroxidation and reported protection against carcinogenesis by antioxidants supports this view (Watenburg, 1978; McCoy et al., 1982; Stich et al., 1989). It is evident from these results that, in DMH-treated rats, the oxidant antioxidant homeostasis is disturbed. Although the possible mechanism of protection against DMBA induced toxicity was not clear in the current study, it is possible that the protective effect of the extract is mediated through antioxidant and/or free radical scavenging activities. Literature has shown medicinal plants with protective properties to mediate their protection via antioxidant and/or free radical scavenging activities due to the high concentration of flavonoids and alkaloids they contain (Olagunju et al., 2009). Tea polyphenols have shown to be strong scavengers against superoxide, hydrogen peroxide, hydroxy radicals, and nitric oxide produced by various chemicals. Chen and Ho (1994) extensively investigated the antioxidant properties of various tea polyphenols. Reports have indicated that tea intake may enhance the levels of antioxidant defense enzymes, such as SOD and catalase. The oral feeding of green tea leaves to rats resulted in enhanced SOD activity in serum and catalase activity in liver, and an increased concentration of glutathione in the liver (Lin et al., 1998). Several studies have found that black tea and green tea offered protection against oxidative damage to red blood cells induced by a variety of inducers (Grinberg et al., 1997; Zhang et al., 1997; Halder and Bhaduri, 1998). Tea tannin and its metabolites act as comutagens and antimutagens, both based on inhibition and promotion of DNA excision repair property (Imanishi et al., 1991). Selenium compounds are capable of inhibiting, carcinogen-induced covalent DNA adduct formation and DNA oxidative damage, DNA methylation, micronuclei induction, chromosomal aberrations, and cancer (An et al., 1988; Hu, 1989; Khalil and Maslat, 1990; Chen, 1992; Combs and Gray, 1998; Zhang and Xiao, 1998; Bayoumy, 1999; Ip, 1998; Sinha et al., 1999; Biswas et al., 1999).
The structure of tea polyphenols might consist of strong metal ion chelators, such as iron and copper, which are required for generation of reactive oxygen radicals by means of Fenton and Haber-Weiss reactions. Miller et al. (1996) have shown that the antioxidant properties of theaflavins and their gallate ester might through chelating iron and copper. Other than organic components which may be present in the ethanolic extract in the present study, inorganic substances may also be having beneficial anticancer effects.

d. Effect of ethanolic extract of stem bark of Bridelia retusa (EESBB) on expression and phosphorylation of p53 protein in mammary tissue of DMBA treated rats.

p53 is a critical mediator of cellular responses to genotoxic stress. Following DNA damage, there is a posttranscriptional induction of p53 protein and subsequent activation of p53. Ultimately, activation of p53 leads to either G1 arrest or cell death. The mechanism by which p53 becomes activated is unknown. Phosphorylation is one potential mechanism by which cells might regulate the activity of p53 after genotoxic stress. In vivo experiments have demonstrated clearly that p53 is a phosphoprotein (Ko and Prives, 1996). The p53 tumor suppressor protein is a critical regulator of cell cycle progression that responds to DNA damage and certain other cellular stresses by arresting cell cycle progression or by inducing apoptosis (Agarwal et al., 1998; Giaccia and Kastan, 1998; Prives, 1998). These responses are important for preserving the integrity of the genome of a cell, thus preventing the transformation of a normal cell into a tumor cell. p53 normally is a short-lived protein that is maintained at low levels in unstressed cells. After cells are exposed to DNA damaging agents, nucleotide depletion, or hypoxia, the p53 protein is transiently stabilized and accumulates in the nucleus. DNA damage also activates p53 as a transcription factor, which in turn induces or represses the transcription of several genes. Both stabilization of the p53 protein and activation of its sequence-specific DNA binding ability are widely believed to be mediated, at least in part, by post-translational modifications to the p53 protein.

In the present study, the total protein expression observed between 51 and 66 kDa for all the samples which on immunoprecipitation was observed at 51 kDa after performing SDS-PAGE and on immunoblotting while for p53 and its phosphorylated form was
observed at 50 kDa and 66 kDa. The p53 protein consists of 393 amino acids giving a molecular mass of 53,000 Daltons (Kirsch and Kastan, 1998), thus indicating expression of p53 in all groups. The nuclear phosphoprotein p53 acts as a tumor suppressor protein, arresting cell cycle progression or inducing apoptosis in response to DNA damage or other cell stresses. The polyclonal antibody to p53, a synthetic peptide of 367-381 amino acids (SHLKSKKGQSTSRHK) of human p53 was used as immunogen in the present study. This peptide is highly conserved (90-95%) among many mammalian species. Nuclear protein p53, plays an essential role in the regulation of cell cycle, specifically in the transition from G0 to G1. The p53 is a DNA-binding protein containing DNA-binding, oligomerization and transcription activation domains. It is postulated to bind as a tetramer to p53-binding site and activate expression of downstream genes that inhibit growth and/or invasion, and thus function as a tumor suppressor. Mutations in the evolutionary cascade codons of the p53 tumor suppressor genes are common in diverse types of human cancer which includes cancer of colon, lung, esophagus, breast, liver, brain, reticulo-endothelial tissues, and hemapoietic tissue. The p53 maps 17p13 region of the human chromosome (Igenex, San Diego).

