5. RESULTS

SECTION 1

In-vitro cell line studies of extracts on MCF-10A human breast epithelial cell line and MCF-7, MDA-MB-453, MDA-MB-231 human breast cancer cell lines

5.1. Effect of extracts on MCF-10A human breast epithelial cell line by MTT assay

The cell viability effect on MCF-10A non-cancerous cell line was studied for various extracts (aqueous, methanol, butanol and ethyl acetate) of *Butea monosperma* flowers (AEBM; MEBM; BEBM and EAEBM), *Lycopersicon esculentum* fruits (AELE; MELE; BELE and EAELE) and *Cassia fistula* pods (AECF; MECF; BECF and EAECF).

In current research, none of the extracts of medicinal plant under investigation possess cytotoxic action on non-tumorigenic human breast epithelial cell line MCF-10A at 72 hours as depicted by no significant changes in cell viability in presence of various extracts as compared to MCF-10 A control cell line. (Figure 5.1- 5.3)

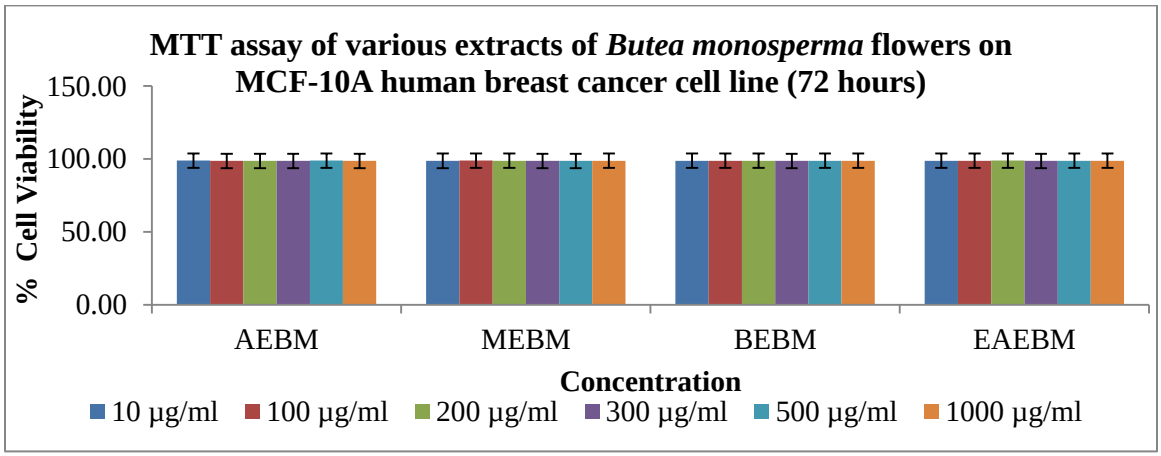


Figure 5.1: Effect of various extracts of *Butea monosperma* flowers on cell viability of MCF-10A human breast epithelial cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

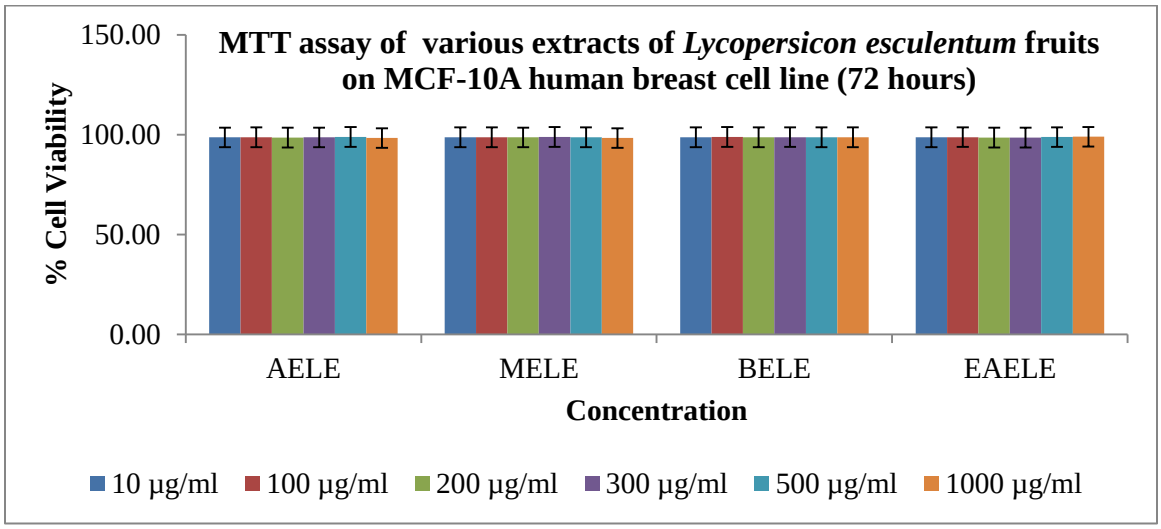


Figure 5.2: Effect of various extracts of *Lycopersicon esculentum* fruits on cell viability of MCF-10A human breast epithelial cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

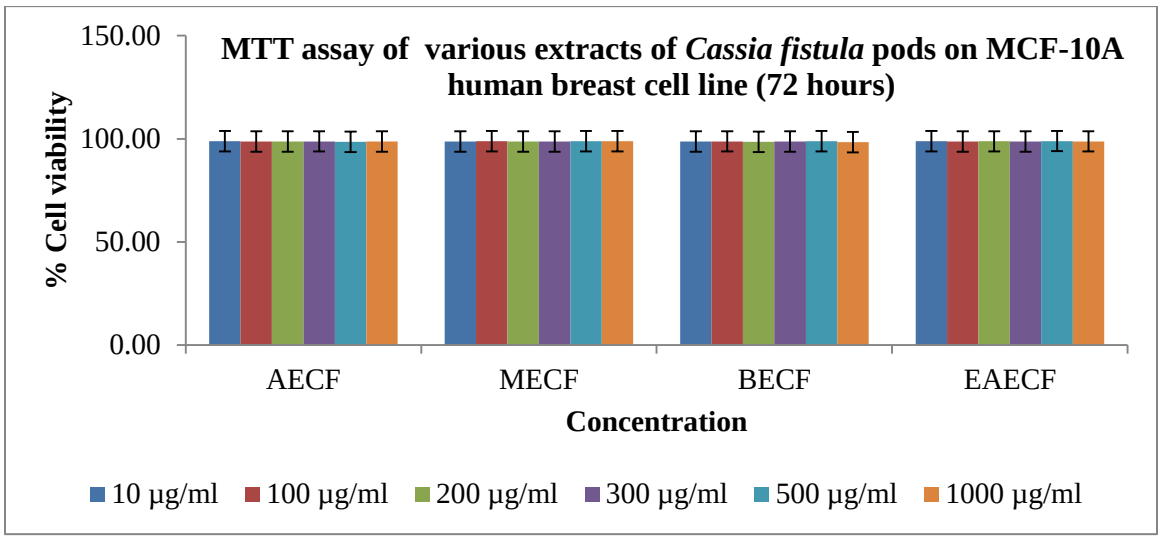


Figure 5.3: Effect of various extracts of *Cassia fistula* pods on cell viability of MCF-10A human breast epithelial cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

5.2. Effect of extracts on cell proliferation of MCF-7, MDA-MB-453, MDA-MB-231 human breast cancer cell lines by MTT Assay

A. *Butea monosperma* flowers

The cell proliferative study was performed on hormone positive (ER +ve) MCF-7; HER-2 positive MDA-MB-453 and triple negative (ER –ve, PR –ve, HER-2 –ve) MDA-MB-231 human breast cancer cell lines. The maximum cell cytotoxicity was observed in MCF-7 cell line which was found to be dose- and time- dependent. On MCF-7, the extracts exhibited antiproliferative activity in following order: MEBM> AEBM> BEBM> EAEBM. (Figure 5.4) On MDA-MB-453 and MDA-MB-231 cell line, only MEBM appeared to be cytotoxic at 72 hours (at 1000 µg/ml). (Figure 5.5) On MDA-MB-453 and MDA-MB-231 cell line, all other extracts (AEBM, BEBM and EAEBM) the inhibition of cell proliferation was below 50%. (Figure 5.6)

B. *Lycopersicon esculentum* fruits

In-vitro experimental studies with different extracts of *Lycopersicon esculentum* fruits at various concentrations (10-1000 µg/ml) and at various time points (24 h, 48h and 72 h) revealed EAELE to be most potent followed by MELE, AELE and BELE in inhibiting proliferation of MCF-7 human breast cancer cell line. (Figure 5.7) The potential of cell proliferation was lacking in other two cell lines *viz.* MDA-MB 453 and MDA-MB-231 as % cell inhibition observed was below 50%. (Figure 5.8 and 5.9)

C. *Cassia fistula* pods

On treatment with different extracts of *Cassia fistula* pods in MCF-7 cell line, at various concentrations such as 10, 100, 200, 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. (Figure 5.10) The % cell inhibition was below 50% which suggests lack of anti-proliferative effect on MDA-MB-453 and MDA-MB-231 breast cancer cells. (Figure 5.11 and 5.12)

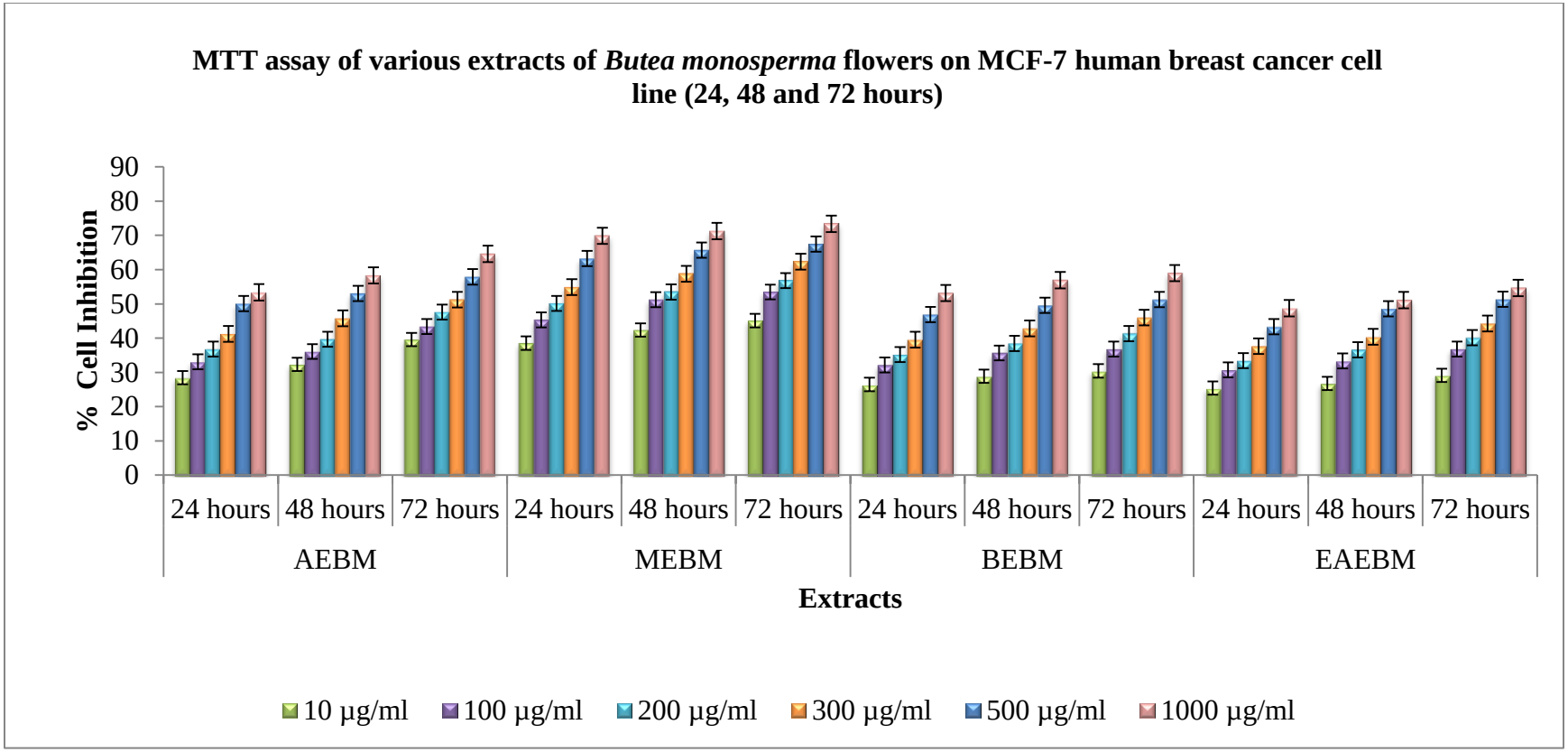


Figure 5.4: Effect of various extracts of *Butea monosperma* flowers on cell proliferation of MCF-7 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

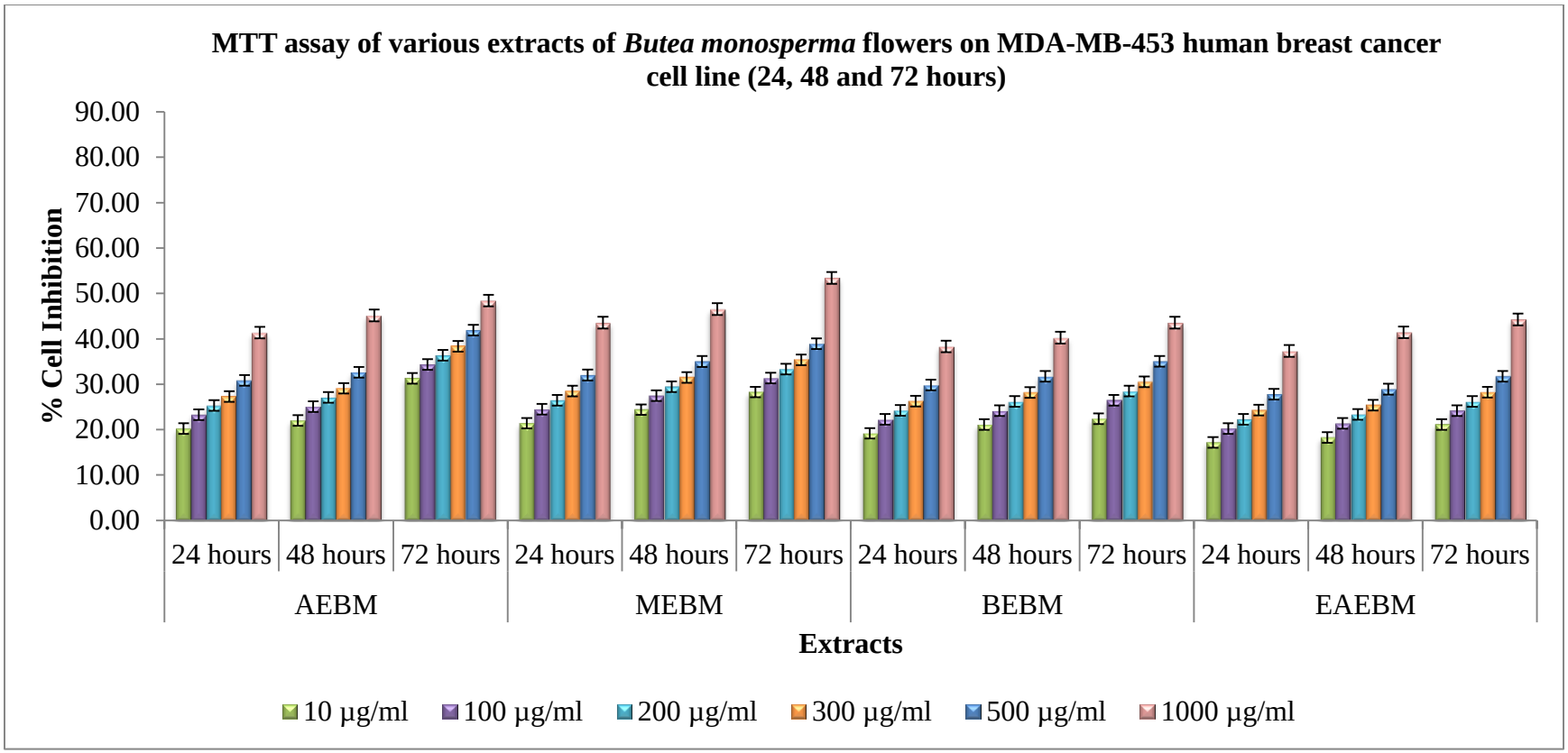


Figure 5.5: Effect of various extracts of *Butea monosperma* flowers on cell proliferation of MDA-MB-453 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

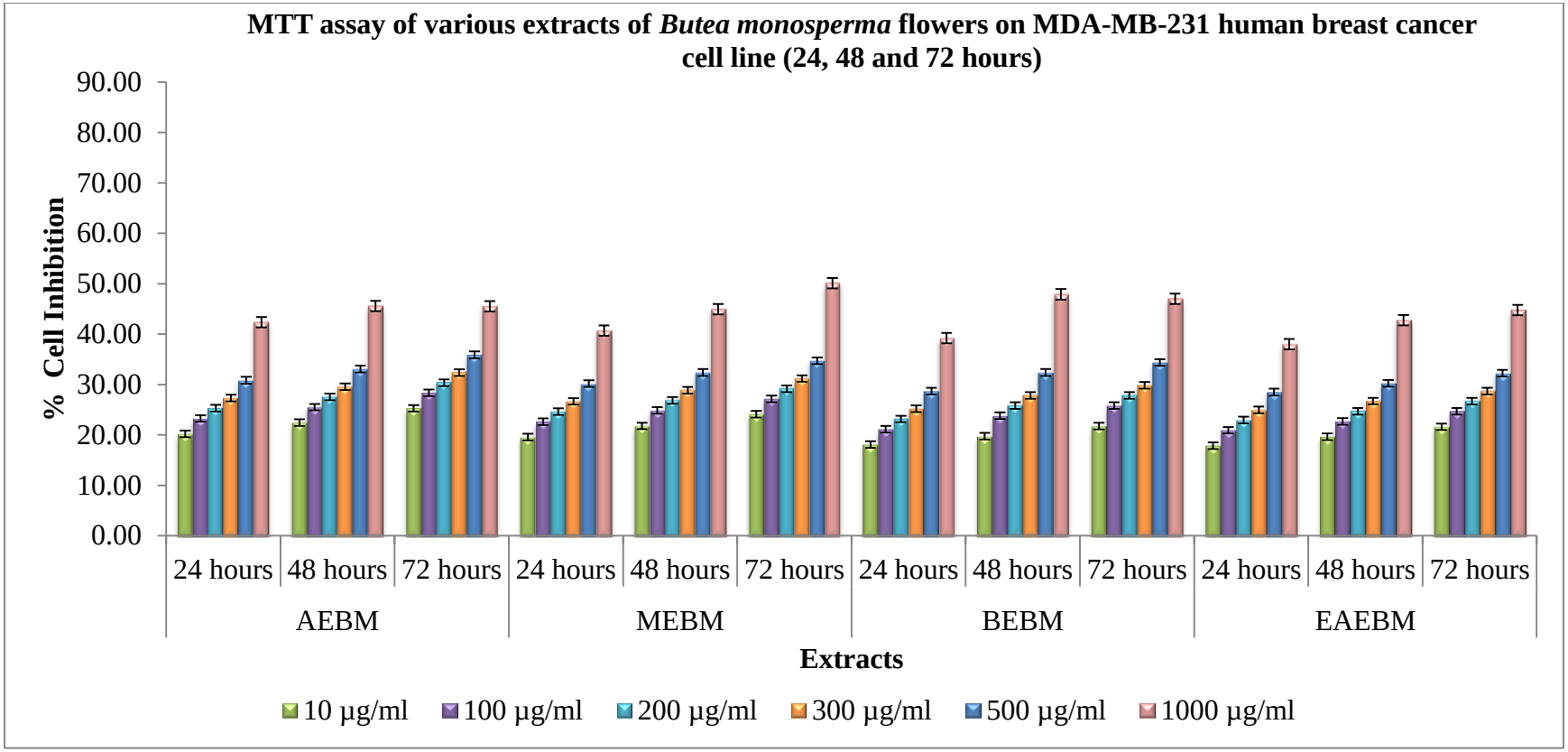


Figure 5.6: Effect of various extracts of *Butea monosperma* flowers on cell proliferation of MDA-MB-231 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

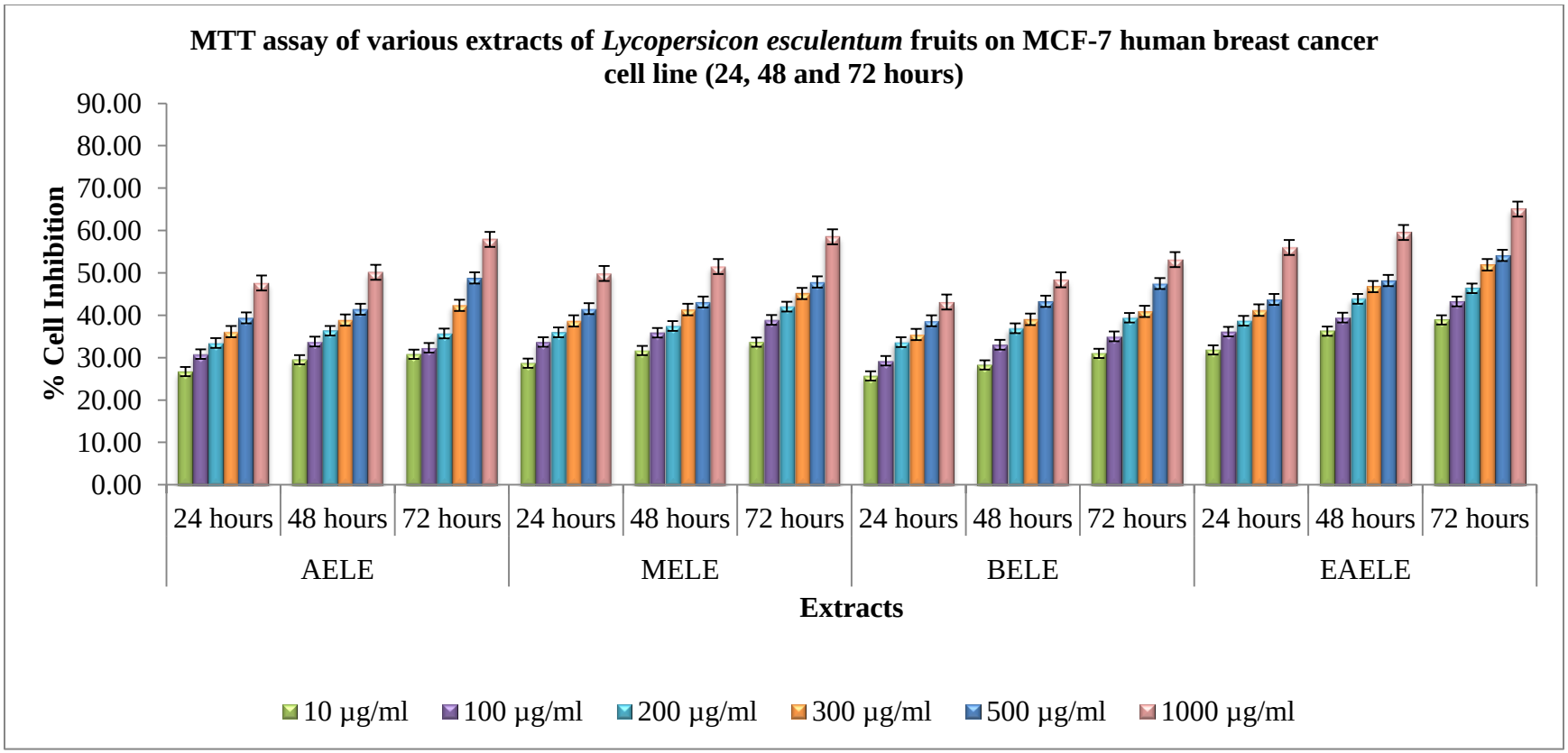


Figure 5.7: Effect of various extracts of *Lycopersicon esculentum* fruits on cell proliferation of MCF-7 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

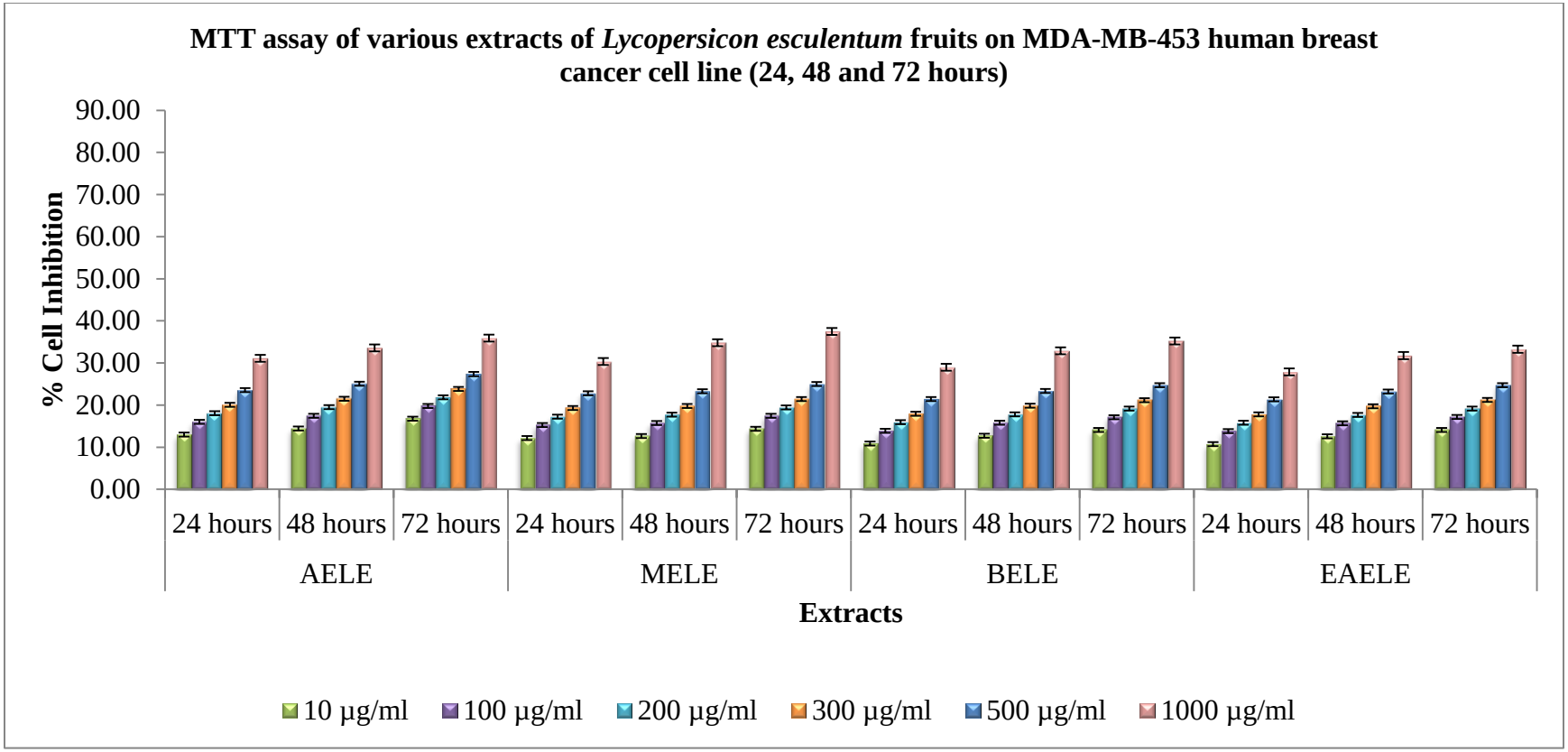


Figure 5.8: Effect of various extracts of *Lycopersicon esculentum* fruits on cell proliferation of MDA-MB-453 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

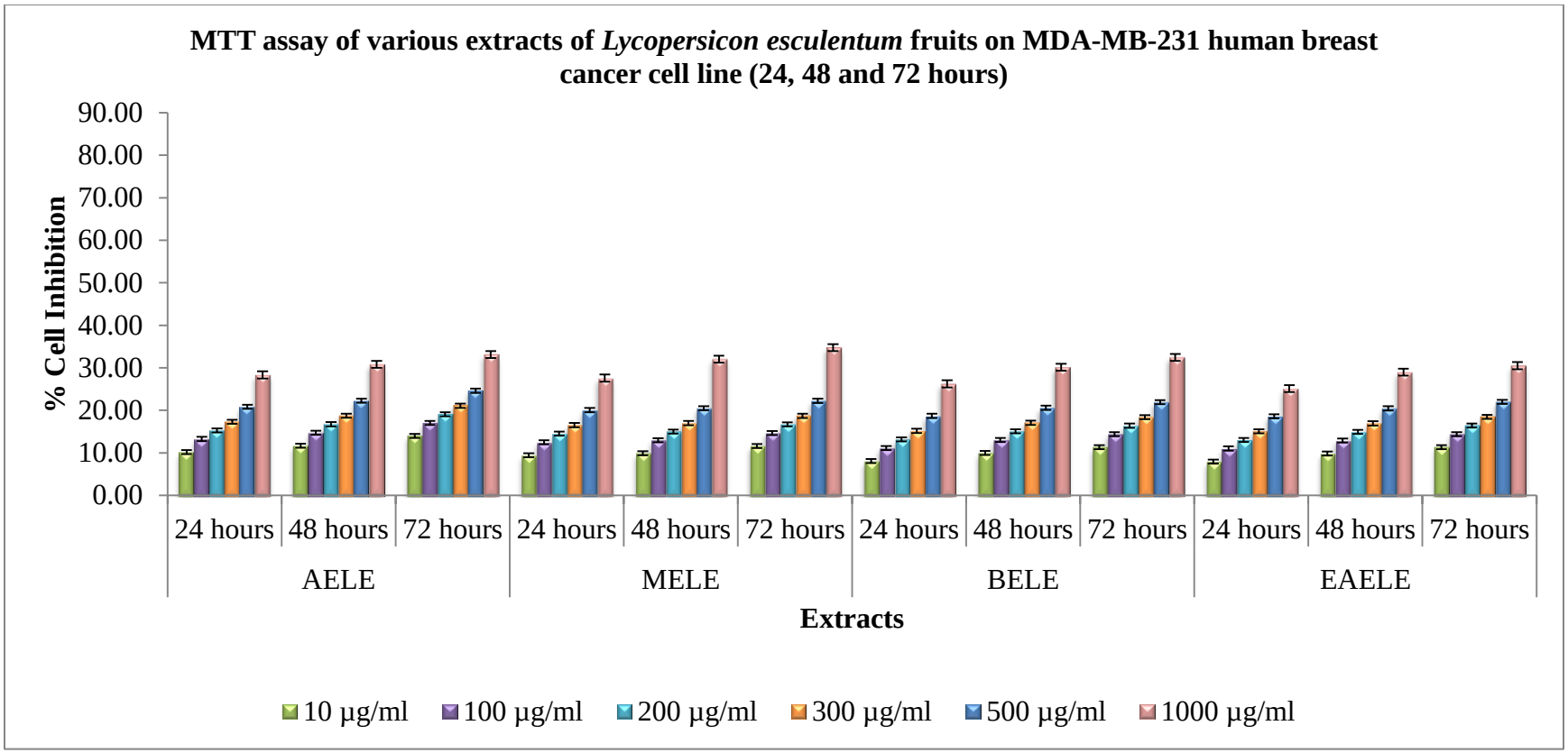


Figure 5.9: Effect of various extracts of *Lycopersicon esculentum* fruits on cell proliferation of MDA-MB-231 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

