Pharmacological evaluation of some medicinal plants in breast cancer

Synopsis of the Ph.D. thesis submitted to
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IAEC Approval of Animal Studies

Animal husbandry, handling and treatments were performed as per the CPCSEA guidelines. Animals were provided pelleted diet and water *ad libitum*. They were housed in polypropylene cages with paddy husk as bedding material at 22 ± 2°C temperature and 50-75% relative humidity under 12hr light/dark cycle. The animals were monitored daily for any sign of distress and mortality. All the mentioned studies were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Dept., Faculty of Tech. & Engg., The M. S. University of Baroda *vide* the protocol number mentioned below:

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1. INTRODUCTION

Breast cancer may be defined as a malignant proliferation of a cellular constituent of the breast.[1] Breast cancer continues to be the most frequently occurring cancer in women around the world. The increased incidence, mortality, economic costs is a burden shared among women globally. Breast cancer continues to be a major public health problem in developed as well as developing countries. Breast cancer alone is expected to account for 30% all new cancer diagnoses in women in 2016.[2] Amongst all cancer, it is the cancer of breast alone which is expected to cross the figure of 100,000 by the year 2020.[3]

Epidemiologic studies have identified a number of risk factors that are associated with an increased risk of a woman developing breast cancer. Women who have a first-degree relative (mother or sister) with breast cancer have a twofold to three fold increased risk of developing breast cancer. Factors like early first menarche, late first pregnancy and menopause, use of oral contraceptives and hormone replacement therapy, alcohol consumption have all been consistently associated with an increased risk of breast cancer.[1] Cancer is a disease characterized by extensive genomic abnormalities and aberrations in gene expression.[4] Tumor-specific mutations, DNA amplifications and translocations can all distort the normal programs of gene expression and function, resulting in unregulated activity of apoptosis, angiogenesis and cell proliferation. The genes which might be involved in breast cancer are BRCA 1 and BRCA 2, EGFR/ErbB1 (Epidermal Growth Factor Receptor), HER-2/neu (Human EGFR Related), TNF-α (Tumor Necrosis Factor- α), IGF (Insulin like Growth Factor) and VEGF (Vascular Endothelial Growth Factor). [1, 4]

Breast cancer is either invasive (infiltrating ductal carcinoma and infiltrating lobular carcinoma) or noninvasive/in situ (ductal carcinoma in situ and lobular carcinoma in situ).[5]

Based on the stage of diagnosis, breast cancer is treated with a multidisciplinary approach involving surgery, radiation and systemic therapy including chemotherapy or hormonal therapy.[1] The biology and behavior of breast cancer affects the treatment plan. For both Ductual carcinoma in-situ (DCIS) and early-stage invasive breast cancer, surgery is recommended. Surgical procedures for breast cancer can cause short-term pain and tenderness in the treated area. Also, the skin in the breast area may feel tight, and the muscles of the arm may feel stiff or weak. Surgery involving lymph nodes, may cause lymphedema in later stage of life.[6, 7] Surgery alone may increase the chances of relapse. For larger cancers, or those that are growing more quickly, systemic treatment is given before surgery to shrink the tumor size or after surgery to prevent recurrence. Generally, combination of chemotherapeutic agents are used for early stage and locally advanced breast cancers. The side effects of chemotherapy depend on the individual, the drug(s) used, the schedule and dose used. These side effects can include fatigue, risk of infection, nausea, vomiting, mouth sores, hair loss, anorexia, diarrhea and bone marrow suppression. Hormonal therapy, also called endocrine therapy, is an effective treatment for most tumors that test positive for either estrogen or progesterone receptors. It is a valuable option for the treatment in postmenopausal women. Tamoxifen is the most commonly used drug. Despite the fact that Tamoxifen has been the mainstream treatment for over 20 years its long haul

utilization is related with a few decency concerns and may prompt expanded danger of endometrial malignancy and thromboembolic complexities. Moreover, numerous patients who at first react to treatment with endocrine operators, in the long run relapse with resistant disease.[8] Aromatase inhibitors are also used in postmenopausal women but longer use increased odds of developing cardiovascular disease and bone fractures.[9] Radiation therapy often helps to lower the recurrence risk. It can cause side effects, including fatigue, swelling of the breast, redness and/or skin discoloration/hyperpigmentation and pain/burning in the skin where the radiation was directed, sometimes with blistering or peeling.[1, 5]

Unfortunately, in spite of improved diagnostic skills and breakthrough in effective treatment, breast cancer continues to be the leading cause of cancer deaths among women worldwide.

Since antiquated times, plants and plant-derived compounds have furnished tremendous backing in conventional medication framework, and have been used as source of new potential drugs in modern pharmaceutical industries. Several new studies have discovered that most patients on cancer therapy are concurrently self-medicating with one or several complementary and alternative medicines.[10] Among complementary and alternative medicines, herbal medicine is the most commonly used group of treatment. Herbal treatment is the oldest used system of medicine in the world with more than 2000 years history.[11] Certain herbs defend the body from malignancy by augmenting detoxification or cleaning the body. Some biological response modifiers, derivatives of herbs, are recognized to hinder the growth of cancer by modifying the activity of precise hormones and enzymes, while other herbs diminish lethal side effects and complications of chemotherapy and radiotherapy.[12] . Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products and the plant kingdom has been the most significant source. Phytoconstituents resulting from the herbs such as *Vinca rosea, Taxus species, Allium sativum, Panax pseudoginseng, Taxus wallichiana, Tinospora cordifolia, Viscum album, Withania somnifera, Zingiber officinale* etc. have been used in numerous preparations to assist the body to battle cancer more efficiently and also decrease the harmful side effects of chemotherapy and radiotherapy. A study of women being treated for early stage breast cancer showed that 10.6% had been using one or more complementary and alternative medicines at the time of diagnosis, while an additional 28.1% began using complementary and alternative medicines (including herbal remedies) after surgery.[10] Vinca alkaloids, Docetaxel and Paclitaxel who hold their names in FDA approved list for treatment of breast cancer, have had their origin from natural sources. However, all these drugs are not breast cancer specific. Hormone receptor and HER2/neu status are two prognostic and/or predictive factors for selection of systemic adjuvant therapy. None of the above mentioned drug act via modulation of receptors or hormones associated with breast cancer.[1] Furthermore, chemotherapy has seen a gradual transition from the long and passionately advocated mono-substance therapy toward a multidrug therapy.[13] It is becoming increasingly obvious through observation that many diseases possess a multi-causal etiology and a complex pathophysiology, which can be treated more effectively with well-chosen drug combinations than with a single drug.[14] A living plant is a complex system with thousands of interacting chemicals, and many of them work together in ***synergy*** –

creating a greater impact than the effect of consuming isolated components in single molecule pharmaceuticals.[14] There are several different ways they may work in synergy. They may help stabilize each other, potentiate or enhance each other, or modify the impact of certain elements. They may help make a constituent more water soluble or protect it from stomach acids. All of these are ways that the synergies created in taking in a whole plant can produce radically different results than taking the chemically isolated ingredients.[14] Generally substantial decrease in toxicity is observed when whole plant extracts are compared with individual molecules derived from plant. For example, non-glycyrrhizin components of Liquorice extract reduces intestinal absorption of glycyrrhizin thus attenuating toxicity.[14] Furthermore, many plants contain substances that inhibit multi-drug resistance (MDR).[15] Herbal medicines can sometimes be grown and produced locally, at lower cost, by or close to those who need them.

Thus, an attempt was made in the current study to identify an easily available common herb and evaluate its potential in the treatment or prevention of breast cancer. The present study focuses on three plants namely *Butea monosperma* flowers, *Cassia fistula* pods and *Lycopersicon esculentum* fruit.

Butea monosperma (Palash) traditionally employed intensively as folklore remedy for a wide spectrum of liver diseases in India. The century old healing system, Ayurvedic medicine, has utilized flowers, bark, leaves, gum and even the seeds of *B.monosperma* to prepare herbal remedies. Practically every part of *Butea monosperma* have been reported to be associated with various remedial properties such as, anti-diarrhoeal,[16] antiestrogenic activity, [17-20] anti-implantation and anti-ovulatory activity,[21] anthelminthic, bactericidal and fungicidal influence[22] and antitumor property against hepatic carcinoma.[17] The methanolic extract of powdered *Butea monosperma* has shown activity against tumor promotion related events of carcinogenesis in rat liver and the protective activity of plant might be due to two major constituents viz isobutrin and butrin.[17, 23] On hepatic carcinoma, treatment with aqueous extract of flowers of *Butea monosperma*. inhibited cell proliferation and accumulation of cells in G1 phase.[19] This was accompanied by induction of apoptotic cell death.[18, 19] The important active principles of *B.monosperma* are butin, butein, butrin, isobutrin, palasitrin, oreopsin and isocoreopsin, chalcones, and aurones triterpene phenolics constituent.[17] Butein shows antiproliferative effect on wide range of human tumor cells including breast carcinoma. [18] The plant polyphenol butein inhibits testosterone-induced proliferation in breast cancer cells expressing aromatase.[24] Furthermore, Butein also possess free radical scavenging activity. [18, 19] Phytoconstituents like quercetin,[25, 26] genistein,[27] medicarpin, lupeol[28] and lupenone[29] are proven to be antitumor agent. Quercetin and genistein are reported to have tyrosine kinase inhibitory activity.[4]

Second plant, *Cassia fistula* was found to be potent anticancer agent on human colon cancer cell line.[30] It is widely used in traditional medicinal system of India and has been reported to possess hepatoprotective, anti-inflammatory, antitussive, antifungal and antibacterial. Oral administration of *Cassia fistula* bark extract to DMBA painted animals completely prevented the formation of oral squamous cell carcinoma.[31, 32] Rhein component from flower is found to be anticarcinogenic.[30] Moreover, *Cassia fistula* possess antiestrogenic activity.[33] It also contain

lupeol which is proven to be antitumor agent.[28] Seeds and pulp treated human cancer cell lines showed up-regulation of p53 and Bax genes, down-regulation of Bcl-2 gene and increased caspase-3, 7 & 10 and -9 enzymes activities.[34]

Third plant, *Lycopersicon esculentum* Mill are one of the most widely used and versatile fruit. Epidemiologic studies suggest that consumption of tomato and tomato-based products reduces the risk of chronic diseases such as cardiovascular disease and cancer. Carotenoids have been found to inhibit the growth of several cancer cell lines including, prostate cancer cells, lung, mammary, two human colon cancer cell lines and leukemia cancer cells. [35-39] Tomato-rich diet could help protect at-risk postmenopausal women from breast cancer, according to new research from The Ohio State University Comprehensive Cancer Center. A diet rich in tomato-based products may help reduce the risk of pancreatic cancer, according to a study from The University of Montreal.[40] A number of studies have been conducted that indicate that the high levels of lycopene in tomatoes works to reduce your chances of developing prostate, colorectal and stomach cancer.[36, 37, 40] Aqueous extract of tomato is found to reduce the expression of TNF-α and IL-1β in LPS-activated macrophages.[41] Tomatoes contain all four major carotenoids: alpha- and beta-carotene, lutein, and lycopene. [40] Lycopene is the best antioxidant amongst all.[40, 42] Studies suggests that tomato extract and lycopene inhibit doxorubicin-induced cardiotoxicity and might serve as a combination chemotherapeutic agent with doxorubicin to limit its cardiotoxic effects.[43] Dietary lycopene and tomato extract supplementations inhibit nonalcoholic steatohepatitis-promoted hepatocarcinogenesis in rats.[44] It interferes in insulin-like growth factor 1 signaling and inhibits VEGF.[45] Studies suggested it to be potent aromatase inhibitor.[46]

The aim of present study was to contribute commonly used medicinal herbs for better health prospects in the individuals suffering from breast cancer.