Low levels of p53 were observed in control samples of the present study which were increased in tissue samples of rats bearing tumors. High levels of p53 protein are a common feature of many human neoplasias (Crawford et al., 1984; Cattoretti et al., 1988; Bartek et al., 1991; Iggo et al., 1990) as also seen in present study. They are associated with poor prognosis in several tumour types including breast and gastric cancer (Martin et al., 1992; Thor et al., 1992). In the present study, the level of expression was lesser in controls. The molecular mechanisms underlying these elevated levels of p53 protein are therefore of great practical and theoretical interest. While in many cases a close correlation has been shown between mutation of the p53 gene and accumulation of high levels of p53 in tumours (Bartek et al., 1990; Iggo et al., 1990; Rodrigues et al., 1990; Bennett et al., 1991; Davidoff et al., 1991) it is now clear that this is not the only mechanism responsible for the enhanced expression of the protein. The normal protein can also occur at elevated levels in cells exposed to DNA damaging agents (Hall et al., 1992; Lu et al., 1992), cells transformed by papovaviruses (Oren et al., 1981; Sarnow et al., 1982) and adenoviruses, and in the normal
cells of certain cancer family patients (Barnes et al., 1992). Elevated levels of normal p53 have also been demonstrated in certain non-virally transformed cells (Casey et al., 1991; Lu et al., 1992). The p53 tumor suppressor protein plays a major role in cellular response to DNA damage and other genomic aberrations. Activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis (Levine, 1997). The increasing level of p53 in eurycomanone-treated HeLa cells could be possibly due to the increasing of p53 stability has been reported from western blots (Mahfudh and Pihie, 2008) as also seen in the present study. The half-life of p53 was increased, because the p53 protein was found at an elevated level and maintained at high levels throughout the experiment. This is consistent with the previous study that p53 is a short-lived protein with a half-life of ~5-20 min (Giaccia and Kartan, 1998), however, following the exposure to DNA damage, the half-life of p53 protein was increases by several folds (Maki and Howley, 1997). The DNA damages could be recognized by p53, therefore the p53 level was increased and apoptosis was induced. The previous report stated that activation of p53 could be invoked by as little as one DNA double-stranded break (Lakin and Jackson, 1999). This DNA damage could induce dramatically increase of p53 stability as the signal could be amplified in the cells. Previous studies also reported that apoptosis in HeLa cells induced by xanthorrhizol (Ismail et al., 2005), staurosporin (Bernard et al., 2003), apigenin (Zheng et al., 2005), vitamin C (Reddy et al., 2001), carboplatin (Singh et al., 2007) and cisplatin (Maldonado et al., 1997) involved the increasing level of p53 as also seen in the present study. Thus the increasing levels of p53 may play an important role in increasing the susceptibility of cells to undergo apoptosis.

The western blotting with anti-p53 antibody showed expression for all the groups. Control group showed no phosphorylation on western blotting with antiphosphotyrosine antibodies while with groups treated with 100 and 150 mg of ethanolic extract to the tumor bearing rats showed lower levels of phosphorylation. The p53-Phosphorylation was observed in samples of DMBA treated rats as well as cancer bearing rats treated with standard drug and 50 mg of ethanolic extract at 50 kDa. Level of phosphorylation was comparatively less in case of samples of tumor bearing rats treated with 100 and 150 mg of ethanolic extract and the expression was observed at 66 kDa. The peptide affinity purified
polyclonal antibody to p53 (Ser315) was generated by immunizing rabbits with synthetic phosphopeptide corresponding to human p53 around the phosphorylation site of serine 315 (S-S-SP-P-Q) (Igenex, San Diego). *In vitro* studies have identified multiple sites of phosphorylation within the amino and carboxyl termini within p53. Although several kinases have been shown to phosphorylate specific sites within p53 in vitro, it has not yet been demonstrated that phosphorylation at these sites is important for the function and regulation of p53 *in vivo*. Interestingly, demonstration of that simultaneous mutation of several serines within the amino terminus of p53 has a biologic effect, namely, a reduction in the ability of the recombinant, overexpressed p53 to suppress transformation as well as a decrease in the ability of p53 to transactivate a reporter construct has been reported (Mayr *et al.* 1995). In a present study, the overexpressed p53 indicates DNA stress due to DMBA and suppression of the stress due to extracts which showed higher levels of p53 in comparison to cancer treated group. In the present study, phosphorylation was not observed in control but DMBA treated samples showed expression which decreased on treatment especially at 150 mg of ethanol extract. Antibodies that recognize specific phosphorylated sites on p53 were developed, and studies with these antibodies have demonstrated that in most cases these sites are phosphorylated in response to stress or DNA-damage (Shieh *et al.*, 1997; Canman *et al.*, 1998; Meek, 1998; Bulavin *et al.*, 1999; Higashimoto *et al.*, 2000; Oda *et al.*, 2000), thus indicating the possible DNA damage in the present study.

p53 is phosphorylated at multiple sites *in vivo* and by several different protein kinases *in vitro* (Meek, 1994; Milczarek *et al.*, 1997). DNA damage induces phosphorylation of p53 at Ser15 and Ser20 and leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2 (Shieh *et al.*, 1997) due to which levels may have increased in the present study. MDM2 inhibits p53 accumulation by targeting it for ubiquitination and proteasomal degradation (Honda *et al.*, 1997; Chehab *et al.*, 1999). p53 can be phosphorylated by ATM, ATR and DNA-PK at Ser15 and Ser37, in the present study SER15 phosphorylation was studied. Phosphorylation impairs the ability of MDM2 to bind p53, promoting both the accumulation and activation of p53 in response to DNA damage (Shieh *et al.*, 1997; Tibbetts *et al.*, 1999) as may be the case in the present study. Chk2 and Chk1 can phosphorylate p53 at Ser20, enhancing its tetramerization, stability and
activity (Shieh et al., 1999; Hirao et al., 2000). p53 is phosphorylated at Ser392 in vivo (Hao et al., 1996; Lu et al., 1997) and by CAK in vitro (Lu et al., 1997). Phosphorylation of p53 at Ser392 is increased in human tumors (Ullrich et al., 1993) and has been reported to influence the growth suppressor function, DNA binding and transcriptional activation of p53 (Hao et al., 1996; Lohrum and Scheidtmann, 1996; Kohn, 1999). p53 is phosphorylated at Ser6 and Ser9 by CK1δ and CK1ε both in vitro and in vivo (Knippschild et al., 1997; Kohn, 1999). Phosphorylation of p53 at Ser46 regulates the ability of p53 to induce apoptosis (Oda et al., 2000). In vivo phosphorylation at Ser315 has been observed following UV-irradiation, and a Ser315Ala mutant p53 has reduced activity as a transcription factor (Ito et al., 2001). Aurora A phosphorylates p53 at Ser315 in a cell cycle-dependent manner leading to MDM2-mediated ubiquitination/degradation of p53 (Sakaguchi et al., 1998). In the present study, p53-Phosphorylation was observed in the samples of all groups except control group and the level of phosphorylation was comparatively less on case of samples of ethanolic extract treated groups best at 150 mg.