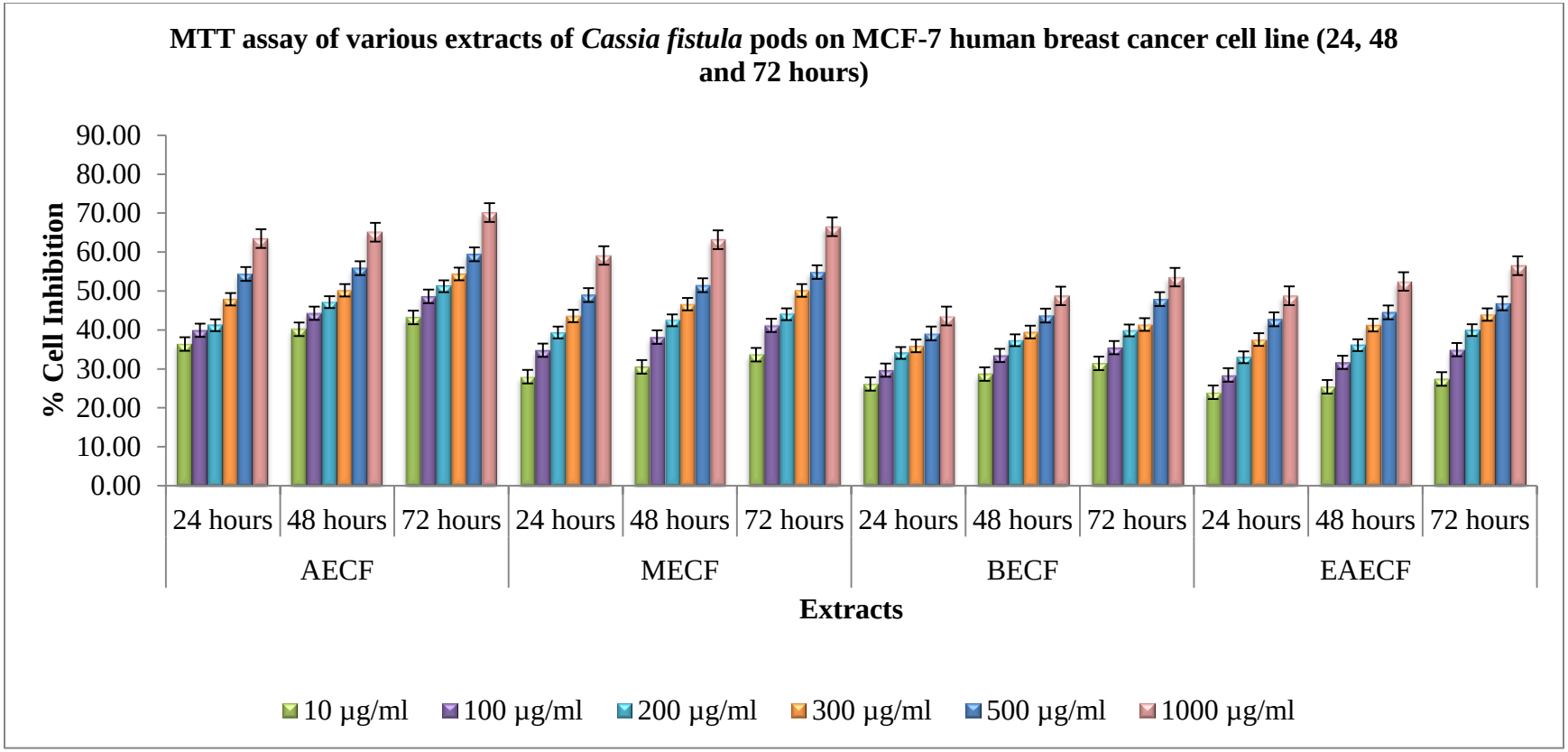


Figure 5.10: Effect of various extracts of *Cassia fistula* pods on cell proliferation of MCF-7 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

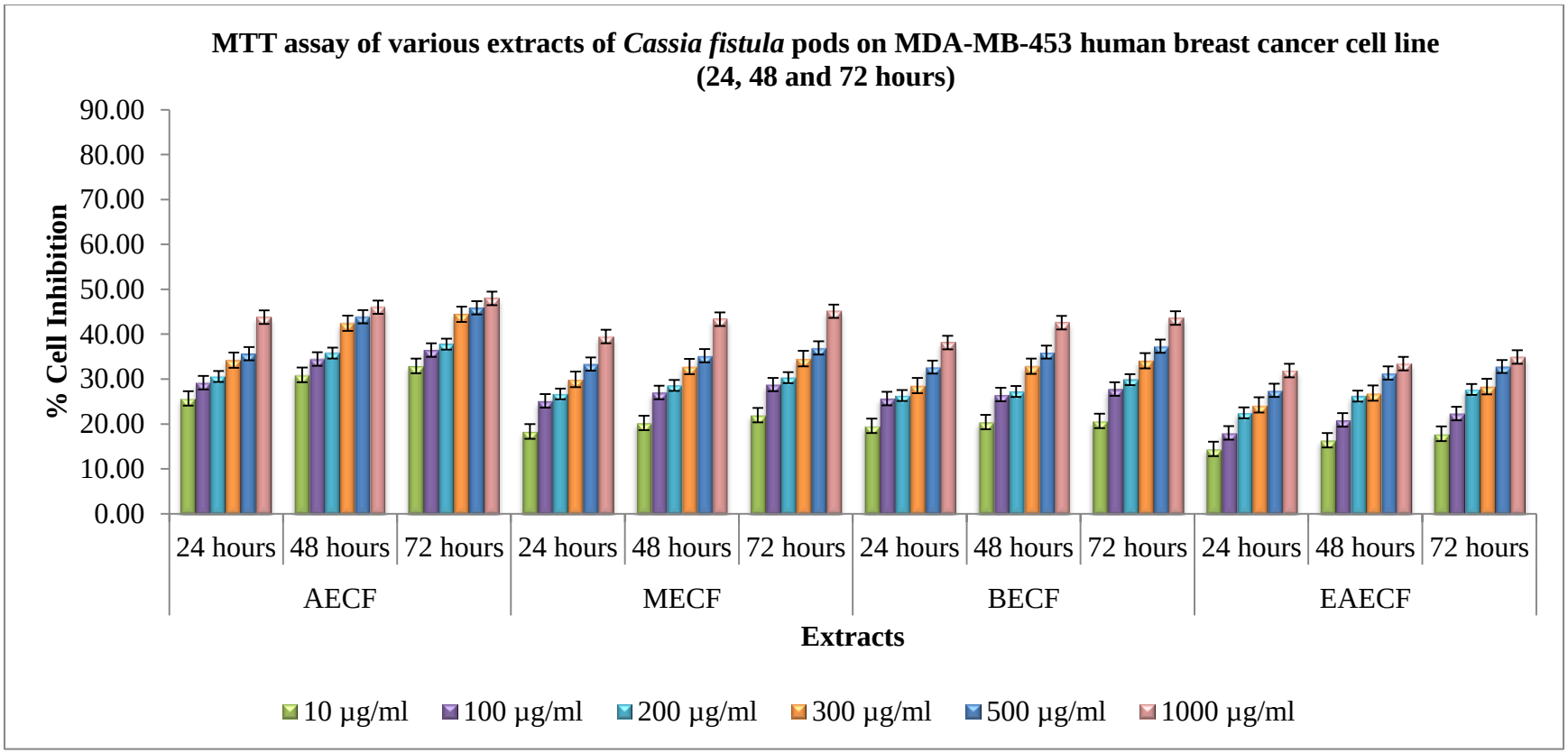


Figure 5.11: Effect of various extracts of *Cassia fistula* pods on cell proliferation of MDA-MB-453 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

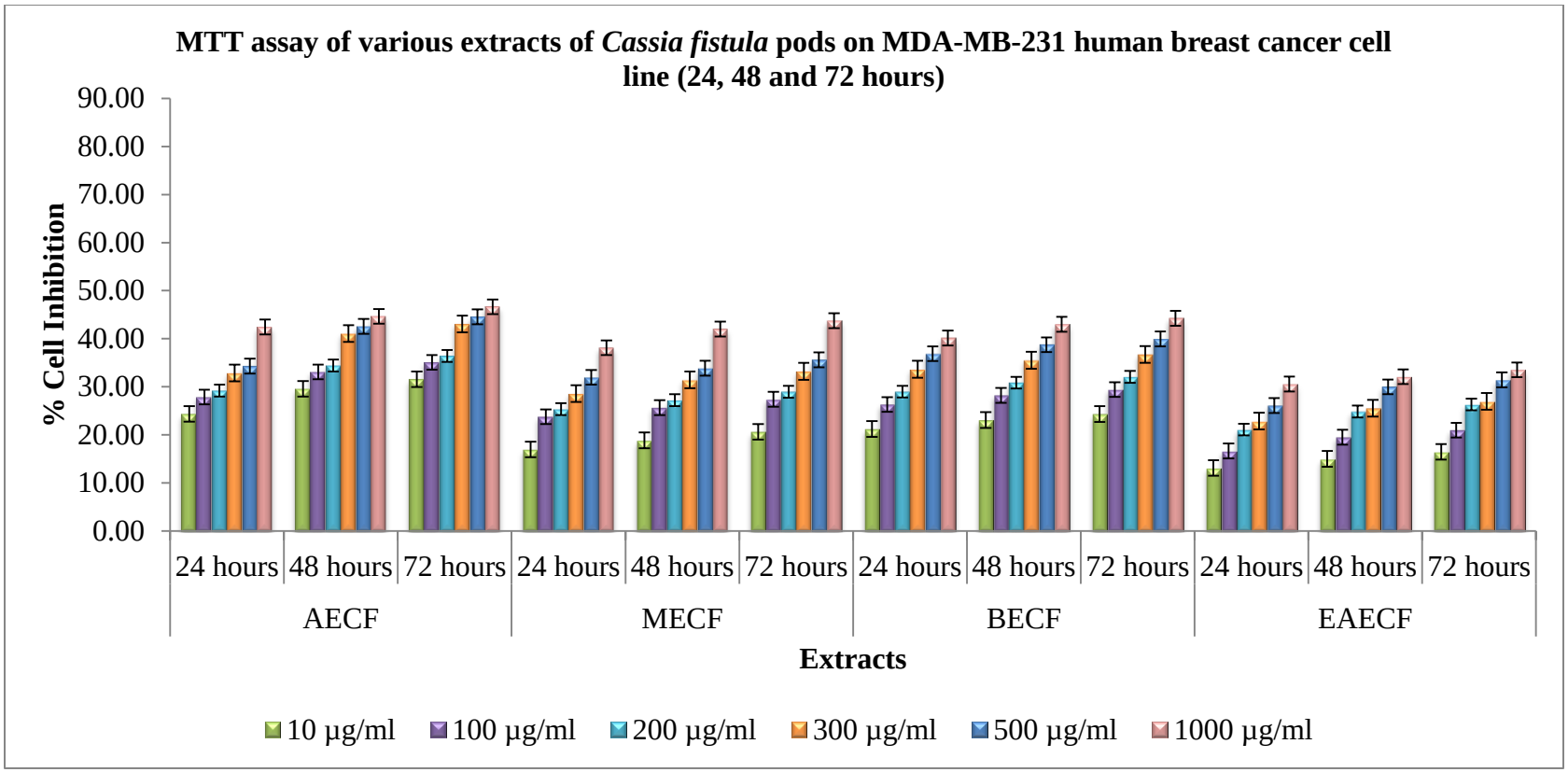


Figure 5.12: Effect of various extracts of *Cassia fistula* pods on cell proliferation of MDA-MB-231 human breast cancer cell line

Values are expressed as Mean ± SEM of 3 independent experiments in triplicate.

5.3. Comparison of IC_{50} values of various extracts of *Butea monosperma* flowers, *Lycopersicon esculentum* fruits and *Cassia fistula* pods

- On basis of IC_{50} values on MCF-7 cell line (72 hours), it is observed that extracts of *Butea monosperma* flowers 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). (Table 5.1)
- At 72 hours, the IC_{50} values of EAELE (215 µg/ml) on MCF-7 cell line was lowest amongst all extracts of *Lycopersicon esculentum* fruits. It was followed by MELE (IC_{50}:560 µg/ml). The IC_{50} value at 72 h 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. (Table 5.1)
- 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 of *Cassia fistula* pods. 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. (Table 5.1)
- In the present study, 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*. (Table 5.1)

Cell viability of non-tumorigenic MCF-10A 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 suggesting no cytotoxicity on normal breast cells.

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.

Table 5.1: IC_{50} values of various extracts of *Butea monosperma* flowers; *Lycopersicon esculentum* fruits and *Cassia fistula* pods on human breast cancer cell lines (MCF-7; MDA-MB-453 and MDA-MB-231)

Cell lines	Hours	Extracts of *Butea monosperma* flowers				Extracts of *Lycopersicon esculentum* fruits				Extracts of *Cassia fistula* pods			
		AEBM	MEBM	BEBM	EAEBM	AELE	MELE	BELE	EAELE	AECF	MECF	BECF	EAECF
MCF-7	24	400	186	690	-	-	-	-	751	344	525	-	-
	48	338	86	510	800	1000	893	-	550	300	435	-	830
	72	300	50	450	480	573	560	890	215	134	300	650	660
MDA-MB-453	24	-	-	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	874	-	-	-	-	-	-	-	-	-	-
MDA-MB-231	24	-	-	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	1000	-	-	-	-	-	-	-	-	-	-

All values are in µg/ml. "-" represents that IC_{50} value is above 1000 µg/ml.

SECTION 2

IN-VIVO PREVENTIVE STUDIES AND *IN-VITRO* MECHANISTIC APPROACH

5.4. *In-vivo* evaluation of extracts of *Butea monosperma* flower (MEBM and AEBM) in Methylnitrosourea (MNU) induced mammary carcinogenesis

5.4.1. Effect of MEBM and AEBM on growth rate and relative organ weight in MNU induced mammary carcinogenesis

Body weight, evaluated as % growth rate showed significant difference in model control animals from 42nd day as compared to normal group. From then, the growth rate of model control animals decreased significantly till the end of the experimental period. In vehicle control, the growth rate curve runs parallel to model control suggesting no significant difference. On treatment with MEBM (100, 200 and 400); AEBM (100, 200, 400) and Tamoxifen, the growth rate curve resembles to normal control group and was significantly different from model control group. (Figure 5.13)

Furthermore, increase in relative uteri and liver weights were observed in tumor bearing model control animals as compared to normal control animals ($P<0.001$). All treatment groups significantly improved relative organ weights as compared to model control animals ($P<0.001$) (Table 5.2).

5.4.2. Effect of MEBM and AEBM on feed consumption efficiency in MNU induced mammary carcinogenesis

Food intake, calculated as feed consumption efficiency was significantly reduced in model control animals from day 35th to 63rd as compared to normal control. There was no significant difference found between treatment groups as compared to normal control (Figure 5.14).

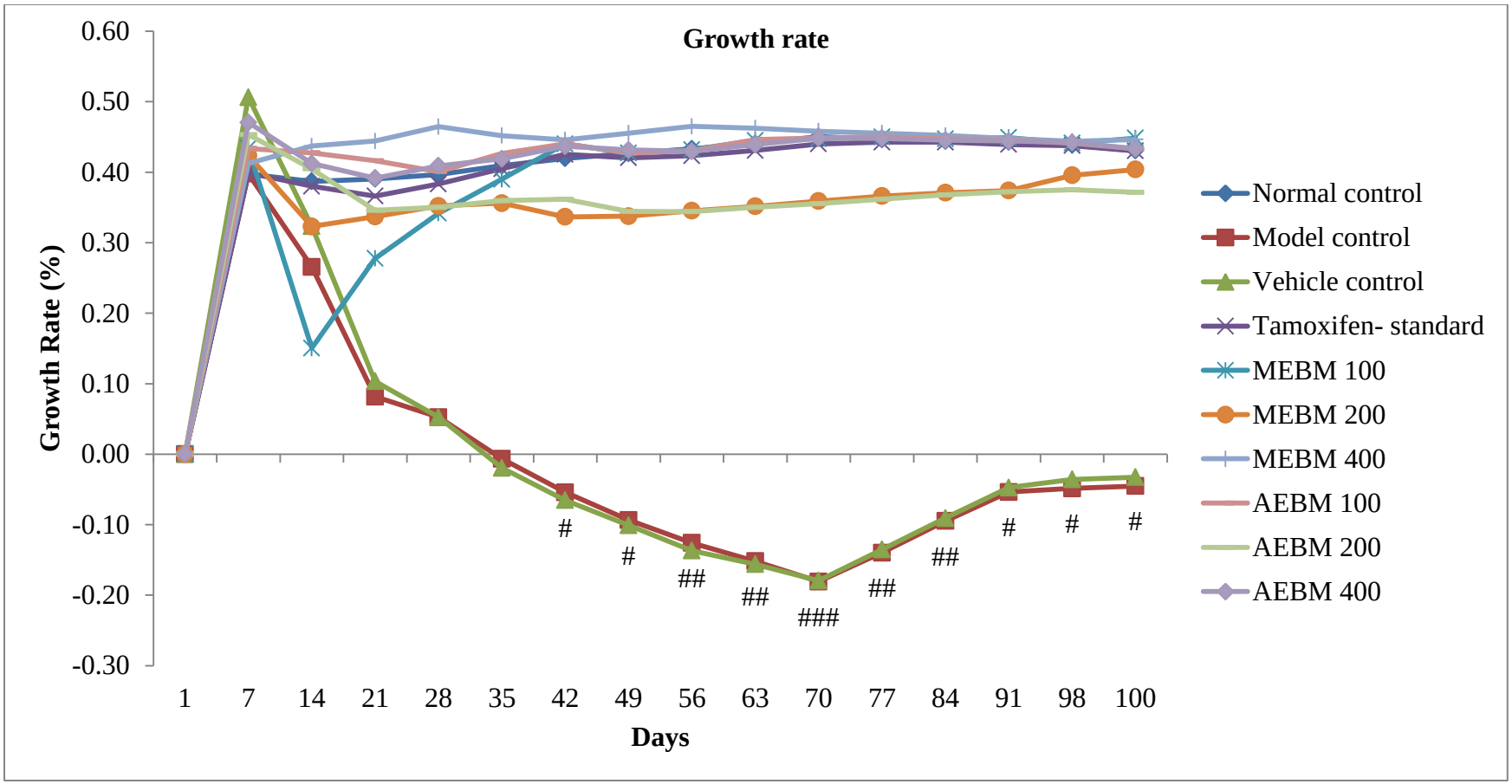


Figure 5.13: Effect of MEBM and AEBM on growth rate in MNU induced mammary carcinogenesis.
Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using two way ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with normal control vs. model control (#P<0.05, ##P<0.01 ###P<0.001)

Table 5.2: Effect of MEBM and AEBM on relative organ weight in MNU induced mammary carcinogenesis

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
Tamoxifen-standard	0.13±0.01***	4.07±0.11***
MEBM 100	0.17±0.01***	4.09±0.09***
MEBM 200	0.19±0.01***	4.24±0.32***
MEBM 400	0.16±0.01***	3.85±0.07***
AEBM 100	0.18±0.01***	4.15±0.09***
AEBM 200	0.20±0.01***	4.51±0.35***
AEBM 400	0.17±0.01***	4.12±0.06***

Values are expressed as 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 normal control vs. model control (###P<0.001), model control vs. all other groups (***P<0.001).

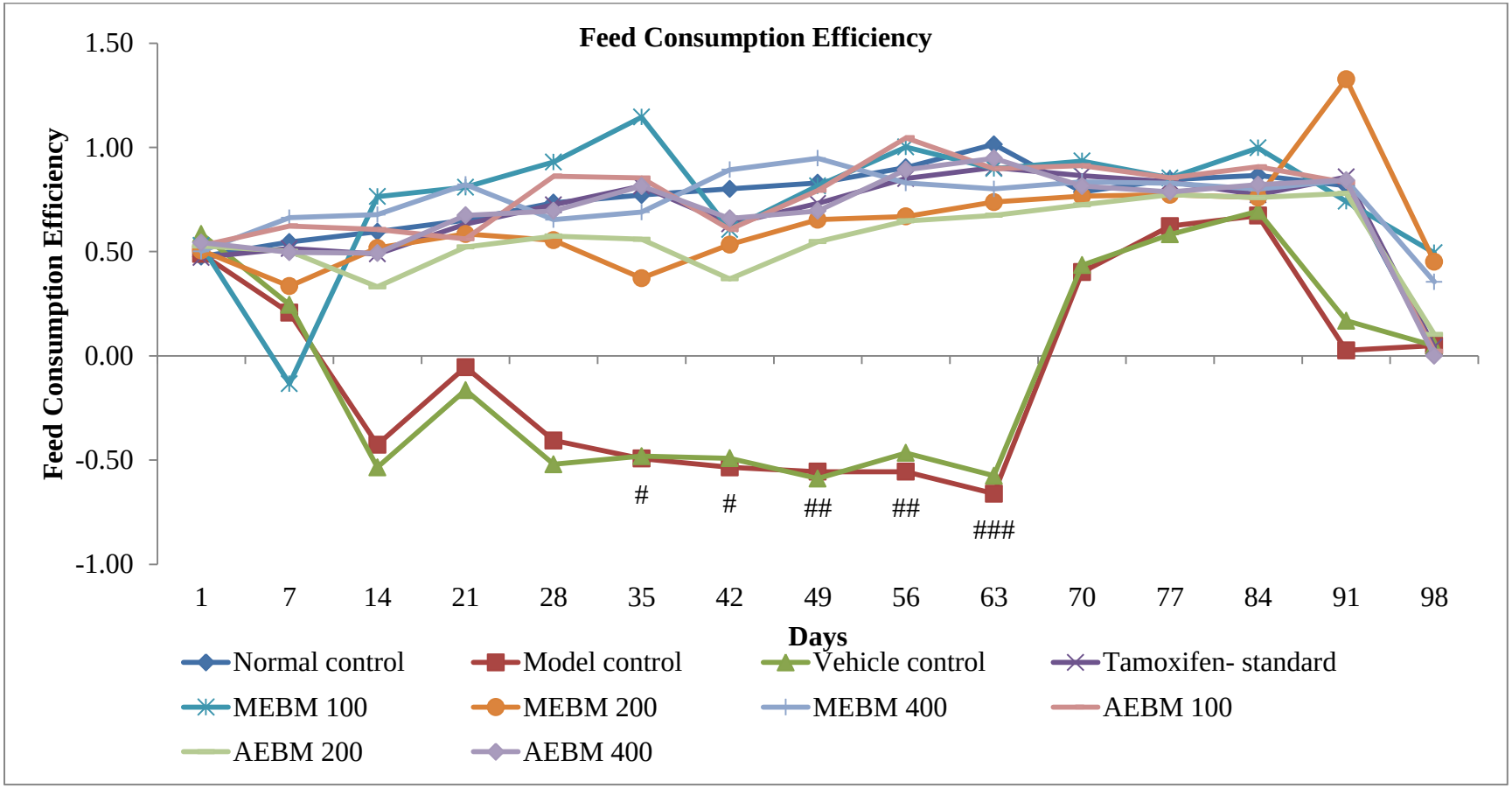


Figure 5.14: Effect of MEBM and AEBM on feed consumption efficiency in MNU induced mammary carcinogenesis.

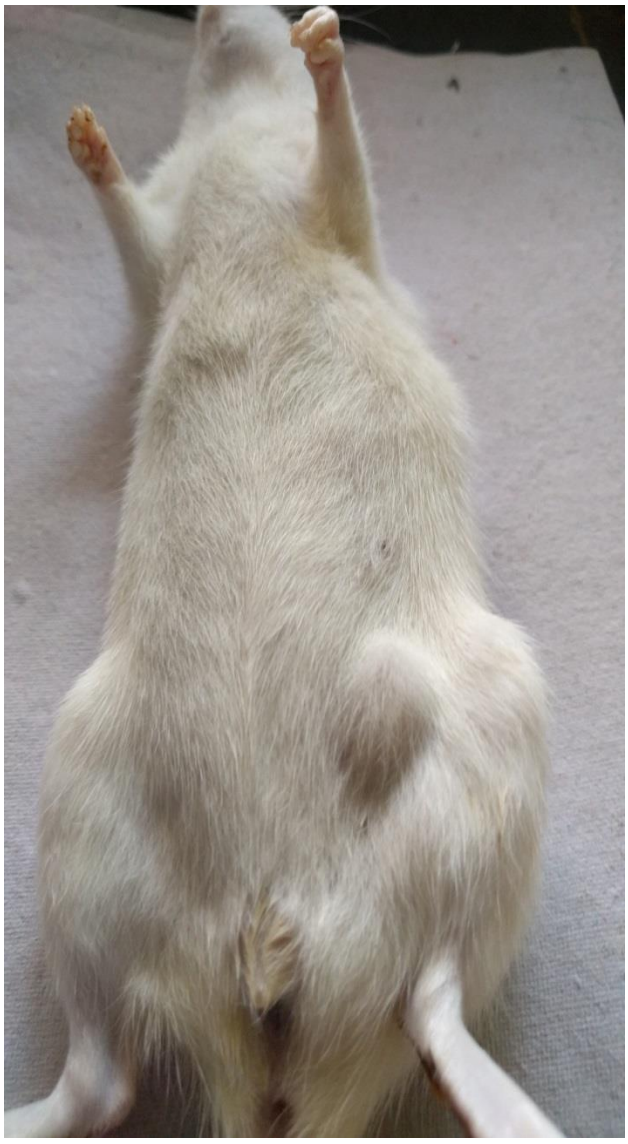
Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using two way ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with normal control vs. model control (#P<0.05, ##P<0.01, ### P<0.001).