2. OBJECTIVES

a) Preparation of extracts of plants (Aqueous, Methanolic, Butanolic and Ethyl acetate)

b) Evaluating the cytotoxicity of various prepared extracts on various *in-vitro* breast cancer cell lines (MCF-7, MDA-MB-453, MDA-MB-231)

c) .*In-vivo* anticarcinogenic activity of selected extracts on N-methyl- N-nitrosourea (MNU) induced rat mammary carcinogenesis model and comparison with standards.

d) Syngenic solid tumor induction by Ehrlich Ascites Carcinoma (EAC) and evaluating molecular aspects.

e) Evaluation of other anti-cancer characteristics of extracts (antiangiogensis, apoptosis and cell metastsis).

3. EXPERIMENTAL WORK

3.1 Chemicals

MCF-7, MDA-MB-231 and MDA-MB-453 human breast cancer cell lines and Ehlrich Ascites Carcinoma (EAC) were procured from NCCS, Pune. Methylnitrosourea (MNU), Propidium iodide were procured from Sigma Aldrich. MTT, DMSO, Culture media, fetal bovine serum, penicillin G-streptomycin solution were procured from Himedia. Annexin V-FITC assay kit was procured from BD sciences.

3.2 Plant procurement

Butea monosperma and *Lycopersicon esculentum* powders were procured from Amines Biotech, Alkapuri, Vadodara. *Cassia fistula* powder was procured from Kisalaya Herbals Ltd., Indore.

3.3 Preparation of extracts

The extracts were prepared by maceration technique. The powder was soaked separately with water, methanol, butanol, and ethyl acetate for 48 h at room temperature with occasional stirring. After filtration through cheese cloth, the filtrates were concentrated using either rotary evaporator (organic solvents) or water bath (water). The extracts were stored at 4°C until use and labeled as follows:

Solvent	***Butea monosperma***	***Cassia fistula***	***Lycopersicon esculentum***
Methanol	MEBM	MECF	MELE
Aqueous	AEBM	AECF	AELE
Butanol	BEBM	BECF	BELE
Ethyl acetate	EAEBM	EAECF	EAELE

3.4 Evaluation of *Butea monosperma* flowers in breast cancer

3.4.1. Cell line and cell culture

All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) -high glucose supplemented with 10% fetal bovine serum (FBS), 1% penicillin G-streptomycin solution. Cells were grown in 37° C in humidified incubator with 5% CO_2.

3.4.2. MTT Assay

MCF-7, MDA-MB-231 and MDA-MB-453 cells ($5x10^6$/well) were seeded into each well of 96-well plates for 24 h. After 24 hours, treatment with various concentrations (10-1000 µg/ml) of extracts was given and plates were incubated for 24, 48, and 72 h. The media containing extracts was discarded after specified time intervals and 5 mg/ml MTT solution (20 µl/well) was added to each well. The cells were incubated for 4 h at 37˚C. The supernatant was aspirated and DMSO (100 µl) was added to the wells. The absorbance was then measured at 570 nm by micro titer plate reader. The percentage of cell inhibition was calculated using the formula [47]

Cell Inhibition (%) = $[[A_{Control} - A_{Treated}] / A_{Control}] \times 100$.

3.4.3. Chemically induced mammary carcinogenesis by Methylnitrosourea (MNU)

Nulliparous Sprague Dawley female rats were obtained from Zydus Research Centre, Ahmedabad. The animals were housed in a group of 6 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12hrs/12hrs light-dark cycle. The animals had free access to conventional laboratory diet and distilled water *ad libitum*.

The experiment was carried out as per guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and The Prevention of Cruelty to Animals act (PCA), 1960. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, The Maharaja Sayajirao University of Baroda (MSU/IAEC/2014-15/1436).

Mammary cancer in rats was induced by a single intraperitoneal injection of 50mg/kg body weight of MNU, dissolved in 0.9% saline adjusted with acetic acid (pH=4).

The rats were randomly divided into ten groups. The normal control (group 1) animals received saline. All groups except group 1 received MNU. Group 2 served as model control. Group 3 (Vehicle control) animals received sesame oil as per the body weight. Group 4 served as standard control and received Tamoxifen (1mg/kg b.w; s.c) [48]. Group 5 to 7 were test groups which received MEBM -100mg/kg; 200 mg/kg and 400mg/kg p.o respectively. Group 8 to 10 were test groups which received AEBM -100mg/kg; 200 mg/kg and 400mg/kg p.o respectively The treatment was given for 100 days starting from first day of MNU injection.[49]

During experimental period, the rats were palpated for tumors every two weeks. Body weight (weekly) and food intake (daily) were measured. At the end of experimental period, blood was withdrawn from retro-orbital plexus from each animal and total blood count was performed. Animals were euthanized humanely for assessing different parameters.

3.4.3.1. Body weight (Growth rate) and Organ Weight

Body weights were measured daily. The growth rate was calculated with the formula:

$$\left(\frac{\text{Final body weight}}{\text{Initial body weight}}\right)^{\left(\frac{1}{\text{Periods}-1}\right)-1}$$

The organ (liver and uteri) wet weight was measured and reported as organ to body weight ratio using formula:

$$\left(\frac{\text{Organ weight}}{\text{Body Weight}}\right) \times 100$$

3.4.3.2. Food intake (Feed consumption efficiency)

The food consumption was measured daily and feed consumption efficiency was calculated by formula:

$$\left(\frac{\text{Weekly body weight gain}}{\text{Weekly food consumption}}\right) \times 100$$

3.4.3.3. Tumor parameters

Tumor parameters involved tumor incidence (number of rats with tumor per total number of rats), number of tumors, tumor multiplicity (average number of tumors per rat), weight, volume (volume = $(\pi/6) \times d1^2 \times d2$; $d1$ is the shortest diameter, and $d2$ is the longest diameter), and latency period (lag time between MNU injection and first tumor appearance) [50-52]

3.4.3.4. Blood count[47]

Blood was collected from retro-orbital plexus and hematological parameters like red blood cells (RBC), white blood cells (WBC) and hemoglobin (Hb) were estimated by fully automated hematology analyzer. (BC-2800Vet Auto Hematology Analyzer)

3.4.3.5. Estrogen and Progesterone receptor expression studies by immunohistochemisty[53-55]

For identification of the presence of Estrogen (ER) and progesterone receptor (PR), standard immunohistochemical procedures were followed. Briefly, tumor sections were rehydrated in PBS and endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide. Tissue sections were blocked with 1.5% normal goat serum solution followed by incubation overnight at 4°C in primary antibody, at 1 Ag/mL, against human ER (rabbit polyclonal ER) or human PR (rabbit polyclonal PR). These antibodies are known to cross-react with rat ER and PR. Sections were incubated with a biotinylated secondary antibody at 1 Ag/mL (goat anti-rabbit IgG) and labeled with a streptavidin-peroxidase. Color development was detected using, 3V-diaminobenzidine as substrate. Tissues were counterstained with Harris hematoxylin, dehydrated in a series of 95% and 100% ethanol, and cleared in xylene, and coverslips were mounted. Positively stained cells were identified by darkly stained nuclei, whereas those stained with a nonspecific rabbit IgG remained blue and stained only with hematoxylin. (Photographed by Nikon Eclipse TS100)

3.4.3.6. Nucleic acid content[56]

The nucleic acids were extracted by the method of Schneider. The breast tissue homogenates were added with 5 mL of 5% TCA to precipitate proteins and nucleic acids. The reaction mixture was centrifuged and the precipitate was heated at 90 °C for 15 min with occasional shaking, which facilitated the quantitative separation of nucleic acids from protein. DNA was estimated by the method of Burton. In this reaction, 3 mL of above sample was mixed with 2 mL of diphenylamine reagent and this was kept in water bath for 10 min to form blue color substance, which was read at 595 nm using spectrometer. The DNA content was expressed as mg/g wet tissue. The level of RNA was estimated by the method of Rawal et al. In this reaction, 3 mL of orcinol-ferric chloride reagent was added with 2 mL of sample. Then, the tubes were heated in water bath for 20 min. The tubes were cooled and the color developed was measured at 595 nm using spectrometer. The RNA content was expressed as mg/g wet tissue.

3.4.4. EAC (EHRLICH ASCITES CARCINOMA) INDUCED SOLID TUMORS

Female BALB/C mice were obtained from Torrent Research Center, Ahmedabad. The animals were housed in a group of 3 per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12hrs/12hrs light-dark cycle. The animals had free access to conventional laboratory diet and distilled water *ad libitum*.

The experiment was carried out as per guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and The Prevention of Cruelty to Animals act (PCA), 1960. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, The Maharaja Sayajirao University of Baroda (MSU/IAEC/2016-17/1639).

The solid tumors were induced by injecting EAC (0.2 ml of 2 X 10^6) sub-cutaneously at the abdomen region in BALB/C female mice. This is taken as day zero and treatment began after 5 days of injecting EAC for period of 30 days. The groups were as follows:

Group I- Normal Control received saline,

Group II-Model Control: EAC (0.2 ml of 2 X 10^6) sub-cutaneously

Group III-Standard Control: EAC (0.2 ml of 2 X 10^6) sub-cutaneously and Tamoxifen (5µg/ml; s.c),

Group IV- Test Group: EAC (0.2 ml of 2 X 10^6) sub-cutaneously and MEBM (400mg/kg; p.o.)

During experimental period, the mice were palpated for tumors every two weeks and body weights were measured. After experimental period, not more than 0.5 ml blood was withdrawn from retro-orbital plexus from each animal and total blood count, inflammatory markers, liver markers were performed. Animals were euthanized humanely for assessing tumor parameters (weight, volume= $(4/3)\pi r^2$, and tumor incidence (number of rats with tumor per total number of rats), mean survival time= ((First death+ last death)/2); % increase in life span= ((Mean survival time of treated group/ Mean survival time of control group)*100), liver wet weight and histopathology.

3.4.5. Scratch motility assay (for cell metastasis)[57]

MCF-7 cells (3.5×10^5 cells/well) were seeded in a 6-well plate and grown till confluent. The confluent cell monolayer was then scratched vertically with a sterile pipette tip, washed twice with PBS and incubated with media containing extracts (Concentration= IC_{50} of extract at 24 h on MCF-7 cell line). The cells in the denuded area was photographed with camera attached to inverted microscope (Nikon Eclipse TS100) and counted at 0- and 24- h incubation. The experiment was performed in triplicates. The percentage inhibition was calculated as follows:

$$\text{Percent inhibition}=100-\left(\left(\frac{no.of\ cells\ in\ denuded\ area\ of\ sample}{no.of\ cells\ in\ denuded\ area\ of\ control}\right) X\ 100\right)$$

3.4.6. Chick Chorioallantoic Membrane (CAM) Assay (for Angiogenesis)[58-60]

On day 0, the fertile chick eggs were placed in a fan-assisted humidified incubator/egg incubator, at 37°C. On day 3, the eggs were removed from the incubator and swabbed with 70% IPA. A

small window was made on the shell at the pointed end of the egg. The eggs were returned to the incubator (37–37.5^{o}C) and incubated till 8th day. On 8th day, a 5mm X 5 mm filter paper disks were used as a carrier for loading extracts. For selection of dose, five concentrations were screened i.e. 10, 20, 30, 40 and 50 µg/ml per filter paper disk. Eggs were sealed and were returned to the incubator for further 48 hr. At the end of the experiment, the eggs were opened and CAM was photographed. The radius of the zone of inhibition of blood vessel growth was visually assessed from the center of each disk to the furthest contiguous area in which tertiary blood vessels were absent. The experiment was repeated with lowest inhibitory concentration.

