6. DISCUSSION

Carcinogenesis is an extremely dynamic, nonlinear process following unpredictable pathways. After immense research into its etiology, pathways and mechanisms, it still remains to be vulnerable disease. Despite the advancements in diagnosis and treatment modalities, the mortality and morbidity associated with cancer is colossal.

For centuries, plant- derived medicines have been a cornerstone of most traditional medical systems with ample of important drugs coming out of it. Multiple studies have found that within one year, up to 90 percent of patients with cancer used a complementary and alternative medicine approach for at least a part of their therapy [286-289] The herbs alter the endogenous pathways and their associated proteins (hormones/enzymes) as well as inconveniences of chemotherapy and radiotherapy.[22] Literature suggests using whole plant extract should be approached rather than using isolated constituents.[21-26] A living plant is a complex system with tons of phytoconstituents working in collaboration creating a majestic effect than isolated components in single molecule pharmaceuticals.[22]

Consequently, an effort was made in the present examination to recognize an effectively accessible regular herb and assess its potential in the treatment or aversion of breast cancer. The three medicinal plants selected for the study were *Butea monosperma* flowers, *Lycopersicon esculentum* fruits and *Cassia fistula* pods. *Butea monosperma* have been reported to be associated with various properties such as antiestrogenic activity, [28-31] apoptotic,[29, 30] free radical scavenging activity[29, 30] and antitumor property against hepatic carcinoma.[28] The *Lycopersicon esculentum* fruit clinically proven to reduce the risk of prostate cancer[37, 38, 41] consists of four major carotenoids: alpha- and

beta-carotene, lutein, and lycopene.[41] Lycopene as properties like aromatase inhibition,[47] and VEGF inhibition.[46] *Cassia fistula* was found to be potent anticancer agent on human colon cancer cell line,[50] and possess antiestrogenic activity.[53]

The selection of various extracts (aqueous, methanol, butanol and ethyl acetate) was based on various *in-vitro* and *in-vivo* anticancer studies as well as presence of reported chemo-preventive phytoconsituents in the extracts. The well-known chemo-preventive phytoconstituents present in various plants are phenolic compounds, tannins, flavonoids, carotenoids and curcuminoids.[290] Literature revealed the presence of some anti-cancer phytoconstituents in selected plant *viz.* Butein, Lycopene, Carotenes, Rhein etc.[28, 259, 291-302]

To scrutinize anticancer potential, one needs to target the four essential hallmarks of cancer (uncontrolled proliferation, sustained angiogenesis, evasion of apoptosis, tissue invasion and metastasis).[303] To begin with, it was planned to screen proficiency of selected plant extracts to halt the uncontrolled cell proliferation. The anti-proliferative study designed in cell lines is the most widely utilized models to recapitulate the science of cancer lines and to test the hypotheses of efficacy of chemotherapeutic agent. [145-148] Thus, in present study, three breast cancer cell lines were selected on the basis of genetics of breast cancer *viz.* MCF-7, MDA-MB-453 and MDA-MB-231.[304]

MCF-7 is the most popular cell line due to its exquisite hormone sensitivity through expression of estrogen receptor, making it perfect for studying hormone response.[304, 305] The HER- 2 positive breast cancer cell line is MDA-MB-453.[306] For triple negative breast cancer cell line, MDA-MB-231 was selected.[304, 306, 307]

To study cell inhibition or cytotoxicity, the widely used cell line based assay is MTT assay.[308] MTT cell proliferation and viability assay is a safe, sensitive, in vitro assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis.[308] The selected extracts of each plant were exposed to human breast cancer cell lines for 24 h, 48 h and 72 hr at wide concentration range (10-1000 µg/ml); above which the cytotoxic potential is not appreciated.

The *Butea monosperma* flower extracts (AEBM, MEBM, BEBM and EAEBM) showed greater cytotoxic potential against MCF-7 hormone positive breast cancer cell line than other two cell lines in time- and dose- dependent manner. At 24 h, in MCF-7 cell line, IC_{50} of AEBM, MEBM and BEBM extracts fell in selected concentration range (10-1000 µg/ml). Prolonging time of exposure of cells of AEBM, MEBM, BEBM and EAEBM from 24 to72 hours, further lower downs the IC_{50} values (MEBM (50 µg/ml) > AEBM (300 µg/ml) > BEBM (450 µg/ml) > EAEBM (480 µg/ml)). In other two cell lines, only MEBM showed cell inhibitory activity at 72 h. All other extracts (AEBM, BEBM and EAEBM) were not found to be anti-proliferative on MDA-MB-453 and MDA-MB-231 human breast cancer cell line. **The maximum inhibition of cell proliferation was observed with MEBM and AEBM as depicted by lowest IC_{50} values on MCF-7 estrogen positive human breast cancer cell line.**

The *Lycopersicon esculentum* fruits extracts (AELE, MELE, BELE and EAELE) showed highest cytotoxic activity against MCF-7 cell line. At 24 hours, IC_{50} value of EAELE was observed to be 750 µg/ml. Other extracts *viz.* AELE, MELE and BELE does not exhibited IC_{50} values in selected concentration range (10-1000 µg/ml). At 48 hours, IC_{50} values of EAELE further decreased to 550 µg/ml, followed by MELE (893 µg/ml) and AELE

(1000 µg/ml). BELE failed to display cell inhibition at 48 hours in selected concentration range (10- 1000 µg/ml). At 72 h, the highest cell inhibition was obtained by EAELE and MELE with IC_{50} values 215 µg/ml and 560 µg/ml. The AELE, MELE, BELE and EAELE failed to manifest anti-proliferative effect on HER-2 positive MDA-MB-453 and triple negative MDA-MB-231 cell lines as portrayed from IC_{50} values. **The comparison of IC_{50} values of selected extracts (AELE, MELE, BELE and EAELE) on MCF-7 human breast cancer cell line, the maximum cytotoxic potential was observed with EAELE and MELE.**

The *Cassia fistula* pods extracts (AECF, MECF, BECF and EAECF) showed cytotoxicity only against MCF-7 breast cancer cell line perceived from IC_{50} values. For other two cell lines *viz.* MDA-MB-453 and MDA-MB-231, IC_{50} values were above the concentration range (10- 1000 µg/ml) selected for the studies. Hence, they lack cytotoxic potential against MDA-MB-453 and MDA-MB-231 human breast cancer cell lines. The enhancement of cytotoxicity with increasing time of exposure (24h, 48h and 72h) was depicted by IC_{50} value of AECF (344, 300 and 134 µg/ml respectively) and MECF (525, 435 and 300µg/ml respectively). The IC_{50} values of BECF (650 µg/ml) and EAECF (660µg/ml) on MCF-7 human breast cancer cell line were higher than AECF and MECF at 72 hours. **The maximal anti-proliferative effect was observed with AECF and MECF in MCF-7 estrogen positive human breast cancer cell line as illustrated by lowest IC_{50} values.**

Thus, in present study, IC_{50} value of extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) on MCF-7 cells was found significantly less than that of MDA-MB-231 and MDA-MB-453 cells, which indicated that the extracts of said medicinal plants were more potent inhibitors of

hormone positive breast cancer cells than other types of breast cancer cells *in vitro*.