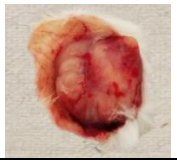
5.4.3. Effect of MEBM and AEBM on tumor parameters in MNU induced mammary carcinogenesis

Injecting MNU (50mg/kg b.w; i.p.) to nulliparous SD female rats resulted in mammary tumors. Tumor was induced after 45.17±9.21 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. (Figure 5.15)

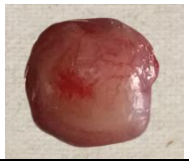
Oral administration of MEBM (100, 200 and 400) and AEBM (100, 200 and 400) to MNU injected rats significantly decreased the mammary cancer. The tumor incidence was reduced to 33.33% by administration of MEBM 400. The weights and volume were significantly curtailed in all treatment groups. The effect of MEBM was found to be dose dependent. (r^2= 0.98)

Tumor latency period was prolonged in Tamoxifen (82.17±16.44; P<0.001), MEBM (100, 200 and 400: 74.33±1.65; 78.66±1.65 and 82.5±2.89 respectively) and AEBM (100, 200 and 400: 72.17±1.42; 75.80±12.75 and 79.40±13.30 respectively) treated groups as compared to model group. It is worth noting that tumor incidence and number of tumors was lower in MEBM treated group than Tamoxifen treated group. No significant changes were observed in vehicle control animals as compared to model control animals. (Figure 5.15) (Table 5.3)



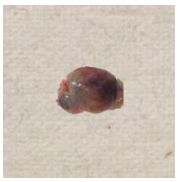


Model Control

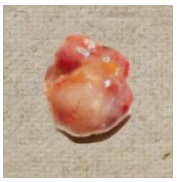


Vehicle Control

Tamoxifen-standard

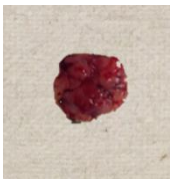


MEBM 100

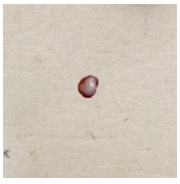


AEBM 100

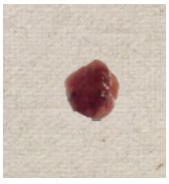
MEBM 200



AEBM 200



MEBM 400



AEBM 400

TUMOR IN RAT

Figure 5.15: Representative images of tumors in model and treated groups in MNU induced mammary carcinogenesis

Table 5.3: Effect of MEBM and AEBM on tumor parameters in MNU induced mammary carcinogenesis

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
Tamoxifen-standard	5	5	0.83	0.89±0.29***	1.95±6.33***	82.17±16.43***
MEBM 100	3	3	1	2.28±0.63***	8.08±4.26***	74.33±1.65***
MEBM 200	3	3	0.75	1.32±0.49***	3.89±2.00***	78.76±1.65***
MEBM 400	2	2	0.67	0.78±0.38***	3.67±1.23***	82.50±1.44***
AEBM 100	6	6	1	3.43±0.63*	24.77±5.87**	72.17±1.42***
AEBM 200	5	5	0.83	2.16±0.76***	10.63±4.14**	75.80±12.75***
AEBM 400	5	5	0.83	1.54±0.59***	8.08±3.22***	79.40±13.3***

Values are expressed as 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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001)

5.4.4. Effect of MEBM and AEBM on hematological parameters in MNU induced mammary carcinogenesis

Untreated MNU group showed significant decrease ($P<0.001$) in number of total RBC count (5.57±0.17 X 10^6/µL), Hb (9.63±0.24 g/L), whereas significant increase in total WBC count (13.63±0.13 X 10^3/µL) was observed as compared to normal group indicating the diseased state. In normal control animals, the total RBC count: 7.11±0.02 X 10^6/µL, Hb: 13.27±0.08 g/L; total WBC count: 6.37±0.06 X 10^3/µL were observed. Upon treatment with MEBM (100, 200 and 400) and AEBM (100, 200 and 400), all hematological parameters were significantly restored as compared to model control ($P<0.001$). Tamoxifen failed to restore total RBC count and Hb levels (total RBC count: 5.40±0.12 X 10^6/µL, Hb: 8.5±0.22 g/L). However, significant difference in total WBC count (7.30±0.11 X 10^3/µL) was observed in Tamoxifen treated group as compared to model group. (Table 5.4)

5.4.5. Effect of MEBM and AEBM on nucleic acid contents in MNU induced mammary carcinogenesis

In present study, within the tumor tissues of cancer bearing animals of model control group, significantly increased ($P<0.05$) levels of nucleic acids (DNA: 5.72±1.02; RNA: 4.69±1.07) were observed when compared to normal control animals (DNA: 3.66±1.03; RNA: 2.47±1.42). These rises were significantly attenuated in treatment groups ($P<0.05$). The % reduction in DNA content in Tamoxifen; MEBM (100, 200 and 400) and AEBM (100, 200 and 400) treated groups was found to be 33.5%, 30.05%, 30.54%, 31.53% 24.14%, 26.60% and 32.02%, respectively as compared to model control. The % reduction in RNA content in Tamoxifen; MEBM (100, 200 and 400) and AEBM (100, 200 and 400) treated groups was found to be 40.21%, 29.29%, 31.20%, 39.28% 31.55%, 32.36%, 33% respectively as compared to model control. (Figure 5.16)

Table 5.4: Effect of MEBM and AEBM on hematological parameters in MNU induced mammary carcinogenesis.

Groups	Total WBC count (X 10^3/µL)	Total RBC count (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
Tamoxifen-standard	7.30±0.11***	5.40±0.12	8.5±0.22
MEBM 100	9.27±0.25***	6.39±0.18*	12.23±0.12***
MEBM 200	9.00±0.25***	6.55±0.11**	12.43±0.12***
MEBM 400	7.43±0.13***	6.87±0.06***	12.40±0.18***
AEBM 100	10.10±0.04***	6.34±0.07*	11.90±0.19***
AEBM 200	9.77±0.02***	6.48±0.13*	11.93±0.27***
AEBM 400	8.43±0.13***	6.63±0.21**	12.10±0.04***

Values are expressed as 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 model control vs normal control (### P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001)

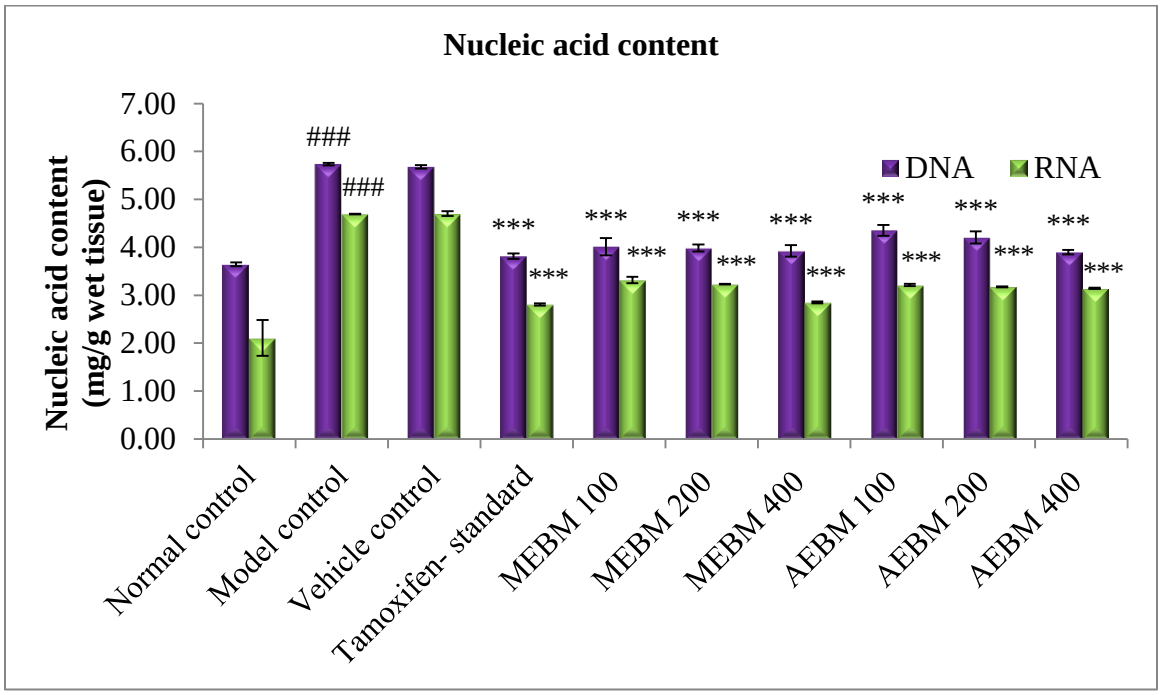


Figure 5.16: Effect of MEBM and AEBM on nucleic acid content in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

5.4.6. Effect of MEBM and AEBM on lipid peroxidation (MDA) and antioxidant enzyme levels in MNU induced mammary carcinogenesis

Model control animals showed significant (P<0.001) increase in MDA levels (3.76±0.06) as compared to normal control animals (1.61±0.08). Treatment with Tamoxifen and extracts, significantly (P<0.001) decreased MDA levels (Tamoxifen: 1.85±0.06; MEBM 100, 200 and 400: 2.56±0.01, 2.38±0.01, 1.97±0.01; AEBM 100, 200 and 400: 2.74±0.06, 2.56±0.02, 2.15±0.27 respectively) as compared to model control. (Figure 5.17)

Model control animals showed significant (P<0.001) decrease in GSH levels (2.86±0.12) as compared to normal control animals (6.55±0.16). Treatment with Tamoxifen and extracts (P<0.001) significantly prevented this decrease in

GSH levels (Tamoxifen: 5.13±0.22; MEBM 100, 200 and 400: 4.73±0.29, 5.03±0.29, 5.27 ±0.21; AEBM 100, 200 and 400: 4.46±0.04, 4.78±0.15, 5.09 ±0.24 respectively) as compared to model control. The effect of MEBM on GSH levels was dose dependent (r^2=0.99) (Figure 5.18)

Model control animals showed significant (P<0.001) decrease in SOD levels (5.39±0.06) as compared to normal control animals (12.35 ±0.03). Treatment with Tamoxifen and extracts (P<0.001) significantly prevented this decrease in SOD levels (Tamoxifen: 11.32±0.18; MEBM 100, 200 and 400: 6.24±2.7, 6.8±2.3, 7.2±2.2; AEBM 100, 200 and 400: 4.07±0.11, 4.28±0.24, 4.32±0.09 respectively) as compared to model control. (Figure 5.19).

Model control animals showed significant (P<0.001) decrease in Catalase activity (19.28±0.25) as compared to normal control animals (39.56±0.15). Tamoxifen significantly (P<0.01) prevented this decrease in Catalase activity (30.08±0.17) as compared to model control. Treatment with MEBM (200 and 400) significantly (P<0.01) prevented this decrease in Catalase activity (25.56±1.4 and 29.24±3.2 respectively) as compared to model control. No significant difference was found on treatment with AEBM as compared to model control. (Figure 5.20)

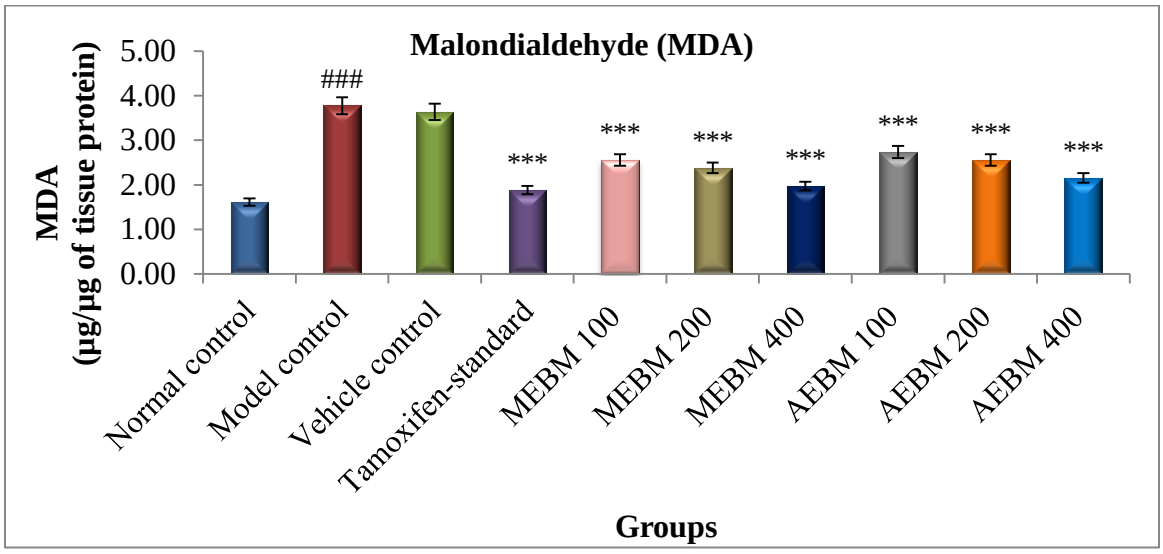


Figure 5.17: Effect of MEBM and AEBM on MDA levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

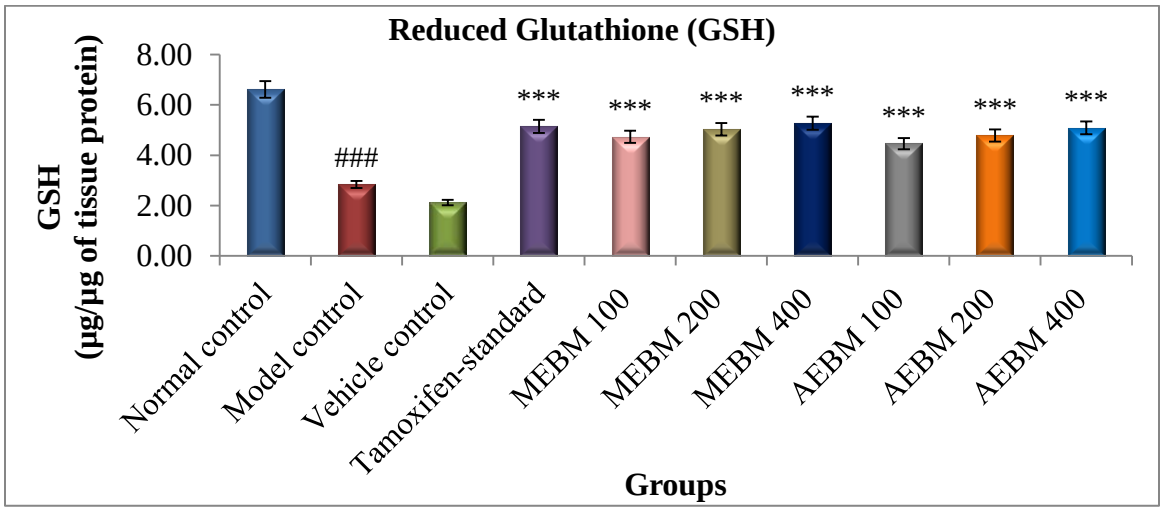


Figure 5.18: Effect of MEBM and AEBM on GSH levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

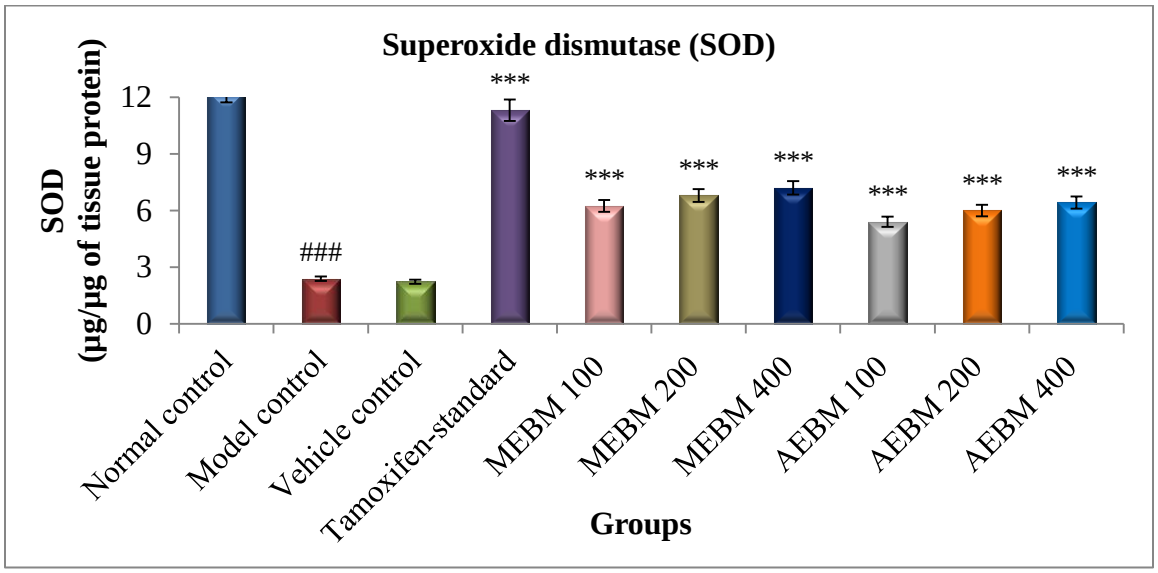


Figure 5.19: Effect of MEBM and AEBM on SOD levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

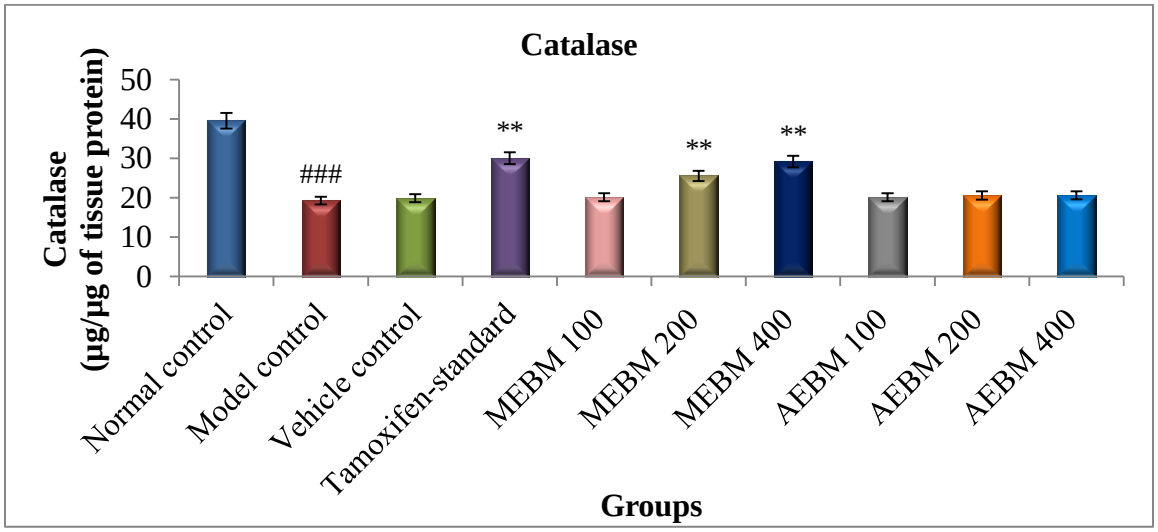


Figure 5.20: Effect of MEBM and AEBM on Catalase levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

5.4.7. Comparison between MEBM and AEBM in MNU induced mammary carcinogenesis

Contemplating on tumor parameters, the most potent extract was found to be MEBM as compared to AEBM in MNU induced mammary carcinogensis. 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. (Table 5.3) The tumor incidence and burden in MEBM treated group was abbreviated as compared to Tamoxifen treated group. Subsequently, it is inferred that MEBM is superior to AEBM. The dose dependent effect was observed in MEBM, hence estrogen and progesterone receptor expression were done in highest dose.

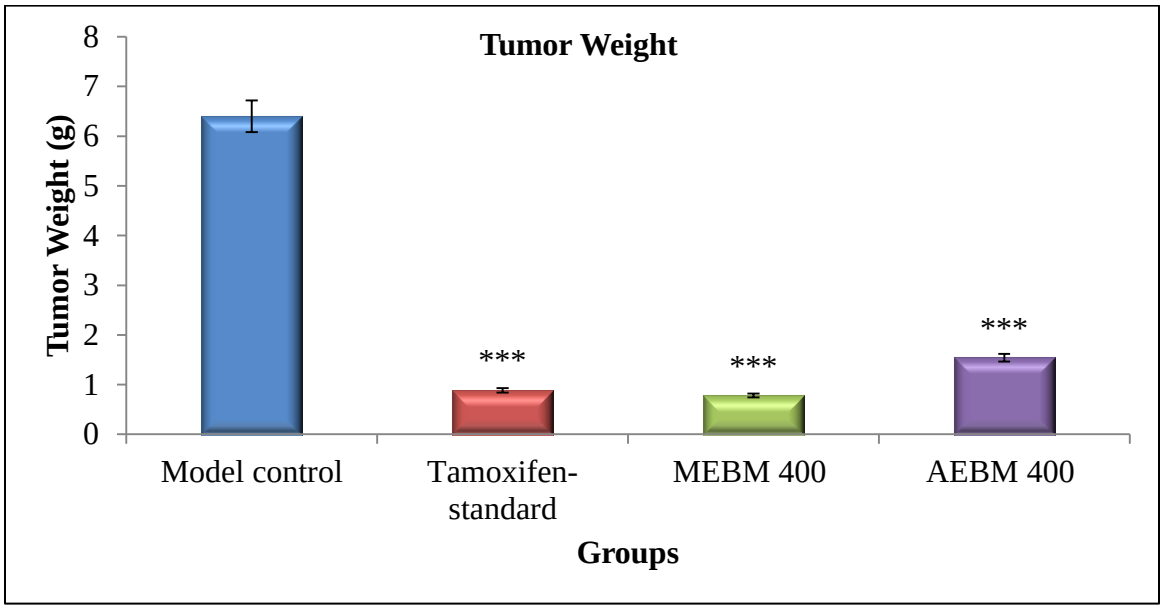


Figure 5.21: Effect of MEBM and AEBM on tumor parameters in MNU induced mammary carcinogenesis

Values are expressed as 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 model control vs. all other groups (**P<0.01, ***P<0.001)

5.4.8. Effect of MEBM and AEBM on estrogen and progesterone receptor expressions in MNU induced mammary carcinogenesis

To confirm *in-vitro* results and support *in-vivo* results, estrogen and progesterone receptor's immunohistochemical analysis was performed in breast tumor tissues of experimental groups in MNU induced mammary carcinogenesis. The immunohistochemistry analysis revealed that the breast tumor tissue of cancer bearing animals expressed significantly higher number of positively stained (brown colored) nuclei (ER: 60% ± 1.03 and PR: 73.34%± 1.29) as compared to normal control animals (ER- 30.56% ± 0.98; PR-43.66% ± 1.38) (Figure 5.22). The % positively stained ER cells for MEBM 400, AEBM 400 and Tamoxifen were found to be 38.23% ± 1.43; 42% ± 1.13 and 31.66% ± 0.89 respectively. The % positively stained PR cells for MEBM 400, AEBM 400 and Tamoxifen were found to be 50.14 % ± 1.32; 56.66% ± 2.69 and 46.66% ± 1.78 respectively (Figure 5.23). Figure 5.24 shows % decrease in estrogen and progesterone expressions of treated group as compared to model control animals.

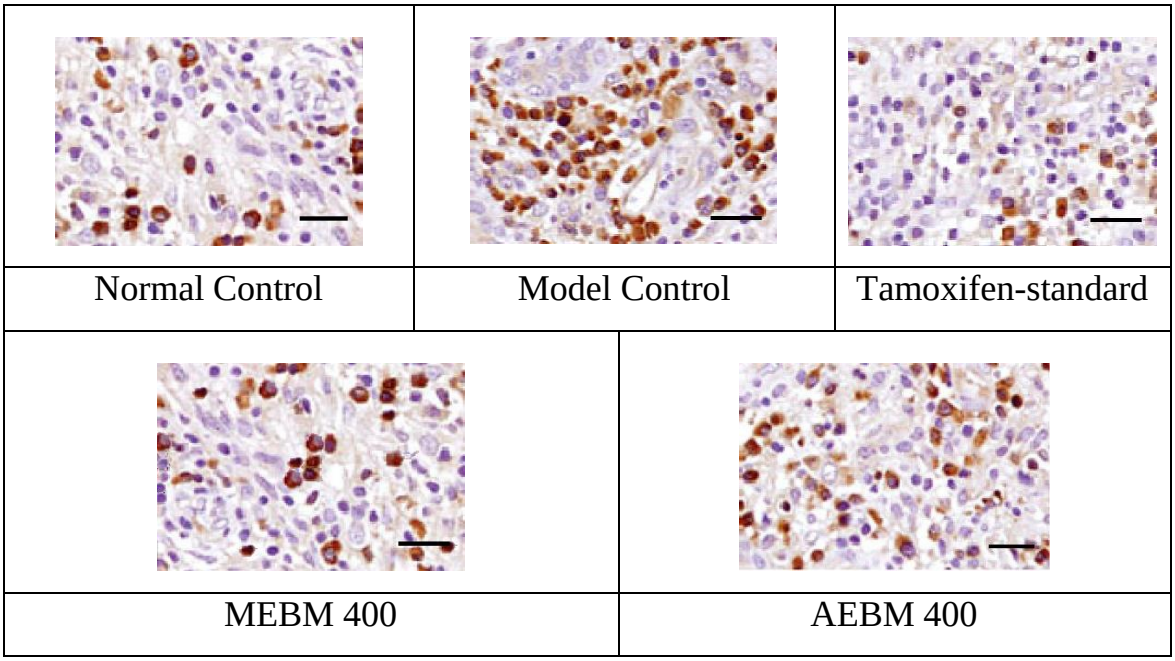


Figure 5.22: Effect of MEBM and AEBM on estrogen receptor expression in MNU induced mammary carcinogenesis.

The photographs are taken in Nikon Eclipse TS100. Magnification: 40X. Scale bar: 100µm.

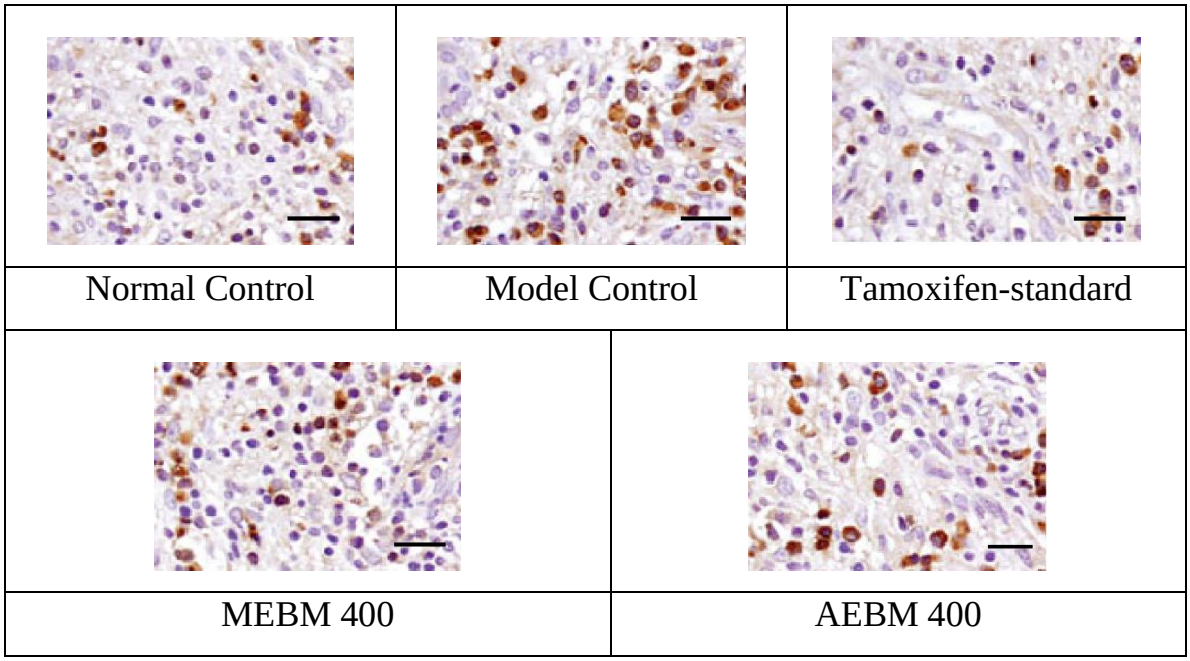


Figure 5.23: Effect of MEBM and AEBM on progesterone receptor expression in MNU induced mammary carcinogenesis.

The photographs are taken in Nikon Eclipse TS100. Magnification: 40X. Scale bar: 100µm.

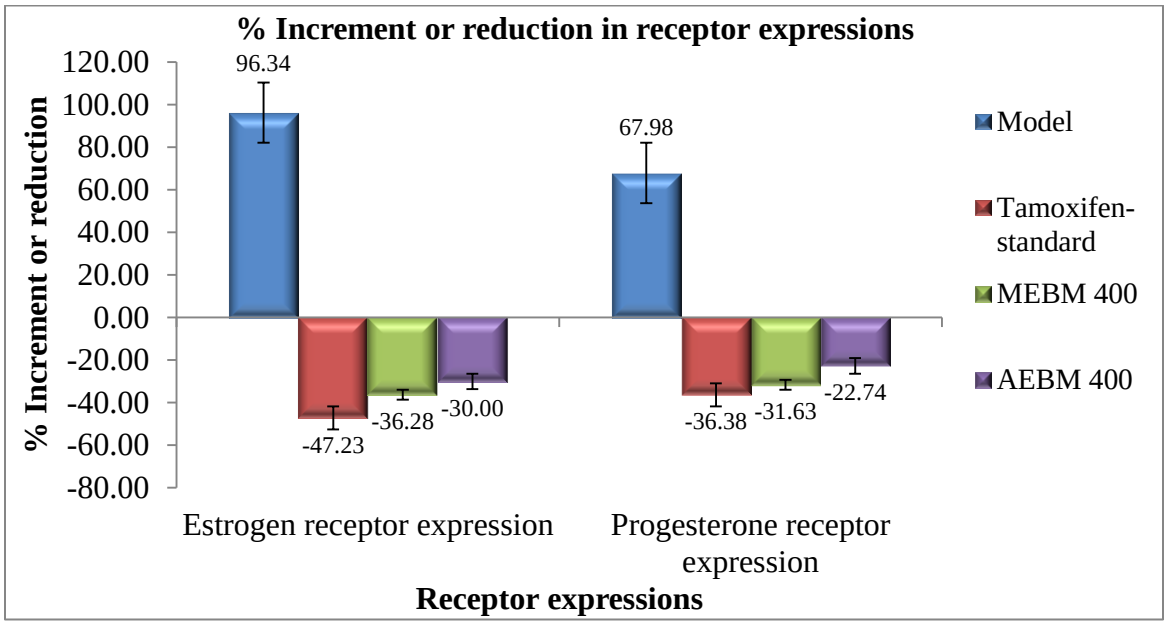


Figure 5.24: Effect of MEBM and AEBM on % increment or reduction in estrogen and progesterone receptor expressions in MNU induced mammary carcinogenesis

The % increment in model control animals is with respect to normal control animals. The % reduction in treated groups is with respect to model control animals.

5.5. *In-vivo* evaluation of extracts of *Lycopersicon esculentum* fruits (EAELE and MELE) in Methylnitrosourea (MNU) induced mammary carcinogenesis

5.5.1. Effect of EAELE and MELE on growth rate and relative organ weight in MNU induced mammary carcinogenesis

When body weight was assessed as % growth rate, significant difference in model control animals was found from 42nd day as compared to normal control. From then, the growth rate of model control animals decreased significantly till the end of the experimental period. In vehicle control, the growth rate curve runs parallel to model control recommending no significant difference. On treatment with EAELE (100, 200 and 400); MELE (100, 200 and 400) and Tamoxifen, the growth rate curve was non-significantly different from normal control but was significantly different from model control. (Figure 5.25)

Moreover, relative uteri and liver weights were increased in model control animals as compared to normal control animals ($P<0.001$). In contrast, treated groups significantly improved relative organ weights as compared to model control animals ($P<0.001$) (Table 5.5).