The tumor suppressor protein p53 regulates the cellular response to DNA damage by mediating cell cycle arrest, DNA repair, and cell death (Shieh et al., 1997; Shieh et al., 1999). The mechanisms involved in p53-mediated cell death remains controversial, and regulation of the p53 function is complicated. Phosphorylation at the Ser-15 residue of p53 is critical for p53-dependent transactivation. In addition, accumulation of p53 protein by inhibiting the interaction between p53 and MDM2, stimulates p53-dependent transactivation (Momand et al., 1992). In response to stress signals, the levels of p53 protein are rapidly increased, and activity is enhanced after phosphorylation at the Ser-15 residue, resulting in the upregulation of the downstream genes, including the cyclin-dependent kinase inhibitor p21WAF1/CIP1 and the proapoptotic gene Bax. In turn, increased levels of Bax induce mitochondrial depolarization, release of cytochrome c, and activation of caspase cascade, leading to apoptosis (Levine, 1997; Jimenez et al., 1999). Activation of p53 is a critical component in the cellular response to DNA damage as seen in the present study. It will be important to determine if the specific phosphorylation of p53, affects the cell that undergoes a G1 checkpoint arrest versus apoptosis in response to stress. In addition, it will be important to delineate whether denovo p53 phosphorylation also occurs when cells are
grown either under stress. Finally, identification of the kinases that phosphorylate p53 \textit{in vivo} will further elucidate the signal transduction pathway initiated by cells in response to DNA damage. Several kinases have been implicated in signal transduction pathways initiated by stress. These kinases are potential candidates that may be involved, directly or indirectly, in the \textit{in vivo} post-translational modification of p53. p53 is a critical mediator of cellular responses to genotoxic stress. Following DNA damage, there is a posttranscriptional induction of p53 protein and subsequent activation of p53. Ultimately, activation of p53 leads to either G1 arrest or cell death. The mechanism by which p53 becomes activated is unknown. Phosphorylation is one potential mechanism by which cells might regulate the activity of p53 after genotoxic stress. \textit{In vivo} experiments have demonstrated clearly that p53 is a phosphoprotein (Ko and Prives 1996) which was also indicated in present study.

Apoptosis is one of the body's most potent defences against cancer; the pathogenesis of many forms of this degenerative disease is closely connected with aberrantly regulated apoptotic cell death. Different mechanisms that regulate apoptosis associated with mediators that trigger or inhibit cell death have led to the development of therapeutic strategies against cancer like targeting p53. It is now known that various triterpenes are able to intervene in such processes as DNA repair, cell proliferation, cell differentiation, angiogenesis and apoptosis (Ikeda et al., 2003; Reyes et al., 2006; Dzubak et al., 2006). Whilst in previous studies have demonstrated clearly that maslinic acid did not affect the apoptosis of normal cells (Reyes et al., 2006) we are able to propose here a plausible molecular mechanism by which this triterpene might induce its anti-tumoral and pro-apoptotic effects upon HT29 colon-cancer cells. The over-expression of p53 in HT29 cells could well explain cell-cycle arrest, cell differentiation and the late induction of genotoxicity which maybe so in the present study. According to a quantitative model, genes involved in growth arrest contain high-affinity p53 binding sites in their promoter, while low-affinity sites are present in the promoter of apoptosis-related genes (Chen et al., 1996). This is in line with observations that increased levels or activity of p53 can lead to the onset of apoptosis, presumably by achieving a certain threshold level. Moreover, p53 mutants with marginally altered conformations retain sufficient activity to induce growth arrest but not apoptosis, presumably because they can still interact only with high-affinity sites. However, despite the
degenerative nature of p53 binding sequences, the apoptotic targets of p53 do not necessarily contain low-affinity promoters (Kaeser and Iggo, 2002). It is evident that curcumin can induce selective cancer cell killing in a p53-dependent manner, but impaired p53 expression or activity is associated with a variety of neoplastic transformations. Increasing reports are indicating that curcumin can block cell cycle progression or even apoptosis in a p53-independent manner as well, especially in the cells that lack functional p53 (Park et al., 2002). In a study, T. arjuna extract-treated human hepatoma cell line (HepG2 cells) showed upstream regulation of p53 protein expression after exposed to the concentrations of 60 and 100 mg/L for 48 hrs as also seen in the present study, highest at 150 mg of ethanolic extract. Hence, T. arjuna extract may possibly enhance the susceptibility of HepG2 cells to apoptosis by attenuating the tumor suppressor protein which maybe so in the present study (Sivalokanathan et al., 2006). Thus indicating the therapeutic possibilities of ethanolic extract in dose dependent manner on p53 protein expression and activiation which may be attributed to the phytoconstituents.

It was reported that certain products from plants can induce apoptosis in neoplastic cells but not in normal cells (Hirano et al., 1995; Chiao et al., 1995). Studies have shown that cytotoxic effect of the phenolic compounds on different tumors is mediated through apoptosis. For instance, gallic acid selectively induces cell death in various transformed cell lines such as PLC/PRF/5 (human hepatoma), HL-60, RG (human promyelocytic leukemia) and P-388D1 (mouse lymphoid neoplasma) (Inaoue et al., 1994). It has been reported that several putative compounds isolated from T. arjuna such as tannic acid, especially flavonoid (luteolin) are potent antitumor promoters and inhibitors of a series of solid tumors and leukemia (Gali et al., 1991; Matsukawa et al., 1993) and reported to induce apoptosis in HepG2 cells as well as inhibit growth of osteosarcoma (U2OS) and glioblastoma (U251) (Nagpal et al., 2000). Previous results showed that, ethanolic extracts of 799 plants had potential for effectively reducing adriamycin induced cellular senescence in human fibroblasts, endothelial cells and smooth vascular cells measured by western blots using antibodies against p53. Among 799 plant extracts, those from Rhei Rhizoma, Cirsii Radix and Plantagnis Semen repressed adriamycin-induced cellular senescence in fibroblasts. Extracts of Cinnamomi Cortex and Cinnamomi Cortex Spissus were effective in
endothelial cells. Extracts of *Euonymi Lignum Suberalatum*, *Salicis Radicis Cortex*, *Polygoni aviculari Herba* and *Chaenomelis langenariae Radix* reduced senescence in vascular smooth muscle cells. These results suggested the effectiveness of plant extracts in reducing cellular senescence of human cells (Yang *et al.*, 2010) contrary to which present study intiated cell death of cancer cells as indicated by increased p53.