The anti-proliferative effect of extracts in MTT assay on cancer cell line is justifiable and rational only if it is found to be non-toxic to normal non-malignant human cells. Thus, to detect safety towards normal breast tissues, the extracts were exposed to MCF-10A human epithelial breast cell line for 72 hours. In current research, none of the extracts of said medicinal plants possess cytotoxic action on non-malignant MCF-10A human epithelial breast cell line. **Thus, it can be indisputably concluded that the extracts are selectively cytotoxic to human breast cancer cell line MCF-7 and safe in normal human breast epithelial cell line MCF-10A.**

To further validate the anti-cancer effect of extracts, testing of the extracts using robust preclinical models is the next pivotal step. The *in-vivo* mammary carcinogenesis was induced in rats by injecting Methylnitrosourea (MNU). Moreover, elucidating the effect on other alterations of cancer cell physiology *viz*. angiogenesis, evasion of apoptosis, metastasis and oxidative stress, the *in-vitro* assays were commenced. For assessment of *in-vivo* anticancer potential in MNU actuated mammary cancer and mechanisms *in-vitro* the extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) were narrowed down to two pe plant under investigation based on IC_{50} values obtained in MTT assay.

As said, “An ounce of prevention is worth a pound of cure”. The small preventive efforts can substantially decrease or eliminate negative outcome. Hence, the preventive studies were designed in female Sprague Dawley rats by intra peritoneal administration of N-methyl N- nitrosourea (MNU).[149] MNU is a water soluble direct alkylating carcinogen,[149] and highly specific carcinogen for mammary gland inducing hormone positive adenocarcinomas which are

similar to humans.[151, 153, 154] Literature survey reveals that at age of 50 days, injecting 50mg/kg b.w (i.p.) MNU to nulliparous Sprague Dawley (SD) female rats induces mammary carcinoma.[156-158]

In present study, injecting MNU (50mg/kg b.w; i.p.) to nulliparous SD female resulted in mammary tumors. Tumor was induced after 45 days post MNU injection. Five out of six rats developed mammary carcinoma suggesting 83.33% tumor incidence. Tumor burden i.e. total number of tumors in MNU injected groups was found to be 6. One rat developed two mammary tumors. Tumor multiplicity i.e. number of tumors per rat was found to be 1. Tumor weight and volume were found to be 6.4±1.47 g and 99.29 ±3.19 mm^3 respectively. The data proposed successful induction of mammary carcinomas in MNU injected rats.

Various studies proved that flavonoids present in *Butea monosperma* flowers extracts are anti-carcinogenic.[28, 236] The results of present investigation are in line with the reported literature. Oral administration of extracts to SD rats bearing MNU induced mammary cancer significantly decreased the tumor parameters. The tumor incidence was reduced to 33.33% by administration of MEBM 400. With AEBM (200 and 400), the tumor incidence found was tantamount to Tamoxifen. It is worth noting that tumor incidence and tumor burden was lower in MEBM than Tamoxifen. The tumor weight was three times lesser in MEBM while two times reduction was seen with AEBM. The tumor volume with MEBM 100 indicated ten-fold diminished over model control. The curtailment in tumor volume with AEBM ranged from four-fold to ten-fold. The latency period was prolonged in all treatment groups with 99.99% confidence level. The latency period with MEBM 400 was similar to that of Tamoxifen. **Thus, the results demonstrated MEBM and AEBM to possess anti-tumor activity *in-vivo* in MNU induced mammary carcinoma.**

Scientists reported carotenoids to be anti-carcinogenic[298] and *Lycopersicon esculentum* fruit contains four carotenoids.[41] Furthermore, studies on humans suggest inverse correlation risk of breast cancer and lycopene.[309] The treatment with EAELE 400 showed reduction in tumor incidence similar to Tamoxifen. The decline in tumor weight with EAELE (100, 200 and 400) ranges from five-fold to three-fold, while that of MELE (100, 200 and 400) was three to two-folds. The 95.97 % depletion in tumor volume was observed with EAELE 400 while 87.71% depletion was seen with MELE 400. The latency period was significantly prolonged with EAELE (100, 200 and 400) and MELE (100, 200 and 400). **Therefore, it can be inferred that in MNU induced mammary carcinoma, EAELE (100, 200 and 400) and MELE (100, 200 and 400) possess anti-proliferative activity.**

Aviello *et al.* proved that anthraquninone class of compounds present in *Cassia* species possess anti-proliferative effect against human colon adenocarcinoma cell line.[302] The treatment with AECF (100, 200 and 400) and MECF (100, 200 and 400) appreciably decreased tumor weight; with remarkable two-fold decrease with AECF 400 treated animals. Greater than three-fold decrease was observed in tumor volume with AECF 100 and MECF 100. The latency period was delayed in AECF (100, 200 and 400) and MECF (100, 200 and 400). **Hence, in present study it can be deduced that AECF (100, 200 and 400) and MECF (100, 200 and 400) have beneficial activity against mammary carcinoma induced by MNU in female rats.**

The results indisputably confirm induction of mammary carcinoma by single injection of MNU (50mg/kg; i.p) to nulliparous female Sprague Dawley rats of 50 days. The extracts *viz.* MEBM, AEBM, EAELE, MELE, AECF and MECF possess tumor growth inhibitory activity *in-vivo* in MNU induced mammary carcinoma which might be contributed to chemo-

preventive phytoconstituents *viz*. flavonoids, tannins, phenolic compounds, carotenoids and anthraquinones.

Numerous studies has reported significant reduction in the body weight and conversely an extensive increment in the organ and as well as tumor weight in cancer patients.[310] Weight loss along with anemia and anorexia is termed as cachexia. It is observed in 80% of patients.[311] Weight loss in cancer patients serve as prognostic marker and it is due to equal loss of both adipose tissue and skeletal muscle mass [310]

In present study, the body weight was expressed as % growth rate. In cancer bearing untreated animals, the induction was confirmed by de-escalation of growth curve and increase in relative organ weight. The growth curve of treated groups (Tamoxifen, MEBM, AEBM, EAELE, MELE, AECF and MECF) runs parallel to normal animals and showed no significant difference in relative organ weight as compared to normal animals. **This goes in the confirmation with the toxicity studies performed by various researchers depicting safety of extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) on normal growth in rats.**

Anorexia is an imperative factor causing weight reduction in cancer patients. The homeostatic loop of body weight and food intake is disrupted in cancer patients.[310, 311] In current research, food intake was illustrated as feed consumption efficiency. The feed consumption efficiency was significantly reduced in model control animals as compared to normal control. In treated groups (Tamoxifen, MEBM, AEBM, EAELE, MELE, AECF and MECF), the feed consumption curve is parallel to normal control group. **The decrease in feed consumption efficiency of MNU injected untreated animals proposes tumor induction and utilization of nutrients in tumor growth. The aversion**

of these changes by extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) is very well perceptible in tumor parameters.