5.5.2. Effect of EAELE and MELE on feed consumption efficiency in MNU induced mammary carcinogenesis

Food intake was calculated as feed consumption efficiency. When compared with normal control animals, the feed consumption efficiency was significantly reduced in model control animals from day 35th to 63rd. In treated groups, the feed consumption efficiency was non-significantly different from normal control. (Figure 5.26).

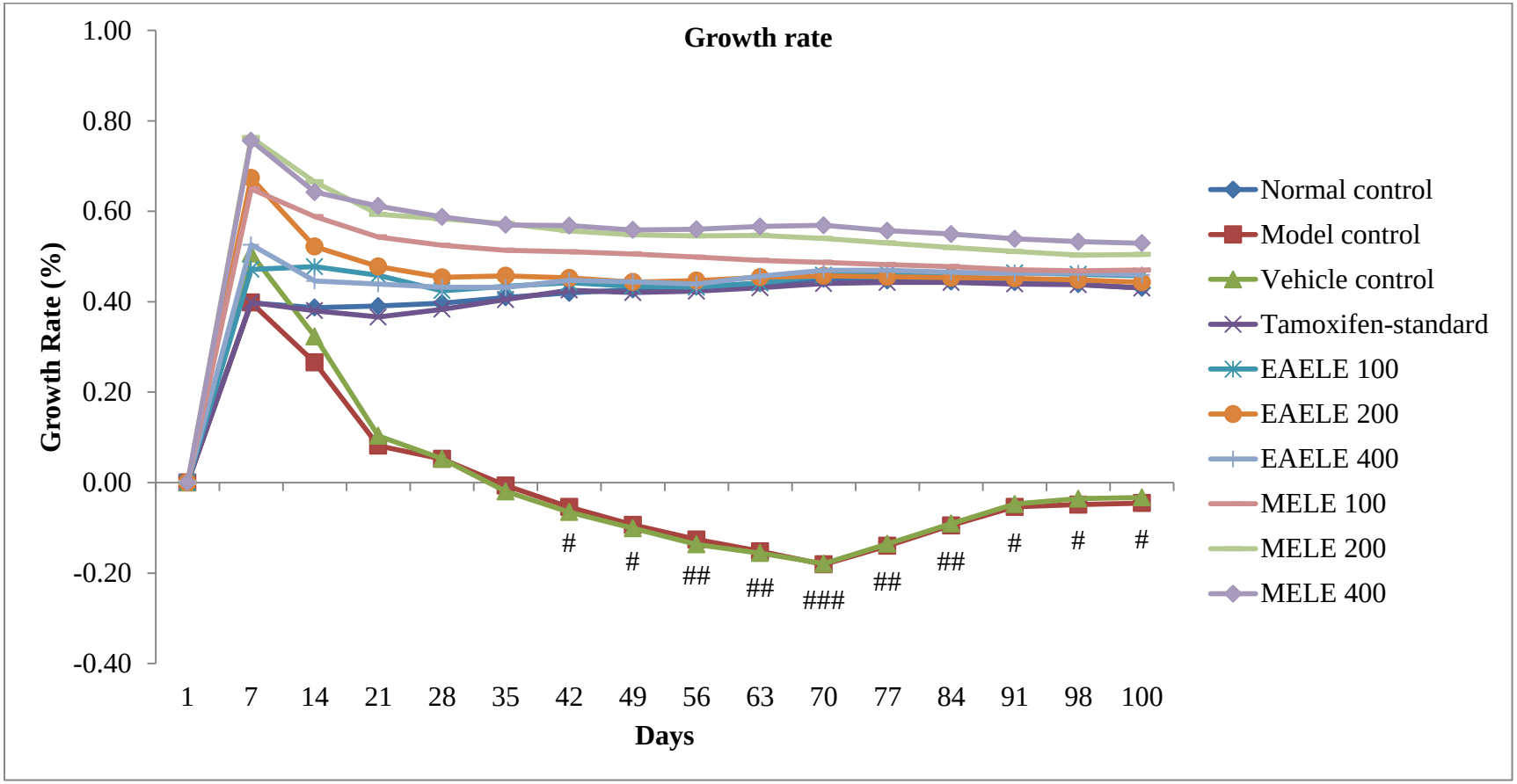


Figure 5.25: Effect of EAELE and MELE on growth rate in MNU induced mammary carcinogenesis.

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

Table 5.5: Effect of EAELE and MELE on relative organ weight in MNU induced mammary carcinogenesis

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
Tamoxifen-standard	0.13±0.01***	4.07±0.11***
EAELE 100	0.17±0.01***	4.08±0.07***
EAELE 200	0.17±0.01***	4.02±0.06***
EAELE 400	0.18±0.01***	3.91±0.03***
MELE 100	0.17±0.01***	4.06±0.09***
MELE 200	0.15±0.01***	3.87±0.08***
MELE 400	0.15±0.01***	3.79±0.09***

Values are expressed as 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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

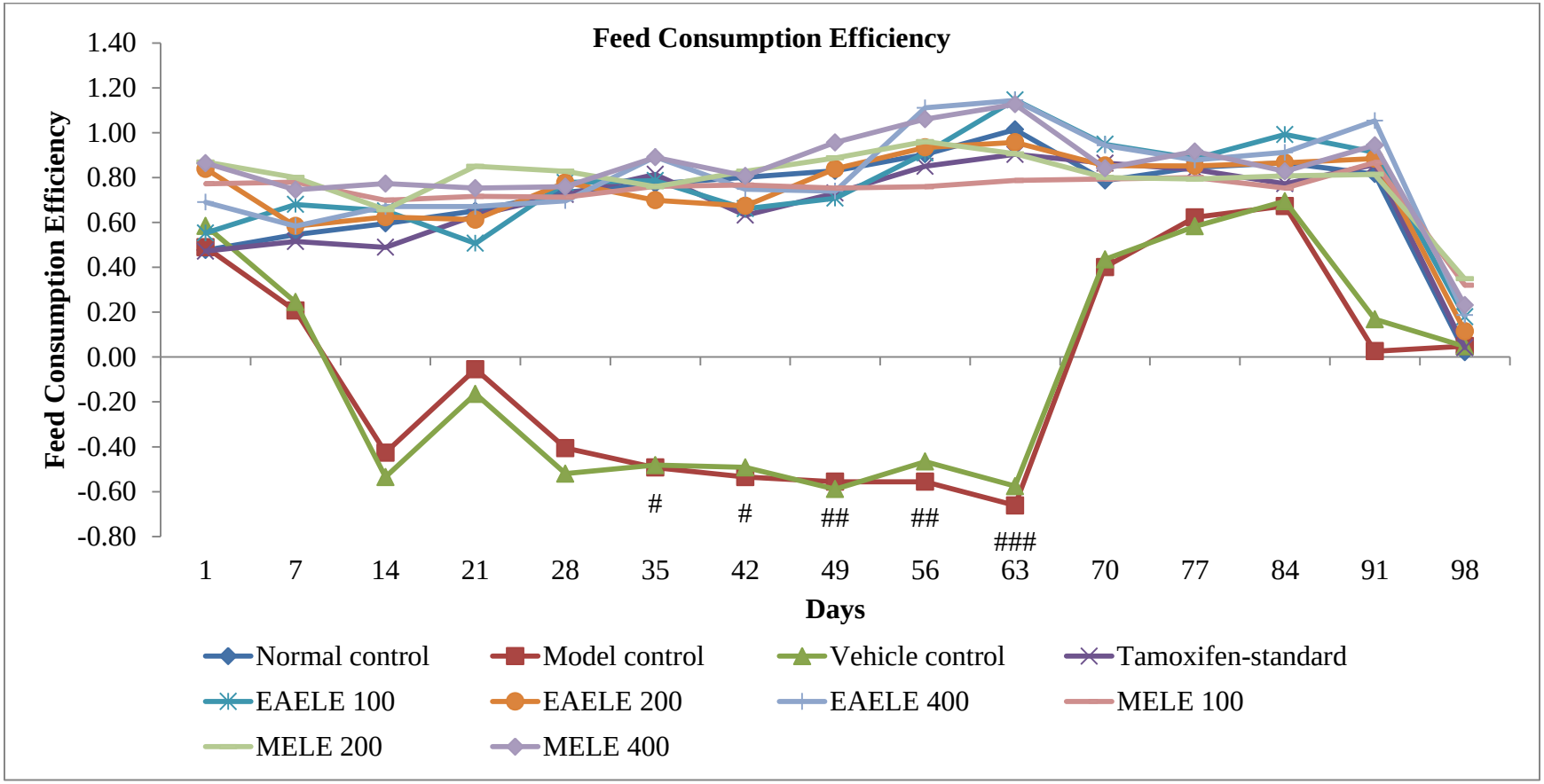


Figure 5.26: Effect of EAELE and MELE on feed consumption efficiency in MNU induced mammary carcinogenesis.

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

5.5.3. Effect of EAELE and MELE on tumor parameters in MNU induced mammary carcinogenesis

The mammary tumors were induced by single injection of MNU (50mg/kg b.w; i.p.) to female rats. The lag period of tumor induction was 45.17±9.21 days. Five out of six rats developed mammary carcinoma suggesting 83.33% tumor incidence. Total tumor burden was found to be 6 in MNU treated animals. 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 model control animals were found to be 6.4±1.47 g and 99.29 ±3.19 mm^3. The successful model of mammary carcinoma was induced by MNU. (Figure 5.27) On treatment with EAELE (100, 200 and 400) and MELE (100, 200 and 400), significant decreased in the tumor growth was observed. The weights and volume were significantly curtailed in all treatment groups. Tumor multiplicity was decreased by two doses of EAELE (200 and 400). The lag period for tumor development was found to be delayed in Tamoxifen (82.17±16.44; $P<0.001$), EAELE (100, 200 and 400: 71.33±1.63; 73.00±12.24 and 77.40±1.18 respectively) and MELE (100, 200 and 400: 70.5±1.73; 74.80±1.64 and 76.80±2.03 respectively) treatment groups as compared to model group. (Table 5.6) No significant changes were observed in vehicle control animals as compared to model control group. (Figure 5.27)

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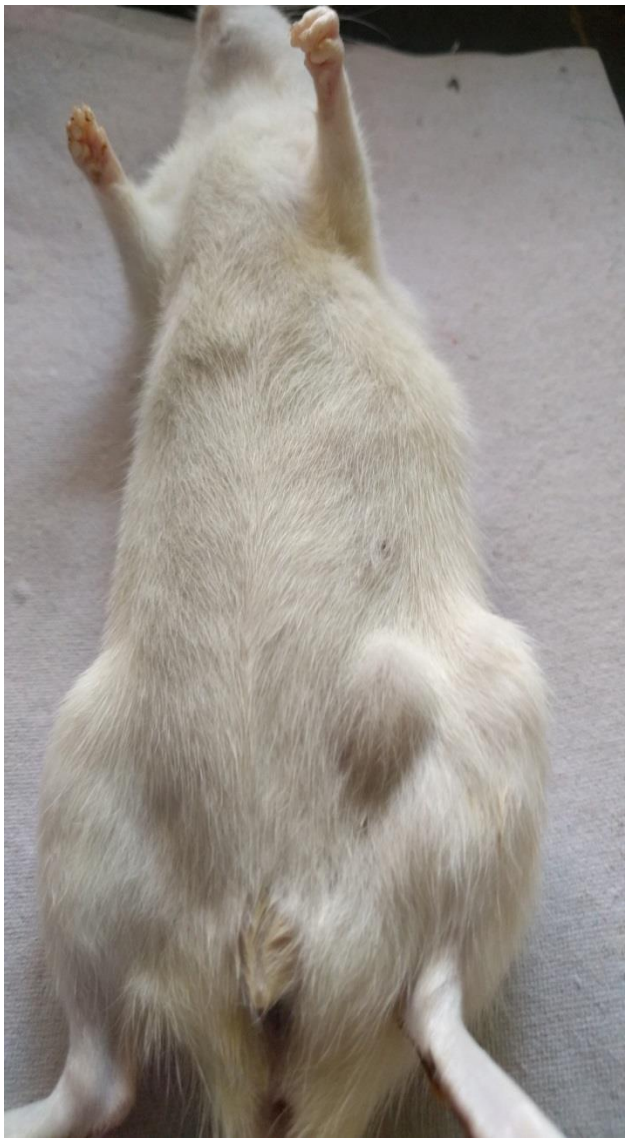
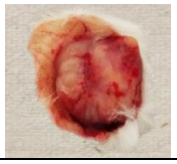
<tr><td rowspan="8">  </td><td colspan="2">  </td></tr>
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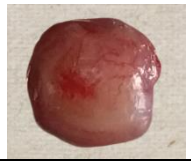
<tr><td colspan="2">  </td></tr>
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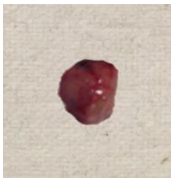
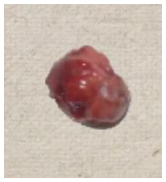
<tr><td>  </td><td>  </td></tr>
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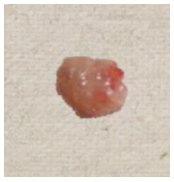
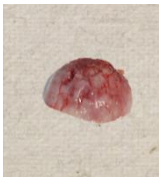
<tr><td>  </td><td>  </td></tr>
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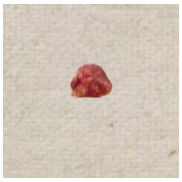
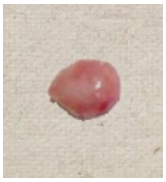
<tr><td>  </td><td>  </td></tr>
<tr><td>TUMOR IN RAT</td><td>EAELE 400</td><td>MELE 400</td></tr>
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Figure 5.27: Representative images of tumors in model and treated groups in MNU induced mammary carcinogenesis

Table 5.6: Effect of EAELE and MELE on tumor parameters in MNU induced mammary carcinogenesis

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
Tamoxifen-standard	5	5	0.83	0.89±0.29***	1.95±6.33***	82.17±16.43***
EAELE 100	6	6	1	3.43±0.63*	13.32±3.96***	71.33±1.63***
EAELE 200	5	5	0.83	2.16±0.76***	9.95±4.66***	73.00±12.24***
EAELE 400	5	5	0.83	1.54±0.59***	4.04±1.45***	77.40±1.18***
MELE 100	6	6	1	3.00±0.65*	21.48±6.97***	70.5±1.73***
MELE 200	6	6	1	2.63±0.61**	15.86±4.38***	74.80±1.64***
MELE 400	6	6	1	2.19±0.51***	12.20±4.58***	76.80±2.03***

Values are expressed as 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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001).

5.5.4. Effect of EAELE and MELE on hematological parameters in MNU induced mammary carcinogenesis

The model control animals showed significant decrease ($P<0.001$) in number of total RBC count (5.57±0.17 X 10^6/µL), Hb (9.63±0.24 g/L) as compared to normal group. The increase in total WBC count (13.63±0.13 X 10^3/µL) was observed as compared to normal group. In normal control animals, total RBC count: 7.11±0.02 X 10^6/µL, Hb: 13.27±0.08 g/L; total WBC count: 6.37±0.06 X 10^3/µL were observed. Upon treatment with EAELE (100,200 and 400), all hematological parameters were significantly restored as compared to model control ($P<0.001$). MELE (100,200 and 400) failed to treat anemic condition. Tamoxifen failed to restore total RBC count and Hb levels (total RBC count: 5.40±0.12 X 10^6/µL, Hb: 8.5±0.22 g/L) as compared to model group. However, significant difference in total WBC count (7.30±0.11 X 10^3/µL) was observed in Tamoxifen treated group as compared to model group. (Table 5.7)

5.5.5. Effect of EAELE and MELE on nucleic acid contents in MNU induced mammary carcinogenesis

The DNA (5.72±1.02) and RNA (4.69±1.07) levels were significantly increased ($P<0.05$) in model control animals as compared to normal control animals (DNA: 3.66±1.03; RNA: 2.47±1.42). The increment was significantly and dose dependently subsided by EAELE ($P<0.001$). Only MELE 400 ($P<0.05$) showed significant attenuation in nucleic acid levels as compared to model control animals. The % reduction in DNA content in Tamoxifen; EAELE (100, 200, 400) and MELE 400 was found to be 33.5%, 22.17%, 27.09%, 29.06% and 5.42% respectively as compared to model control. The % reduction in RNA content in Tamoxifen; EAELE (100, 200, 400) and MELE 400 was found to be 40.21%, 25.51%, 26.66%, 31.26% and 13.40% respectively as compared to model control. (Figure 5.28)

Table 5.7: Effect of EAELE and MELE on hematological parameter in MNU induced mammary carcinogenesis.

Groups	Total WBC count (X 10^3/µL)	Total RBC count (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
Tamoxifen-standard	7.30±0.11***	5.40±0.12	8.5±0.22
EAELE 100	9.21±0.15***	6.24±0.05*	11.6±0.11***
EAELE 200	8.1±0.05***	6.38±0.05**	11.87±0.10***
EAELE 400	7.77±0.06***	6.77±0.06***	12.23±0.17***
MELE 100	11.±0.33**	5.40±0.08	10.2±0.04
MELE 200	10.8±0.04***	5.63±0.10	10.23±0.15
AEBM 400	10.2±0.04***	5.73±0.02	10.17±0.12

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 model control vs normal control (### P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001).

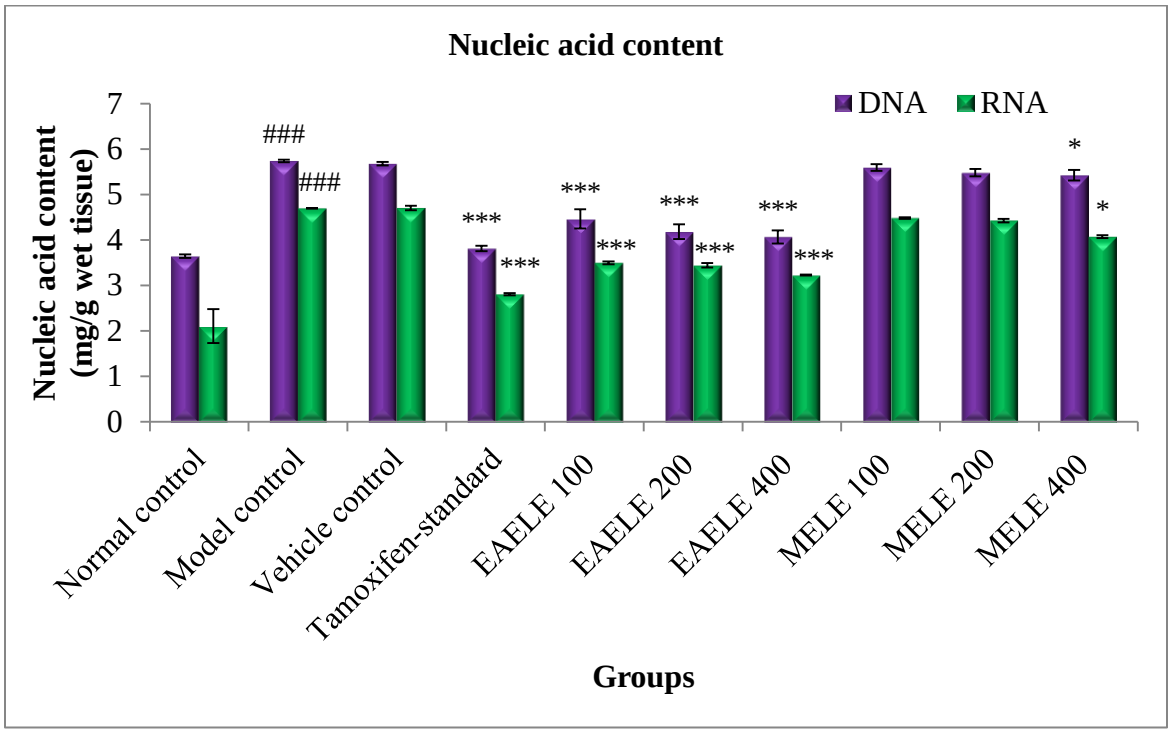


Figure 5.28: Effect of EAELE and MELE on nucleic acid content in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05 ***P<0.001).

5.5.6. Effect of EAELE and MELE on lipid peroxidation (MDA) and antioxidant enzyme levels in MNU induced mammary carcinogenesis

The lipid peroxidation in model control (3.76±0.06) animals was significantly higher (P<0.001) than normal control animals (1.61±0.08). Treatment with Tamoxifen, EAELE and MELE significantly decreased lipid peroxidation as compared to model control. (Tamoxifen: 1.85±0.06; EAELE 100, 200 and 400: 2.83±0.13, 2.51±0.21, 2.24±0.35; MELE 100, 200 and 400: 3.01±0.26, 2.74±0.12, 2.56±0.27 respectively). The dose dependent decrease was seen in EAELE (r^2=0.99) (Figure 5.29)

The GSH level were significantly decreased in model control animals (2.86±0.12) as compared to normal control animals (6.55±0.16) ($P<0.001$). The EAELE (100,200 and 400) and MELE (200 and 400) significantly prevented this decrease in GSH levels in dose dependent manner ($r^2=0.99$) as compared to model control animals. (Tamoxifen: 5.13±0.22; EAELE 100, 200 and 400: 3.52±0.35, 4.58±0.25, 5.56 ±0.44; MELE 200 and 400: 3.51±0.35, 4.36 ±0.32 respectively). (Figure 5.30)

The SOD levels were significantly lower ($P<0.001$) in model control animals (5.39±0.06) as compared to normal control animals (12.35 ±0.03). The treated groups showed significantly higher enzyme levels as compared to model control animals. (Tamoxifen: 11.32±0.18; EAELE 100, 200 and 400: 7.24±2.0, 7.8±1.8, 8.1±2.1; MELE 100, 200 and 400: 5.12±2.6, 5.18±2.3, 5.45±2.3 respectively) (Figure 5.31)

Model control animals showed significant ($P<0.001$) decrease in Catalase activity (19.28±0.25) as compared to normal control animals (39.56±0.15). Tamoxifen significantly ($P<0.001$) prevented this decrease in Catalase activity (30.08±0.17). Treatment with EAELE (100, 200 and 400: 22.59±2.4; 27.12±1.7and 30.65±1.9 respectively) significantly and dose dependently ($r^2=0.99$) prevented this decrease in Catalase activity as compared to model control animals. MELE (200 and 400: 22.56±2.4 and 23.24±2.2 respectively) significantly prevented decline in Catalase activity as compared to model control animals. (Figure 5.32)

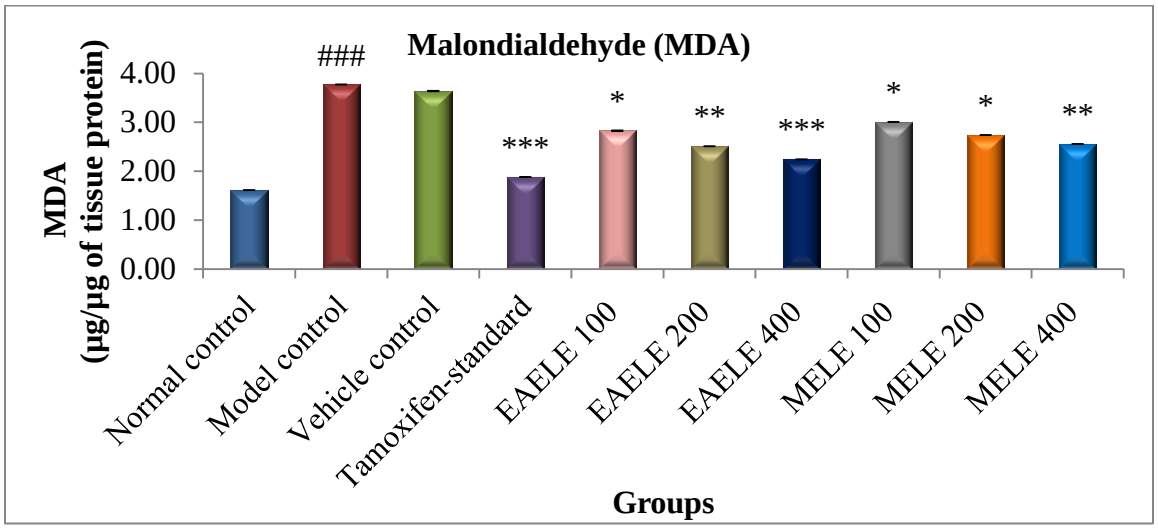


Figure 5.29: Effect of EAELE and MELE on MDA levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001).

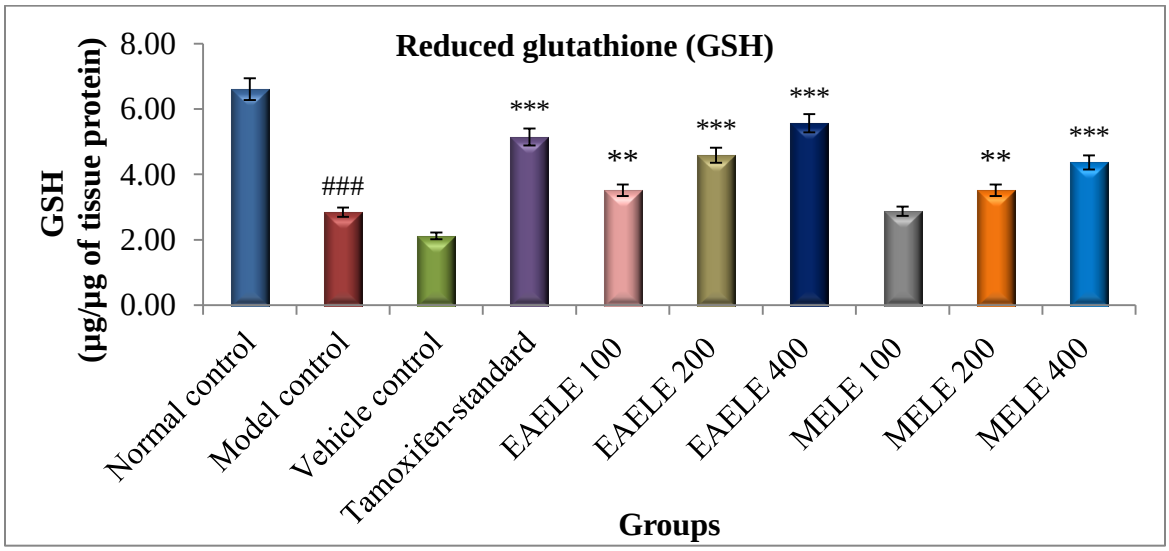


Figure 5.30: Effect of EAELE and MELE on GSH levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs all other groups (*P<0.05, **P<0.01, ***P<0.001).

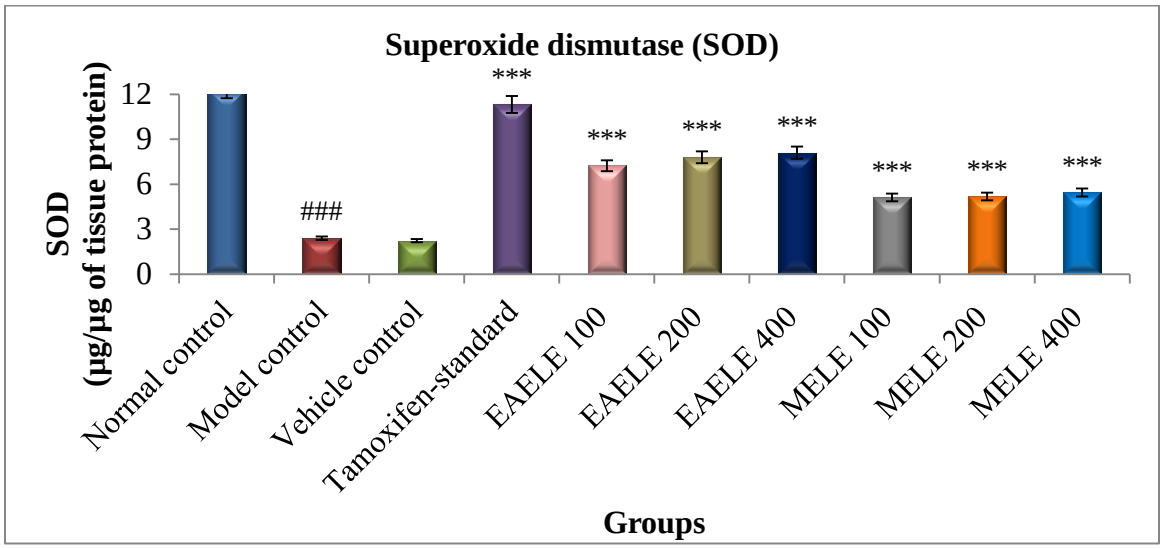


Figure 5.31: Effect of EAELE and MELE on SOD levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

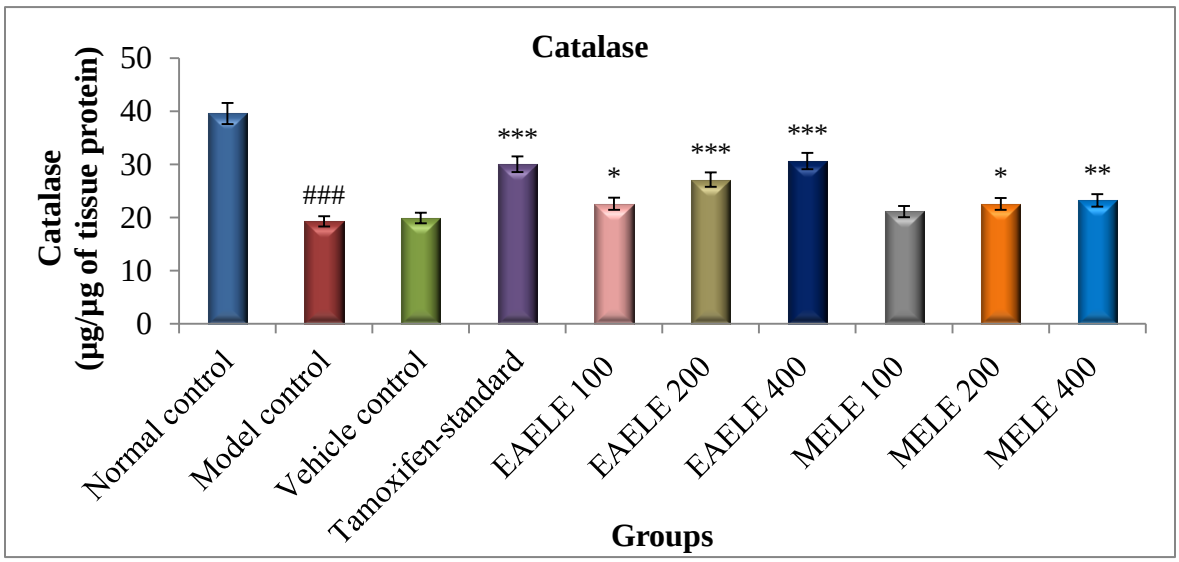


Figure 5.32: Effect of EAELE and MELE on Catalase levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs all other groups (*P<0.05, **P<0.01, ***P<0.001).