3.4.7. Annexin V-FITC binding assay[61]

Apoptosis was studied using Annexin V-FITC/ propidium iodide (PI) double staining and analysis by flow cytometry. The adhered cells were incubated with extracts (Concentration= IC_{50} of extract at 24 h on MCF-7 cell line) for 24 h. After 24 h, the cells were removed with 200 µl of 2.5% (v/v) trypsin, followed by the addition of 1 ml of DMEM containing 10% (v/v) FBS and centrifuged at 311 ×g for 4 min. The supernatant was removed, and the cells were incubated with 500 µl of binding buffer (0.01 M Hepes/NaOH, pH 7.4, containing 0.14 M NaCl and 2.5 mM $CaCl_2$). The suspensions were transferred to tubes and centrifuged at 311 ×g for 6 min. The cells were resuspended with 50 µl of binding buffer with 3 µl of annexin V/FITC and 5 µl of PI (50 µg/ml). The cells were incubated at room temperature for 30 min with the addition of 300 µl of binding buffer and analyzed in a flow cytometer (Analyzed by FACSDiva Version 6.1.3).

3.4.8. RESULTS AND DISCUSSION

3.4.8.1. MTT Assay

The cancer cell acquires certain peculiar capabilities, the sustained proliferation being the most striking one. The capacity of anticancer drug to hinder cell multiplication can be investigated using MTT assay on cancer cell lines. To estimate this property, different human breast cancer cell lines *viz.* MCF-7 (estrogen receptor positive), MDA-MB-453 (HER2 positive) and MDA-MB-231 (triple negative) were used [62].

In present study, the maximum cell death was observed in MCF-7 cell line which was found to be dose- and time- dependent. On basis of IC_{50} values on MCF-7 cell line (72 hours), it is observed that extracts exhibited anti-proliferative activity in following order: MEBM (50 µg/ml) > AEBM (300 µg/ml) > BEBM (450 µg/ml) > EAEBM (480 µg/ml). On MDA-MB-453 and MDA-MB-231, IC_{50} of methanolic extract at 72 hours was found to be 874 µg/ml and 1000 µg/ml. For all other extracts, IC_{50} were greater than 1000 µg/ml. (Table 1)

Extracts	**IC_{50} for MCF-7**			**IC_{50} for MDA-MB-231**	**IC_{50} for MDA-MB-453**
	24 h (µg/ml)	**48 h (µg/ml)**	**72 h (µg/ml)**	**72 h (µg/ml)**	**72 h (µg/ml)**
MEBM	186	86	50	1000	874
AEBM	400	338	300	>1000	>1000
BEBM	690	510	450	>1000	>1000
EAEBM	>1000	800	480	>1000	>1000

Table 1: IC_{50} value of various extracts of *Butea monosperma* flowers on human cancer cell lines

In-vivo anticancer potential evaluation and cell lines studies (like Annexin V FITC binding assay and Scratch Motility assay) with the aim of elucidating the effect of test compound on other peculiar cancer cell characteristics (*viz*. apoptosis and metastasis) were planned following MTT assay. For the same, the extracts from various test plants were narrowed down to two based on the results of MTT.

3.4.8.2. MNU induced mammary carcinogenesis

3.4.8.2.1. Effect on Growth Rate and relative organ weight in MNU induced mammary carcinogenesis

When body weight was evaluated as % growth rate, significant difference was found from 42 day. From then, the growth rate of model control animals decreased significantly ($P<0.05$) till the end. In vehicle control, the growth rate curve runs parallel to model control suggesting no significant difference. On treatment with extracts and Tamoxifen, the growth rate curve resembles to normal control and was significantly different from model control. (Figure 1)

Furthermore, increase in relative uteri and liver weights were observed in tumor bearing group 2 animals as compared to normal control animals ($P<0.001$). All treatment groups significantly improved relative organ weights when compared to model control animals ($P<0.001$) (Table 2). This result can be linked to anti-migratory effect found in scratch motility assay advocating no metastasis in other organs.

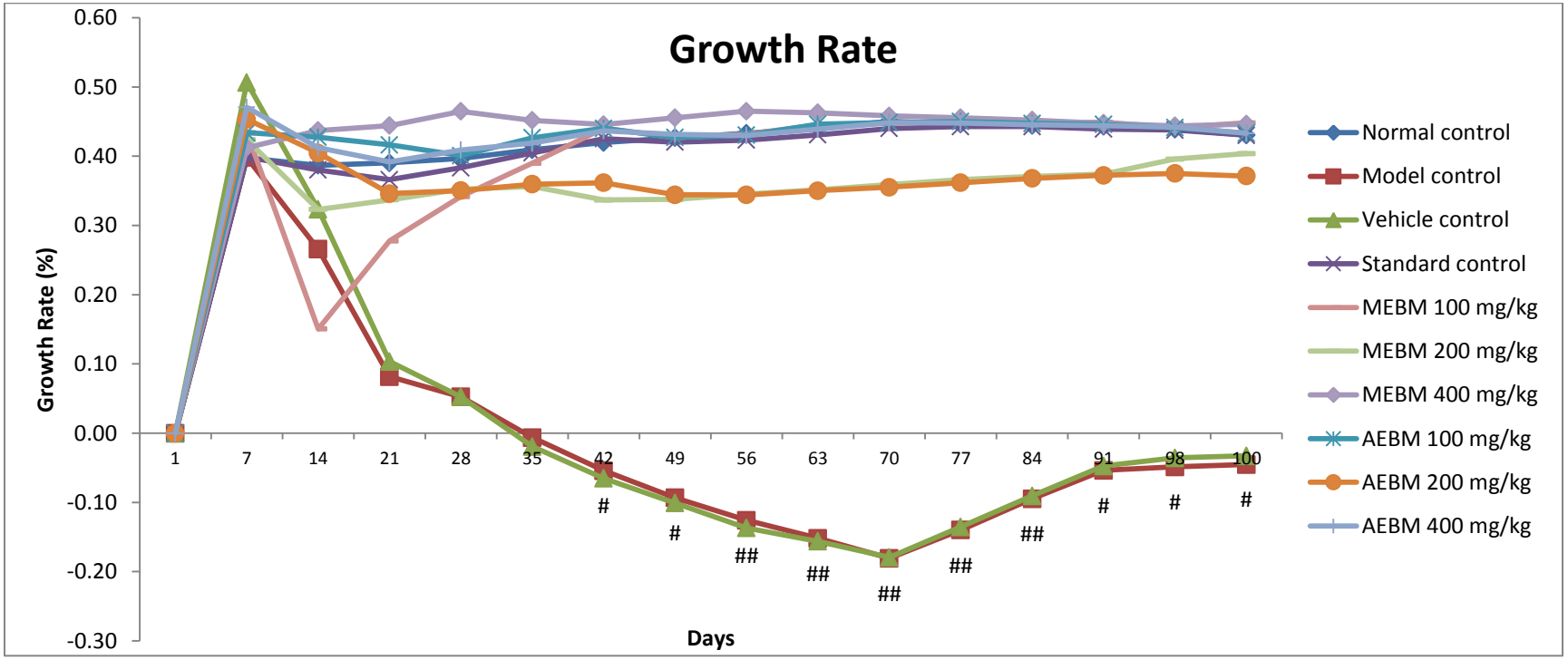


Figure 1: Effect of *Butea monosperma* flower extracts on growth rate in MNU induced mammary carcinogenesis.

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with #P<0.05 normal control vs. model control ##P<0.01 normal control vs. model.

Groups	Relative Uteri Weight (g)	Relative Liver Weight (g)
Normal control	0.18±0.01	4.08±0.08
Model control	0.28±0.02$^{###}$	5.75±0.34$^{###}$
Vehicle control	0.26±0.03	6.03±0.26
Standard control	0.13±0.01***	4.07±0.11***
MEBM 100 mg/kg	0.17±0.01***	4.09±0.09***
MEBM 200 mg/kg	0.19±0.01***	4.24±0.32***
MEBM 400 mg/kg	0.16±0.01***	3.85±0.07***
AEBM 100 mg/kg	0.18±0.01***	4.15±0.09***
AEBM 200 mg/kg	0.20±0.01**	4.51±0.35***
AEBM 400 mg/kg	0.17±0.01***	4.12±0.06***

Table 2: Effect of *Butea monosperma* flower extracts on relative organ weight in MNU induced mammary carcinogenesis

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control, **P<0.01 model control vs. all other groups, ***P<0.001 model control vs. all other groups.

3.4.8.2.2. Effect on Feed Consumption Efficiency in MNU induced mammary carcinogenesis

Food intake was calculated as feed consumption efficiency. The feed consumption efficiency was significantly reduced in model control animals from day 35 to 63 as compared to normal control. There was no significant difference found between treatment groups as compared to normal control (Figure 2). This can be correlated to depletion in body weight. Additionally, it proposes tumor induction and utilization of nutrients in tumor growth.

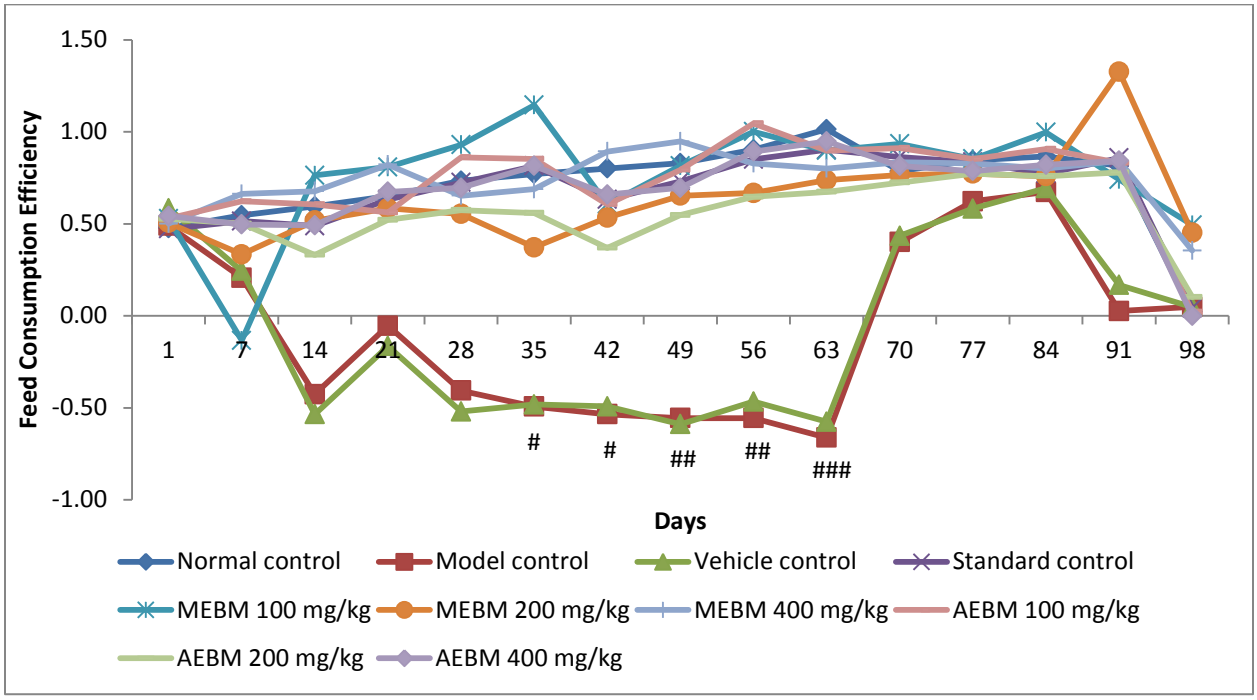


Figure 2. Effect of *Butea monosperma* flower extracts on feed consumption efficiency in MNU induced mammary carcinogenesis.