The overall protective effects of *B. retusa* on DMBA induced weight loss, decreased tumor size, enzymes activity, VEGF levels, antioxidants status, histological and protein p53 expression studies was evident in the present study. Most phytochemicals are known to exert their anticarcinogenic effects by scavenging oxygen free radicals and enhancing antioxidant levels (Johnson, 1997). Assay of circulatory biomarkers has emerged as a reliable method for screening putative chemopreventive agents. Circulatory levels of lipid peroxidation and antioxidants are reliable indicators, because they reflect the bioavailability as well as increased utilization to counter lipid peroxidation (Nagini *et al.*, 1997). Antioxidants like phenolic compounds can affect carcinogen metabolism and reduce their bioavailability or their metabolic activation, this can also reduce the DNA damage. Squalene and β-sitosterol, major components of a non-saponifiable fraction, have been proposed as potential anticancer agents (Nakagawa *et al.*, 1985; Murakoshi *et al.*, 1992; Katdare *et al.*, 1997; Owen *et al.*, 2004). Selenium has been effective against 7,12-dimethylbenz[a]anthracene (DMBA)-croton oil-induced skin tumors (Shamberger and Rudolf, 1966; Riley, 1969; Shamberger, 1970) and against N-2-fluorenylacetamide (FAA)-induced liver and mammary tumors (Clayton and Baumann, 1949; Harr *et al.*, 1972; Harr *et al.*, 1973). Vitamin E reduced the number of DMBA-croton oil induced skin tumors (Shamberger and Rudolf, 1966; Shamberger, 1970) and the number of fibrosarcomas induced by 3-methylcholanthrene (Jaffe, 1946; Haber and Wissler, 1962). Ascorbic acid reduced DMBA-croton oilinduced skin tumors (Shamberger and Rudolf, 1966), reduced uroepithelial carcinomas (Schlegel *et al.*, 1969), and inhibited growth of sarcoma 180 (Yamafuji *et al.*, 1971). Although not every antioxidant experiment was positive against carcinogenesis (17), the large number of positive effects (Jaffe, 1946; Clayton and Baumann, 1949; Haber and Wissler, 1962; Shamberger and Rudolf, 1966; Frankfurt *et al.*, 1967; Epstein *et al.*, 1967; Riley, 1969; Schlege *et al.*, 1969; Shamberger, 1970; Yamafuji *et al.*, 1971).
al., 1971; Wattenberg, 1972; Harr et al., 1972; Harr et al., 1973; Ulland et al., 1973 Wattenberg, 1973;) may indicate some general effect of antioxidants against carcinogenesis.

Medicinal plants may exert the carcinogenic potential by modulating carcinogen detoxification, inhibiting lipid peroxidation, or by improving in vivo circulatory antioxidants defense mechanism (Sudbo et al., 2003). Environmental factors are recognized to play a major role in the etiology of various cancers that account for over 80% of human malignancies. When cells are exposed to a carcinogenic agent, the carcinogen interacts with DNA directly, or via the formation of reactive oxygen species (ROS), resulting in chromosomal abnormalities and compromised host antioxidant defense mechanisms that eventually culminates in neoplastic transformation (Das, 2002). It follows that cancer control can be achieved by decreasing the rate of DNA damage and enhancing antioxidant defenses. Previous study revealed that the i) levels of LPO, in terms of thiobarbituric acid reactive substances (TBARS), ii) activities of detoxifying enzymes and iii) activities of antioxidant enzymes in the RBCs were considered to be more sensitive to oxidative stress (Della Rovere et al., 2000; Marlin et al., 1997). ROS formed during DMBA metabolism can diffuse from the site of generation to other targets within the cells or even propagate the injury outside to intact cells. These ROS produce deleterious effects by initiating lipid peroxidation directly or by acting as second messengers for the primary free radicals that initiate lipid peroxidation (Das, 2002). Thus, enhanced hepatic lipid peroxidation in DMBA-treated animals may be attributed to excessive generation of ROS exacerbated by decreased efficiency of host antioxidant defense mechanisms. Enhanced LPO associated with depletion in detoxifying and antioxidant (CAT, SOD) enzymes in the RBCs were observed in previous study (Devasena et al., 2006). These are the characteristic findings in malignant transformation (Rovere et al., 2000; Ray et al., 2000).

Every antioxidant, including vitamin antioxidants, is a redox agent, protecting against ROS in some circumstances, but also promoting ROS generation in others (Herbert, 1996). Herbert (1994 and 1995) has reported that antioxidant vitamin supplements at pharmacological levels might promote heart disease, cancer, and liver and kidney disease. Breinholt et al. (1999) observed that the activities of antioxidant enzymes in RBC decreased
concurrently with an increase in the antioxidant potential due to administered flavonoids which may also be the case in present study. Hence, Breinholt et al., (1999) have hypothesized that the antioxidant enzymes in RBC were downregulated by genistein in response to an improved antioxidant status of the RBC due to the increase of high antioxidant potential from genistein supplementation. Others observed that genistein could suppress 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated production in vitro and in vivo and inhibit superoxide anion formation by the xanthine-xanthine oxidase system (Wei et al., 1993 and Wei et al., 1995). Our study investigated the 1) levels of LPO, in terms of thiobarbituric acid reactive substances (TBARS), 2) activities of antioxidant enzymes in the RBCs which are considered to be more sensitive to oxidative stress (Devasena et al., 2006; Della Rovere et al., 2000; Marlin et al., 1997). Enhanced LPO associated with depletion in antioxidant (CAT, SOD) enzymes in the RBCs were observed in our study. These are the characteristic findings in malignant transformation (Della Rovere et al., 2000; Ray et al., 2000). It is evident from these results that, in DMBA treated rats, the oxidant-antioxidant homeostasis is disturbed. The induction of these enzymes by the ethanolic extract of *B. retusa* extract represents a promising strategy in enhancement of antioxidant system enzymes which affords protection against cellular damage and inhibits cancer.