5.5.7. Comparison between EAELE and MELE in MNU induced mammary carcinogenesis

Considering tumor parameters and nucleic acid content, the most potent extract was concluded to be EAELE. The tumor burden was 5 in EAELE 400 treated group while all animals developed tumors in MELE 400 treated 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. (Figure 5.33) All doses of EAELE significantly tapered nucleic acid rise while only MELE 400 significantly significantly truncated this rise with confidence level of 95%. (Figure 5.34) Hence, it is concluded that EAELE is superior to MELE in hindering mammary carcinogenesis. The non-significant difference was observed amongst treatment doses of extracts which infers that increasing the dose will not increase the effect. So, the estrogen and progesterone expressions were measured in highest dose of EAELE and MELE.

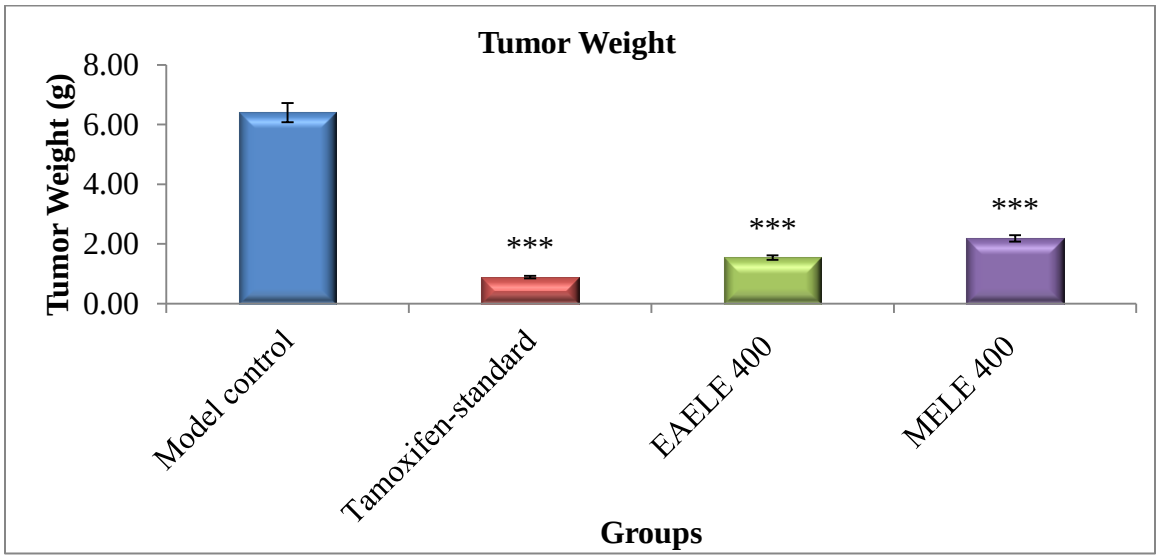


Figure 5.33: Effect of EAELE and MELE on tumor parameters in MNU induced mammary carcinogenesis

Values are expressed as 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 model control vs. all other groups (**P<0.01, ***P<0.001).

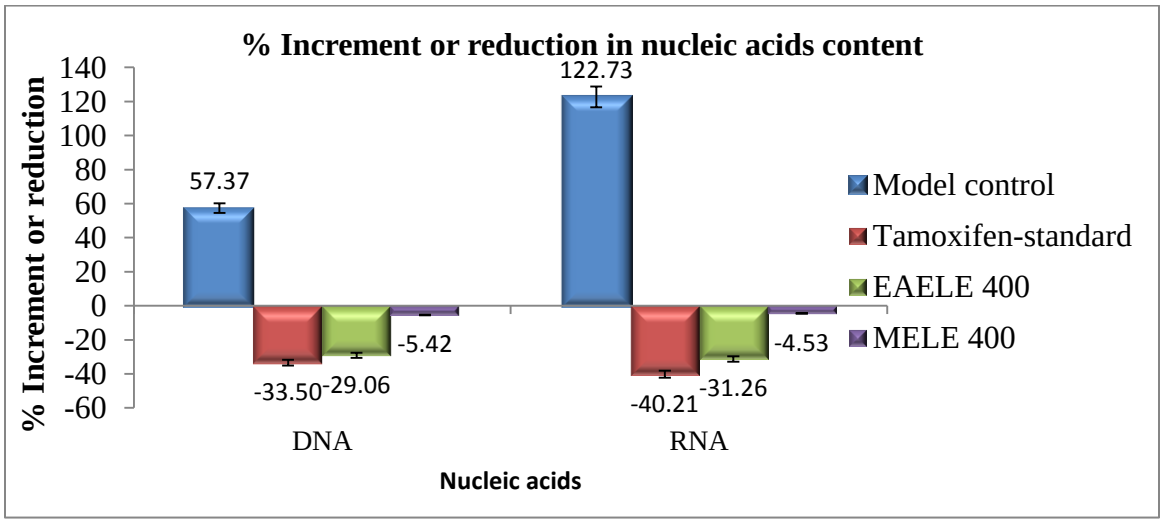


Figure 5.34: Effect of EAELE and MELE on % increment or reduction in nucleic acid levels in MNU induced mammary carcinogenesis

The % increment in model control animals is with respect to normal control animals. The % reduction in treated groups is with respect to model control animals.

5.5.8. Effect of EAELE and MELE on estrogen and progesterone receptor expressions in MNU induced mammary carcinogenesis

The model control animals (ER- 60% ± 1.03 and PR- 73.34%± 1.29) showed significantly higher number of positively stained (brown colored) nuclei as compared to normal control animals (ER- 30.56% ± 0.98; PR-43.66% ± 1.38). The % positively stained ER cells for EAELE 400, MELE 400 and Tamoxifen treated groups were found to be 45.23% ± 2.13; 52% ± 2.57 and 31.66% ± 0.89 respectively (Figure 5.36). The % positively stained PR cells for EAELE 400, MELE 400 and Tamoxifen treated groups were found to be 55.49 % ± 2.18; 62.12% ± 3.91 and 46.66% ± 1.78 respectively (Figure 5.37). The percentage decrease in treated groups as compared to model group is shown in Figure 5.37.

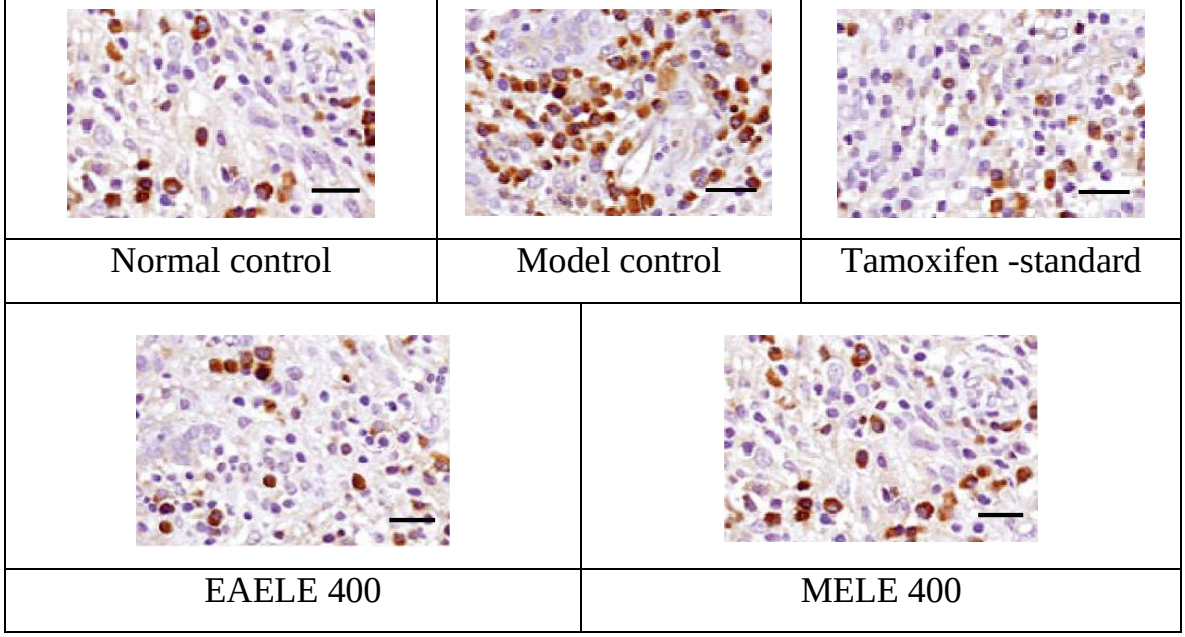


Figure 5.35: Effect of EAELE and MELE on estrogen receptor expression in MNU induced mammary carcinogenesis.
The photographs are taken in Nikon Eclipse TS100. Magnification: 40X. Scale bar: 100µm.

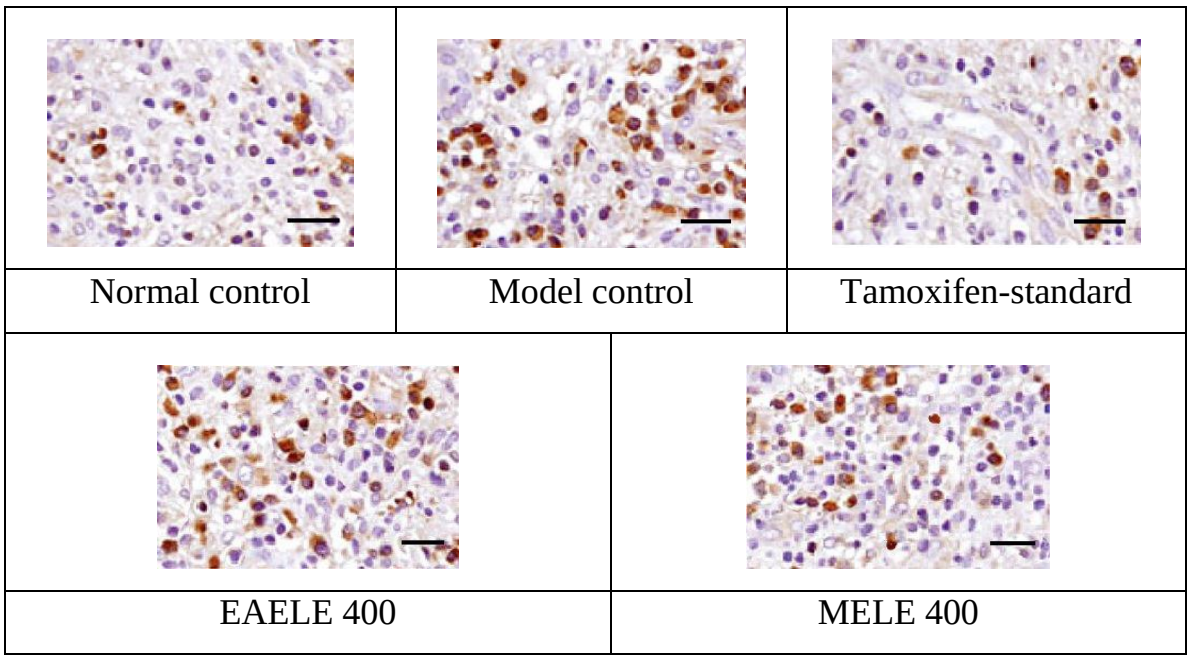


Figure 5.36: Effect of EAELE and MELE on progesterone receptor expression in MNU induced mammary carcinogenesis.
The photographs are taken in Nikon Eclipse TS100. Magnification: 40X. Scale bar: 100µm.

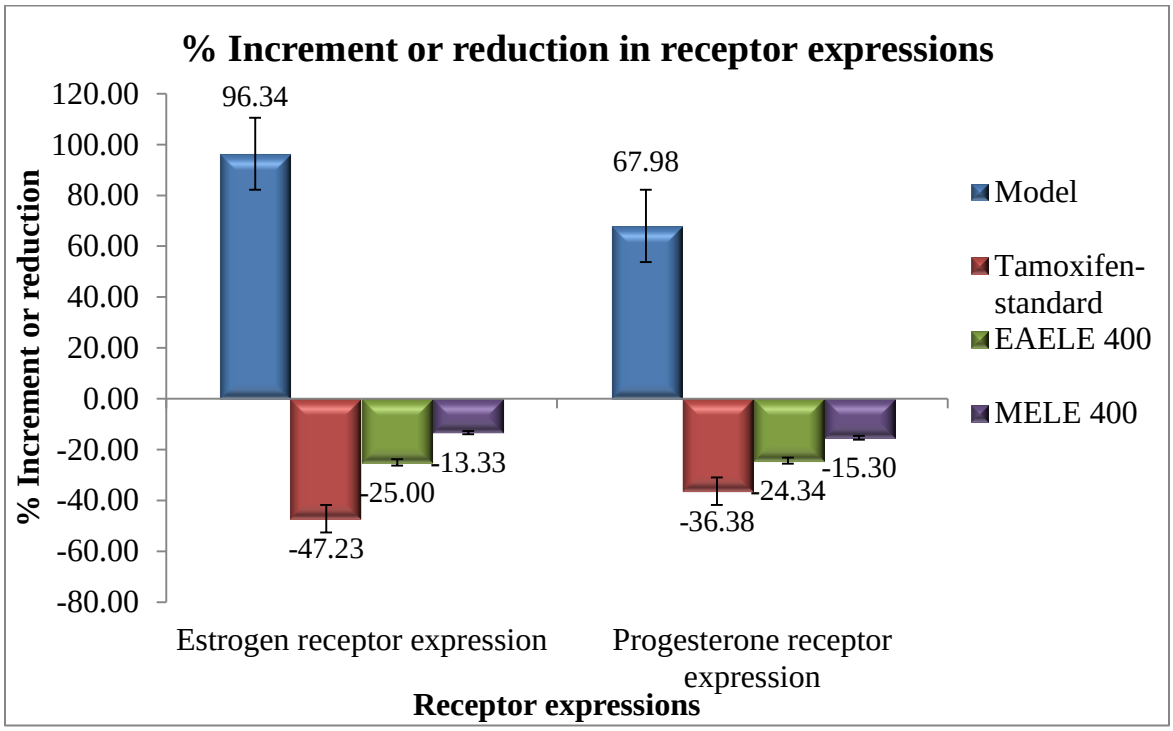


Figure 5.37: Effect of EAELE and MELE on % increment or reduction in estrogen and progesterone receptor expressions in MNU induced mammary carcinogenesis

The % increment in model control animals is with respect to normal control animals. The % reduction in treated groups is with respect to model control animals.

5.6. *In-vivo* evaluation of AECF and MECF in Methylnitrosourea (MNU) induced mammary carcinogenesis

5.6.1. Effect of AECF and MECF on growth rate and relative organ weight in MNU induced mammary carcinogenesis

The % growth rate was significantly different from 42nd day in model control animals when compared to normal control. The significant decrease was observed till the end of the experimental period. The growth rate curve of treated groups ran parallel to normal control and was significantly different from model control. (Figure 5.38)

The relative organ (uteri and liver) weights was increased in model control animals as compared to normal control animals ($P<0.001$). Tamoxifen, AECF and MECF significantly improved relative organ weights when compared to model control animals ($P<0.001$) (Table 5.8).

5.6.2. Effect of AECF and MECF 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 35th to 63rd as compared to normal control. There was no significant difference found between treatment groups as compared to normal control (Figure 5.39).

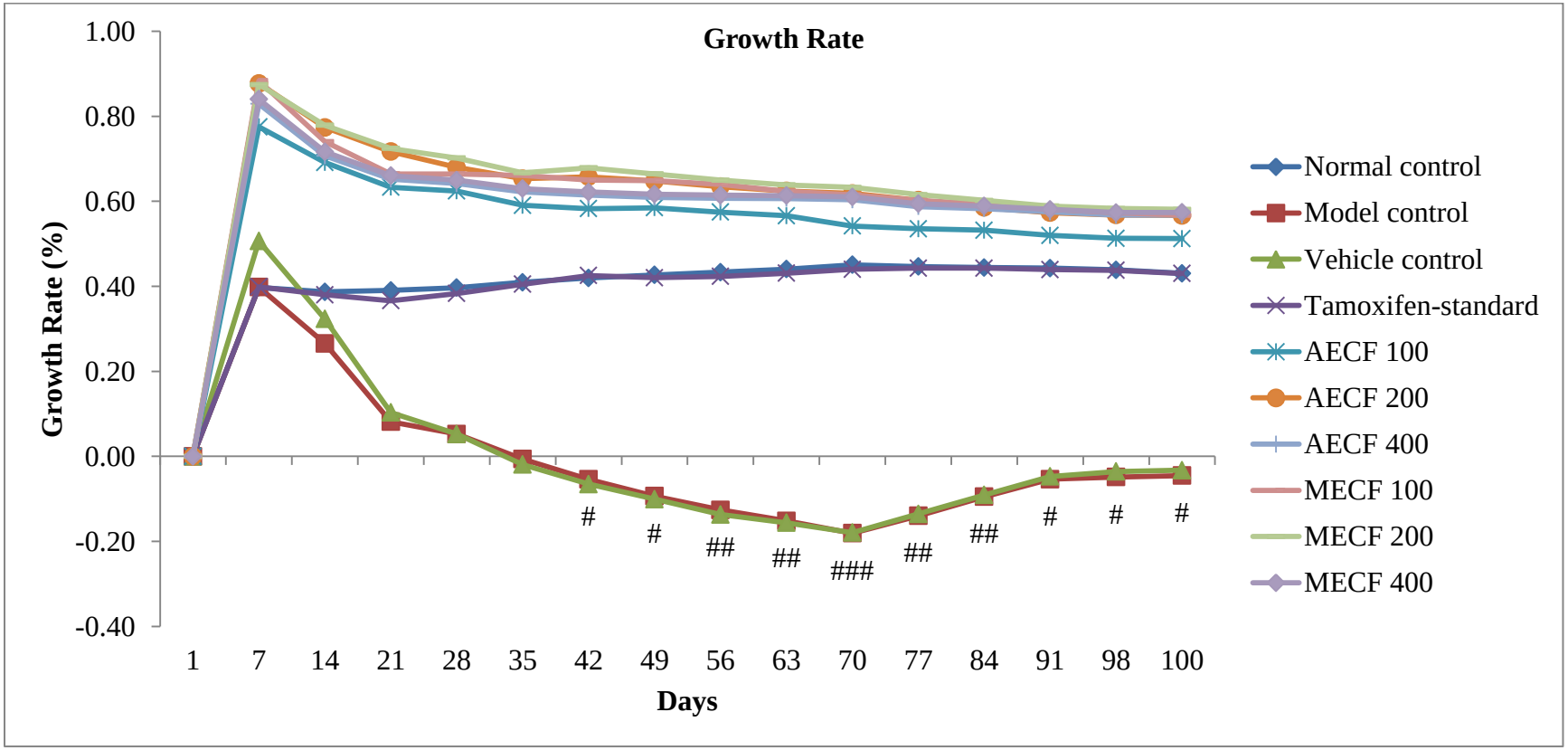


Figure 5.38: Effect of AECF and MECF on growth rate in MNU induced mammary carcinogenesis.
Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using two way ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with normal control vs. model control (#P<0.05, ##P<0.01, ###P<0.001).

Table 5.8: Effect of AECF and MECF on relative organ weight in MNU induced mammary carcinogenesis

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
Tamoxifen-standard	0.13±0.01***	3.80±0.11***
AECF 100	0.16±0.01***	3.59±0.09***
AECF 200	0.15±0.01***	3.60±0.12***
AECF 400	0.14±0.01***	3.85±0.07***
MECF 100	0.16±0.01***	3.69±0.17***
MECF 200	0.16±0.01***	3.64±0.13***
MECF 400	0.15±0.01***	3.60±0.12***

Values are expressed as 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 normal control vs. model control (###P<0.001), model control vs. all other groups (***P<0.001).

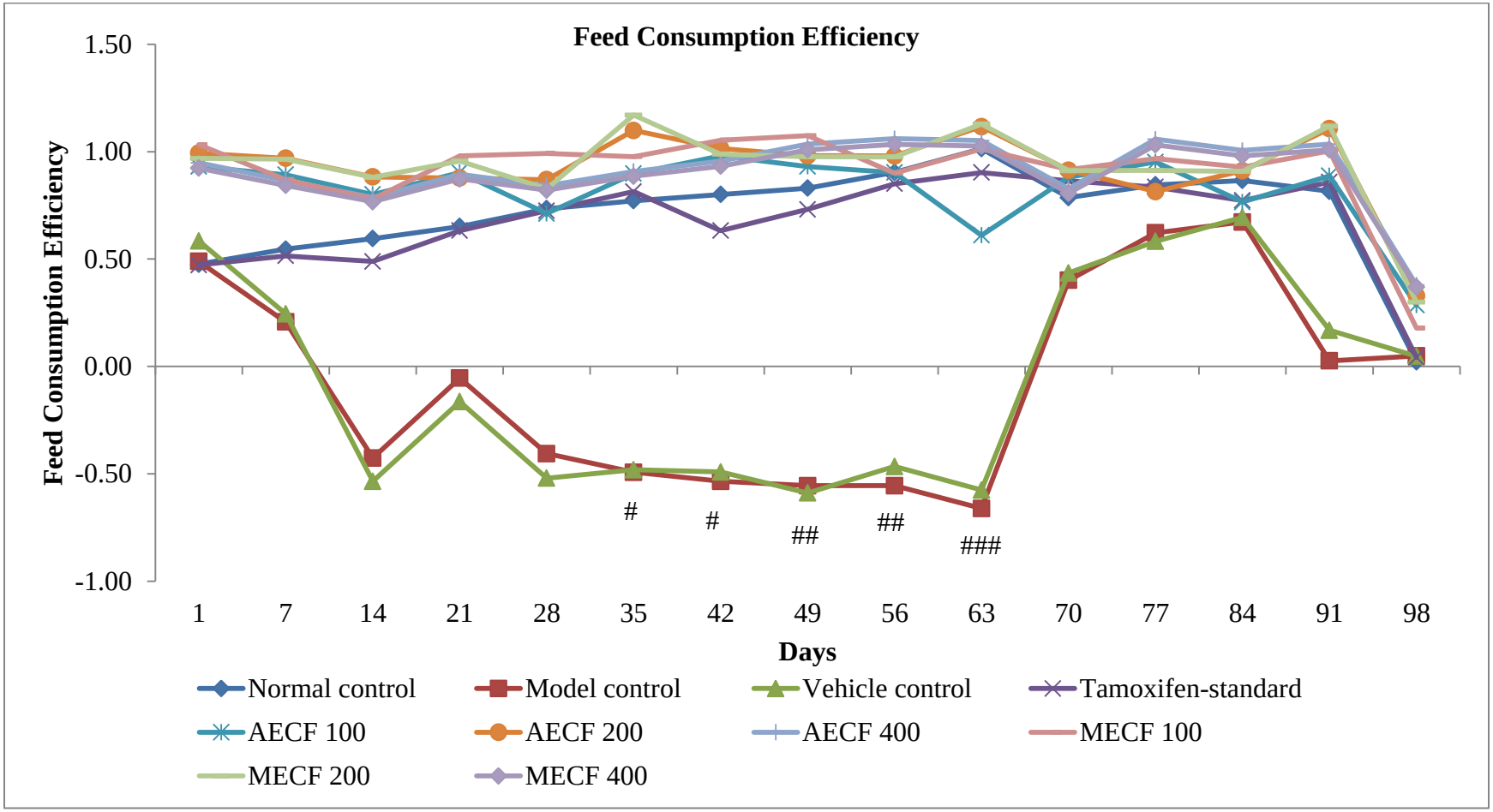


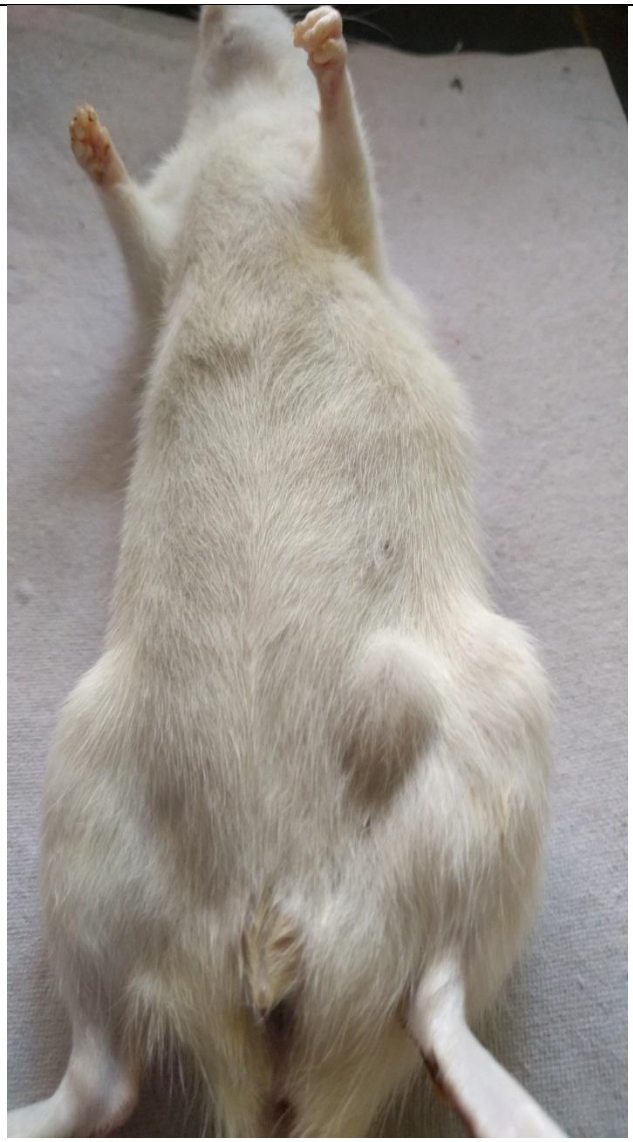
Figure 5.39: Effect of AECF and MECF on feed consumption efficiency in MNU induced mammary carcinogenesis.

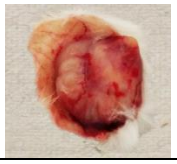
Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using two way ANOVA analysis followed by Bonferroni's's Post hoc test. Significant values were compared with normal control vs. model control (#P<0.05, ##P<0.01, ### P<0.001).

5.6.3. Effect of AECF and MECF on tumor parameters in MNU induced mammary carcinogenesis

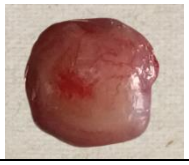
The model control animals successfully developed mammary cancer in 45.17±9.21 days. The tumor incidence was 83.33%. The total number of tumors in model control animals was 6. One rat developed two mammary tumors. Tumor multiplicity i.e. number of tumors per rat was found to be 1. The tumor weight and volume in model control animals were found to be 6.4±1.47 g and 99.29 ±3.19 mm^3. (Figure 5.40)

The tumor weight was significantly decreased in AECF (100, 200 and 400) treated animals while mid and MECF 400 treated animals showed significant decrease as compared to model group. The tumor volume was significantly decreased in all treatment groups as compared to model control animals. The appearance of first tumor post MNU injection was prolonged in treated animals as compared to model control animals (Tamoxifen (82.17±16.44; $P<0.001$), AECF (100, 200 and 400: 66.83±1.45; 67.17±2.76 and 72.83±2.32 respectively) and MECF (100, 200 and 400: 72.17±1.42; 75.80±12.75 and 79.40±13.3 respectively). (Table 5.9)





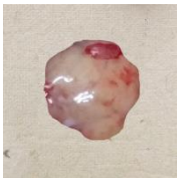
Model Control



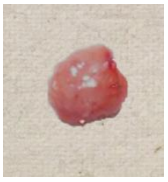
Vehicle Control

Tamoxifen-standard

AECF 100



MECF 100



AECF 200

MECF 200

AECF 400

MECF 400

TUMOR IN RAT

Figure 5.40: Representative images of tumors in model and treated groups in MNU induced mammary carcinogenesis

Table 5.9: Effect of AECF and MECF on tumor parameters in MNU induced mammary carcinogenesis

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	$6.4\pm 1.46^{\#\#\#}$	$99.29\pm 1.18^{\#\#\#}$	$45.17\pm9.21^{\#\#\#}$
Vehicle control	6	6	1	6.2 ± 1.55	89.74 ± 1.18	53.23 ± 19.41
Tamoxifen-standard	5	5	0.83	$0.89\pm0.29^{***}$	$1.95\pm6.33^{***}$	$82.17\pm16.43^{***}$
AECF 100	6	6	1	$3.06\pm0.62^{*}$	$28.08\pm4.86^{***}$	$66.83\pm1.45^{**}$
AECF 200	6	6	1	$2.93\pm0.59^{*}$	$21.65\pm3.97^{***}$	$67.17\pm2.76^{**}$
AECF 400	6	6	1	$2.63\pm0.50^{**}$	$15.71\pm1.98^{***}$	$72.83\pm2.32^{***}$
MECF 100	6	6	1	4.03 ± 0.75	$31.13\pm7.69^{***}$	$72.17\pm1.42^{***}$
MECF 200	6	6	1	$3.47\pm0.78^{*}$	$27.99\pm10.54^{***}$	$75.80\pm12.75^{***}$
MECF 400	6	6	1	$2.97\pm0.59^{*}$	$17.66\pm6.66^{***}$	$79.40\pm13.3^{***}$

Values are expressed as 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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001).