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's's Post hoc test. Significant values were compared with #P<0.05 normal control vs. model control ##P<0.01 normal control vs. model control ### P<0.001 normal control vs. model control.

3.4.8.2.3. Effect on Tumor Parameters in MNU induced mammary carcinogenesis

Injecting MNU (50mg/kg b.w; i.p.) to nulliparous SD female resulted in mammary tumors without metastasis. Tumor was induced after 45 days post MNU injection. Five out of six rats developed mammary carcinoma suggesting 83.33% tumor incidence. 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. The weight and volume of tumor in positive control animals were found to be 6.4±1.47 g and 99.29 ±3.19 mm^3. The data proposed successful induction of mammary carcinomas in MNU injected rats.

Oral administration of SD rats bearing MNU induced mammary cancer with extracts significantly decreased the mammary cancer. The tumor incidence was reduced to 33.33% by administration of highest dose of MEBM. The weights and volume were significantly curtailed in all treatment groups. Tamoxifen (82.17±16.44; P<0.001), all doses of MEBM (100mg/kg: 74.33±1.65; 200mg/kg: 78.66±1.65 and 400mg/kg: 82.5±2.89) and AEBM (100mg/kg: 72.17±1.42; 200mg/kg: 75.80±12.75 and 400mg/kg: 79.40±13.30) prolonged latency period. It is worth noting that tumor incidence and number of tumors was lower in MEBM than Tamoxifen.

No significant changes were observed in group 3 vehicle control animals. The results indisputably confirm that extracts inhibit cancer cell proliferation *in-vivo*. (Table 3)

Groups	Tumor Incidence	Total number of tumors	Tumor multiplicity	Tumor weight (g)	Tumor volume (mm^3)	Tumor latency period (days)
Normal control	0	0	0	0	0	0
Model control	5	6	1	6.4± 1.46$^{###}$	99.29± 1.18$^{###}$	45.17±9.21$^{###}$
Vehicle control	6	6	1	6.2± 1.55	89.74± 1.18	53.23±19.41
Standard control	5	5	0.83	0.89±0.29***	1.95±6.33***	82.17±16.43**
MEBM 100 mg/kg	3	3	1	2.28±0.63**	8.08±4.26***	74.33±1.65***
MEBM 200 mg/kg	3	3	0.75	1.32±0.49**	3.89±2.00***	78.76±1.65***
MEBM 400 mg/kg	2	2	0.75	0.78±0.38***	3.67±1.23***	82.50±1.44***
AEBM 100 mg/kg	6	6	1	3.43±0.63	24.77±5.87**	72.17±1.42***
AEBM 200 mg/kg	5	5	0.83	2.16±0.76*	10.63±4.14**	75.80±12.75***
AEBM 400 mg/kg	5	5	0.83	1.54±0.59***	8.08±3.22***	79.40±13.3***

Table 3: Effect of *Butea monosperma* flower extracts on tumor parameters in MNU induced mammary carcinogenesis

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control; *P<0.05 model control vs. all other groups; **P<0.01 model control vs. all other groups; ***P<0.001 model control vs. all other groups

3.4.8.2.4. Effect on Hematological parameters in MNU induced mammary carcinogenesis

Untreated MNU group showed significant decrease (P<0.001) in number of RBC (5.57±0.17 X 106/µL), Hb (9.63±0.24 g/L) indicating a tendency of anemia, whereas concomitant increase in WBC (13.63±0.13 X 103/µL) was observed indicating the diseased state. In normal control animals, RBC: 7.11±0.02 X 106/µL, Hb: 13.27±0.08 g/L; WBC: 6.37±0.06 X 103/µL was observed. Upon treatment with extracts, all hematological parameters were significantly restored

as compared to model control (P<0.001). Tamoxifen failed to restore all hematological parameters (RBC: 5.40±0.12 X 106/µL, Hb: 8.5±0.22 g/L; WBC: 7.30±0.11 X 103/µL) (Table 4)

Groups	WBC (X 10^3/µL)	RBC (X 10^6/µL)	Hb (g/L)
Normal control	6.37±0.06	7.11±0.02	13.27±0.08
Model control	13.63±0.13###	5.57±0.17###	9.63±0.24###
Vehicle control	13.23±0.29	5.27±0.06	5.57±0.17
Standard control	7.30±0.11***	5.40±0.12	8.5±0.22
MEBM 100 mg/kg	9.27±0.25***	6.39±0.18*	12.23±0.12***
MEBM 200 mg/kg	9.00±0.25***	6.55±0.11**	12.43±0.12***
MEBM 400 mg/kg	7.43±0.13***	6.87±0.06***	12.40±0.18***
AEBM 100 mg/kg	10.10±0.04***	6.34±0.07*	11.90±0.19***
AEBM 200 mg/kg	9.77±0.02***	6.48±0.13*	11.93±0.27***
AEBM 400 mg/kg	8.43±0.13***	6.63±0.21**	12.10±0.04***

Table 4: Effect of *Butea monosperma* flower extracts on Complete Blood Count in MNU induced mammary carcinogenesis.

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ### P<0.001 model control vs normal control; *P<0.05 model control vs. all other groups; **P<0.01 model control vs. all other groups; ***P<0.001 model control vs. all other groups

3.4.8.2.5. Effect on Estrogen and Progesterone receptor expressions in MNU induced mammary carcinogenesis

To confirm *in-vitro* results, estrogen and progesterone receptor's immunohistochemical analysis was performed. ER activation in breast and uterus enhances cell proliferation which is necessary for growth and maintenance of tissues.[63] 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. Literature revealed that MNU induces estrogen dependent tumors.[63] Overexpression of ER can be due to binding of growth factors also like IGF-I, IGF II, EGF and TGF. PR contributes to proliferative signals in normal and neoplastic mammary gland which has been observed in animal and human studies. Progesterone acts via sensitizing mammary cancer cells to the actions of growth factors by upregulating target genes of signal transduction pathways [64]. Our findings are in line with previous studies demonstrating that MNU injected rats showed increased expression levels of ER and PR [63, 65]. (Figure 3 and 4) It revealed that the breast tumor tissue of cancer bearing group 2 animals expressed significantly higher number of positive stained (brown colored) nuclei (60% ± 1.03 and 73.34%± 1.29) as compared to normal control animals (ER- 30.56% ± 0.98; PR-43.66% ± 1.38). The % positive stained ER cells for MEBM, AEBM and Tamoxifen were found to be

38.23% ± 1.43; 42% ± 1.13 and 31.66% ± 0.89 respectively. The % positive stained PR cells for MEBM, AEBM and Tamoxifen were found to be 50.14 % ± 1.32; 56.66% ± 2.69 and 46.66% ± 1.78 respectively. No significant difference was found between vehicle control and positive control animals. This match-up with *in-vitro* studies suggesting that the extract induce cytotoxic effect by a mechanism involved with estrogen and progesterone receptor modulation.

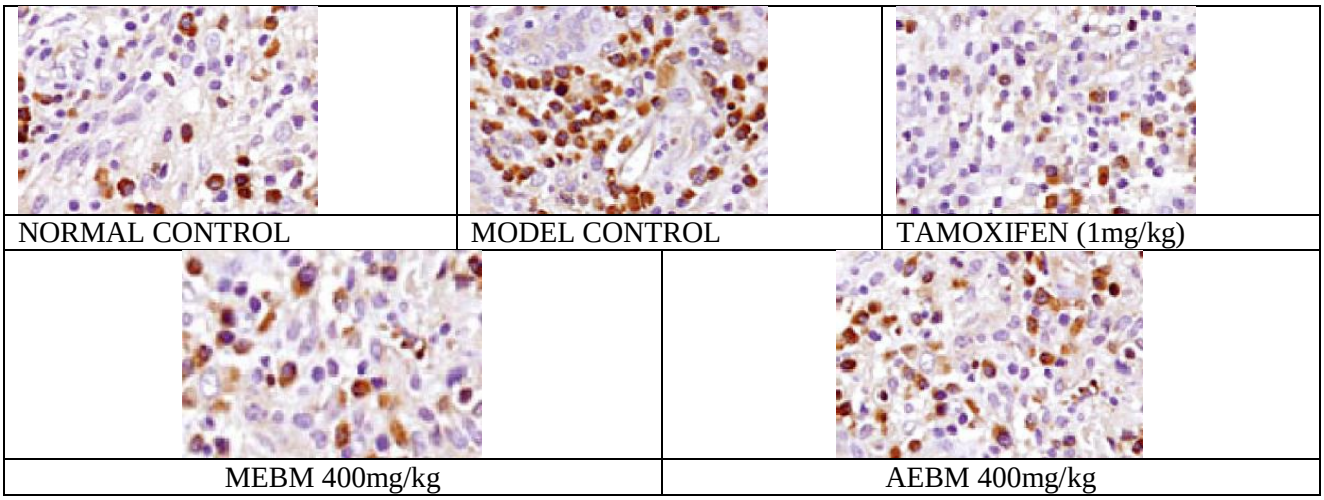


Figure 3: Effect of *Butea monosperma* flower extracts on estrogen receptor expression in MNU induced mammary carcinogenesis. Magnification: 40x

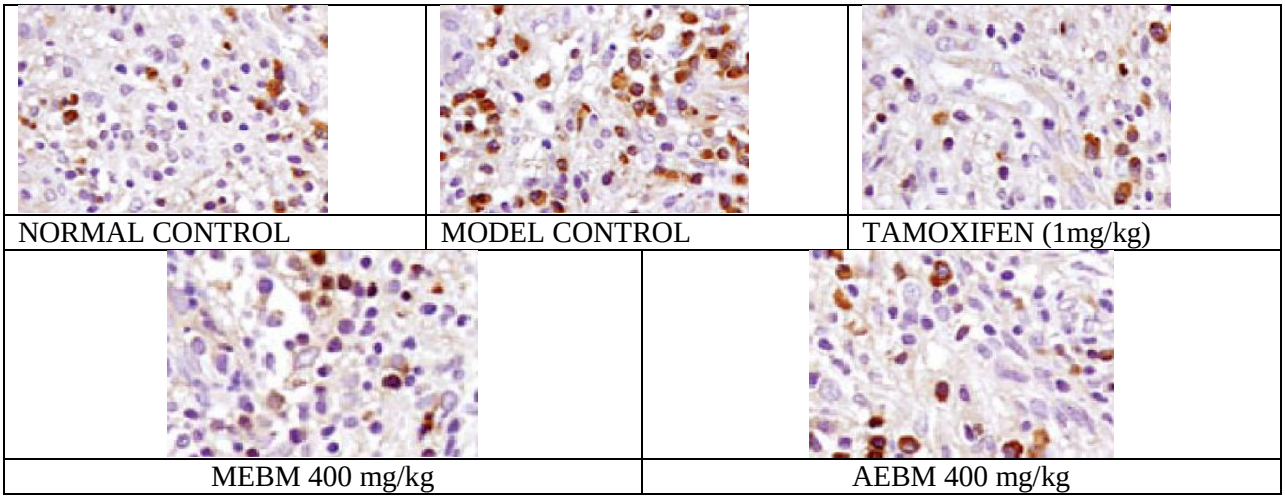


Figure 4: Effect of *Butea monosperma* flower extracts on progesterone receptor expression in MNU induced mammary carcinogenesis. Magnification: 40x

3.4.8.2.6. Effect on Nucleic Acid contents in MNU induced mammary carcinogenesis

Nucleic acids damage is a delicate marker and imminent biological target for many initiators of carcinogenesis [66]. Increase of DNA adducts generation and oxidative base injuries have been described in the nearby normal tissue and tumor tissues of breast cancer patients [67]. These findings demonstrate that an accumulation of damaged DNA may promote to breast carcinogenesis. Consequently, evaluating DNA content is the essential part in tumorigenesis [68].

