7. SUMMARY AND CONCLUSION

7.1. Aim of study

Carcinogenesis is an extremely dynamic, nonlinear process following unpredictable pathways. 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. 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, apoptotic, free radical scavenging activity and antitumor property against hepatic carcinoma. *Lycopersicon esculentum* fruit clinically proven to reduce the risk of prostate cancer consists of all four major carotenoids: alpha- and beta-carotene, lutein, and lycopene. Lycopene as properties like aromatase and VEGF inhibition. *Cassia fistula* was found to be potent anticancer agent on human colon cancer cell line and possess antiestrogenic activity.

With this background, the present study was aimed to evaluate *Butea monosperma* flowers, *Lycopersicon esculentum* fruits and *Cassia fistula* pods in breast cancer.

The objectives of the study were:

1. Evaluating the effect of various extracts on cell viability in normal epithelial breast cell line *in-vitro* (MCF-10A).

2. Evaluating the cytotoxicity of various extracts *in-vitro* on human breast cancer cell lines (estrogen positive MCF-7, HER-2 positive MDA-MB-453, triple negative MDA-MB-231).

3. *In-vivo* preventive study of extracts in N-methyl- N-nitrosourea (MNU) induced mammary carcinogenesis.

4. *In-vitro* mechanistic assay for angiogenesis (Chick chorioallantoic membrane assay); apoptosis (Annexin V- FITC binding assay), oxidative stress (DCFH-DA assay) and metastasis (Scratch motility assay)

5. Evaluating the curative proficiency of extracts against syngeneic model (Ehlrich Ascites Carcinoma induced solid mammary tumors).

7.2. Work Plan

- 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).
- In present study, the four extracts (aqueous, methanol, butanol and ethyl acetate) of each plant were screened to halt the uncontrolled cell proliferation in human breast cancer cell lines by MTT assay. The extracts were screened for cell proliferation and safety in non-cancerous MCF-10A human breast epithelial cell lines.
- The two potent extracts found on basis of IC_{50} values in MTT assay were screened for their preventive effect in MNU induced mammary carcinogenesis as well as for mechanistic approach in *in-vitro* assays viz. Chick Chorioallantoic Membrane (CAM) assay of angiogenesis, Annexin V- FITC binding assay of apoptosis, scratch motility assay of metastasis and DCFH-DA assay for oxidative stress.

- The curative proficiency of best extract of each plant was studied in syngeneic model; Ehlrich Ascites Carcinoma (EAC) induced solid mammary tumors.

7.3. *In-vitro* cell line study (MTT assay)

- To study antiproliferative effect three breast cancer cell lines were selected based on genetics of breast cancer viz. estrogen positive MCF-7, HER-2 positive MDA-MB-453 and triple negative (ER-ve, PR-ve, HER-2 –ve) MDA-MB-231.
- 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 MEBM at 72 hours was found to be 874 µg/ml and 1000 µg/ml. IC_{50} were greater than 1000 µg/ml for all the remaining extracts (AEBM, BEBM, EAEBM).
- At 72 hours, the IC_{50} values of EAELE (215 µg/ml) on MCF-7 cell line was lowest amongst all extracts. It was followed by MELE (IC_{50}:560 µg/ml). The IC_{50} of AELE and BELE was 573 µg/ml and 890 µg/ml. However, IC_{50} values for EAELE, MELE, AELE and BELE on other two cell lines (MDA-MB-453 and MDA-MB-231) does not fall in selected concentration range (10-1000 µg/ml), suggesting lack of anti-proliferative effect of extracts on them.
- The IC_{50} value of AECF at 72 hours on MCF-7 cell line was found to be 134 µg/ml which is lowest amongst all extracts. The IC_{50} of MECF, BECF and EAECF was 300 µg/ml, 650 µg/ml and 660 µg/ml on MCF-7 cells at 72 hours. In HER-2 positive and triple negative cell lines, the IC_{50} values of AECF, MECF, BECF and EAECF were above 1000 µg/ml, suggesting lack of cytotoxicity on them.

- In current research, on the exposure of different extracts of *Butea monosperma* flowers (AEBM, MEBM, BEBM and EAEBM), *Lycopersicon esculentum* fruits (AELE; MELE; BELE; EAELE) and *Cassia fistula* pods (AECF; MECF; BECF; EAECF) for 24 h, 48 h and 72h to different cell lines, the highest decrease in cell proliferation was found in MCF-7 cell line.
- The IC_{50} value of various extracts 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 estrogen positive breast cancer cells than other types of breast cancer cells in vitro.
- Cell viability of non-tumorigenic MCF-10 A human breast epithelial cell line was unaffected by various extracts of three plants selected for the study viz. *Butea monosperma* flowers; *Lycopersicon esculentum* fruits and *Cassia fistula* pods.
- To further confirm above results, *in-vivo* preventive studies of two most potent extracts of *Butea monosperma* flowers (MEBM and AEBM), *Lycopersicon esculentum* fruits (EAELE and MELE) and *Cassia fistula* pods (AECF and MECF) were performed in nulliparous female Sprague Dawley rats by injecting carcinogen MNU, known to experimentally induced hormone positive mammary cancer. The effect on various mechanisms (angiogenesis, apoptosis, metastasis and oxidative stress) of two potent extracts of medicinal plants was further studied in MCF-7 human breast cancer cell line at concentration equal to IC_{50} of respective extracts.