Among the many factors influencing development of tumor by MNU, oxidative stress has crucial role in initiation of mammary carcinoma. [268] Also, oxidative stress is one of the intersecting factors amongst all cancer. The free radicals generated due to oxidative stress causes peroxidation of cell membrane.[312, 313] Lipid peroxidation has been implicated in several pathologic conditions including aging, hepatotoxicity, hemolysis and cancer. The reactive oxygen species formed damages intracellular molecules like DNA, RNA, lipids and proteins. They cause nicks in DNA and malfunctions in repair mechanism of DNA.[312, 314] Malondialdehyde (MDA) is the major end product of lipid peroxidation. Studies suggest that cancer patients have high levels of DNA-MDA adduct.[315] Also, the increased DNA content in breast cancer might be due to the escalation of DNA synthesizing enzyme expression with repression of many enzymes related to differentiated cell function. RNA levels were additionally observed to be expanded in the malignant condition. The atypical increase in DNA content leads to an increased transcription with increased RNA content of tumor cells.[316]

Khanzode and colleagues have also reported that the oxidative stress generated in cancer conditions are due to an imbalance between pro-oxidants and antioxidants status and it seems to play an important role in breast cancer.[317] It is reported that the enzymatic and non-enzymatic antioxidants are the form of the first line defense mechanism to scavenge excessively generated ROS in the system. Hence, antioxidant status has been suggested as a useful tool in estimating risk of oxidative damage induced carcinogenesis. SOD, CAT and GSH act mutually and constitute the enzymatic anti-oxidative defense mechanism against reactive oxygen species.[268]

In the present investigation, the increased levels of MDA and nucleic acids were observed in breast homogenates of MNU treated animals.[313] This may be due to the free radicals induced by MNU during tumor carcinogensis.[268] **The decreased activities of antioxidant enzymes (GSH, SOD and Catalase) in cancer bearing untreated group is probably due to exhaustion of these enzymes to scavenge excessively generated reactive oxygen species produced by MNU during mammary carcinoma development.**

The numerous studies proved selected medicinal plant as potent antioxidant. The hydroalcholic extract of *Butea monoseprma* was proven to be antioxidant in diabetic mice.[318] Sehrawat *et. al.* proved Butein from flowers of said plant imparts free radical scavenging and anti- oxidative properties better than Butrin.[29] The study also proved reversal of imbalance of antioxidant enzymes by treatment of Butein.[29] Tons of studies proved carotenoids as quenchers of singlet oxygen and scavengers of other reactive oxygen species.[319-321] Lycopene present in *Lycopersicon esculentum* is one of the most potent antioxidants amongst carotenoids[39, 322] and has been suggested to prevent carcinogenesis by protecting critical biomolecules including proteins, lipids and DNA.[323, 324] One of the study disclosed that aqueous extract of *Cassia fistula* flowers appreciably decreased peroxidation products in heart tissues of diabetic rats.[254] The methanol bark extract was also reported to be anti-oxidant in Wistar rats where the decreased activities of key antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione in diabetic rats were brought back to near normal range upon treatment.[252]

The reports of present study are in harmony with the previous studies. MEBM (100,200 and 400) and AEBM (100,200 and 400) attenuated lipid peroxidation by 32.22%, 36.99%, 47.73%, 27.45%, 32.22% and 42.96% respectively as

compared to cancer bearing untreated animals. EAELE (100,200 and 400) and MELE (200 and 400) decreased MDA levels by 25.06%, 33.41%, 40.57%, 27.45% and 32.22% respectively. AECF (200 and 400) and MECF (200 and 400) decreased MDA levels by 26.25%, 38.16%, 28.63% and 42.95% respectively as compared to model control. The percentage rise in nucleic acid was attenuated by all doses (100, 200 and 400) of MEBM, AEBM, EAELE and AECF. The MELE 400 was effective to decrease nucleic acids levels. MECF showed no effect. **The decrease in lipid peroxidation depicted by MDA values and attenuation in nucleic acid levels by extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) may be contributed to phytoconstituents *viz*. flavonoids, tannins, carotenoids, phenolic compounds etc. who combat the oxidative stress generated by MNU during tumorigenesis.**

The percentage rise in GSH levels by MEBM (100,200 and 400) and AEBM (100,200 and 400) were 66.56%, 77.13%, 85.58%, 57.05%, 68.32% and 79.24% respectively as compared to MNU untreated group. The percentage rise in SOD levels with MEBM (100,200 and 400) and AEBM (100,200 and 400) were above 125% as compared to model group. The Catalase levels were significantly different only in MEBM (200 and 400) as compared to model group and the noted percentage rise was 32.54% and 51.62%.

The EAELE (100,200 and 400) and MELE (200 and 400) significantly increased GSH level as compared to model group. The percentage rise noted was 23.90%, 61.43%, 95.84%, 23.73% and 53.69% respectively. The percentage rise in SOD levels with EAELE (100,200 and 400) and MELE (100,200 and 400) were above 100% as compared to model group. The percentage rise of Catalase by EAELE (100,200 and 400) and MELE (200 and 400) was observed to be 17.13%, 40.62%, 58.93%, 16.98% and 20.50%.

The AECF (200 and 400) and MECF (200 and 400) significantly increased GSH level as compared to model group. The percentage rise in GSH levels noted was 46.35, 48.40%, 49.37% and 80.45% respectively. The percentage rise in SOD levels with AECF (100,200 and 400) and MECF (100,200 and 400) were 74.92%, 83.70%, 89.56%, 74.81%, 90.06% and 114.43% as compared to model group. No significant change was observed in Catalase activity.

Thus, MEBM (100,200 and 400) and EAELE (100,200 and 400) reinstated all the above mentioned antioxidant enzyme levels *viz.* GSH, SOD and Catalase in significant proportion as compared to model group, thus combating the deleterious effect of MNU, evident by dwindled tumor parameters. AEBM (100,200 and 400), AECF (200 and 400) and MECF (200 and 400) possess beneficial effect on GSH. SOD levels were restored by all the extracts with 99.99% level of significance. The drop in catalase levels were restrained by MEBM (200 and 400), EAELE (100,200 and 400) and MELE (200 and 400). The positive effect on antioxidant status can be contributed to presence of chemical constituents like flavonoids, phenolic compounds, tannins, carotenoids etc.