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 [69]. In present study, within the tumor tissues of group 2 cancer bearing animals, the significant increased ($P<0.05$) levels of nucleic acids (DNA: 5.72±1.02; RNA: 4.69±1.07) were observed when compared to group 1 control animals (DNA: 3.66±1.03; RNA: 2.47±1.42). Ironically, these rises were attenuated by treatment groups ($P<0.05$) dose dependently. The % reduction in DNA content in Tamoxifen; AEBM (100, 200, 400mg/kg) and MEBM (100, 200, 400 mg/kg) was found to be 33.5%, 24.14%, 26.60%, 32.02%, 30.05%, 30.54% and 31.53% respectively. The % reduction in RNA content in Tamoxifen; AEBM (100, 200, 400mg/kg) and MEBM (100, 200, 400 mg/kg) was found to be 40.21%, 31.55%, 32.36%, 33%, 29.29%, 31.20% and 39.28% respectively. (Figure 5) This intimates anti-tumor property of extracts. Treatment slow down the progression of tumor growth and size which can be linked to decrease in nucleic acid content of treated groups..

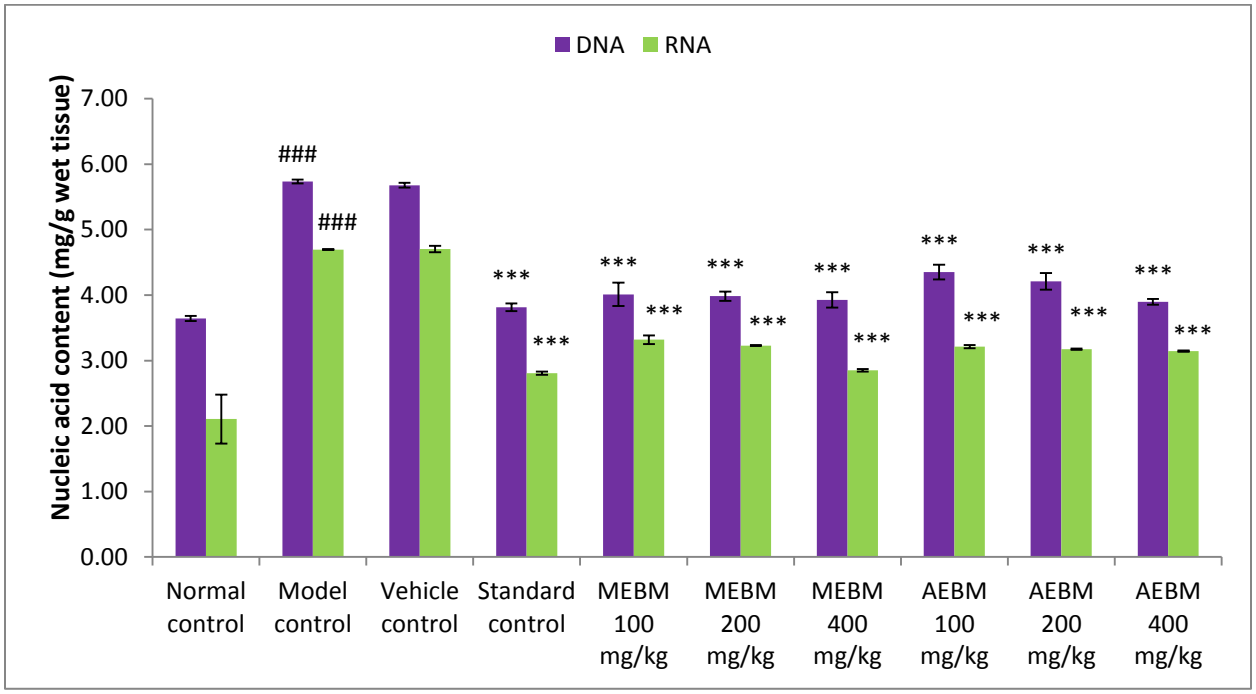


Figure 5: Effect of *Butea monosperma* flower extracts on nucleic acid content in MNU induced mammary carcinogenesis

Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using One Way ANOVA analysis followed by Bonferroni's post hoc test. Significant values were compared with ###$P<0.001$ normal control vs. model control; ***$P<0.001$ model control vs. all other groups.

For evaluating molecular aspects, the extracts were narrowed down to one based on the results of MNU induced mammary carcinogenesis.

3.4.8.3. EAC induced solid tumors

3.4.8.3.1. Effect on Body weight and relative liver weight in EAC induced solid tumors

The significant increase in body weight was found in tumor bearing group II animals up to 40g. The fluid in peritoneal cavity was observed during the experimental period. Upon treatment with standard and MEBM, the body weight was non-significantly different from normal control animals. No fluid accumulation was observed in treatment groups. The increased in relative liver weights were observed in tumor bearing group II animals as compared to normal control animals. ($P<0.001$) Tamoxifen and MEBM treated groups significantly improved relative liver weights when compared to model control animals ($P<0.001$) (Table 4)

Groups	Body Weight (g)	Relative liver weight
Normal control	34.67±0.25	5.20±0.17
Model control	40±11.54$^{###}$	9.36±0.75$^{###}$
Standard control	34.55±0.85	5.52±0.28***
MEBM	33±0.26	5.55±0.20***

Table 4: Effect of MEBM on body weight and tumor parameters in EAC induced solid tumors

Values represent Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###$P<0.001$ normal control vs. model control; ***$P<0.001$ model control vs. all other groups

3.4.8.3.2. Effect on Tumor Parameters in EAC induced solid tumors

Upon treatment with MEBM (from 5 day until 30 day), a significant reduction in the tumor volume was observed compared to untreated control animals bearing tumor. By 30th day, MEBM treated animals showed no tumor, unlike untreated tumor animals. More importantly, there was significant increase in the lifespan of MEBM administered animals. The tumor volume was staggeringly decreased in treatment group (82.61% tumor reduction; $P<0.001$) as compared to Group II cancer bearing animals. Tumor weight of cancer bearing animals were found to be 2.23±0.56 ($P<0.001$) which was truncated in MEBM treated animals 0.2±0.18 ($P<0.001$). Mean survival time for cancer bearing group II animals was 17 days whereas it was extended to 29.5 days for MEBM treated animals. (Table 5)

Groups	Tumor weight (g)	Tumor Volume (mm^3)	% Tumor reduction	Mean Survival Time	% Increase in Life span
Normal control	-	-	-	30	-
Model control	2.23±0.56	54.21±12.56	-	17###	-
Standard control	0	0	100%	30***	76.47
MEBM	0.20±0.18***	9.43***	82.61%	29.5***	73.53

Table 5: Effect of MEBM on body weight and tumor parameters in EAC induced solid tumors

Values represent Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control; ***P<0.001 model control vs. all other groups

3.4.8.3.3. Effect on Liver markers in EAC induced solid tumors

Liver is the main site for metabolism and detoxification. The metastasis of breast cancer to liver is common in humans which are depicted by increase in serum AST and ALT levels. In untreated EAC induced solid tumors, both AST (97.24 ±1.12; P<0.001) and ALT (76.02 ±1.23; P<0.001) levels were significantly elevated. The increase in liver enzymes in model control might be due to generalized destruction of liver cells. Treatment with MEBM altered liver enzymes levels and restored them to that of normal group. (AST: 22.99 ±1.09 and ALT (16.804 ±1.45; P<0.001). The animals treated with Tamoxifen showed significant decrease in AST (53.04 ±2.12; P<0.001) and ALT (35.36 ±1.63; P<0.001) levels but it was higher than that of MEBM treated animals. This finding can be correlated with the anti- migratory effect in scratch motility assay and anti-angiogenic effect in CAM assay. (Figure 6)

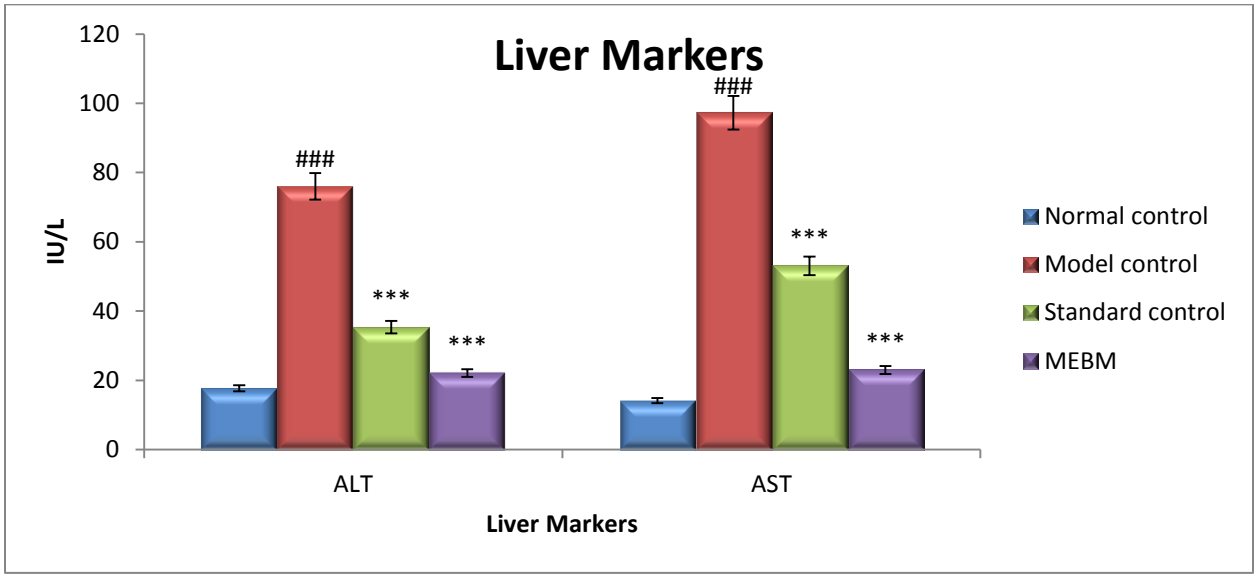


Figure 6: Effect of MEBM on liver markers in EAC induced solid tumors

Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control; ***P<0.001 model control vs. all other groups

3.4.8.3.4. Effect on Hematological parameters in EAC induced solid tumors

The results are in harmony with MNU induced mammary carcinogenesis. WBC (8.3 ±1.09; P<0.001) was found to be increased in untreated EAC injected mice whereas RBC (6.75±2.13; P<0.001) and HB (10.7 ±1.39; P<0.001) was found to be decreased as compared to normal control animals (WBC: 5.1 ±1.91; RBC: 8.3± 2.19 and HB: 11.5 ±2.35; P<0.001). Chemotherapy in cancer causes anemia due to reduction in RBCs count. So treatment should enhance the RBCs count to normal. In this study, the treatment with MEBM increased RBC count (8.62± 2.19; P<0.001) along with Hb (10.9 ±2.35; P<0.001). Concomitant decrease in WBC was observed upon treatment. (5.8± 2.19; P<0.001). Myelosuppression was observed with Tamoxifen treatment.