7.4. *In-vivo* MNU induced mammary carcinogenesis and *in-vitro* mechanistic studies

- In MNU induced mammary carcinogenesis, it was deduced that MEBM 400 is superior to AEBM 400 in forestalling breast cancer. The tumor was evident only in 2 animals in highest dose of MEBM group while 5 animals developed tumors in highest dose of AEBM group. The tumor incidence and burden in MEBM treated group was abbreviated as compared to Tamoxifen treated group. The 36% reduction in estrogen receptor expression was seen with MEBM 400, while 30% was seen with AEBM 400 treatment as compared to model group. Likewise, 31.63% reduction in progesterone receptor expression was seen with MEBM 400, while 22.74% was seen with AEBM 400 treatment as compared to model group. The MEBM in Chick Chorioallantoic Membrane assay was found to hostile angiogenesis. No anti-angiogenic effect was seen with AEBM. The apoptotic rate with MEBM was 2% higher than AEBM. The significant 11.85% higher anti-migratory effect was observed in MEBM as compared to AEBM. All these results suggest MEBM tackle breast cancer more aggressively and showed significantly higher anti-cancer potential than aqueous extract.
- In preventive studies, EAELE 400 is significantly better than MELE 400 in hindering mammary carcinogenesis. The tumor burden was 5 in highest dose of EAELE group while all animals developed tumors in highest dose of MELE group. The tumor incidence and burden of EAELE was similar to Tamoxifen treated group. The tumor weight was 41.67% lessened in EAELE group as compared to MELE. All doses of EAELE significantly tapered nucleic acid rise while only MELE 400 significantly significantly truncated this rise with confidence level of 95%. The estrogen levels in EAELE 400 were reduced by 25% while in MELE 400 it was only 13.33% when compared with model control animals. Similarly, the decline in progesterone levels was higher in EAELE 400 (24.34%) than MELE 400

(15.30%) when compared to model control animals. No anti-angiogenic effect was seen with MELE while EAELE showed anti-angiogenic effect. The apoptotic rate was 3.7% higher in EAELE than MELE. The cell motility was inhibited in greater extent by EAELE as compared to MELE. The outcomes of in-vivo preventive studies and in-vitro mechanistic studies demonstrated EAELE to be more potent against breast cancer as compared to MELE.

- Scrutinizing tumor parameters and nucleic acid content, AECF was found to be better candidate against MNU induced mammary carcinogenesis. The 25% and 14.1% reduction in estrogen and progesterone receptor expression was seen with AECF 400 respectively as compared to model group. The total of 10% estrogen expressions was reduced by MECF 400 as compared to model group. Negligible effect was seen on progesterone receptor expressions in MECF 400 when compared to model group. In in-vitro mechanistic studies, anti-angiogenic effect was seen in AECF only. Also, the apoptotic rate with AECF was significantly higher (11.7%) than MECF. The anti-migratory effect was 5.92% higher in AECF than MECF. The AECF wins the race in handling breast cancer more efficiently.
- Based on results of preventive studies and *in-vitro* mechanistic assays, the curative proficiency of MEBM, EAELE and AECF was further studied in syngeneic model of mice.

7.5. Curative proficiency of MEBM, EAELE and AECF in EAC induced mammary carcinogenesis

- The curative therapeutic approach with the insights into mechanisms was studied in EAC induced solid mammary cancer in mice. Considering tumor parameters, the extracts showed significant difference from model control animals. The tumor weight was significantly diminished in all treatment

groups *viz.* MEBM, EAELE and AECF as compared to model control animals and also was non-significantly different from Tamoxifen treated animals. The % tumor volume reduction was highest with MEBM. The percentage increase in life span was only 2.94% less in MEBM as compared to Tamoxifen. In stark contrast, the 14.71% difference in life span was seen between AECF and Tamoxifen treated animals. The order of curative proficiency is MEBM> EAELE> AECF. These outcomes can be attributed to anti- angiogenic potential of extract which halt the tumor growth in starting phase.

- While addressing anti-angiogenic mechanisms *in-vivo*, the VEGF levels were assessed. All extracts possess significantly ($P<0.001$) decreased VEGF levels as compared to model control animals affirming anti-angiogenic capability of extract *in-vivo*. However, the order of anti-angiogenic potential was MEBM> EAELE> AECF. This suggests that all extracts can be used as a preventive therapy in breast cancer or they reduced the risk of breast cancer if consumed.
- The tumor suppressor gene expressions are of much more importance in obstructing tumor genesis. The intensification in p53 expressions by MEBM (35%) treatment was non-significantly higher than Tamoxifen (34%). The % increase in tumor suppressor gene by EAELE and AECF was same (30%).
- While focusing on apoptosis pathway, Caspase-9 levels were quantified. The Caspase-9 levels were significantly decreased by MEBM and EAELE with confidence levels 99.9%. The 95% confidence level was achieved in AECF treated animals when compared to model control. These outcomes suggest that MEBM, EAELE and AECF induce apoptosis via p53 –

Caspase-9 cascade activation and can be used in early stages of breast cancer.

- While targeting anti-metastatic potential of extracts *in-vivo,* liver enzymes, cytokine levels and lysosomal enzyme levels were assessed. The animals treated with MEBM showed curtailment in AST and ALT levels which was significantly lower than Tamoxifen. The decline in liver enzymes by EAELE was significantly lower from MEBM and Tamoxifen. No significant difference was observed in AECF treated animals and model control. The TNF-α level was non-significantly different between Tamoxifen and MEBM. The significant higher TNF- α and IL-6 level was recorded in EAELE and AECF treated groups when compared to Tamoxifen and MEBM. The lysosomal enzyme level was significantly ($P<0.01$) higher in AECF as compared to MEBM. As such, no significant different was found between MEBM and EAELE. These results indicate that both MEBM and EAELE significantly abrogate metastasis progression as compared to model control. The results of MEBM and EAELE were comparable to Tamoxifen. AECF possess no activity against metastasis *in-vivo.* Thus, it can be concluded that MEBM and EAELE might be used in invasive breast cancer.