Worldwide, over ten million cancer cases, with over six million deaths, were estimated in the year 2000 (Parkin, 2001). Drug discovery from medicinal plants has played an important role in the treatment of cancer (Butler, 2004). Vinca alkaloids, epipodophyllotoxins, taxanes and camptothecins are the four main classes of anticancer agents from plants currently in clinical use and natural products isolated from medicinal plants can remain as essential components in the search for new medicines (Balunas and Kinghorn, 2005). The inhibitory action of these compounds are related to several steps involved in the carcinogenic process namely prevention of formation of carcinogens from procarcinogens, induction of coordinated enzyme response and scavenging the active metabolite of the carcinogen. The underlying molecular mechanisms by which dietary factors influence the development of cancer are poorly understood. However, a large number of naturally occurring chemicals have been shown to protect against carcinogenesis as may have been the case in the present study. Indoles and isothiocyanates (found in
cruciferous vegetables) flavonoids (in citrus fruits), coumarins (found in legumes) and organosulphurs (in garlic and onion) are some of the phytochemicals that can prevent chemical carcinogenesis (Kelly et al., 2000, Cristina et al., 2009). Courmarins and flavonoids were also detected in the present study. These compounds appear to confer resistance against carcinogenesis through their ability to generate a chemical signal that stimulates increased expression of protective antioxidant and detoxification enzymes in specific organs of the host. Numerous reviews revealing the merits and possible risk of compounds of diverse categories such as carotenoids, dithiolthiones, flavonoids, glucosinolates, isothiocyanates, allylsulphonydrlys, fermentable fibres and other bioactive food components have been found to influence experimentally induced cancers in recent years (Gill and Cross, 2000; Abdullah and Gruber, 2000). Alcoholic extract of *Piper longum* and piperine was found to significantly inhibit the growth of solid tumor induced by Dalton’s lymphoma ascites (DLA) cells and ascites tumor induced by Ehrlich ascites carcinoma (EAC) cells. The results of previous findings indicated that the *Piper longum* and piperine could act as a non-toxic immunomodulator and posses antitumor property (Sunila and Kuttan, 2004). In one of our previous studies using the polysaccharides isolated from *Tinospora cordifolia*, maximum inhibition was obtained when the animals were pre-treated with the extract prior to tumour induction but simultaneous administration also reduced the tumour nodule formation. The long and short term in vitro experiments have shown that the polysaccharide fraction was neither directly toxic to the tumour cells or inhibited the proliferation as well (Leyon and Kuttan, 2004). These results are indicative of the involvement of the immune system in the reduction of the metastatic potential of the drug treated animals. Since the polysaccharide fraction is a specific mitogen of B-cells (Sainis et al., 1997) the tumour reduction obtained may be mediated B-cell or non-specific immune cells such as NK-cell mediated.

*Aegle marmelos, Holarrhena antidysenterica, Punica granatum, Cyperus rotundus, Emblica officinalis*, and *Plumbago zeylanica* can be used as anti-diarrhoeals when diarrhoea becomes one of the complications of cancer cachexia. *Terminalia chebula* could be useful against chronic constipation and digestive disorders which are common in cancer patients resulting in loss of appetite. *Eclipta prostrata, Emblica officinalis, Withania somnifera* and
*Piper longum* can be directed to correct nausea and vomiting (Nayak, 2002). The above-mentioned herbs have proven to be powerful immunostimulants, helpful to manage pain and ache strengthens mental faculties and helps to manage insomnia or sleeplessness due to stress (Bahru, 2000). The therapy includes recommendations for lifestyle and use of specific foods and herbs which are very helpful not only in preventing the progression of the disease but also makes the patients feel better and comfortable in overcoming the symptoms. An herbal combination of *Withania sominifera*, *Asparagus racemosa*, *Hydrocotyle asiatica*, *Nardostachys jatamansi*, *Elettaria cardamomum*, *Tribulus terrestris*, *Zingiber officinalis* and *Eclipta alba* could also be useful in the treatment of anxiety, tension and insomnia. *Ocimum sanctum* is beneficial against stress and depression during cancer. *Curcuma longa*, *Zingiber officinale*, *Glycyrrhiza glabra*, *Terminalia chebula*, *Ocimum sanctum* and *Adhatoda vasica* are used to control cough and shortness of breath especially for lung cancer patients (Nayak, 2002). Thus, indicating total positive effects of herbs on the entire body system. Karthikeyan *et al.* (1999) suggested that orally administered extract of *Ocimum sanctum* may have the ability to prevent early stages in development of DMBA induced carcinogenesis in hamsters. The oral treatment with its alcoholic leaf extract elevated activities of cytochrome P-450, aryl hydrocarbon hydroxylase, etc. in the liver which important in detoxification of carcinogens and mutagens (Banerjee *et al.*, 1996). Prashar *et al.* (1998) suggested that its leaf extract blocks or suppresses the events associated with chemical carcinogenesis by inhibiting metabolic activation of carcinogen. One or combinational effects as seen in the above reports may be responsible for the protective effect of ethanolic extract in the present study. However, there can be pointed some limitations on the extrapolation of these results due to evident differences between animals and humans, including when considering food habits. In opposite to laboratory animals, people don’t eat isolated food but meals and have complex dietary habits with several synergies between nutrients (Terry *et al.*, 2001). Several interaction mechanisms and also other lifestyle factors like physical activity, smoking or alcohol consumption may affect the promotion or protective effects of specific nutrients. Hence there can be more aspects that effect the cancer formation and promotion in nature which also needs to be considered.
The inhibition of VEGF expression and signalling in tumors is a promising therapeutic strategy. Tumor-induced angiogenesis is largely dependent on VEGF, and studies have demonstrated that anti-VEGF antibodies successfully inhibit both angiogenesis and tumor growth (Eatock et al., 2000). The first anti-VEGF drug, bevacizumab, was approved by Food and Drug Administration in 2004. In all phase trials, the drug was reported to be well-tolerated, and increased the response and survival rates of patients (Tortora et al., 2004). In contrast to VEGF inhibition in tumors and other diseases, administration of additional VEGF could potentially treat disorders that result in restricted or limited blood supply. For example, it may be a therapeutic agent for chronic limb ischaemia, which is often caused by obstructive atherosclerosis and has a high mortality rate; other potential therapeutic applications include the treatment of coronary insufficiency and restenosis (Ferrara and Davis-Smyth, 1997). Understanding the distribution of VEGF in human body is vital for the prognosis and treatment of cancer and other disorders. It is especially important for designing and understanding anti-VEGF therapeutics. However, important variations across studies, including methods of sample collection, patient selection, measurement units, statistical analysis and data interpretation, make it difficult to obtain a global view of VEGF distribution. There has been no comprehensive review of the literature that elucidates the relative concentrations of VEGF in such body compartments as blood, normal tissues and organs, and tumour, nor one that gives detailed overview of VEGF levels across various cancer types. The present study was aimed to view the protective effects of ethanolic extract of B. retusa on VEGF levels and summarize the results in comparison to the standard drug available in market for treatment of cancer. An additional motivation and aim of the present study is to provide a solid basis for quantitative, systems biology studies of the VEGF system in health and disease (Mac Gabhann and Popel, 2006; Mac Gabhann et al., 2006). Angiogenesis inhibition is a target for anticancer therapy. In this scenario, the definitions of the actual cellular producers of VEGF in quantities sufficient to promote tumor growth and of the molecular mechanisms involved in stimulating those cells to produce VEGF are fundamental. The VEGF levels in the present study were lowered on treatment of extract which showed comparatively lesser beneficial effects to that of standard drug, cyclophosphamide. Evaluating the exact
mechanism for the reduced levels and protection provided by the extract and the standard drug needs further investigation.