3.4.8.3.5. Effect on Inflammatory markers in EAC induced solid tumors

Inflammation is one of the factors of tumor progression, metastasis, treatment resistance and relapse.[70] IL-6 and Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine involved in apoptosis, inflammation, and immunity. The increased level of TNF-α was possibly linked to the activation of NF-κB, which plays a crucial role in inflammation and carcinogenesis.[71] IL-6 signaling has been linked to both pro- and antiapoptotic activity in breast cancer cells.[72] In group II cancer bearing animals, TNF- α and IL-6 were significantly elevated compared to normal control animals. The beneficial effect on cytokine levels were observed in estrogen antagonist, Tamoxifen treated group III. MEBM decreased elevated levels of cytokines, thereby inhibiting tumor progression. This effect can be correlated with anti-estrogenic effect. The curtailing effects

on inflammatory markers are also responsible for decrease in tumor progression and metastasis. (Figure 7)

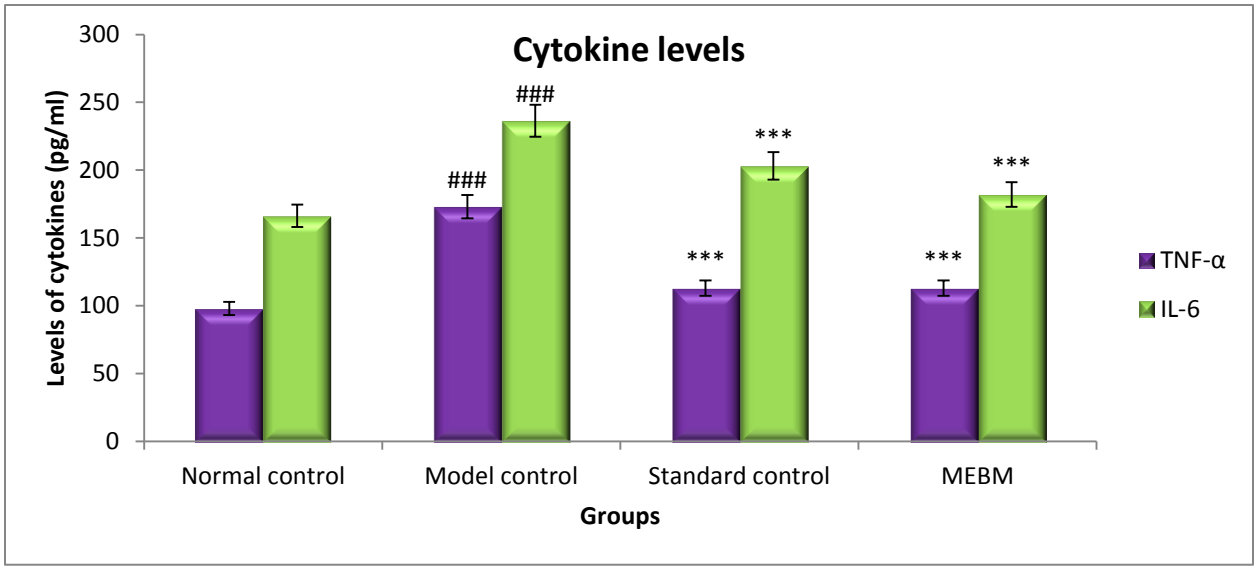


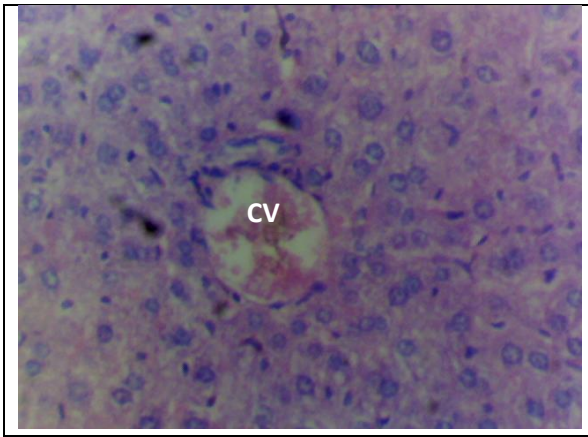
Figure 7: Effect of MEBM on cytokines level in EAC induced solid tumors

Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control; ***P<0.001 model control vs. all other groups

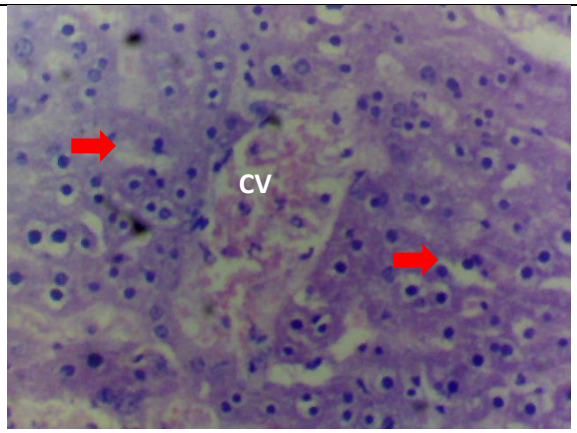
3.4.8.3.6. Effect on Histology of liver and kidney in EAC induced solid tumors

The control liver shows normal structure of hepatocytes and central vein. Enormous changes are seen in liver of model control mice. Enlarged and congested central vein, infiltration of tumor cells mixed with leukocytes. The fatty degeneration of hepatic tissue is observed. After treatment with MEBM, normal architecture can be seen but fatty degeneration is still found. Treatment with Tamoxifen showed enlarged central vein with less fatty degeneration.

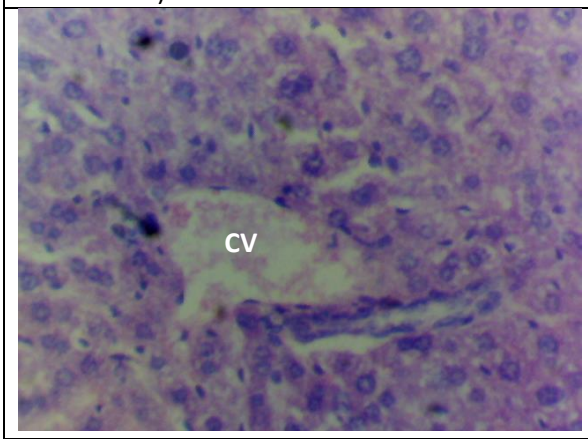
The control kidney shows normal histological features. The cancer bearing Group II animals showed congested renal vein, degenerated renal tubule and narrow glomerular space. The kidney of treated animals showed normal glomeruli and renal tubule. (Figure 8) Improvement in histopathological changes in structure of liver and kidney is of prime importance suggesting anti-metastatic potential of extract.