5.6.4. Effect of AECF and MECF on hematological parameters in MNU induced mammary carcinogenesis

The MNU injected untreated animals showed anemic condition as compared to normal group (total RBC count- 5.57±0.17 X 10^6/µL, Hb- 9.63±0.24 g/L; P<0.001). The increase in total WBC count (13.63±0.13 X 10^3/µL) was observed in model control animals as compared to normal group. In normal control animals, total RBC count: 7.11±0.02 X 10^6/µL, Hb: 13.27±0.08 g/L; total WBC count: 6.37±0.06 X 10^3/µL were observed. The total WBC count was significantly decreased with AECF (100, 200 and 400) (P<0.001) and two doses of MECF (200 and 400) (P<0.05) as compared to model control. Treatment with AECF (100, 200 and 400), MECF (100, 200 and 400) and Tamoxifen failed to restore total RBC count and Hemoglobin level. (Table 5.10)

5.6.5. Effect of AECF and MECF on nucleic acid contents in MNU induced mammary carcinogenesis

In model control, the significantly increased (P<0.05) levels of nucleic acids (DNA: 5.72±1.02; RNA: 4.69±1.07) were observed when compared to normal control animals (DNA: 3.66±1.03; RNA: 2.47±1.42). AECF dose dependently and significantly attenuated nucleic acid rise as compared to model control animals (P<0.05). The % reduction in DNA content in Tamoxifen and AECF (100, 200, 400) was found to be 33.5%, 5.91%, 7.39% and 12.81% respectively as compared to model control. The % reduction in RNA content in Tamoxifen and AECF (100, 200, 400) was found to be 40.21%, 9.36%, 13.02% and 14.88% respectively as compared to model control. No significant difference was observed with MECF (100, 200 and 400) as compared to model control animals. (Figure 5.41)

Table 5.10: Effect of AECF and MECF on hematological parameters in MNU induced mammary carcinogenesis.

Groups	Total WBC count (X 10^3/µL)	Total RBC count (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
Tamoxifen-standard	7.30±0.11***	5.40±0.12	8.5±0.22
AECF 100	9.67±0.10***	5.83±0.19	9.73±0.12
AECF 200	9.40±0.15***	5.67±0.21	9.70±0.12
AECF 400	9.07±0.05***	5.65±0.20	9.90±0.18
MECF 100	11.83±0.45	5.60±0.08	9.30±0.04
MECF 200	10.97±0.21*	5.70±0.07	9.50±0.04
MECF 400	10.90±0.69*	5.73±0.17	9.53±0.10

Values are expressed as 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 model control vs normal control (### P<0.001); model control vs. all other groups (*P<0.05, ***P<0.001).

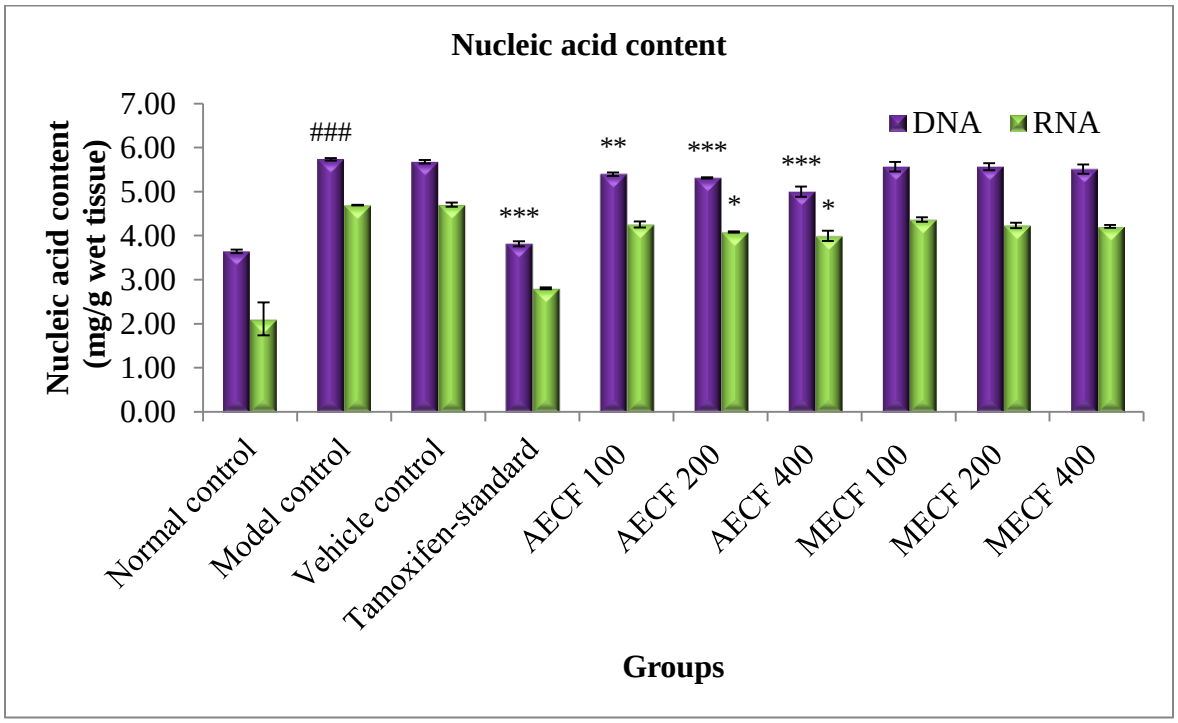


Figure 5.41: Effect of AECF and MECF on nucleic acid content in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001).

5.6.6. Effect of AECF and MECF on lipid peroxidation (MDA) and antioxidant enzyme levels in MNU induced mammary carcinogenesis

The significant (P<0.001) rise in MDA levels (3.76±0.06) was observed in model control animals as compared to normal control animals (1.61±0.08). Treatment with Tamoxifen, AECF (200 and 400), and MECF (200 and 400) significantly decreased MDA levels as compared to model control group (Tamoxifen: 1.85±0.06; AECF 200 and 400: 2.78±0.01, 2.33±0.01; MECF 200 and 400: 2.65±0.12, 2.15±0.17 respectively) (Figure 5.42).

The reduced glutathione (GSH) was significant (P<0.001) decreased in model control (2.86±0.12) as compared to normal control animals (6.55±0.16). The treated groups significantly (P<0.01) hindered this attenuation in GSH levels

when compared with untreated MNU group. (Tamoxifen: 5.13±0.22; AECF 200 and 400: 4.16±0.25, 4.21 ±0.22; MECF 200 and 400: 4.24±0.11, 5.12 ±0.15 respectively) (Figure 5.43)

Model control animals showed significant ($P<0.001$) decrease in SOD levels (5.39±0.06) as compared to normal control animals (12.35 ±0.03). Treatment with Tamoxifen and extracts ($P<0.001$) significantly prevented this decrease in SOD levels when compared with model control animals (Tamoxifen: 11.32±0.18; AECF 100, 200 and 400: 4.18±2.6, 4.39±2.3, 4.53±2.2; MECF 100, 200 and 400: 4.17±2.11, 4.54±1.24, 5.12±2.12 respectively) (Figure 5.44).

Model control animals showed significant ($P<0.001$) decrease in Catalase activity (19.28±0.25) as compared to normal control animals (39.56±0.15). Tamoxifen significantly ($P<0.001$) prevented this decrease in Catalase activity (30.08±0.17). No significant difference was found on treatment with AECF (100, 200 and 400) and MECF (100, 200 and 400) in animals as compared to model control (Figure 5.45).

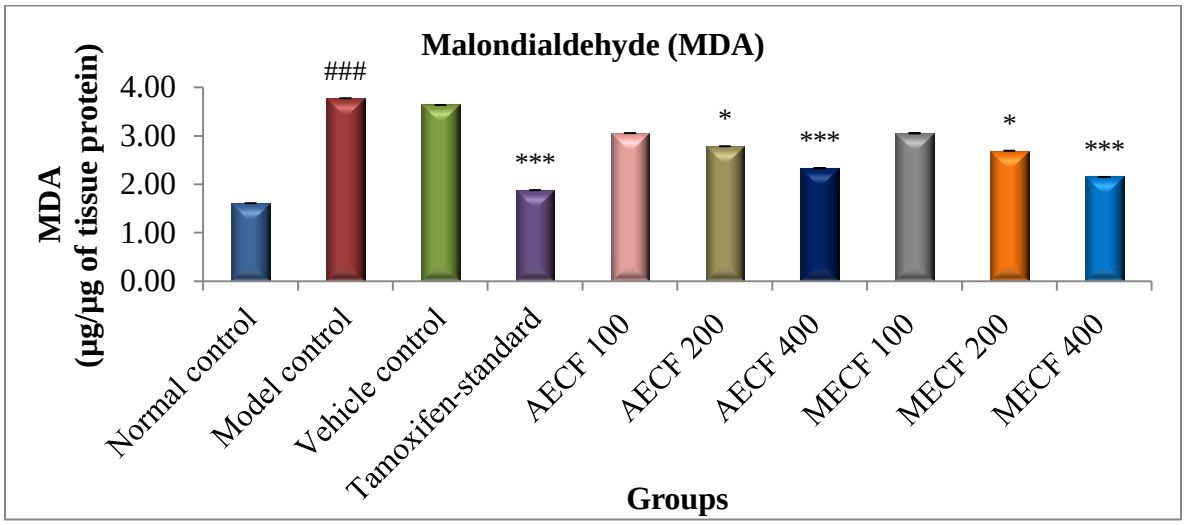


Figure 5.42: Effect of AECF and MECF on MDA levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

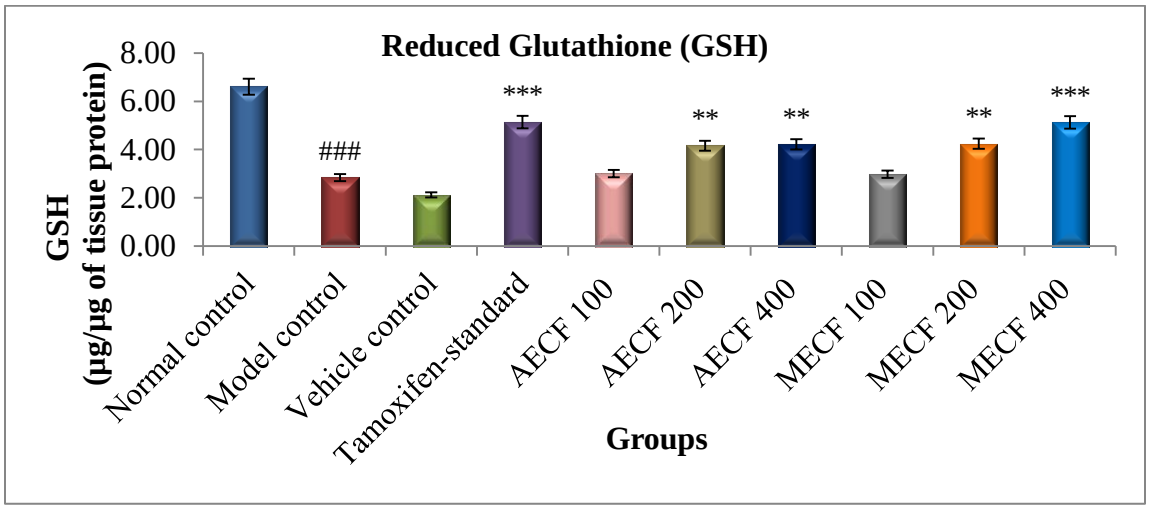


Figure 5.43: Effect of AECF and MECF on GSH levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

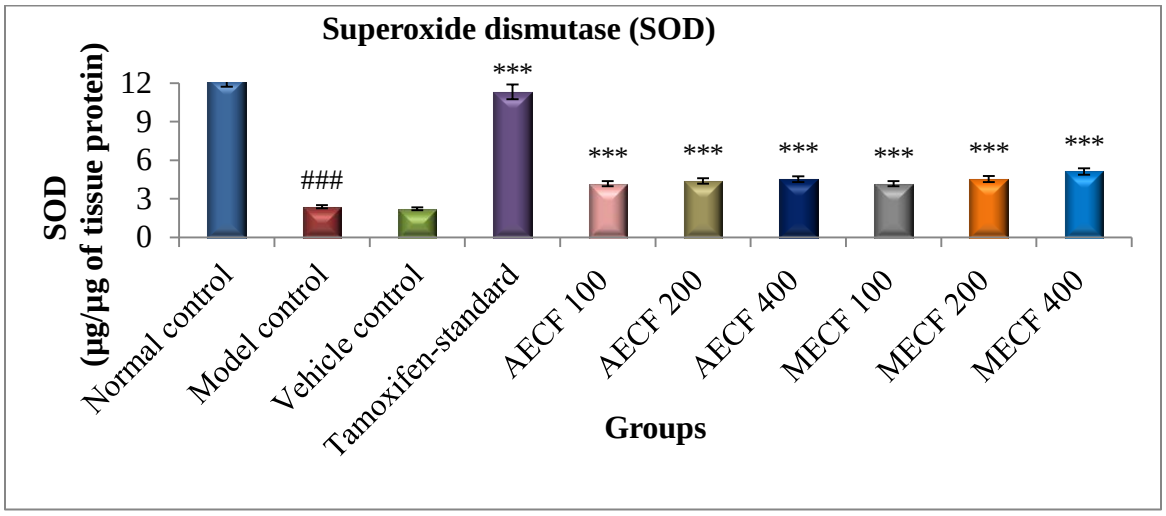


Figure 5.44: Effect of AECF and MECF on SOD levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001), model control vs. all other groups (***P<0.001).

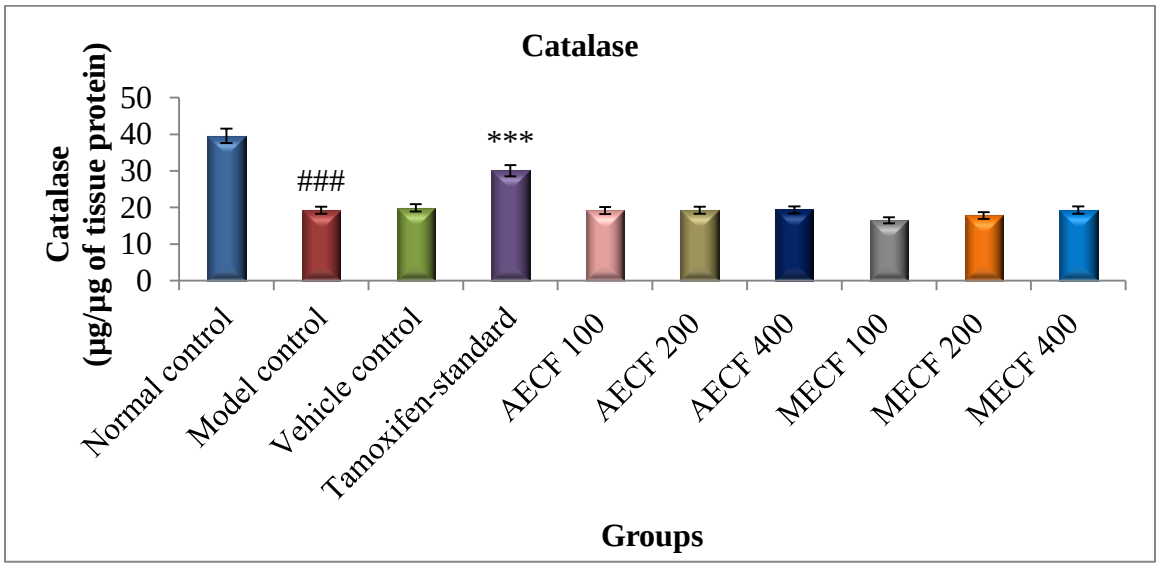


Figure 5.45: Effect of AECF and MECF on Catalase levels in MNU induced mammary carcinogenesis

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

5.6.7. Comparison between AECF and MECF in MNU induced mammary carcinogenesis

Scrutinizing tumor parameters and nucleic acid content, the most potent extract was observed to be AECF. The tumor weight was significantly reduced by all the three doses of AECF as compared to model control animals. The 11.54% tumor weight was reduced in AECF 400 as compared to MECF 400. (Figure 5.46) The reduction in DNA levels was observed in all treatment doses of AECF as compared to model control animals. The reduction in RNA levels was observed in AECF (200 and 400) as compared to model control animals (Figure 5.47) No significant difference was observed in nucleic acid levels in MECF treated groups. All this suggests AECF as better candidate against MNU induced mammary carcinogenesis. There was non-significant difference between treatment doses and hence, to compare anti-estrogenic and anti-progesterogenic effect, the immunohistochemistry was performed in highest doses of AECF and MECF.

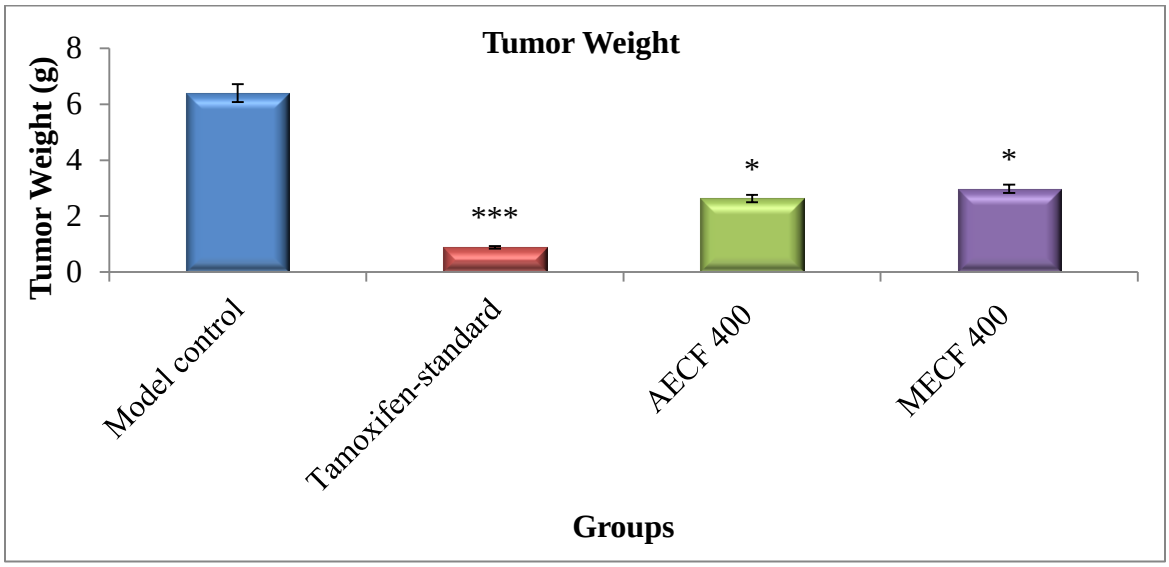


Figure 5.46: Effect of AECF and MECF on tumor parameters in MNU induced mammary carcinogenesis

Values are expressed as 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 model control vs. all other groups (**P<0.01 ***P<0.001).

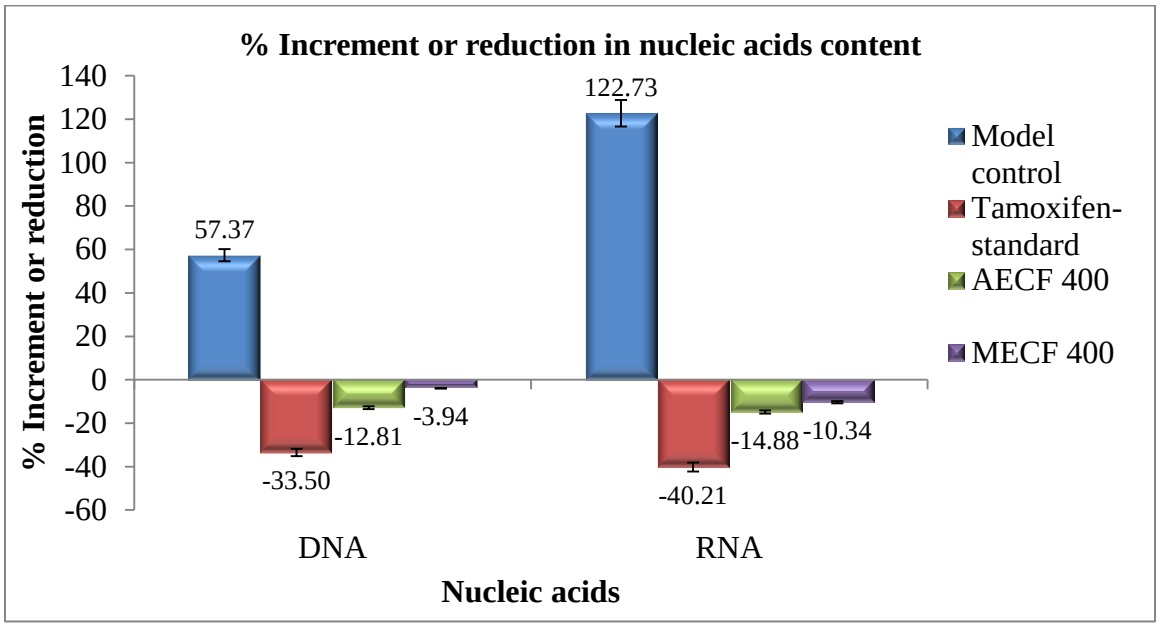


Figure 5.47: Effect of AECF and MECF on % increment or reduction in nucleic acid levels in MNU induced mammary carcinogenesis

The % increment in model control animals is with respect to normal control animals. The % reduction in treated groups is with respect to model control animals.

5.6.8. Effect of AECF and MECF on estrogen and progesterone receptor expressions in MNU induced mammary carcinogenesis

The immunohistochemistry analysis of model control tumor samples showed significantly higher expressions of hormones (ER-60% ± 1.03 and PR-73.34%± 1.29) as compared to normal control animals (ER- 30.56% ± 0.98; PR-43.66% ± 1.38). The % positively stained (brown color) ER cells for AECF 400, MECF 400 and Tamoxifen were found to be 45% ± 1.12; 54% ± 2.43 and 31.66% ± 0.89 respectively. (Figure 5.48) The % positively stained PR cells for AECF 400, MECF 400 and Tamoxifen were found to be 62% ± 2.16; 70% ± 1.85 and 46.66% ± 1.78 respectively. (Figure 5.49) The percentage decrease in treated groups compared to model control is shown in Figure 5.50.

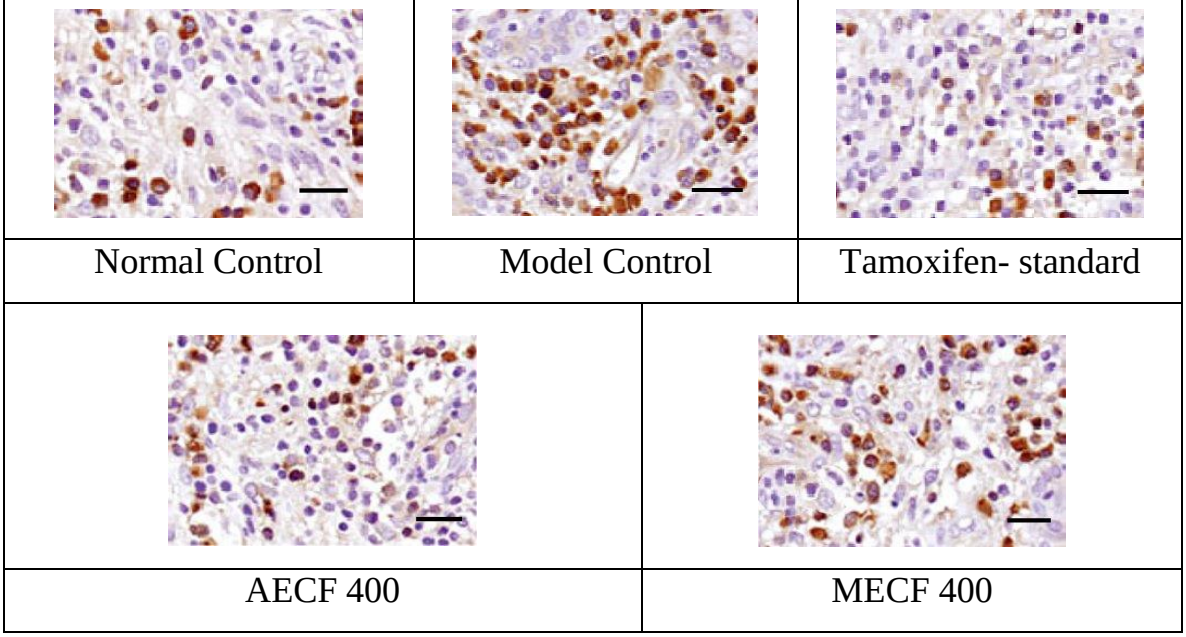


Figure 5.48: Effect of AECF and MECF on estrogen receptor expression in MNU induced mammary carcinogenesis.

The photographs are taken in Nikon Eclipse TS100. Magnification: 40X. Scale bar: 100µm.

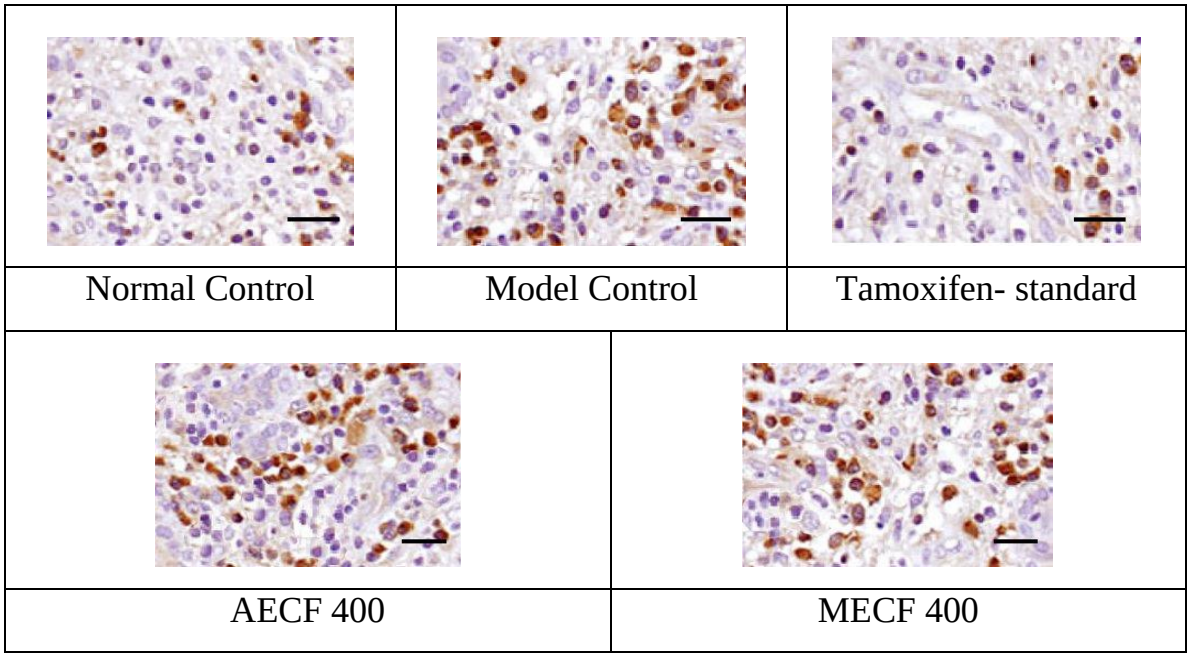


Figure 5.49: Effect of AECF and MECF on progestrogene receptor expression in MNU induced mammary carcinogenesis.

The photographs are taken in Nikon Eclipse TS100. Magnification: 40X. Scale bar: 100µm.