The transcriptional activation of p53 is critical for initiating an early response to genotoxic stress. p53 has an arsenal of target genes at its disposal and may even possess some selectivity towards a particular fate. The convergence of multiple signals stimulating several p53 activators could be required for a full transcriptional activation response. Perhaps the phosphorylation ‘profile’ of p53 drives its selectivity towards a particular cellular response. A unique combination of phosphorylated residues could also serve as a framework for further posttranslational modifications. Together, these previous findings continue to paint a complex portrait of p53 activation, with different types of genotoxic stress activating specific pathways that may work cooperatively for maximal activation (Brooks and Gu, 2003). It also highlights the intricacy of an assortment of pathways and networks all converging on a commonly shared protein of the cell, p53 (2). Though only SER315 phosphonylation was studied for identifying active p53 in the present study, other amino acids may be also subjected to phosphorylation. The acetylation levels of p53 are significantly enhanced in vivo in response to almost every type of stress, which correlates well with its activation and stabilization induced by stress (10). Along with phosphorylation acetylation is also a form of activitaion that was not studied in the present study but needs further investigation. Betulinic acid, a triterpene of Betula alba has been reported to possese anti-HIV, antihelmentic, anticancer, antibacterial, antimalarial, etc (Yogeeswari and Sriram, 2005) indicating the importance of phytoconstituents in treatment of diseases which may have been so in the present study that needs further investigation. Some of the dietary agents that are known to modulate p53 activity are curcumin (Han et al., 1999), resveratrol (Huang et al., 1999), EGCG (Gupta et al., 2000), indole-3-carbinol (Hong et al., 2002), and silibinin (Gu et al., 2005).

In addition to causing p53 to accumulate, DNA damage is widely believed to activate p53 as a transcription factor through post-translational mechanisms. In support of this hypothesis, microinjection of damaged DNA or a monoclonal antibody to the carboxyl terminus of p53 activated p53-specific transcription and induced p53-dependent cell-cycle
arrest (Hupp and Lane, 1995; Huang et al., 1996). p53 may be activated as a transcription factor in response to a DNA damage-inducing agent has been reported. p53 is modified at several additional sites, and these may function independently or in conjunction with acetylation to activate p53 in response to UV or other agents; modifications to other components also may be important (Sakaguchi et al., 1998). It is clear that numerous agents in fruits and vegetables can interfere with multiple cell-signaling pathways. More clinical trials are also needed to validate the usefulness of these agents either alone or in combination with existing therapy. Thus in the present study, the extract was capable of increasing total p53 and decreasing phosphorylated p53 indicating its protective role at molecular level. Previous advances in understanding the initial accumulation and activation of p53 have added a layer of complexity to this pathway (Brooks and Gu, 2003). Thus further investigation in studying the effects B. retusa extracts on p53 protein expression. Considering the breadth of complexity in the regulation of p53 function, advances continue to show the multitude of mechanisms the cell uses to stabilize and activate this essential protein.