The carcinogen MNU is reported to produce mammary tumors by oxidative stress which is hormone positive *viz.* estrogen and progesterone. [88, 91] Also clinically, the foremost step in breast cancer diagnosis and treatment is clarifying hormone receptor status as treatment modality and outcome depends on it.[325] The immunohistochemistry is used most often to find out if cancer cells have estrogen and progesterone receptors.[326, 327] With this overview and to confirm *in-vitro* results, estrogen and progesterone receptor's immunohistochemistry was performed in tumor tissues in MNU induced mammary carcinogen model. ER activation in breast and uterus enhances cell

proliferation which is necessary for growth and maintenance of tissues.[328] When the response to estrogens by the endocrine system is deregulated, ER activation might eventually result in tumor formation. Studies suggests that reduced levels of ER-α in the mammary gland predict low breast cancer risk. Overexpression of ER can be due to binding of growth factors also like IGF-I, IGF II, EGF and TGF. More than two-third patients manifest expression of both estrogen and progesterone receptor. One of the clinical studies suggests that combination of Selective Estrogen Receptor Modulator (SERM) or Selective Progesterone Receptor Modulator (SPRM) elicited 70% tumor size regression as compared to individual therapies.[329] Moreover, progesterone acts via sensitizing mammary cancer cells to the actions of growth factors by upregulating target genes of signal transduction pathways.[330]

The findings of present study are in line with previous studies[88, 91] demonstrating that MNU injected model control rats showed increased expression levels of ER (96.34%) and PR (67.98%) as compared to normal group. **Thus, the single injection of MNU (50mg/kg; i.p) to nulliparous female Sprague Dawley rats produced mammary tumors expressing estrogen and progesterone receptors.**

The studies on Butein suggests inhibition of testosterone- induced proliferation in breast cancer cells expressing aromatase, proving its role in inhibition of estrogen synthesis as aromatase inhibitor.[35] Various reported studies suggested alcoholic extract of said flowers exhibited antiestrogenic activity.[31, 331] The MEBM and AEBM treated animals exhibited 36.29% and 30% decrement in expression of estrogen receptor in tumor tissues respectively as compared to model group. The regression of progesterone levels in MEBM and AEBM treated animals is 31.63% and 22.74% respectively as compared to model group.

Literature revealed lycopene and other carotenoids possess anti-estrogenic activity.[48, 49] Moreover, the leaves of *Lycopersicon esculentum* is found to be aromatase inhibitor, and thus how also it might reduce estrogen levels in present study.[332] The leaves and fruits contain same chemical constituents like lycopene and carotenoids.[333] The ER and PR percentage decrease in EAELE and MELE treated animals were 25%, 13.33%, 24.34% and 15.30% respectively as compared to model group.

The studies on *Cassia fistula* seeds of said plant is reported to be antiestrogenic in overietomized female rats.[53] In present study, the % decrease in estrogen levels with AECF and MECF was found to be 25% and 10% respectively while % PR decrease was found to be 14.10% and 4.55% respectively as compared to model group.

The results of *in-vivo* studies match up with *in-vitro* studies suggesting that the extracts (MEBM, AEBM, EAELE, MELE and AECF) induce cytotoxic effect by a mechanism involved with estrogen and progesterone receptor modulation.

Having established the anti-proliferative action in MCF-7 cell line and decrease in tumor burden as well as hormone (estrogen and progesterone) expressions, it was necessary to explore effect on other manifestations of cancer cell physiology.

The primitive step in tumorgenesis is neovascularization or angiogenesis. The cancer cells are able to kick start angiogenesis, assuring that cancer cells are in continuous supply of oxygen and nutrients to grow. This key procedure also fill in as means for tumor cells to metastasize.[334] It can be suggested that angiogenesis may act as a separate individual prognostic factor for breast cancer in humans.[335] Among diverse range of models to study anti-angiogenic

potential, chick embryo models is widely used.[336] For studies, the use of chick embryo models is facilitated by the existence in avian species of a specialized respiratory tissue, named the "chorioallantoic membrane" that allows gas exchange between the atmosphere and embryo.[337] In the chick embryo, the chorioallantois is formed between days 4 and 5 of development, when the outer mesodermal layer of the allantois fuses with the mesodermal lining of the chorion, and a network of blood vessels is gradually formed between the two layers.[337] The middle portion of chorioallantoic membrane is fully developed by 8 or 9 day and is ready to response to proangiogenic or antiangiogenic stimuli. The vasculature keeps on growing till day 12 of embryonic stage. Furthermore, the chick embryo is naturally immune-incompetent till day 17 allowing grafting of different species origin.[336, 337]

In present study, for selection of dose, five concentrations were screened i.e. 10, 20, 30, 40 and 50 µg/ml. The experiment was repeated with the lowest inhibitory concentration and chorioallantoic membrane was photographed. The 3± 0.18 mm, 2.5 ± 0.45 mm and 1 ± 0.75 mm zone of inhibition is seen with MEBM (20 µg/ml), EAELE (20 µg/ml) and AECF (30 µg/ml) respectively. Tumor growth is dependent on angiogenesis. Inhibiting tumor angiogenesis halt the tumor growth and metastatic potential of tumors *in-vivo.* AEBM, MELE and MECF showed no significant anti-angiogenic potency within the selected concentration range. **Out of six extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) screened for anti-angiogenic property using CAM assay, only three *viz.* MEBM, EAELE and AECF demonstrated decrease in neovascularization of chorioallantoic membrane in the order - MEBM (20 µg/ml) >EAELE (20 µg/ml)> AECF (20 µg/ml) as depicted from zone of inhibition.**

The clinical and laboratory evidences suggest that cytotoxic chemotherapy and anti-angiogenic therapy are very much dependent on induction of apoptosis.[338] Cell apoptosis is a typical physiological procedure of efficiently controlled cell demise for keeping up stable inner condition of the entire living being.[339] Apoptosis happens regularly during development, aging and as a defense mechanism such as immune reaction or when cells are damaged by disease or noxious agents. In diseases like cancer, too little apoptosis occurs, resulting in malignant cells that will not die.[340] The huge amount of literature suggests that defects in apoptotic pathways play a crucial role in carcinogenesis and resistance to chemotherapeutic drugs.[341] The machinery of apoptosis is perplexing and includes many signaling pathways. Apoptosis can be activated in a cell through either the caspase-intervened extrinsic or else intrinsic pathways. Both pathways focalize to activate the effector apoptotic caspases resulting ultimately in morphological and biochemical cellular alterations, characteristics of apoptosis. The numerous new treatment procedures focusing on apoptosis pathways are possible and might be utilized as a part of the treatment of various types of cancer.