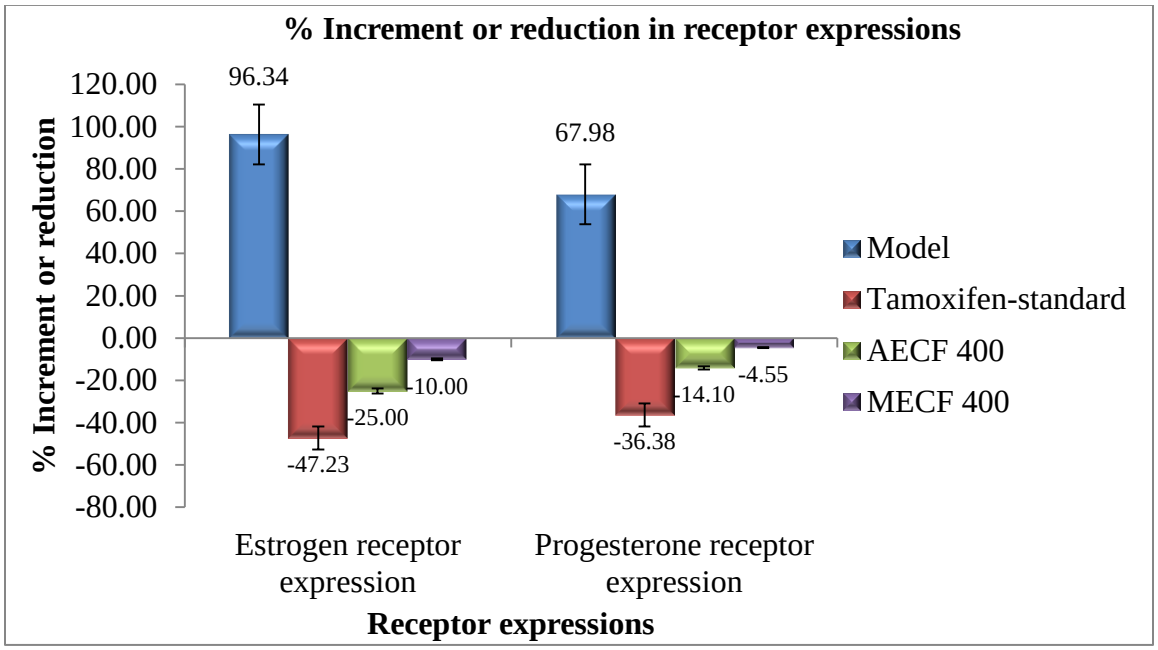
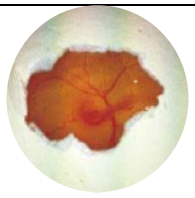
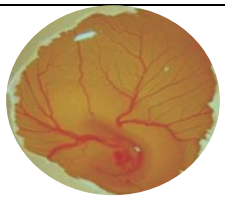
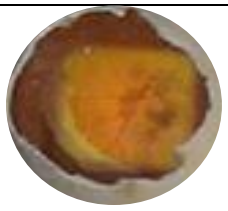
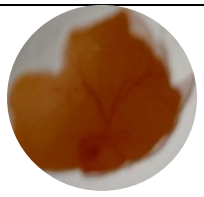


Figure 5.50: Effect of AECF and MECF on % increment or reduction in estrogen and progesterone receptor expressions in MNU induced mammary carcinogenesis

The % increment in model control animals is with respect to normal control animals. The % reduction in treated groups is with respect to model control animals.

5.7. Effect of extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) on angiogenesis (Chick Chorioallantoic Membrane Assay)

Anti-angiogenic potential of extracts was studied using Chick Chorioallantoic Membrane (CAM) of fertilized eggs. 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 3 ± 0.18 mm zone of inhibition is seen with MEBM (20 µg/ml). The 2.5 ± 0.45 mm zone of inhibition is seen with EAELE (20 µg/ml). The 1± 0.75 mm zone of inhibition is seen with AECF (30 µg/ml). AEBM, MECF and MELE showed no significant anti-angiogenic potency in selected concentration range (10-50 µg/ml). (Figure 5.51)

	
(a) Control Group (PBS) 0 h	(b) Control Group (PBS) 48 h
	
(c) MEBM 0 h	(d) MEBM 48 h
	
(e) AEBM 0 h	(f) AEBM 48 h

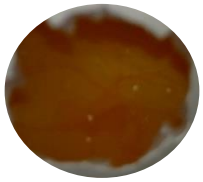
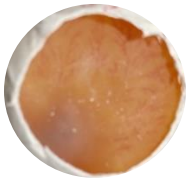
	
(g) EAELE 0 h	(h) EAELE 48 h
	
(i) MELE 0 h	(j) MELE 48 h
	
(k) AECF 0 h	(l) AECF 48 h
	
(m) MECF 0 h	(n) MECF 48 h

Figure 5.51: Effect of extracts on angiogenesis in the Chick Chorioallantoic Membrane of fertilized eggs

(a) Control group 0 h (b) Control group after 48 h- no hindrance in growth and neovascularization of CAM was observed. (c) MEBM 0 h (d) MEBM 48 h- Zone of inhibition with loss of blood vessels. (e) AEBM 0 h (f) AEBM 48 h- Formation of new blood vessels (g) EAELE 0 h (h) EAELE 48 h- Zone of inhibition with no neovascularisation. (i) MELE 0 h (j) MELE 48 h- Formation of new blood vessels (k) AECF 0h (l) AECF 48 h- Zone of inhibition with loss of blood vessels. (m) MECF 0 h (n) MECF 48 h- Formation of new blood vessels.

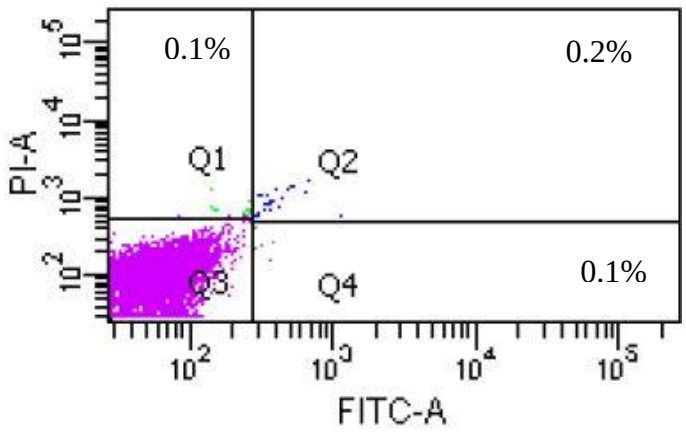
5.8. Effect of extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) on apoptosis (Annexin V-FITC PI double staining assay)

In present research, the treatment of MCF-7 cells with MEBM and AEBM for 24 h resulted in significant increase 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%) was reduced.

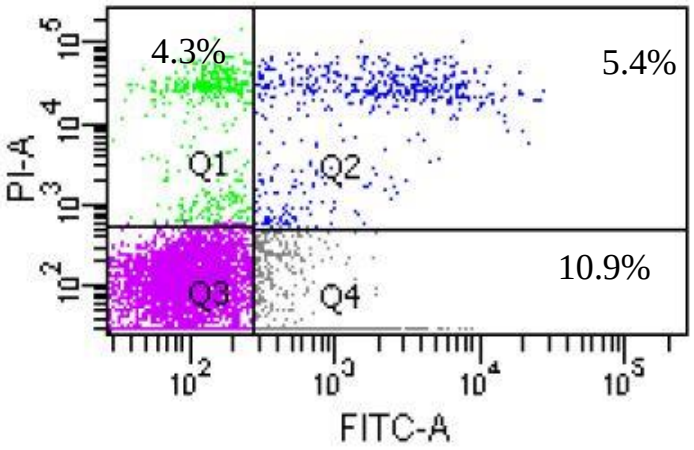
In EAELE and MELE treated MCF-7 cells, the increase was observed in ratios of early (EAELE: 9.00%; MELE: 5.9%) and late apoptosis (EAELE: 4.7%; MELE: 4.2%) cells, while the percentage of viable cells (EAELE: 59.4%; MELE: 63.7%) was reduced.

On treatment with AECF and MECF for 24 h; the increase percentage of early (AECF: 5.9%; MECF: 3.8%) and late apoptosis (AECF: 4.6%; MECF: 3.1%) population was observed. The percentage of viable cells (AECF: 55.6%; MECF: 63.6%) was reduced.

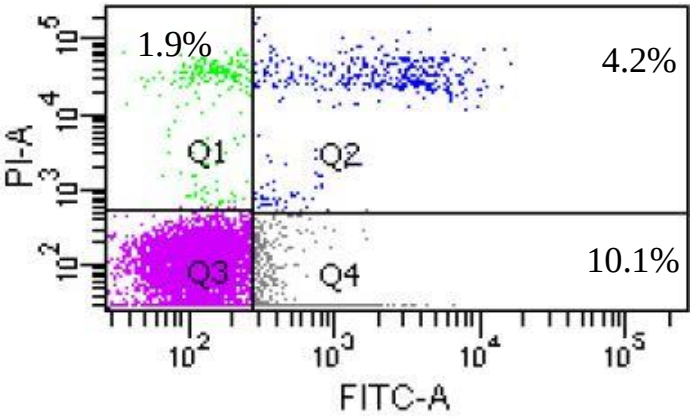
The viable cell in control wells are 72.8%; while the cells in early apoptotic phase is 0.1% and in late apoptotic phase is 0.2%. (Fig. 5.52)



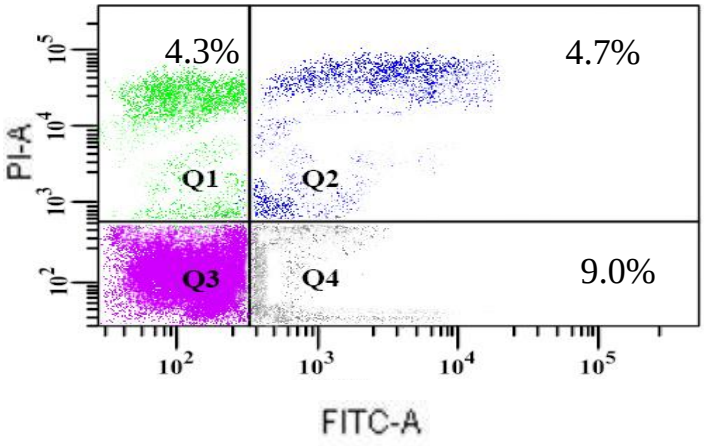
a) Control



b) MEBM



c) AEBM



d) EAELE

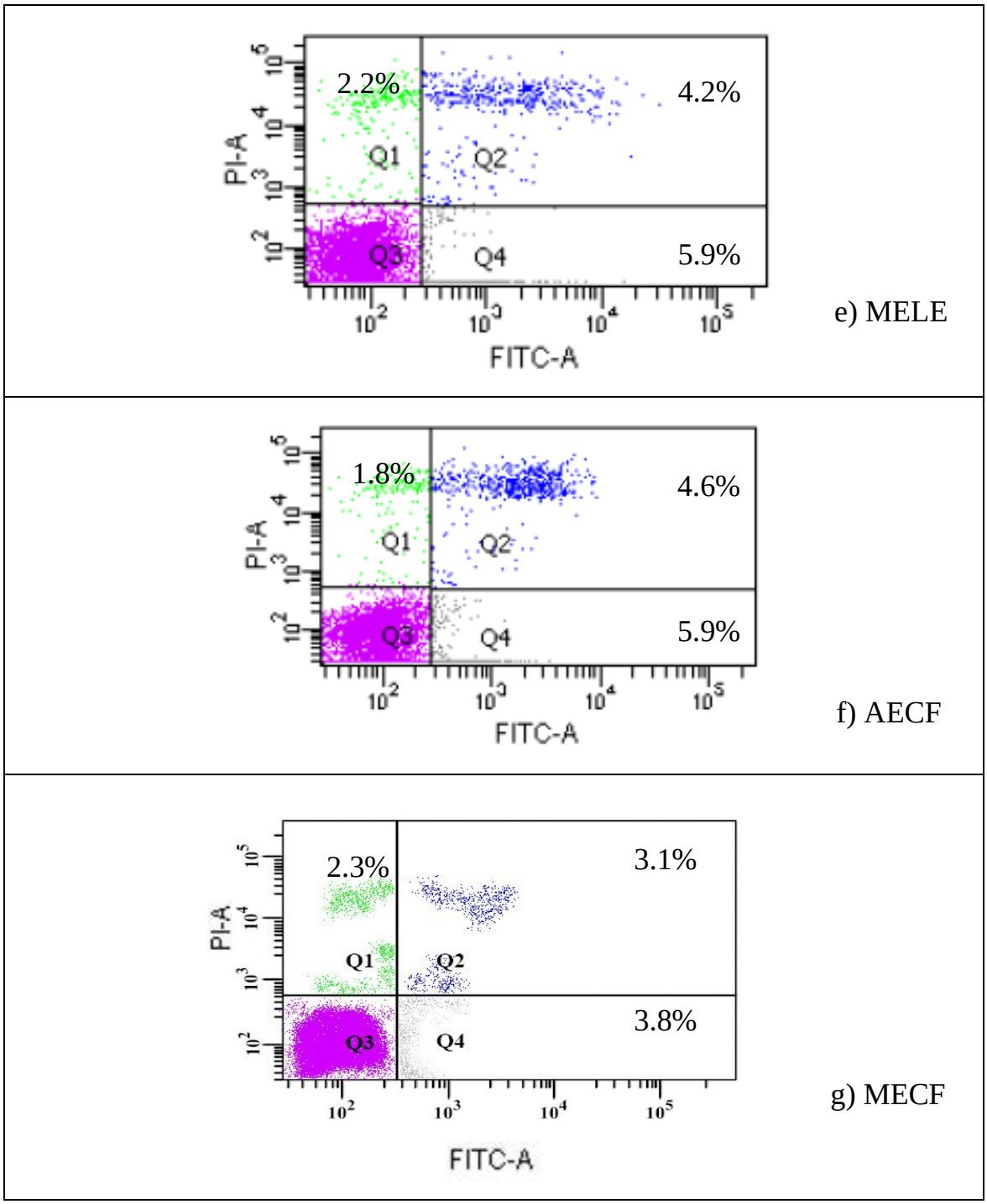
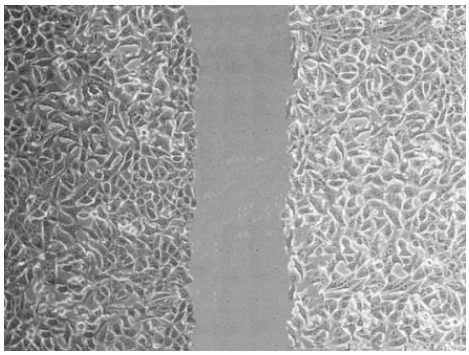


Figure 5. 52: Effect of extracts on the apoptosis on MCF-7 human breast cancer cell line

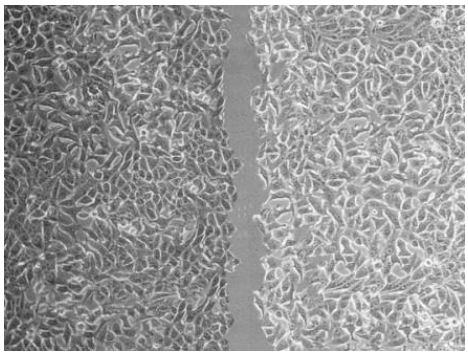
Q1: Necrosis; Q2: Late Apoptosis; Q3: Viable cells; Q4: Early Apoptosis; FITC-A and PI-A: dyes. (a) Control MCF-7 cells. MCF-7 cells treated with (b) MEBM and (c) AEBM. (d) EAELE (e) MELE (f) AECF (g) MECF Percentage of apoptotic and necrotic population is increased in treated cells. The analysis was done by FACS Diva Version 6.1.3

5.9. Effect of extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) on cell inhibition on Scratch Motility Assay

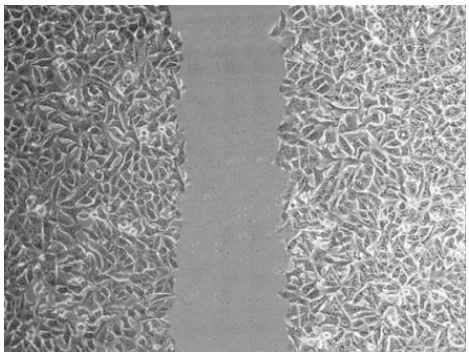
In present investigation, the scratch motility assay displayed the ability of plant extracts to suppress migration of MCF-7 cells in a denuded area. The inhibition of MCF-7 migration in denuded area with MEBM was 69.26 ± 1.76 % which is significantly higher ($P<0.05$) than that of AEBM (57.41 ± 2.03%). EAELE showed 67.04 ± 2.03% inhibition of MCF-7 cell motility which was higher than that of MELE (61.48 ± 1.45%). The MCF-7 cell motility was inhibited to 61.11 ± 1.15 % by AECF which is higher than that of MECF (55.19 ± 2.91%). (Fig.5.53)



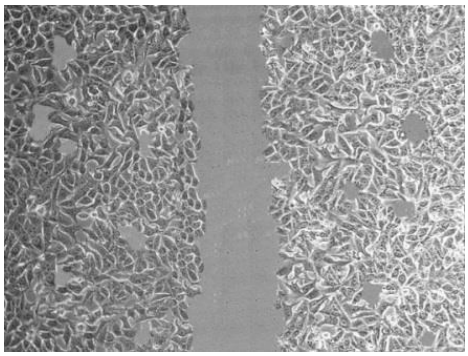
a) CONTROL- 0 h



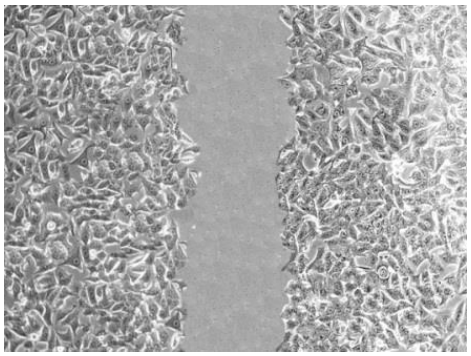
b) CONTROL- 24 h



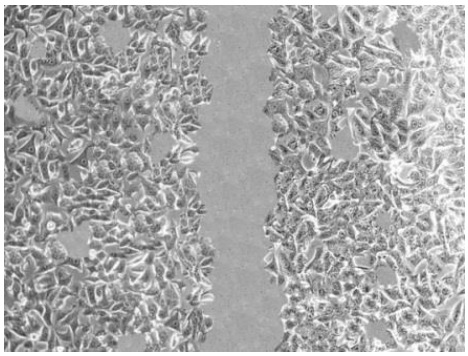
c) MEBM - 0 h



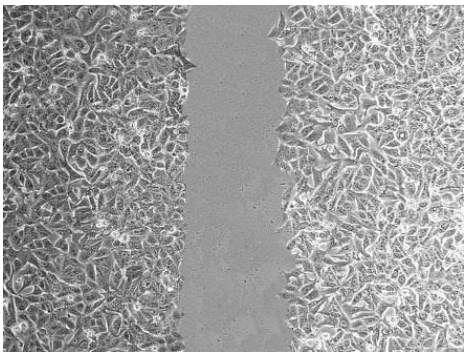
d) MEBM - 24 h



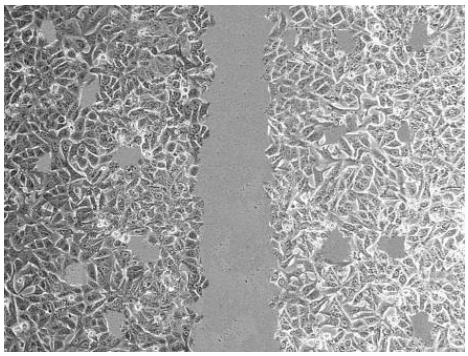
e) AEBM - 0 h



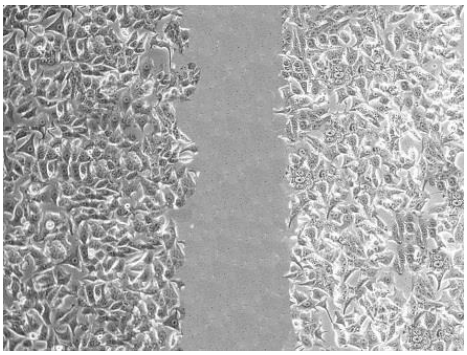
f) AEBM – 24 h



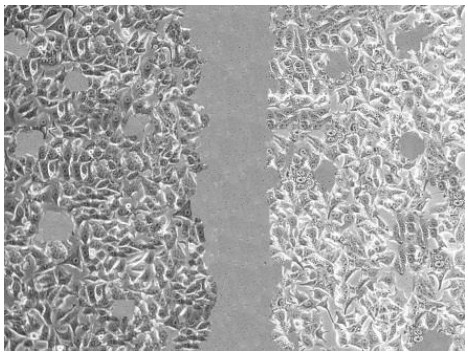
g) EAELE - 0 h



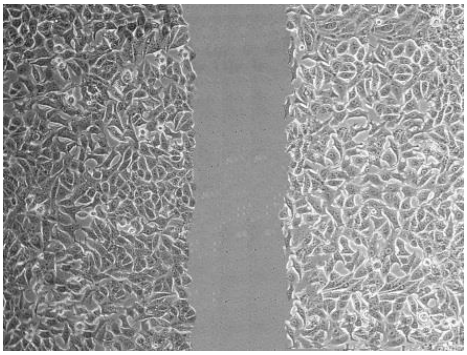
h) EAELE - 24 h



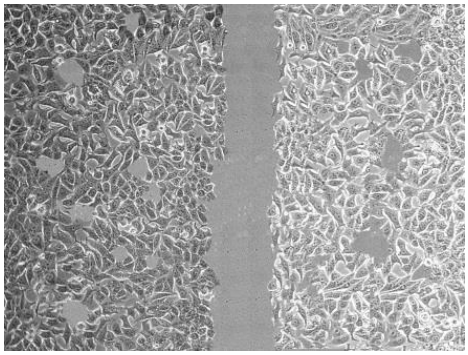
i) MELE - 0 h



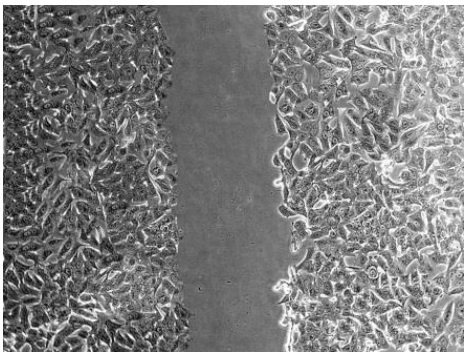
j) MELE – 24 h



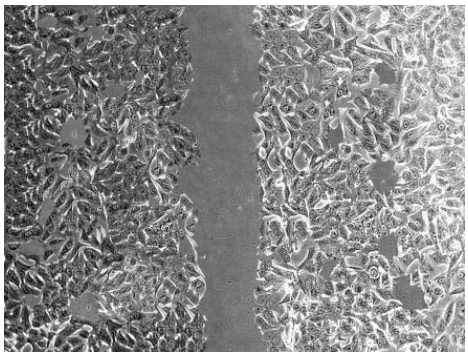
k) AECF - 0 h



l) AECF - 24 h



m) MECF - 0 h



n) MECF – 24 h

Figure 5. 53: Effect of extracts on the cell migration (metastasis) on MCF-7 human breast cancer cell line.

Scratch wound closure was monitored over a time course of 24 h after treatment with extracts.

(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 (g) EAELE- 0 h- MCF-7 cells with scratch (h) EAELE 24 h- Inhibition of cell migration and less restoration of monolayer in scratched area as compared to control cells. (i) MELE- 0 h- MCF-7 cells with scratch (j) MELE 24 h- Inhibition of cell migration and less restoration of monolayer in scratched area as compared to control cells but more as compared to EAELE (k) AECF- 0 h- MCF-7 cells with scratch (l) AECF 24 h- Inhibition of cell migration and less restoration of monolayer in scratched area as compared to control cells. (m) MECF- 0 h- MCF-7 cells with scratch (n) MECF 24 h- Inhibition of cell migration and less restoration of monolayer in scratched area as compared to control cells but more as compared to AECF.

5.10. Effect of extracts (MEBM, AEBM, EAELE, MELE, AECF and MECF) on oxidative stress (ROS generation) (DCFH-DA Assay)

None of the extracts stimulated reactive oxygen species formation in MCF-7 human breast cancer cell line. The fluorescence was non-significantly different from control cells. (Figure 5.54)

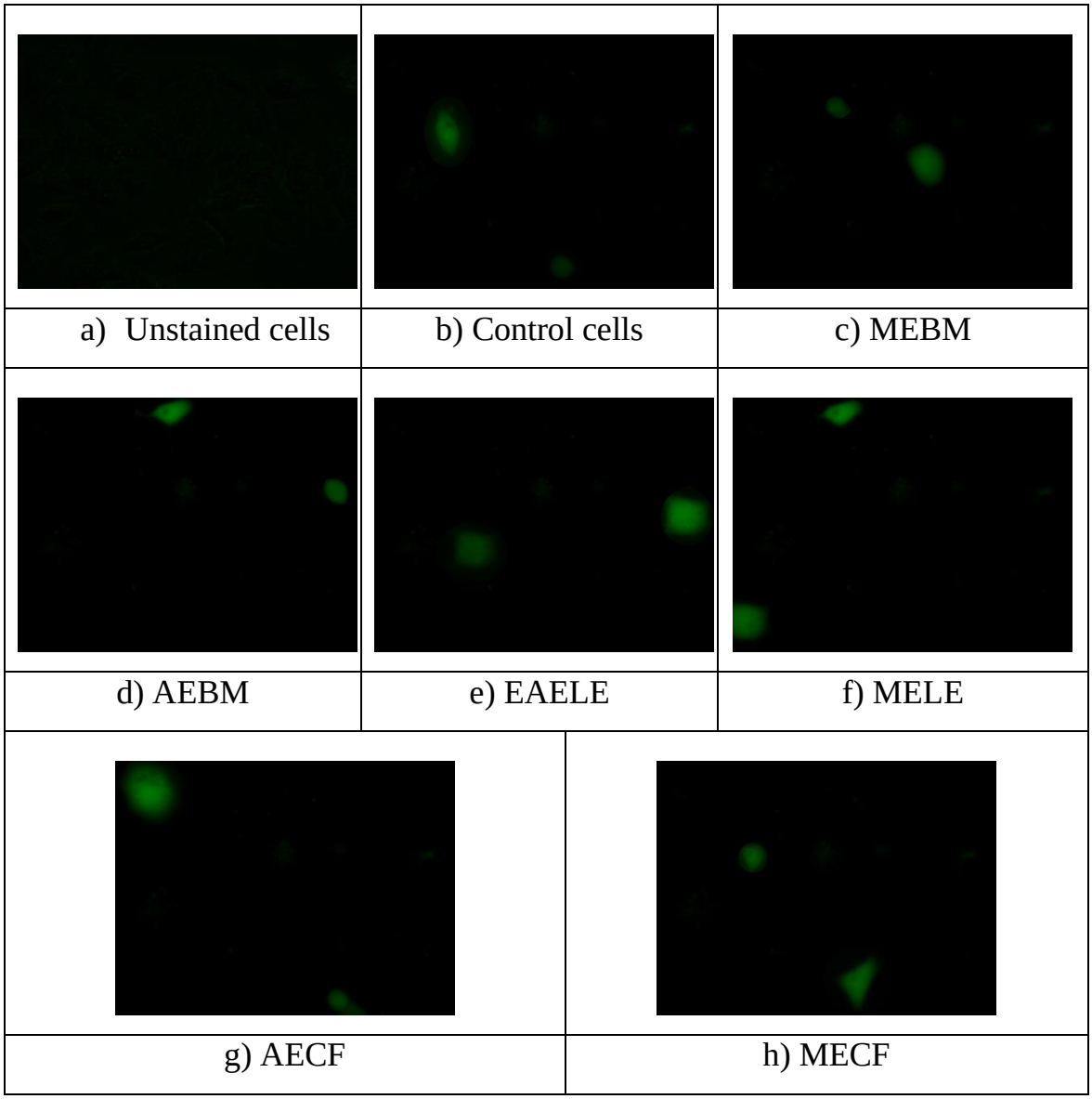


Figure 5.54: Effect of extracts on the oxidative stress on MCF-7 human breast cancer cell line.

a) Unstained cells; b) Control cells – Only DCFH; c) Cells treated with MEBM and DCFH; d) Cells treated with AEBM and DCFH; e) Cells treated with EAELE and DCFH; f) Cells treated with MELE and DCFH; g) Cells treated with AECF and DCFH; h) Cells treated with MECF and DCFH

5.11. Comparison between two potent extracts studied in MNU induced mammary carcinogenesis and in *in-vitro* mechanistic studies

- In MNU induced mammary carcinogenesis, it was deduced that MEBM 400 is superior to AEBM 400 in forestalling breast cancer. (Figure 5.21 and 5.22) 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. (Figure 5.24) 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. (Figure 5.24) The MEBM in Chick Chorioallantoic Membrane assay was found to hostile angiogenesis. No anti-angiogenic effect was seen with AEBM. (Figure 5.51) The apoptotic rate with MEBM was 2% higher than AEBM. (Figure 5.52) The significant 11.85% higher anti-migratory effect was observed in MEBM as compared to AEBM. (Figure 5.53) All these results suggest MEBM tackle breast cancer more aggressively and showed significantly higher anti-cancer potential than aqueous extract.
- In preventive studies, looking at tumor parameters and nucleic acid levels EAELE 400 is significantly better than MELE 400 in hindering mammary carcinogenesis. (Figure 5.33 and 5.34) The estrogen levels in EAELE 400 were reduced by 25% while in MELE 400 it was only 13.33% reduced 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. (Figure 5.37) No anti-angiogenic effect was seen with MELE while EAELE showed anti-angiogenic effect. (Figure 5.51) The apoptotic rate was 3.7% higher in EAELE than MELE. (Figure 5.52) The cell motility

was inhibited in greater extent by EAELE as compared to MELE. (Figure 5.53) 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. (Figure 5.46 and 5.47) 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. (Figure 5.50) In *in-vitro* mechanistic studies, anti-angiogenic effect was seen in AECF only. (Figure 5.51) Also, the apoptotic rate with AECF was significantly higher (11.7%) than MECF. (Figure 5.52). The anti-migratory effect was 5.92% higher in AECF than MECF (Figure 5.53). The AECF wins the race in handling breast cancer more efficiently.
- ***Based on these, the curative proficiency of MEBM, EAELE and AECF was further studied in syngeneic model of mice***.