The earlier experimental results indicated that aqueous methanolic extract of G. lucidum possesses significant protective effect against DMBA-induced mammary tumour and antipromotional effect against skin tumour (Lakshmi et al., 2009). G. lucidum with its antitumour activity and the polysaccharides with a broad spectrum of immune modulating activities may represent a novel immunotherapeutic agent in cancer therapy (Zhou et al., 2007). Hence, further studies have to be conducted to find out the exact mechanism of the ethanolic extract of B. retusa. Experiments in Sprague Dawley rats showed that feeding broccoli, cabbage, Brussels sprouts protected against 7, 12-dimethylbenz(a)anthracene (DMBA) induced mammary tumors (Stoewsand, 1988). Dietary garlic supplementation has shown to protect against experimentally induced cancers at various sites in animal models (Ip et al., 1992). Previous results presented that provide a rational for the anti-tumor activity of garlic oil against DMBA-induced sub-maxillary salivary glands carcinogenesis in rats. The garlic oil seems to exert adjuvant effect on various defense mechanisms of the host against chemically-induced carcinogenesis through increased availability and utilization of
beta-carotene (Ziu et al., 1994). Consumption of tea on a regular basis has been associated with reduced risk of several forms of cancer in human populations and mouse models. There is strong evidence linking green tea use to reduction in cancer risk in parts of Asia (Dreosti et al., 1997). These previous reports have reported that green tea catechins exhibited many biological functions, including anti-carcinogenic activity. However, the molecular mechanisms of those flavonoids action were unclear. Though, these reports have described the anti-carcinogenic effects of tea flavonoids and the molecular mechanisms which may be involved which may also be the case in present study since flavonoids were detected. All these studies indicate that the phytoconstituents may be responsible for anticancer activity of the various extracts used in the investigation which may also be the case in present study. Thus the exact mechanism for such protective activity may be one or combinational of many. There is still much that remains unknown about the in vivo molecular mechanisms of ethanolic extract that affects mammary carcinogenesis. Hence initiating further research on the ethanolic extract of B. retusa and elucidating the constituent(s) as well as mechanism(s) becomes important at pharmaceutical view point.
SUMMARY AND CONCLUSION

Female Sprague Dawley rats (45-50 days) were treated with DMBA to induce mammary cancer and the protective role of ethanolic extract of stem bark of *B. retusa* was investigated which was compared to cyclophosphamide (standard drug). The change in body weight, tumor size and organs weight as well as oxidative stress caused by DMBA in mammary tissue, liver, kidney, erythrocytes and serum was studied which were identified to be brought to normal values by both ethanolic extracts.

1. The weight loss due to DMBA was not significant but was enhanced by treatment with plant extract in the present study. The toxic effect of DMBA in the present study was observed by decrease in the weight of liver and kidney. The weight loss may be due to less consumption of food and cancer cachexia which was rectified by the extract indicating its protective role best at 150 mg/kg which was comparable to standard drug.

2. Histopathological studies revealed damage caused by DMBA to mammary tissue, liver and kidney was reduced on treatment with extract and standard drug. The metastasis was not found in vital organs of liver and kidney, though toxicity due to DMBA was observed. Although, the carcinomas (ductal) retained normal architecture of the gland, there was invasion of the surrounding tissues and the tissue invasion was mostly local. No metastasis was observed in the vital organs like liver and kidney, though signs of toxicity were observed but no appearance of cancerous tissue which may have needed longer time then carried out in the present study.

3. The transaminases (SGOT and SGPT) were significantly increased in DMBA treated group when compared to that of negative control group and the activity in extract treated group was almost equivalent to that of standard drug in the present study. These enzymes are localized in tissues and hence leakage of these enzymes into the serum indicates the extent of damage to the tissue. The extract decreased the levels of these enzymes indicating decrease in tissue lyses. The levels of VEGF responsible for the formation of new blood vessels that helps in tumor development was
EXPLANATION TO PHOTOGRAPHS

Effect of ethanolic extract of stem bark of *Bridelia retusa* (EESBB) on gross appearance of mammary glands of DMBA treated rats.

**Fig. 1** : Gross appearance of the control rat (negative control) showing normal mammary glands.

**Fig. 2** : Gross appearance of DMBA induced mammary tumor bearing rats.

**Fig. 3** : Gross appearance of DMBA induced mammary tumor bearing rats treated with 1 mg/kg (standard drug) cyclophosphamide.

**Fig. 4** : Gross appearance of DMBA induced mammary tumor bearing rats treated with 150 mg/kg ethanol extract.

**Fig. 5** : Gross appearance of DMBA induced mammary tumor bearing rats treated with 100 mg/kg aqueous extract.

**Fig. 6** : Gross appearance of DMBA induced mammary tumor bearing rats treated with 150 mg/kg aqueous extract.
EXPLANATION TO MICROPHOTOGRAPHS

Effect of ethanolic extract of stem bark of *Bridelia retusa* (EESBB) on mammary tissue of DMBA treated rats

**Fig. 1** : T.S. of the mammary tissue of the normal rat (negative control) showing mammary ducts surrounded by adipose tissue.

**Fig. 2** : T.S. of the mammary tissue of the rat (positive control) treated with DMBA, histologic study of the mammary tissue exhibited severe mammary tissue degenerated with severe invasion of ductular tissue by cancer cells and loss of lobular integrity.

**Fig. 3** : T.S. of the mammary tissue of the rats treated with DMBA and cyclophosphamide (standard drug), histologic study of the mammary tissue exhibited moderate regeneration and reduction in necrotic changes. Ducts with distinct epithelium were also visible.

**Fig. 4** : T.S. of the mammary tissue of the rats treated with DMBA and 50 mg/kg ethanol extract, histologic study of the mammary tissue showed moderately decreased necrosis and revival of tissue integrity.

**Fig. 5** : T.S. of the mammary tissue of the rats treated with DMBA and 100 mg/kg aqueous extract for 5 days, histologic study of the mammary tissue revealed moderate degeneration and necrosis. Distinct lobules visible showing lesser invasion of tissue by cancer cells.

**Fig. 6** : T.S. of the mammary tissue of the rats treated DMBA and 150 mg/kg aqueous extract for 5 days, histologic observations revealed moderate regeneration and reduction in necrosis. Ducts with distinct epithelium were also visible.

Photographs original exposures at 100 X

| E   | - Epithelium     |
| D   | - Ducts          |
| DE  | - Ductal epithelium |
| IDC | - Invasive Ductal Carcinoma |
| L   | - Lobules        |
| N   | - Necrosis       |
EXPLANATION TO MICROPHOTOGRAPHS

Effect of ethanolic extract of stem bark of Bridelia retusa (EESBB) on liver of DMBA treated rats.