In current research, to explore apoptotic potential of plant extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF; concentration= IC_{50} value obtained in MTT assay of MCF-7 cells at 24 hours), Annexin- V FITC binding assay in MCF-7 human breast cancer cell line was performed. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V- FITC possesses high affinity for PS and thus binds to it.[342] This is used in conjunction with Propidium Iodide (PI) to identify early apoptotic population. The viable cells with intact plasma membrane exclude PI.[342] The cells which are Annexin V FITC and PI negative

(Quadrant 3) are viable and intact. The cells which are Annexin V FITC positive and PI negative (Quadrant 4) are in early apoptotic phase. The Annexin V FITC and PI positive cells are in late stage apoptosis (Quadrant 2).[342]

In literature, the phytoconstituent Butein isolated from flowers of *Butea monosperma* is proclaimed to induce apoptosis in A549 lung cancer cells[234] and in PCa prostate cancer cell line.[235] In present study, the apoptotic potential of MEBM (16.3%) was higher than AEBM (14.3%) as seen by percentage cell in apoptotic quadrants Q2 and Q4. The cell viability was decreased by MEBM and AEBM when compared to normal cells. **In apoptosis assay, the early apoptotic population in quadrant Q4 is more in MEBM and AEBM suggesting cell death is through some apoptotic pathway and not by necrosis (Quadrant Q1).**

Palozza *et.al.* demonstrated carotenoids to be responsible for modulation of apoptosis signals.[343] In one of his studies he concluded induction of apoptosis in immortalized fibroblasts exposed to tobacco smoke by downregulating signaling proteins.[296] He further proved apoptotic effect of beta-carotene in human colon adenocarcinoma cell lines.[344] The EAELE (13.7%) showed higher population in apoptosis phase (Quadrant Q2 and Q4) than MELE (10.1%) as well as decrease in cell viability in Quadrant Q3 (EAELE: 59.4%; MELE: 63.7%). **The EAELE and MELE target early stages of apoptosis (Quadrant Q4) as discerned by higher percentage of early apoptotic cell populace than late apoptotic cell populace.**

Irshad *et. al.* publicized fruit pulp and seed extract of *Cassia fistula* to be apoptotic on SiHa and MCF-7 cells.[345] On phytochemical analysis they concluded that compounds like rhein, thymol and oleic acid might be

responsible for the said property.[345] Also, literature suggested AECF possessed highest amount of anthraquinones calculated as rhein % w/v.[299] Rhein lysinate was described to downregulate anti-apoptotic proteins, Bcl-2 and cyclin D and upregulate apoptotic protein Bax in malignant glioma U87 cells.[259] The apoptotic potential of AECF (10.5%) was sky-higher than MECF (6.9%) as depicted in quadrant Q2 and Q4. **The cell population treated with AECF in Q4 is greater than Q1 evincing the cell death through apoptosis rather than necrosis.**

Thus, from Annexin V- FITC binding apoptosis assay, it can be inferred that at concentration equal to IC_{50} value obtained in MTT assay on MCF-7 cell line at 24 hours, apoptosis was induced in highest amount by MEBM, EAELE and AECF. The apoptotic effect can also be correlated to decrease in tumor volume in MNU induced mammary carcinoma.

To speculate apoptotic mechanism, the generation of oxidative stress in cancer cells was targeted. The MCF-7 cells were exposed to extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF; concentration= IC_{50} value obtained in MTT assay of MCF-7 cells at 24 hours) in presence of non-fluorescent dye DCFH-DA. DCFH-DA gets oxidized to its highly fluorescent derivative 2',7'-dichlorofluorescein (DCF) by generated reactive oxygen species. The plant extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) doesn't show increase in fluorescence as compared to untreated normal MCF-7 cells. **Hence, the plant extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF; concentration= IC_{50} value obtained in MTT assay of MCF-7 cells at 24 hours) does not produce oxidative stress in MCF-7 cancer cells as revealed by negligible fluorescence in DCFH-DA assay as compared to untreated MCF-7 cancer cells.**

The angiogenesis and evasion of apoptosis by tumor cells leads to crucial step of cancer progression, metastasis, which makes the disease deadly. Hence, to assess anti-metastatic potential of extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF concentration= IC_{50} value obtained in MTT assay of MCF-7 cells at 24 hours), scratch motility assay was commenced in MCF-7 human breast cancer cell line. Scratch assays were first used as models of wound healing for epithelial or mesenchymal cells.[346] Molecules of interest as potential therapeutics are added to the wells and images of cell movement are captured at regular intervals within a 24 hour period for data analysis.[346]

In present research, the denuded area in extract (MEBM, AEBM, EAELE, MELE, AECF and MECF) treated wells was higher than control wells. However, the anti-migratory effect of MEBM was significantly higher (11.85%) than AEBM. EAELE and AECF possess higher (5.56% and 5.92% respectively) metastatic potential than MELE and MECF respectively.

Thus, in scratch motility assay, MEBM, EAELE and AECF are deduced to be potent anti-metastatic on MCF-7 human breast cancer cell line at concentration equal to IC_{50} value obtained in MTT assay of MCF-7 cells at 24 hours. The effect might be attributed to anti-angiogenic and apoptotic phytoconstituents present in extracts namely Butein in MEBM, carotenoids and lycopene in EAELE and Rhein in AECF.

The outcome of preventive studies and *in-vitro* assays motivated us to take a step forward taking up the curative studies. The curative potential of anticancer drugs can be studied by xenograft or allograft (syngeneic model) mouse tumor systems.[165] Xenograft involves transplantation of human cancer cell lines or solid tumors in immunocompromised mice,[162] and so they don't mimic actual patient situation.[164, 165] In syngeneic model, allografts are immortalized from

mice tumor cells, which are engrafted back into same bred with fully active immune system.[164, 166] The rejection of transplant is negligible as the cancer tissues and recipient share ancestry.[164, 166] It represents tumor microenvironment more closely to real life situation.[164, 166] However, complexity of human tumors may or may not be depicted.[167] **The allograft (syngeneic) model was selected for the study with the advantage of full functional and active immunological system.**

Scrutinizing tumor parameters, nucleic acid levels, hormone (estrogen and progesterone) levels in MNU induced mammary carcinogenesis and *in-vitro* studies (CAM assay of angiogenesis, Annexin V- FITC binding assay of apoptosis and scratch motility assay of metastasis), the extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) of each plant were narrowed down to one. The results suggest that among the various extracts screened, MEBM, EAELE and AECF tackle breast cancer more aggressively and showed significantly higher anti-cancer potential than other potent extract of said plants. The effect might be contributed to phytoconstituents possessed by the extract *viz*. flavonoids specifically Butein in MEBM, carotenoids especially lycopene in EAELE and anthraquinone particularly Rhein in AECF. Furthermore, the above mentioned phytoconstituents are reported to be anti-estrogenic.