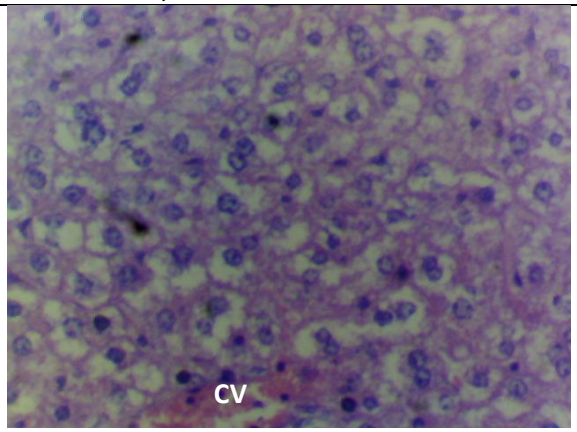
a) NORMAL CONTROL- LIVER



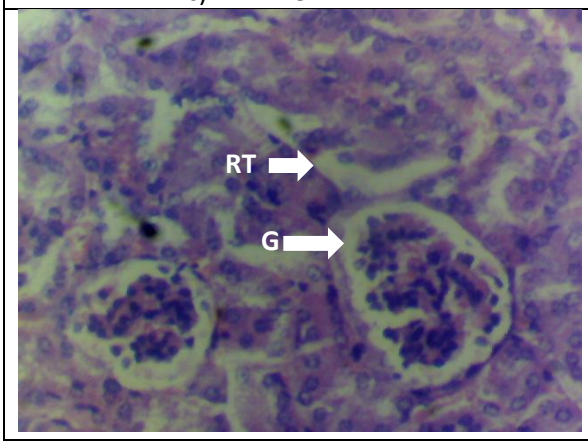
b) MODEL CONTROL-LIVER



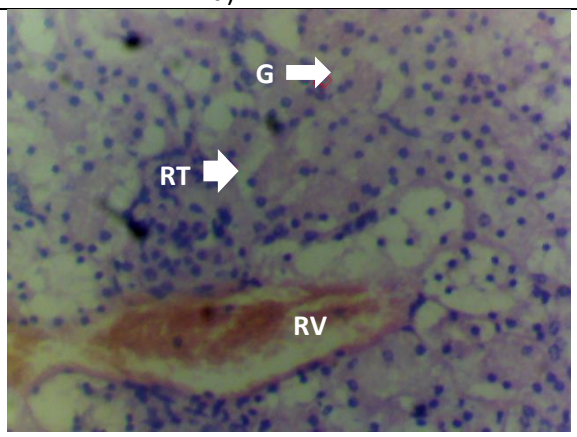
c) TAMOXIFEN- LIVER



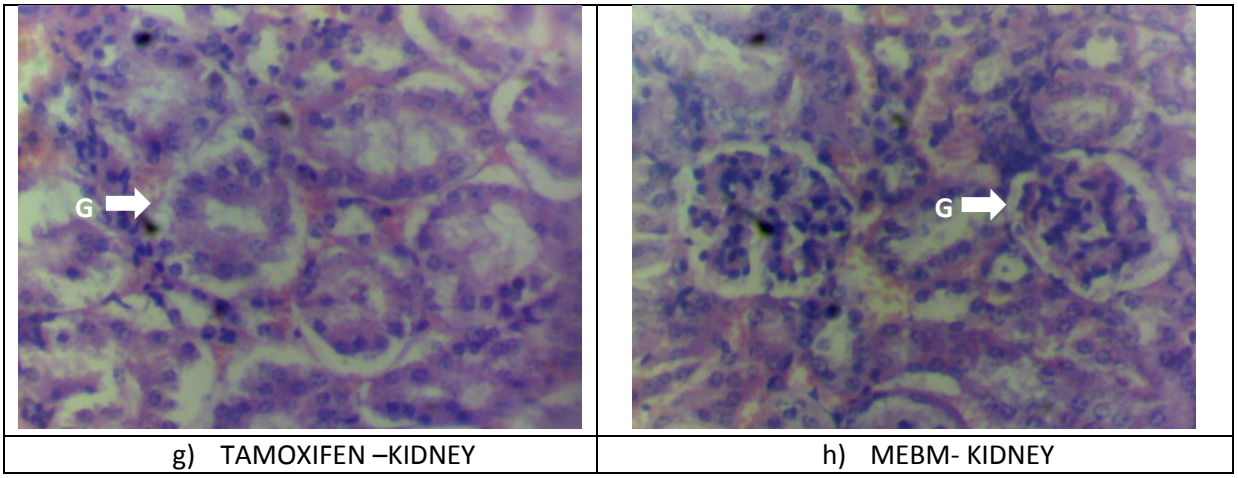
d) MEBM-LIVER



e) NORMAL CONTROL- KIDNEY



f) MODEL CONTROL- KIDNEY



g) TAMOXIFEN –KIDNEY | h) MEBM- KIDNEY

Figure 8: Effect of MEBM on histopathology of liver and kidney in EAC induced solid tumors. a) normal liver: normal structure of hepatocytes and central vein; b) model control liver: Enlarged and congested central vein; fatty degeneration; c) Tamoxifen treated liver: enlarged central vein with less fatty degeneration; d) MEBM treated liver: normal architecture with fatty degeneration e) normal kidney: normal structure of glomeruli and renal tubule f) model control kidney: congested renal vein, degenerated renal tubule and narrow glomerular space g) Tamoxifen treated kidney: normal glomeruli and renal tubule h) MEBM treated kidney: normal glomeruli and renal tubule. CV: Central vein; red arrow: fatty degeneration; RV: renal vein; G: glomeruli; RT: renal tubule. Magnification: 40x

3.4.8.4. Scratch Motility Assay

The fundamental reason that makes cancer so serious is due to its ability to spread in the body. Metastasis is leading cause for mortality in cancer patient. At least half of the patients display clinically noticeable metastasis at the time of diagnosis. The change in cell-cell and cell-matrix adhesion is of supreme importance for cell in metastatic journey.[73] In present investigation, the scratch motility assay displayed the ability of plant extracts to suppress migration of MCF-7 cells in a denuded area. At the concentration equal to IC_{50} of methanolic extract, the inhibition of MCF-7 migration in denuded area was 69.26 ± 1.76 % which is higher than that of aqueous extract (57.41 ± 2.03%). (Figure 9)

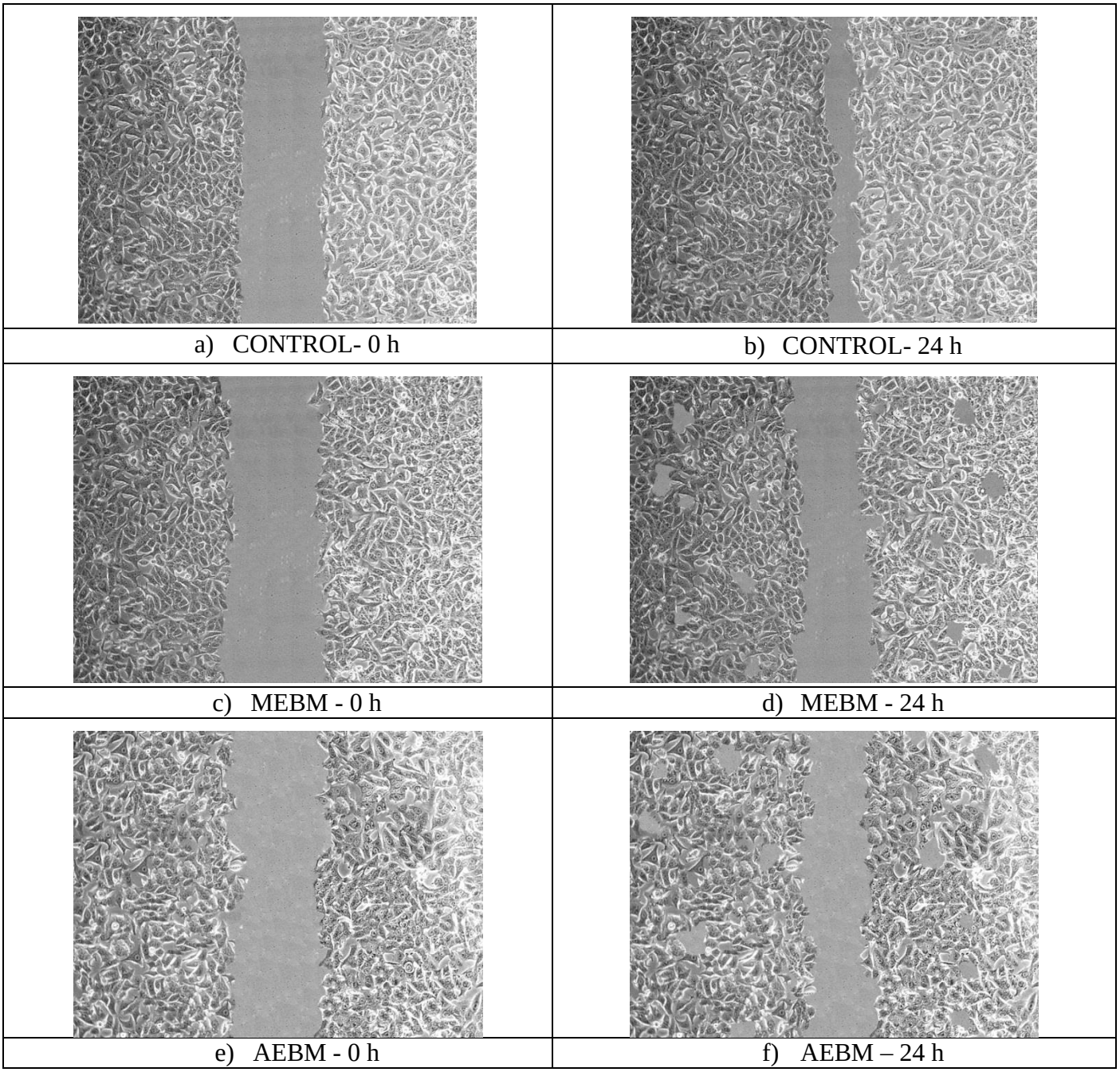


Figure 9. Effect of e on the cell migration (metastasis) on MCF-7 human breast cancer cell line. (a) Control- 0 h- MCF-7 cells with scratch. (b) Control 24 h- Migration of cells and restoration of monolayer in scratched area. (c) MEBM- 0 h- MCF-7 cells with scratch (d) MEBM 24 h- Inhibition of cell migration and less restoration of monolayer in scratched area as compared to control cells. (e) AEBM- 0 h- MCF-7 cells with scratch (f) AEBM 24 h- Inhibition of cell migration and less restoration of monolayer in scratched area as compared to control cells but more as compared to MEBM. The photographs are taken in Nikon Eclipse TS100. Magnification: 10X.

3.4.8.5. Chick Chorioallantoic Membrane Assay

Another abnormal property of cancer cell is sustained angiogenesis. Studies suggest that tumors induce the sprouting of new blood vessels from the surrounding vasculature. This key procedure supply supplements to developing tumor as well as fill in as means for tumor cells to metastasize.[74] 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 CAM was photographed. The 3mm zone of inhibition is seen with MEBM (20 µg/ml). (Fig 10) AEBM showed no significant anti-angiogenic potency.

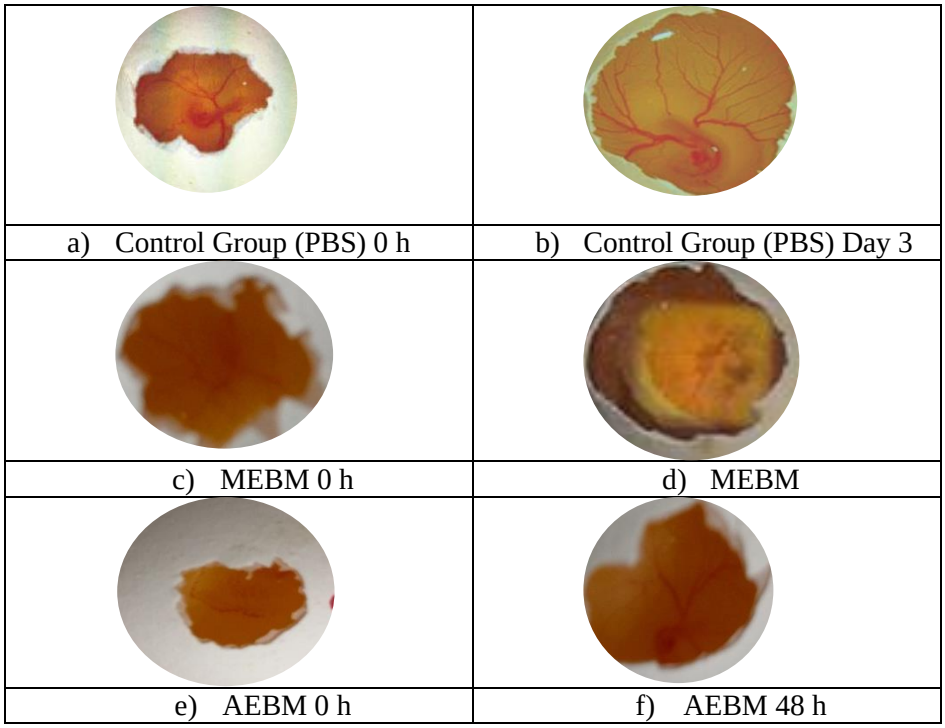


Figure 10. Effect of extracts in Chick Chorioallantoic Membrane Assay a) Control 0 h; b) Control 48 h: neovascularization and proliferation of blood vessels; c) MEBM 0 h; d) MEBM 48 h: zone of inhibition with loss of blood vessels; e) AEBM 0 h; f) AEBM 48 h: no hindrance in growth of blood vessels

3.4.8.6. Annexin V- FITC binding assay

Cell apoptosis is a typical physiological procedure of efficiently controlled cell demise for keeping up stable inner condition of the entire living being [75]. In diseases like cancer, too little apoptosis occurs, resulting in malignant cells that will not die. The balance between cell division and cell death is lost resulting in the cell population which should have died [76]. The huge amount of literature suggests that defects in apoptotic pathways play a crucial role in carcinogenesis and resistance to chemotherapeutic drugs [77]. The numerous new treatment procedures focusing on apoptosis are possible and might be utilized as a part of the treatment of various types of cancer. In present research, the treatment of MCF-7 cells with potent extracts of *Butea monosperma* for

24 h resulted in significant increases in the ratios of early (MEBM: 10.9%; AEBM: 10.1%) and late apoptosis (MEBM: 5.4%; AEBM: 4.2%) cells, while the percentage of viable cells (MEBM: 53.5%; AEBM: 64.8%) reduced. The viable cell, early apoptotic and late apoptotic population in control wells are 72.8%; 0.1% and 0.2% respectively. (Figure 11) This effect can be correlated to decrease in tumor volume. Also, dysregulation of apoptosis leads to failure of tissue size homeostasis resulting in tumor genesis.