Fig. 1 : T.S. of the liver of the control rats showing radially arranged hepatic cords around the central vein. The hepatocytes with centrally located nuclei

Fig. 2 : T.S. of the liver of the rats treated with DMBA, histologic study of the liver exhibited normal structure of liver lobules revealed dilation of central vein and infiltration around portal triad. Necrosis was abundant with loss of radial arrangement of hepatocytes.

Fig. 3 : T.S. of the liver of the rats treated with DMBA and cyclophosphamide, histologic study of the liver exhibited normal structure of liver lobules showing hepatic cords with cuboidal or polyhedral hepatic cells with sinusoids and negligible necrosis.

Fig. 4 : T.S. of the liver of the rats treated with DMBA and 50 mg of ethanol extract, histologic study of the liver revealed decreased dilation of central vein and necrosis.

Fig. 5 : T.S. of the liver of the rats treated with DMBA and 100 mg of ethanolic extract, histologic observations revealed hepatocytes with lesser dilation of central vein and necrosis.

Fig. 6 : T.S. of the liver of the rats treated with DMBA and 150 mg of ethanolic extract, histologic observations revealed hepatocytes with central vein and radial arrangement with minimum necrosis.

Photographs original exposures at 100 X

CV - Central vein
H - Hepatocytes
EXPLANATION TO MICROPHOTOGRAPHS

Effect of ethanolic extract of stem bark of *Bridelia retusa* (EESBB) on kidney of DMBA treated rats.

**Fig. 1** : T.S. of the kidney of the control rats showing cortical tubules with normal arrangement. Thick epithelial cells with prominent glomerulus in Bowman’s capsule.

**Fig. 2** : T.S. of the kidney of the rats treated with DMBA showed glomerular and tubular necrotic damage. Small and atrophied glomeruli, loosely arranged in Bowman’s capsule were also observed.

**Fig. 3** : T.S. of the kidney of the rats treated with DMBA and cyclophosphamide displayed lesser disorganized glomerulus attached to Bowman’s capsule with reduced necrosis.

**Fig. 4** : T.S. of the kidney of the rats treated with DMBA and 50 mg ethanolic extract showed small and atrophied glomeruli, less loosely arranged in Bowman’s capsule and decreased necrosis.

**Fig. 5** : T.S. of the kidney of the rats treated with DMBA and 100 mg ethanolic extract revealed that the glomeruli were less atrophied and attached to Bowman’s capsule with minimum necrosis.

**Fig. 6** : T.S. of the kidney of the rats treated with DMBA and 150 mg revealed that the cortical tubules with glomeruli attached to Bowman’s capsule. The necrotic damage was also reduced.

Photographs original exposure at 100 X

CT - Cortical tubules
G - Glomerulus
BC - Bowman’s capsule
TC - Tubular cell
EXPLANATION TO PHOTOGRAPHS

**Fig 1.** The total proteins were extracted from the mammary gland tissue samples, loaded on SDS-PAGE to obtain expression between 66 and 51 kDa for all the lanes.

**Fig 2.** SDS-PAGE analysis of the samples immunoprecipitated with anti-p53 antibody showed bands at 51 kDa for all the lanes.

PM – protein marker, L1 – Control group, L2 – DMBA treated group 2, L3 – Standard drug (cyclophosphamide) treatment to DMBA exposed group, L4 – 50 mg/kg b.wt. ethanolic extract treatment to DMBA exposed group, L5– 100 mg/kg b.wt. ethanolic extract treatment to DMBA exposed group, L6 – 150 mg/kg b.wt. of ethanolic extract treatment to DMBA exposed group
EXPLANATION TO PHOTOGRAPHS

Fig 3. Nitrocellulose membrane after development with 1X TMB-H₂O₂ (Primary antibody used was p53-Ab) showed bands at 90-50 kDa for all the lanes.

Fig 4. Nitrocellulose membrane after development with 1X TMB-H₂O₂ (Primary antibody used was α- phosphotyrosine -Ab) showed bands below 66 kDa for all the lanes.

PM –protein marker, L1 – Control group, L2 – DMBA treated group 2, L3 – Standard drug (cyclophosphamide) treatment to DMBA exposed group, L4 – 50 mg/kg b.wt. ethanolic extract treatment to DMBA exposed group, L5– 100 mg/kg b.wt. ethanolic extract treatment to DMBA exposed group, L6 – 150 mg/kg b.wt.of ethanolic extract treatment to DMBA exposed group
decreased in dose dependent manner by the extract but the effects of the standard drug was much better

4. The stress caused due to DMBA in the present study was evident by significant increase in lipid peroxidation and decrease SOD and CAT activity in the mammary tissue, liver, kidney erythrocytes and serum. The antioxidant effect of extract was observed by significant decrease in lipid peroxidation and increase in SOD and CAT activity comparable to standard drug. SOD is the first and most the important line of antioxidant enzyme defense against ROS and particularly oxygen radical. SOD demolishes superoxide by converting it into peroxide that can in turn be destroyed by CAT. The possible modulation of the antioxidant system that could decompose the peroxides and thereby offering a protection against lipid peroxidation. This may be due to protection against production of radicals which may have inhibited activity of the enzymes, thus indicating antioxidant property of the extract.

5. The protein p53 expression was increased in all the mammary tissue of cancer bearing rats indicating essential role of this protein against cancer. The active phosphorylated forms of p53 protein was reduced to that of normal on treatment with extract in dose dependent manner with best results at 150 mg of extract which was better than standard drug. Thus indicating positive effects of the extract at molecular levels.

6. Overall the carcinogenesis was reduced in dose dependent manner with 150 mg of ethanolic extract showing results comparatively similar to that of cyclophosphamide (standard drug). The toxic effects of DMBA in mammary tissue, liver, kidney, erythrocytes and serum were brought to normal evident from hithological and study of oxidative stress parameters. This may be due to various substances present in the extract that need further investigation.

The promising results obtained indicate extensive study on this plant will enable to exploit its potentials. The anticancer and antioxidant activity of the plant along with restoration of histology proves therapeutic property of the extract.