The curative study model was induced by injecting 0.2 ml of 2 X 10^6 cells of Ehrlich Ascites Carcinoma (EAC) sub-cutaneously in BALB-C female nulliparous mice. EAC appeared firstly as a spontaneous breast cancer in a female mouse.[280, 347] EAC is referred to as an undifferentiated carcinoma, and has high transplantable capability, rapid proliferation and 100% malignancy.[280] If ascites fluid injected into peritoneal cavity contains the tumor cells, the

ascites form is obtained, but a solid form is obtained when the tumor cells are injected into muscle tissue or sub-cutaneously.[280]

The *Butea monosperma* flowers, *Lycopersicon esculentum* fruits and *Cassia fistula* pods were safe up to 6000 mg/kg[222], 5000mg/kg[223] and 6600mg/kg.[220] The dose of 400 mg/kg was fixed on basis of toxicity data and the anticancer studies of various extract on EAC induced solid mammary carcinoma where the dose ranges from 100-400mg/kg,[224, 283, 348-351] beyond which the curative anticancer potential might not be appreciable.

In current EAC induced tumor model, the significant increase in body weight and fluid in peritoneal cavity was found in EAC injected untreated animals. The tumor weight and volume were rapidly increased till the end of the study in positive control mice. The death was observed from 3 day. The results suggest successful induction of solid mammary tumors in mice by EAC.

The MEBM 400, EAELE 400 and AECF 400 treated animals showed significantly decreased body weight to normal level. No accumulation of fluid in peritoneal cavity was observed in MEBM 400 and EAELE 400. However, in two animals with AECF 400 treatment, fluid accumulation in peritoneal cavity was observed and one of them died on 25 day. The total number of death observed with MEBM 400 (29^{th} day) and EAELE 400 (28^{th} day) treated group was one. The reduction in tumor volume observed was 82.81%, 69.08% and 71.98% in MEBM 400, EAELE 400 and AECF 400 treatment respectively. The anti-tumor activity of MEBM 400, EAELE 400 and AECF 400 was associated with increase in life span of EAC-bearing animals by 73%, 70.59% and 61.76% respectively as compared to model group. Tamoxifen also improved tumor parameters with 76.47% increase in life span as compared to

model control. In Tamoxifen treated animals, no tumors were observed at the end of experiment.

Thus, it is deduced that MEBM 400, EAELE 400 and AECF 400 possess curative anti-cancer potential against EAC induced solid mammary tumor with increase in life span. It can also be inferred that the effect of MEBM 400, EAELE 400 and AECF 400 on tumor parameters in EAC induced solid carcinoma is systemic and not only related to local cytotoxic effect. Moreover, the reported safety in literature of MEBM 400, EAELE 400 and AECF 400 is confirmed by normal body weight of animals.

It was demonstrated that in EAC-bearing mice, extensive formation of new blood vessels provides more nutrients and oxygen supply to the highly divided EAC-cells leading to the induction of growth and proliferation.[347] With positive results in chick chorioallantoic membrane assay, the *in-vivo* anti-angiogenic potential of extracts were studied in EAC induced solid mammary carcinoma through evaluation of protein involved in angiogenesis. One of the most important proteins in the growth of new blood vessels is called vascular endothelial growth factor or VEGF, which is secreted by some cancer cells.[352] VEGF attaches to a protein, or VEGF receptor, on the surface of nearby endothelial cells, and then signals the cell's control centers to begin growth and formation of new blood vessels.[353] It plays important role in tumorigenesis and metastasis.[354] The factor is also responsible for early relapse in primary breast cancer.[354] The protein is much more important before the tumor grows beyond 1-2mm, as it requires nutrients and oxygen to proliferate [355-357] and is upregulated by oncogenic transformation, hypoxia or growth factors.[358, 359] The new network formed in tumor under influence of VEGF is structurally and functionally abnormal leading to suboptimal blood flow in tumor which increases more VEGF production.[217] Studies suggests that VEGF also inhibit

apoptosis via induction of anti-apoptotic factor Bcl-2.[360] VEGF stimulates endothelial cells to secrete various proteolytic enzymes that degrades proteins of extracellular matrix, thus by facilitating metastasis.[361, 362]

In present study, VEGF levels are intensified by 2.5 fold in model group. **Hence, it is inferred that the protein VEGF is responsible for angiogenesis in EAC induced mammary tumor.**

Attenuation in VEGF levels were seen in MEBM, EAELE and AECF treated animals. Previous studies reported Butein found in MEBM was found to inhibit mTOR activation, thus by suppressing expression of angiogenic growth factor.[363] The lycopene found in EAELE is documented to inhibit angiogenesis both *in-vitro* and *in-vivo* by inhibiting MMP-2/uPA system through VEGFR2-mediated PI3K-Akt and ERK/p38 signaling pathways.[295] Previous studies reported that Rhein inhibited the activation of phosphatidylinositol 3-kinase (PI3K), phosphorylated- AKT (p-AKT), and phosphorylated ERK, suppressing *in-vitro* angiogenesis.[364] As compared to model group, MEBM 400, EAELE 400 and AECF 400 showed reduction of VEGF levels by 40.60%, 37.20% and 35.67% respectively. Tamoxifen attenuated VEGF levels by 47.06%.

Thus, the anti-angiogenic potential of MEBM 400, EAELE 400 and AECF 400 is confirmed in EAC induced solid mammary tumor model.

Literature suggests that loss in tumor suppressor gene can increase angiogenic output of tumor cells and also protect them from apoptosis. Numerous studies reported that the p53 tumor suppressor gene, acts via halting angiogenesis or inducing apoptosis in response to variety of stresses like DNA damage, oncogene activation or hypoxia.[365] The p53 induced apoptosis is irreversible and significant regulator in tumor genesis. [353, 365] Various reports demonstrated mutation of p53 in over 50% of human cancers. [366] Symonds *et. al*. proved that

with p53 mutation, there is decrease in apoptosis in transgenic mice. The oncogenic activity of p53 mutation is due to their ability to interfere with p53 dependent apoptosis by a dominant negative mechanism.[367] Ahmed *et. al.* showed decreased expression of p53 gene in cancer bearing mice while treatment with diallyl sulphide induces higher expression of p53 genes.[347]

The finding of present study is in consistent with that, where the p53 gene expression was downturn in cancer bearing untreated animals. The MEBM 400 treatment escalate p53 gene expressions by 10% while with treatment of EAELE 400 and AECF 400, 5% hike was observed in p53 gene expression as compared to model group. Tamoxifen also increase p53 gene expression levels by 9% as compared to model group.

Thus, the increase in expression of p53 tumor suppressor gene by MEBM 400, EAELE 400 and AECF 400 might be responsible in tumor regression which is depicted by reduction in tumor volume and burden. It might also be responsible for anti-angiogenic effect of MEBM 400, EAELE 400 and AECF 400.