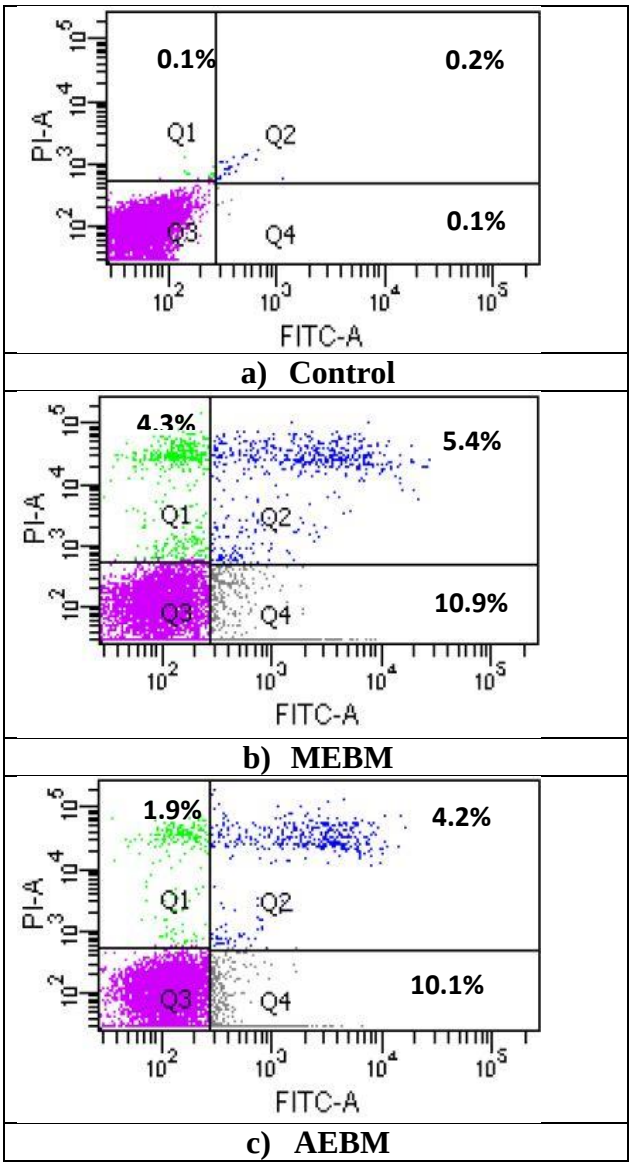


Figure 11: Effect of extracts on the apoptosis on MCF-7 human breast cancer cell line: Q1: Necrosis; Q2: Late Apoptosis; Q3: Viable cells; Q4: Early Apoptosis. (a) Control MCF-7 cells. MCF-7 cells treated with (b) MEBM and (c) AEBM. Percentage of apoptotic and necrotic population is increased in treated cells. The analysis was done by FACSDiva Version 6.1.3

3.5 Evaluation of *Cassia fistula* pods in breast cancer

Experimental procedure for *in-vitro* and *in-vivo* studies were same as described above (Section 3.4). The extracts were given at doses 100mg/kg, 200mg/kg and 400 mg/kg.

3.5.1. RESULTS AND DISCUSSION

3.5.1.1 MTT Assay

On treatment with different extracts of pods of *Cassia fistula* in MCF-7 cell line, at various concentrations such as 10, 100, 300, 500 and 1000 µg/ml for 24 h, 48h and 72 h, the anti-proliferative order was found to be AECF> MECF> BECF> EAECF. (Table 6) However, IC_{50} values observed for other two cell lines suggest lack of anti-proliferative effect of extracts on them.

Extracts	IC_{50} for MCF-7			IC_{50} for MDA-MB-231	IC_{50} for MDA-MB-453
	24 h (µg/ml)	48 h (µg/ml)	72 h (µg/ml)	72 h (µg/ml)	72 h (µg/ml)
MECF	525	435	300	>1000	>1000
AECF	344	300	134	>1000	>1000
BECF	>1000	>1000	650	>1000	>1000
EAECF	>1000	830	660	>1000	>1000

Table 6: IC_{50} value of various extracts of *Cassia fistula* pods on human cancer cell lines

3.4.8.2.7. Effect on Tumor Parameters in MNU induced mammary carcinogenesis

Injecting MNU (50mg/kg b.w; i.p.) to nulliparous SD female resulted in mammary tumors without metastasis. Tumor was induced after 45 days post MNU injection. Five out of six rats developed mammary carcinoma suggesting 83.33% tumor incidence. 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. The weight and volume of tumor in positive control animals were found to be 6.4±1.47 g and 99.29 ±3.19 mm^3. The data proposed successful induction of mammary carcinomas in MNU injected rats.

Oral administration of SD rats bearing MNU induced mammary cancer with extracts significantly decreased the mammary cancer. The weights ($P<0.05$) and volume ($P<0.001$) were significantly curtailed in all treatment groups. Tamoxifen, AECF and MECF prolonged latency period. The results suggest that extracts inhibit cancer cell proliferation *in-vivo*. (Table 7)

Groups	Tumor Incidence	Total number of tumors	Tumor multiplicity	Tumor weight (g)	Tumor volume (mm^3)	Tumor latency period (days)
Normal control	0	0	0	0	0	0
Model control	5	6	1	6.4± 1.46$^{###}$	99.29± 1.18$^{###}$	45.17±9.21$^{###}$
Vehicle control	6	6	1	6.2± 1.55	89.74± 1.18	53.23±19.41
Standard control	5	5	0.83	0.89±0.29** *	1.95±6.33***	82.17±16.43**
AECF 100 mg/kg	6	6	1	3.06±0.62*	8.08±4.86***	66.83±1.45**
AECF 200 mg/kg	6	6	1	2.93±0.59*	21.65±3.97***	67.17±2.76**
AECF 400 mg/kg	6	6	1	2.63±0.50**	15.71±1.98***	72.83±2.32***
MECF 100 mg/kg	6	6	1	4.03±0.75	31.13±7.69***	72.17±1.42***
MECF 200 mg/kg	6	6	1	3.47±0.78*	27.99±10.54***	75.80±12.75**
MECF 400 mg/kg	6	6	1	2.97±0.59*	17.66±6.66***	79.40±13.3***

Table 7: Effect of *Cassia fistula* pods extracts on tumor parameters in MNU induced mammary carcinogenesis

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control; *P<0.05 model control vs. all other groups; **P<0.01 model control vs. all other groups; ***P<0.001 model control vs. all other groups

3.6 Evaluation of *Lycopersicon esculentum* fruit extracts in breast cancer

Experimental procedure for *in-vitro* and *in-vivo* studies were same as described above (Section 3.4). The extracts were given at doses 100mg/kg, 200mg/kg and 400 mg/kg.

3.6.1. RESULTS AND DISCUSSION

3.6.1.1. MTT Assay

In-vitro experimental studies with different extracts (MELE, AELE, BELE and EAELE) of fruits of *Lycopersicon esculentum* at various concentrations and at various time points (24 h, 48h and 72 h), EAELE was observed to be more potent followed by MELE, AELE and BELE in inhibiting proliferation of MCF-7 human breast cancer cell line (Table 8). However, IC_{50} values observed for other two cell lines suggest lack of anti-proliferative effect on estrogen independent cell lines.

Extracts	IC_{50} for MCF-7			IC_{50} for MDA-MB-231	IC_{50} for MDA-MB-453
	24 h (µg/ml)	48 h (µg/ml)	72 h (µg/ml)	72 h (µg/ml)	72 h (µg/ml)
MELE	>1000	893	560	>1000	>1000
AELE	>1000	1000	573	>1000	>1000
BELE	>1000	>1000	890	>1000	>1000
EAELE	751	550	215	>1000	>1000

Table 8: IC_{50} value of various extracts of *Lycopersicon esculentum* fruit on human cancer cell lines

3.4.8.2.8. Effect on Tumor Parameters in MNU induced mammary carcinogenesis

Injecting MNU (50mg/kg b.w; i.p.) to nulliparous SD female resulted in mammary tumors without metastasis. 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. The weight and volume of tumor in positive control animals were found to be 6.4±1.47 g and 99.29 ±3.19 mm^3. The data proposed successful induction of mammary carcinomas in MNU injected rats.

On treatment, ameliorating effect of extracts was observed on tumor parameters. The weights and volume were significantly curtailed in all treatment groups. Latency period was significantly prolonged. Tumor multiplicity was decreased in animals treated with EAELE (200mg/kg and 400mg/kg). The results indisputably confirm that extracts inhibit cancer cell proliferation *in-vivo*. (Table 9)

Groups	Tumor Incidence	Total number of tumors	Tumor multiplicity	Tumor weight (g)	Tumor volume (mm^3)	Tumor latency period (days)
Normal control	0	0	0	0	0	0
Model control	5	6	1	6.4± 1.46$^{###}$	99.29± 1.18$^{###}$	45.17±9.21$^{###}$
Vehicle control	6	6	1	6.2± 1.55	89.74± 1.18	53.23±19.41
Standard control	5	5	0.83	0.89±0.29***	1.95±6.33***	82.17±16.43**
EAELE 100 mg/kg	6	6	1	3.43±0.63*	13.32±3.96***	71.33±1.63*
EAELE 200 mg/kg	5	5	0.83	2.16±0.76***	9.95±4.66***	73.00±12.24
EAELE 400 mg/kg	5	5	0.83	1.54±0.59***	4.04±1.45***	77.40±1.18**
MELE 100 mg/kg	6	6	1	3.00±0.65**	21.48±6.97***	70.5±1.73***
MELE 200 mg/kg	6	6	1	2.63±0.61**	15.86±4.38***	74.80±1.64***
MELE 400 mg/kg	6	6	1	2.19±0.51***	12.20±4.58***	76.80±2.03***

Table 9: Effect of *Lycopersicon esculentum* fruit extracts on tumor parameters in MNU induced mammary carcinogenesis

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control; *P<0.05 model control vs. all other groups; **P<0.01 model control vs. all other groups; ***P<0.001 model control vs. all other groups

4. Salient Features

- Methanol and aqueous extract of *Butea monosperma* flowers exhibited significantly higher anti-proliferative activity against MCF-7 human breast cancer cell line than other two (MDA-MB-453 and MDA-MB-231).
- Chemo-prevention by methanol and aqueous extract of *Butea monosperma* flowers was perceptible by its effect on prevention of mammary tumor induction, abating changes in nucleic acid & receptor status and restoration of hematological parameters.
- Moreover, methanol and aqueous extract of *Butea monosperma* flowers also possessed significantly higher apoptotic rate and prevent metastasis on MCF-7 cells line.
- Methanol extract possess increased tendency to curtail angiogenesis. No anti-angiogenic effect was seen with aqueous extract.
- The results suggest methanol extract of *Butea monosperma* flowers tackle breast cancer more aggressively and showed significantly higher anti-cancer potential than aqueous extract. This effect of methanol extract is also observed in EAC induced solid tumors. The improvement in tumor parameters and restoration of hematological parameters suggests curative potential of extract. Re-instating normal levels of liver enzymes along with reduction of liver weight suggests ameliorating effect on metastasis. The curtailing effects on inflammatory markers are also responsible for decrease in tumor progression and metastasis. The results are assisted by histopathological analysis.
- Ameliorating effect of aqueous and methanol extract of *Cassia fistula* pods were seen on MCF-7 human breast cancer cell line and on tumor parameters *in-vivo.*
- Ethyl acetate and methanol extract of *Lycopersicon esculentum* showed mitigating effect on MCF-7 human breast cancer cell line and on tumor parameters *in-vivo.*

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