In search of apoptotic pathway, caspase cascade was studied in EAC model. Several lines of evidence suggest caspase-9 acts downstream of p53 to promote apoptosis, [365, 368, 369] by release of apoptogenic factors like cytochrome c from the mitochondria.[370] Liberated cytochrome c permits the formation of apoptosome, which comprises of the adapter protein Apaf-1 and caspase 9, which is activated following recruitment into the apoptosome.[371] Activated caspase 9 then cleaves and activates the effector caspases, such as caspases-3 and -7, which perpetrate the death program.[371]

Previous studies of Butein on prostate cancer LNCaP cells showed increased levels of Caspase-3, -8 and -9.[235] The carotenoids present in *Lycopersicon*

esculentum are reported to suppress cyclin D1 levels and increase p21, p27 and p53 levels in human cancer cell lines.[294] The elaborative study on anthraquinone Rhein isolated from Rhubarb induced dose- and time-dependent increase in caspase-9-mediated apoptosis with ROS-mediated activation of NF-κB- and p53- signaling pathways in human breast cancer cells.[372]

In current research, the caspase-9 activity was eight-fold downregulated in tumor bearing animals than normal animals suggesting evasion of apoptosis in EAC induced mammary tumors. The treatment with MEBM 400, EAELE 400 and AECF 400 showed up-regulation of Caspase-9 with confidence level 99.9%, 99% and 95%. Thus, MEBM 400, EAELE 400 and AECF 400 induce apoptosis through death receptor independent intrinsic pathway involving up-regulation of p53 gene and activating downstream caspase-9 cascade. Multiple targets for inducing apoptosis via mitochondrial pathway makes extracts clinically attractive for treating cancer.

With anti-metastatic potential of MEBM, EAELE and AECF in scratch motility assay as well as anti-angiogenic and apoptotic effect of MEBM 400, EAELE 400 and AECF 400 in syngeneic model, the mechanisms of anti-metastasis was studied in EAC induced solid mammary tumors. Metastasis is leading cause for mortality in cancer patient.[373] At least half of the patients display clinically noticeable metastasis at the time of diagnosis.[374] The change in cell-cell and cell-matrix adhesion is of supreme importance for cell in metastatic journey.[374]

The reduction in relative organ weight in both preventive and curative studies in MEBM 400, EAELE 400 and AECF 400 treated animals can be linked to above mentioned facts, advocating no metastasis or treatment of

metastatic tumor in other organs. These further ensured the safety profile of MEBM 400, EAELE 400 and AECF 400.

Literature revealed that the metastasis of breast cancer to liver is common in humans[373] which are preliminary depicted by increase in serum AST and ALT levels.[375] Around 54% of all patients showed AST levels more than twice the upper limit of normal.[375]

The same condition was replicated in EAC inoculation in mice. Two fold rises in AST and ALT levels were observed in model control animals. MEBM 400 and EAELE 400 treated animal leads to nearly normal enzyme levels. The percentage decrease in AST was 76.36% and 36.93% by MEBM 400 and EAELE 400 respectively as compared to model group. The percentage decrease in ALT was 70.93% and 48.84% by MEBM 400 and EAELE 400 respectively as compared to cancer bearing untreated animals. It is worth noting that liver enzyme levels in MEBM 400 treated group were less than Tamoxifen treated group. However, AECF showed no effect of liver enzymes.

Thus, the decrease in liver enzymes in EAC induced mammary tumor can be correlated with the hepato-protective and anti-metastatic potential of MEBM 400 and EAELE 400.

The complex and exceptionally particular metastatic cascade not just relies on the intrinsic characteristics of tumor cells but also the microenvironment of tumor.[376] During an oncogenic event, continuous tissues injury leads to changes in tissue homeostasis, thus leading to inflammatory response which never fades away.[376] The correlation between inflammation and cancer is assisted by populace based studies demonstrating the use of NSAIDS or other anti-inflammatory drugs are associated with diminished occurrence of colorectal, breast, pancreatic and gastric cancers.[376, 377] Inflammation is one of the factors

of tumor progression, metastasis, treatment resistance, poor prognosis and relapse.[378, 379] An inflammatory milieu comprises of infiltrated immune cells, cytokines, chemokines and growth factors. IL-6 and Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine involved in apoptosis, inflammation, and immunity. TNF- α has been reported to be elevated in the blood serum of patients diagnosed with advanced stage breast cancer and correlate with an increased number and size of metastatic sites.[379] The increased level of TNF-α was possibly linked to the activation of NF-κB, which plays a crucial role in inflammation and carcinogenesis.[379] The important roles of ER and PR in invasion and metastasis of breast cancer are probably associated with NF-κB and TNF- α expression.[379] IL-6 signaling has been linked to both pro- and antiapoptotic activity in breast cancer cells.[380] Evidence suggests high level of IL-6 in breast, lung and lymphomas, leading to poor prognosis.[380] Different studies have archived IL-6 production in estrogen positive breast cancer cells.[381] TNF- α and IL-6 are predicted to up-regulate production of VEGF.[358, 381] Also, IL-6 is responsible for conversion of estrogen precursors like estrone to estrogen.[381]

In current research, the cytokines levels (TNF- α: 76.53% and IL-6: 42.08%) outrageously increased in cancer bearing untreated animals as compared to normal groups. **Thus, it can be inferred increased levels of cytokine might be one of the factors contributing to angiogenesis and metastasis in EAC induced mammary tumor.**

During invasion and metastasis, various natural tissue layers i.e. basement membrane and extracellular matrix have to be degraded. Since many layers and substrates are involved in extracellular matrix, the work is done by multiple lysosomal enzymes.[382] Lysosomal enzymes are implicated in tissue remodeling, which occurs during the physiological involution of the uterus, the prostate

gland and the mammary gland. They are released when lysosomes are exposed to ionizing radiation or free radical lipid peroxidation or estrogen.[383, 384] There are approximately forty lysosomal enzymes involved in the hydrolytic pathways for nucleic acids, nucleotide phosphates, proteins and peptides, glycoproteins, gangliosides, etc.[383] Lysosomal enzymes include: many cathepsins, acid phosphatases, glucosidases, galactosidases, glucuronidase, DNAases, ribonucleases, sulfatases, lipases, neuramidase, lysozyme, fucosidase, phosphodiesterases, mannosidase, and glucosaminidase.[383] Studies suggest that high level of these enzymes can be correlated to metastatic potential and is associated with likelihood of poor prognosis.[382, 385, 386] Increase activities of lysosomal enzymes in tumor condition may be due to a defect in the mechanism which regulates activities of these enzymes.[387, 388] The activities of lysosomal enzymes are linked to progression and regression of mammary cancer specially β-D- glucosidase.[389]

In the present study, tremendous elevated (more than 200%) levels of β-glucosidase were found in cancer bearing untreated animals as compared to normal group. **Thus, it is inferred that metastasis of mammary cancer to different organs might have occurred in EAC induced model which is due to increase in β- glucosidase activity responsible for degradation of extracellular matrix.**

The reduction in provocative cytokines and lysosomal enzymes suggests anti-metastatic potential of the MEBM 400 and EAELE 400 in EAC induced solid carcinomas. Previous studies demonstrated Butein present in MEBM inhibited invasion through ERK1/2 and NF-κB pathways in bladder cancer cells are proven by Zhang *et.al.*[390] Rasheed *et. al.* proved Butrin, Isobutrin, and Butein from *Butea monosperma* selectively inhibit NF-κB in activated human mast cell suppressing TNF-α, IL-6 and IL-8.[232] Butein also downregulates

expression of chemokine receptor CXCR4, which can be correlated with inhibition of CXXL 12- induced migration and invasion of breast cancer cells.[391] It function through suppression of NF-kB activation in breast tumor cells.[391] Butein was also shown to curb PMA-induced COX-2 expression in both non-cancerous and cancerous breast cells.[392] Hwang and Lee showed that lycopene at 5 and 10 μM could decrease the gelatinolytic activities of the matrix metalloproteinases MMP-2 and MMP-9 and inhibit the adhesion, invasion and migration of SK-Hep1 cells.[393] Further, Hwang *et. al* confirmed suppression of MMP-9 in SK-Hep1 cells and induction of nm23-H1 metastasis suppressor gene.[297]

In current research, MEBM 400 and EAELE 400 decreased elevated TNF- α levels by 34.68% and 17.53% and IL-6 by 22.97% and 7.56% respectively as compared to model group. Tamoxifen reduced TNF- α levels and IL-6 by 34.68% and 14.05% respectively. It is worth noting that MEBM 400 decreased IL-6 levels in greater amount than Tamoxifen. There was no significant effect observed in AECF 400 as compared to model group.

Thus, the curtailing effects of MEBM 400 and EAELE 400 on inflammatory markers in EAC induced mammary tumors are also responsible for decrease in tumor progression and metastasis. The decrease in cytokine levels can be correlated with anti-estrogenic effect of MEBM 400 and EAELE 400. Furthermore, they also effect by reducing VEGF levels thus by inhibiting angiogenesis. AECF 400 lacks beneficial effect on cytokines levels depicted by non-significant difference than cancer bearing untreated animals.

Upon MEBM 400 and EAELE 400 treatment, the β- glucosidase were reduced in breast cancer-bearing animals by 37.75% and 21.72% as compared to EAC

untreated animals. However, only 12% decrease was seen in AECF 400 treated animals as compared to model group.

Thus, MEBM 400 and EAELE 400 shows anti-metastatic potential depicted by decrease in lysosomal enzyme β- glucosidase level which is responsible for invading extracellular matrix. The effect might be due to the membrane stabilizing property of extracts on lysosomal enzymes, thus by protecting the rapid leakage of enzymes and obstruct the rise in the enzymatic activity. Moreover, VEGF is associated with induction of proteolytic enzymes. The decline of VEGF levels in present study might also be one reason for decreased levels of lysosomal enzyme.

The get in-sights of tissue architect and confirm anti-metastatic potential of extracts, histopathological analysis of liver and kidney was performed. **The histopathological analysis of liver revealed enlarged and congested central vein; infiltration of tumor cells mixed with leukocytes and fatty degeneration of hepatic tissue in EAC induced untreated animals.** Tamoxifen treated groups showed normal liver cells without fatty denegeration. **After treatment with MEBM 400 and EAELE 400, normal architecture can be seen. No leukocytes were observed. However, in EAELE 400, fatty degeneration was observed.**

The kidney of tumor bearing untreated animals shows congested renal vein, degenerated renal tubule and narrow glomerular space. Tamoxifen treated groups showed normal kidney structure. **The kidney of MEBM 400 and EAELE 400 treated animals showed normal glomeruli and renal tubule.**

AECF 400 showed no improvement in liver and kidney architecture. Tumor cells were observed in both liver and kidney. The leukocytes infiltration was observed in liver.

Thus, the normal liver and kidney architecture in histopathological analysis by MEBM 400 and EAELE 400 treatment, confirms no metastasis of mammary cancer in other organs. These confirm the anti-metastatic property of MEBM 400 and EAELE 400 and inevitably proving their curative role in breast cancer. The effect can be contributed to phytoconstituents which decreased VEGF, cytokines and lysosomal enzyme levels.

Chemotherapy in cancer causes anemia due to reduction in RBCs count. So treatment should enhance the RBCs count to normal. Also, studies suggest that complete blood count can predict severity of disease and treatment in cancer patients.[394] Elevated WBC counts predict a worse prognosis in patients with cancer.[394] Anemia (decrease in RBC and Hb) can affect quality of life in cancer patients and is poor prognostic factor. Severe anemia may cause delay in treatment or reduction in dose of drug. An anemia-inducing substance has been reported in cancer, which binds to RBC membrane.[311] It then lowers glucose influx and pyruvate kinase activity, which initiates RBC dysfunction and lysis, leading to anemia.[310, 325]

In present study, EAC injected untreated mice showed increase in total WBC count and decrease in total RBC count as well as Hb levels. The treatment with MEBM 400 and EAELE 400 restored normal levels of blood cells in both preventive and curative studies. AECF and Tamoxifen decreased total WBC count but failed to restore total RBC count and Hb levels.

Thus, it can be deduced that MEBM 400 and EAELE 400 possess anti-cancer property without any side effects on hematological profile.

In conclusion, the three potent extracts for breast cancer therapy were identified *viz.* MEBM, EAELE and AECF. The MEBM, EAELE and AECF exhibited significantly higher anti-proliferative activity against MCF-7 human breast cancer cell line than other two (MDA-MB-453 and MDA-MB-231) and found to be safe in normal human breast epithelial cell line. Chemo-prevention was perceptible by their effect on prevention of mammary tumor induction, abating changes in nucleic acid and combating oxidative stress. The estrogen receptor status was slashed significantly by MEBM, EAELE and AECF. The progesterone levels were significantly decline with MEBM and EAELE treatment. The curative potential of MEBM, EAELE and AECF was evident by improvement of tumor parameters in EAC induced solid mammary tumors. The anti-angiogenic potential of MEBM, EAELE and AECF was observed *in-vitro* in CAM assay and *in-vivo* by downregulation of VEGF expression in EAC induced solid mammary tumors. The apoptotic effect of MEBM, EAELE and AECF was spotted in *in-vitro* Annexin V FITC binding assay. The apoptotic pathway was deduced to be p53-caspase-9 cascade activation. The anti- metastatic capacity of MEBM and EAELE was discernable in *in-vitro* scratch motility assay. It was further vindicated by re-instatement of normal levels of liver enzymes, curtailment of inflammatory markers, decline in lysosomal enzymes and improvement in histo-architech of liver and kidney. Thus, with anti-angiogenic and apoptotic potential, the MEBM, EAELE and AECF can be utilized for early stages of breast therapy regimen. The phenomenal cessation of metastasis makes MEBM and EAELE splendid candidates for late stage breast cancer therapy also.