SECTION 3

Curative potential study of extracts in syngeneic mice model

5.12. *In-vivo* evaluation of MEBM, EAELE and AECF in Ehrlich ascites *carcinoma* (EAC) induced solid tumors

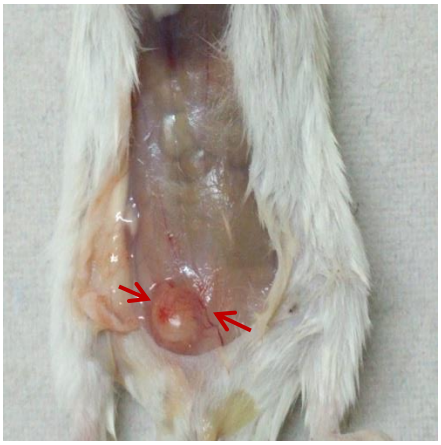
5.12.1. Effect of MEBM, EAELE and AECF on body weight in EAC induced solid tumors

The significant increase in body weight up to 40 g with fluid accumulation in peritoneal cavity was observed in EAC injected model control animals. (Figure 5.55) Upon treatment with Tamoxifen, MEBM 400, EAELE 400 and AECF 400, the body weight was non-significantly different from normal control animals. (Animal weights on 30^{th} day: Normal control- 34.67±0.24g; Tamoxifen- 34.55±0.85g and MEBM- 33±0.26 g). (Table 5.11) No fluid accumulation was observed in Tamoxifen, MEBM 400 and EAELE 400 treatment groups. However, in AECF 400 treated group, fluid accumulation was observed in two animals.

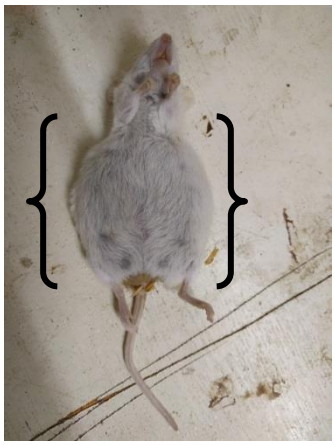
5.12.2. Effect of MEBM, EAELE and AECF on tumor parameters in EAC induced solid tumors

Upon treatment with MEBM 400, EAELE 400 and AECF 400 (from 5 day until 30 day), a significant reduction in the tumor volume (82.61%; 69.08% and 71.98% respectively; $P<0.001$) was observed compared to untreated control animals bearing tumor. Tumor weight of cancer bearing animals were found to be 2.23±0.56 ($P<0.001$) which was significantly truncated in MEBM 400, EAELE 400 and AECF 400 treated animals (0.2±0.18, 0.2±0.16 and 0.36±0.20 respectively; $P<0.001$). There was significant increase in the lifespan of MEBM 400, EAELE 400 and AECF 400 administered animals as compared to model group. Mean survival time for model control animals was 17 days whereas it

was extended to 29.5 days for MEBM 400, 29 days for EAELE 400 and 27.5 days for AECF 400 treated animals. (Table 5.11) (Figure 5.55 and Figure 5.56)



a)



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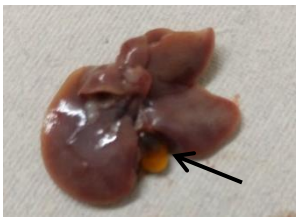
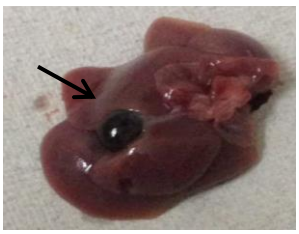


c)



d)

Figure 5.55: EAC induced solid mammary tumors in mice

a) Tumor in EAC injected mice with neovascularization (shown by "→"); b) Peritoneal fluid accumulation (denoted by "{}") in EAC injected mice; c) and d) accumulation of fluid (shown by "→") in liver in EAC injected mice

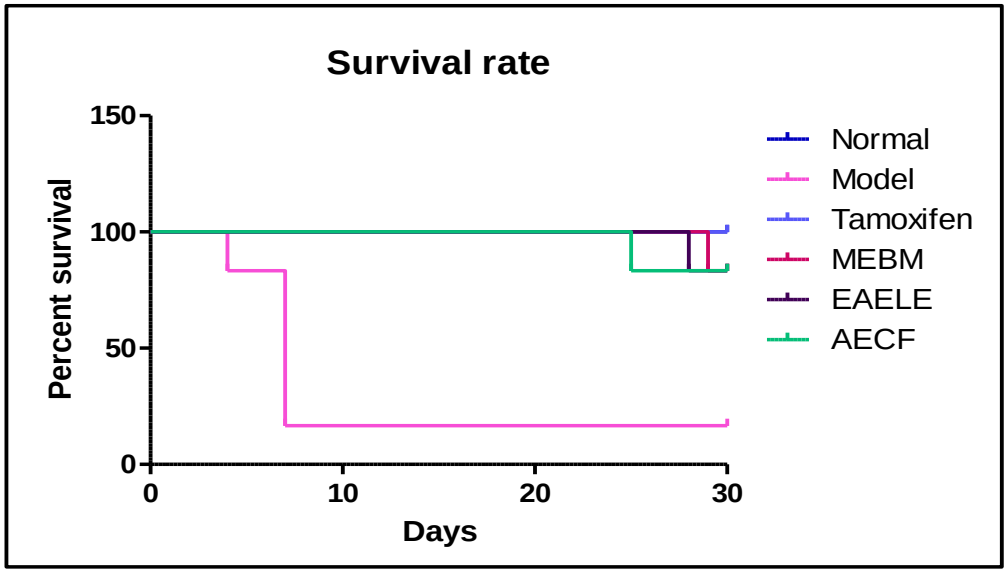


Figure 5.56: Effect of MEBM, EAELE and AECF on survival rate in EAC induced solid tumors

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using survival curve analysis

Table 5.11: Effect of MEBM, EAELE and AECF on body weight and tumor parameters in EAC induced solid tumors

Groups	Body Weight (g)	Tumor weight (g)	Tumor Volume (mm^3)	% Tumor reduction	Mean Survival Time	% Increase in Life span
Normal control	34.67±0.25	-	-	-	30	-
Model control	40±11.54$^{###}$	2.23±0.56	54.21±12.56	-	17$^{###}$	-
Tamoxifen- standard	34.55±0.85***	0	0	100%	30***	76.47%
MEBM 400	33±0.26***	0.20±0.18***	9.43+0.01***	82.61%	29.5***	73.53%
EAELE 400	35.42±0.58***	0.20±0.16***	16.76+0.01***	69.08%	29***	70.59%
AECF 400	37.1±0.26*	0.36±0.20***	15.19+6.35***	71.98%	27.5***	61.76%

Values are expressed as 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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

5.12.3. Effect of MEBM, EAELE and AECF on liver weight in EAC induced solid tumors

Liver weight was found to be increased in EAC injected untreated animals (2.57±0.13) as compared to normal control animals (1.85±0.06), suggesting abnormalities in liver. The significant decrease ($P<0.001$) in liver weight was observed in Tamoxifen, MEBM 400, EAELE 400 and AECF 400 as compared to model control group suggesting improvement in liver. (Tamoxifen- 1.9±0.08g; MEBM 400- 1.83±0.08 g; EAELE 400- 2.06±0.17 g and AECF 400- 2.09±0.08 g respectively)

5.12.4. Effect of MEBM, EAELE and AECF on liver enzymes in EAC induced solid tumors

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 as compared to normal control animals. Treatment with MEBM 400 (AST: 22.99 ±1.09 and ALT: 22.10 ±1.45; $P<0.001$) and EAELE 400 (AST: 61.33 ±1.02 and ALT: 85.30 ±1.11; $P<0.001$) significantly decreased liver enzymes levels as compared to model group. 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 as compared to model group but it was higher than that of MEBM 400 treated animals. No significant improvement was observed with AECF 400 treatment. (Figure 5.57)

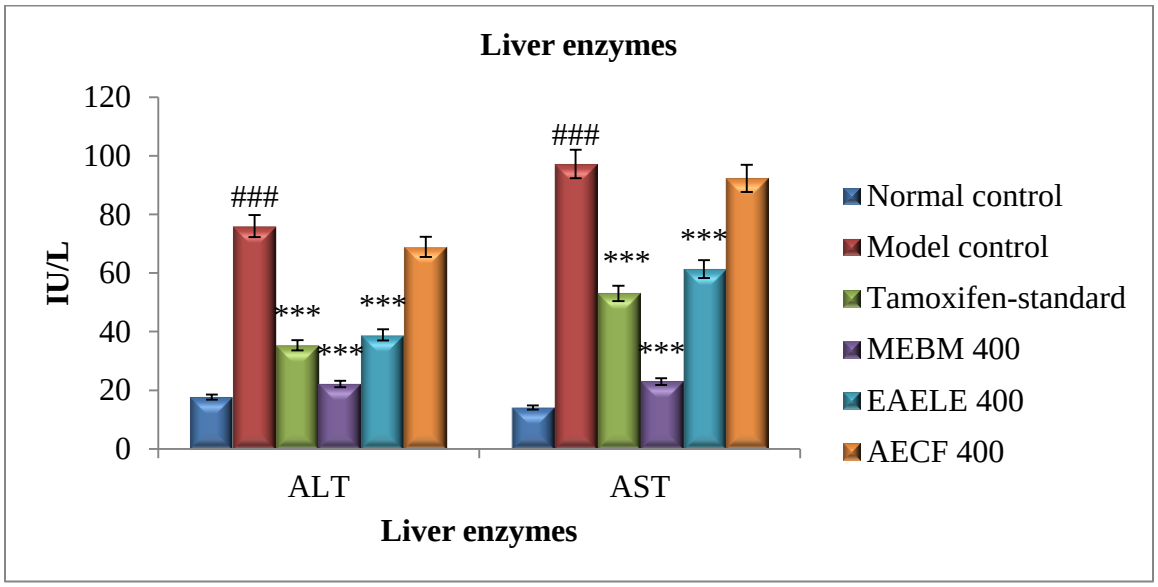


Figure 5.57: Effect of MEBM, EAELE and AECF on liver enzymes in EAC induced solid tumors

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001)

5.12.5. Effect of MEBM, EAELE and AECF on hematological parameters in EAC induced solid tumors

The results are in harmony with MNU induced mammary carcinogenesis. The total WBC count (8.3 ±1.09 X 10^3/µL; P<0.001) was found to be increased in untreated EAC injected mice whereas total RBC count (6.75±2.13 X 10^6/µL; P<0.001) and Hb (9.7 ±1.39; P<0.001) was found to be decreased as compared to normal control animals (total WBC count: 5.1 ±1.91 X 10^3/µL; total RBC count: 8.3± 2.19 X 10^6/µL and Hb: 11.5 ±2.35; P<0.001). In this study, the treatment with MEBM 400 and EAELE 400 increased total RBC count (8.62 ± 2.19 X 10^6/µL and 5.7 ± 1.9 X 10^6/µL respectively; P<0.001) along with Hb (10.9 ±2.35 and 11.0 ± 2.95; P<0.001) as compared to model group. The significantly decrease total WBC count was observed upon treatment with MEBM 400, EAELE 400 and AECF 400 (5.8± 2.19; 5.7± 1.20 and 6.53 ± 2.59

respectively; P<0.001) as compared to model group. No significant difference was observed in total RBC count and Hb levels with AECF 400 (6.62 ± 2.19 X 10^6/µL and Hb: 9.5± 1.38) and Tamoxifen (Total RBC count: 6.92 ± 1.19 X 10^6/µL and Hb: 9.9± 0.98) treatment. However, significant difference in total WBC count (6.30±0.11 X 10^3/µL) was observed in Tamoxifen treated group as compared to model group.

5.12.6. Effect of MEBM, EAELE and AECF on cytokine levels in EAC induced solid tumors

In EAC injected model control animals, TNF- α (173± 1.12; P<0.001) and IL-6 (236.36± 1.45) were significantly (P<0.001) elevated as compared to normal control animals (TNF- α- 98± 0.69 and IL-6- 166.36± 0.98). MEBM 400 (TNF- α- 113± 0.78 and IL-6- 182.07± 1.01; P<0.001) and EAELE 400 (TNF- α- 143± 0.91; P<0.001 and IL-6- 212.5± 1.57; P<0.05) decreased elevated levels of cytokines as compared to model control animals, thereby inhibiting tumor progression. The significant decrease on cytokine levels were observed in Tamoxifen treated group (TNF- α- 113± 0.91 and IL-6- 203.14± 1.7) when compared to model control. The non-significant change was observed after AECF 400 (TNF- α- 162± 1.19 and IL-6- 224.14± 1.22) treatment when compared to model control animals. (Figure 5.58)

5.12.7. Effect of MEBM, EAELE and AECF on Lysosomal enzyme levels (β-Glucosidase) in EAC induced solid tumors

The activity of lysosomal enzyme in tissue homogenate is significantly elevated in model control (48.12± 1.34; P < 0.001) animals when compared with normal control animals (12.43± 0.57). In Tamoxifen (12.14± 0.83; P<0.001), MEBM 400 (29.95± 0.59; P<0.001), EAELE 400 (37.67± 1.01; P<0.01) and AECF 400 (42.33± 1.2; P < 0.05) treated groups, β-glucosidase levels were significantly

decreased when compared with EAC injected model control animals. (Figure 5.59)

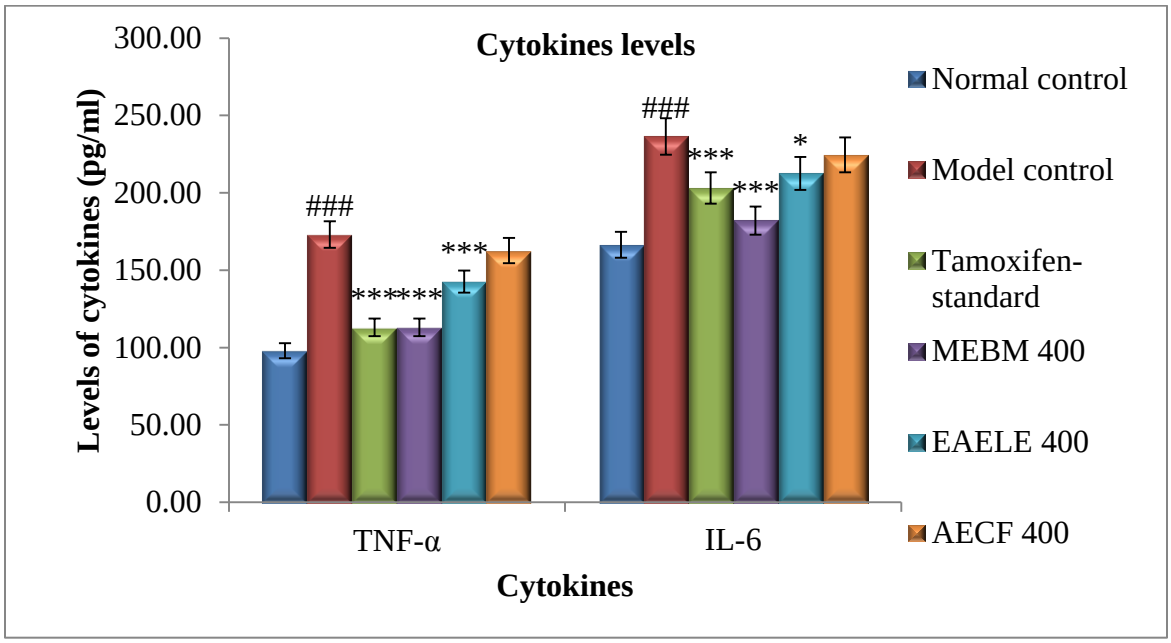


Figure 5.58: Effect of MEBM, EAELE and AECF on cytokines levels in EAC induced solid tumors

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

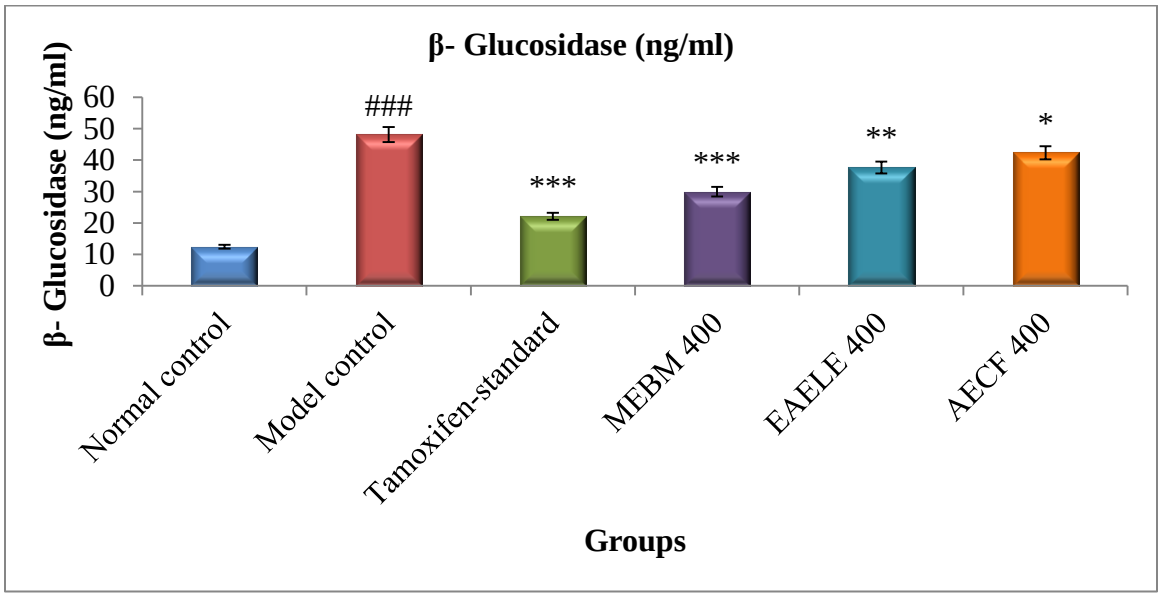


Figure 5.59: Effect of MEBM, EAELE and AECF on lysosomal enzyme (β-Glucosidase) levels in EAC induced solid tumors

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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05; **P<0.01; ***P<0.001).

5.12.8. Effect of MEBM, EAELE and AECF on Vascular endothelial growth factor (VEGF) levels in EAC induced solid tumors

The VEGF levels in breast tissues was significantly increased in EAC injected model control animals (85.30± 1.57; P < 0.001) when compared with normal control animals (34.72± 0.39). In Tamoxifen (45.16± 0.83; P<0.001), MEBM 400 (50.67± 0.54; P<0.001), EAELE 400 (53.57± 0.91; P<0.001) and AECF 400 (54.87± 0.63; P<0.001) treated groups, VEGF was significantly decreased as compared to model control. (Figure 5.60)

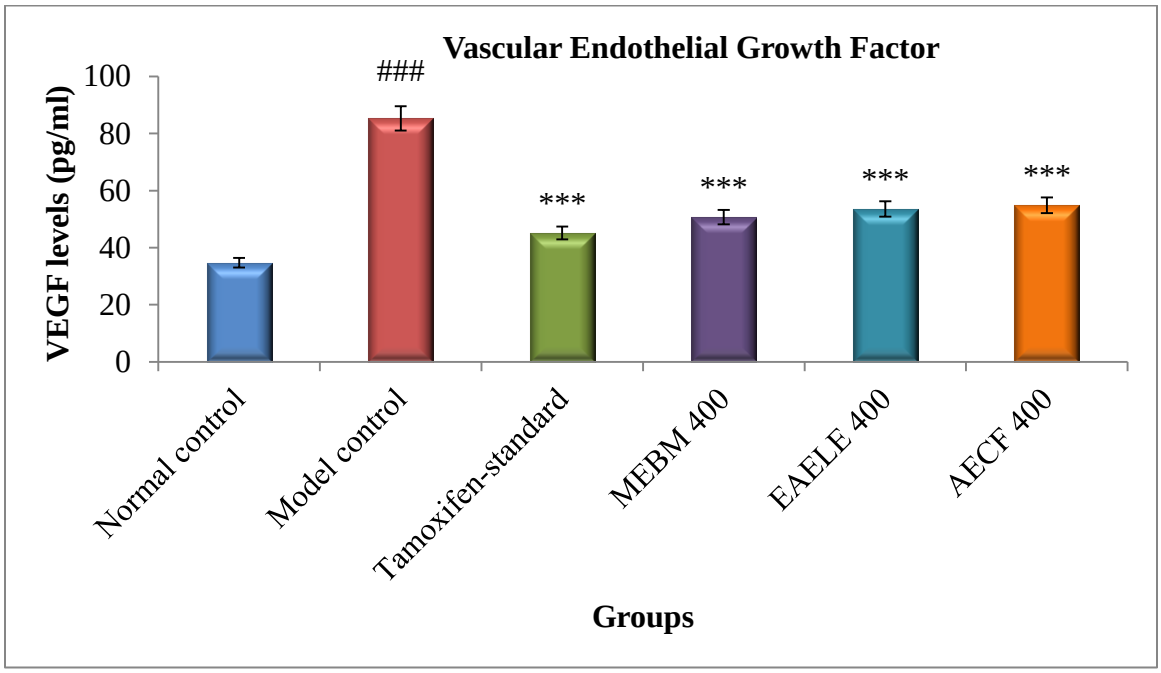


Figure 5.60: Effect of MEBM, EAELE and AECF on VEGF levels in EAC induced solid tumors

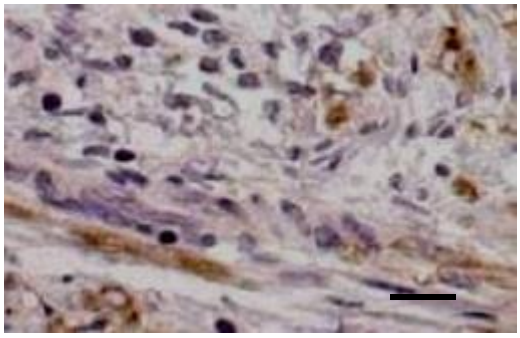
Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using one way ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001).

5.12.9. Effect of MEBM, EAELE and AECF on p53 gene expression (immunohistochemistry) in EAC induced solid tumors

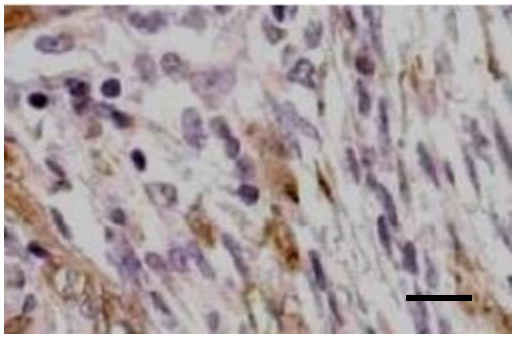
Immunohistochemical analysis of mammary tumor tissue in EAC injected untreated mice revealed significantly lower amount of p53 gene when compared to normal control animals. On the other hand, the expression of p53 gene was found to be significantly higher in MEBM 400, EAELE 400, AECF 400 and Tamoxifen treated animals, when compared to EAC injected untreated mice.

The % stained cells (brown colored stained receptor) in model control were found to be 25% ± 1.03 when compared to normal breast tissue (45%± 1.48). The % stained cells for MEBM 400 and Tamoxifen were found to be 35% ±

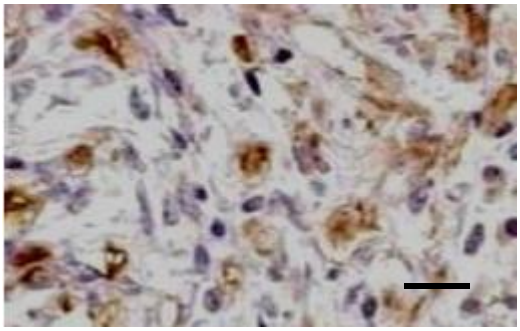
1.86 and 34% ± 1.56 respectively. The % stained cells for EAELE 400 and AECF 400 were found to be 30% ± 1.93 and 30% ± 1.91 respectively. (Figure 5.61)



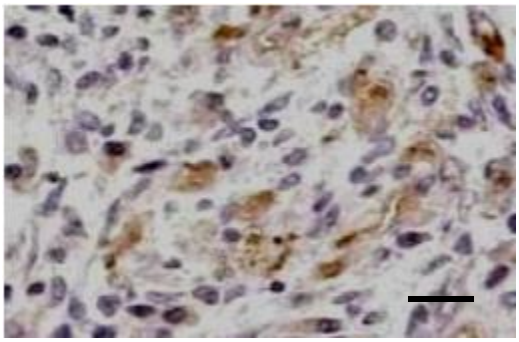
Normal Control



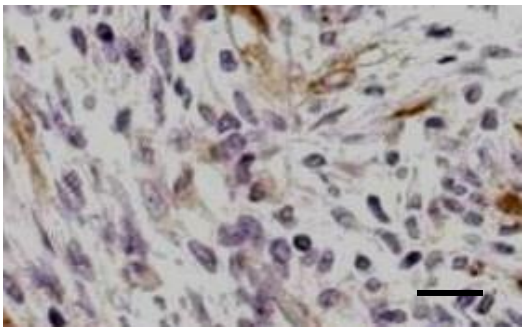
Model Control



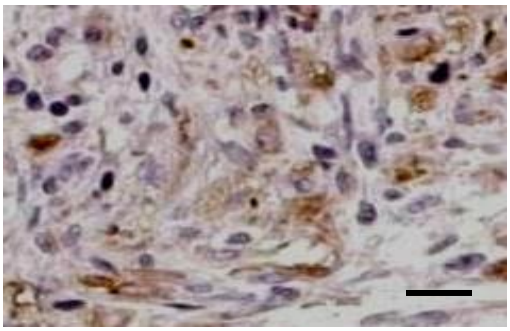
Tamoxifen-standard



MEBM 400



EAELE 400



AECF 400

Figure 5.61: Effect of MEBM, EAELE and AECF on p53 Gene expressions in EAC induced solid tumors

The photographs are taken in Nikon Eclipse TS100. Magnification: 40x. Scale: 100µm

5.12.10. Effect of MEBM, EAELE and AECF on CASPASE-9 in EAC induced solid tumors

The levels of Caspase-9 in tissues is significantly decreased in EAC injected model control animals (0.68± 0.35; P < 0.001) when compared with normal control animals (3.11± 1.10). In Tamoxifen (2.18± 0.87; P < 0.001); MEBM 400 (2.00± 0.95; P < 0.001), EAELE 400 (1.85± 0.79; P < 0.001) and AECF 400 (1.54± 0.65; P < 0.05) treated groups, levels of Caspase-9 were significantly increased when compared with model control animals. (Figure 5.62)

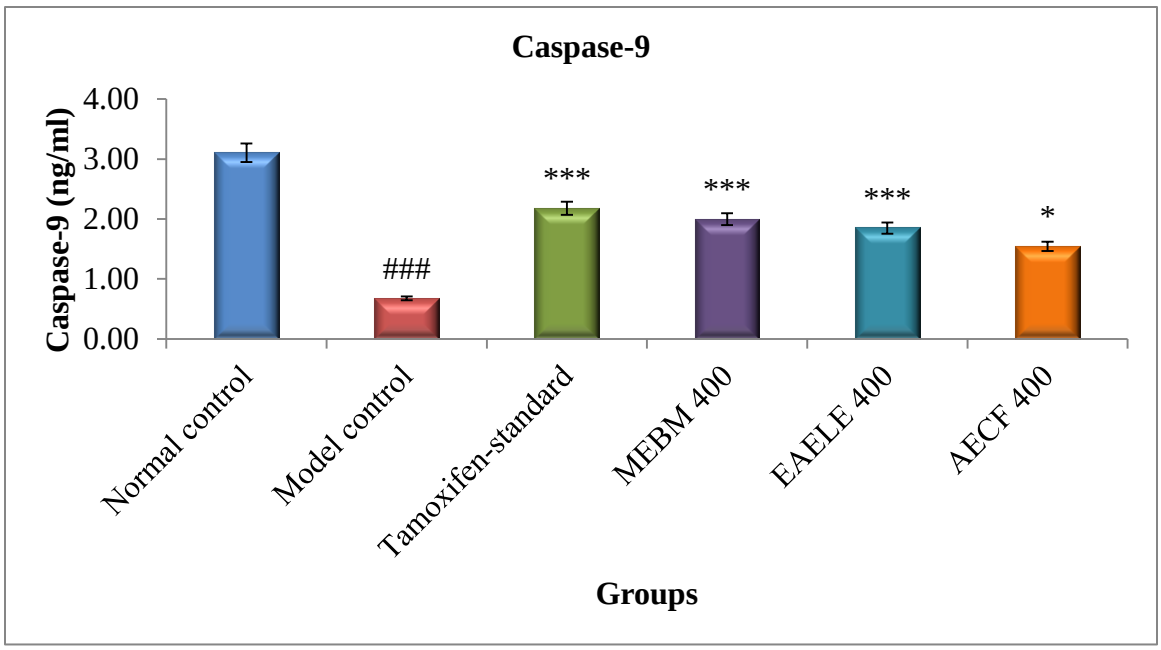


Figure 5.62: Effect of MEBM, EAELE and AECF on Caspase-9 in EAC induced solid tumors

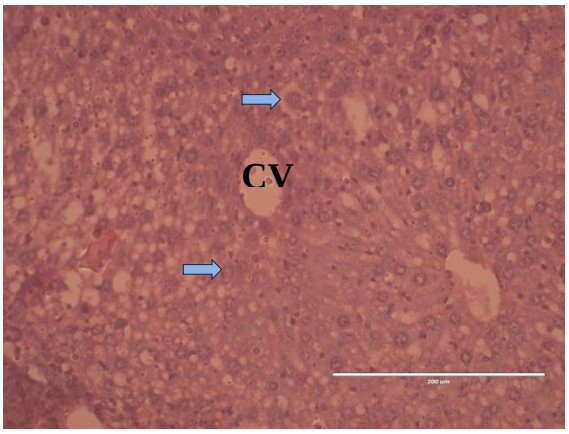
Each bar represents Mean ± SEM of 6 animals. Values are statistically evaluated using one way ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05, ***P<0.001).

5.12.11. Effect of MEBM, EAELE and AECF on histopathology of organs in EAC induced solid tumors

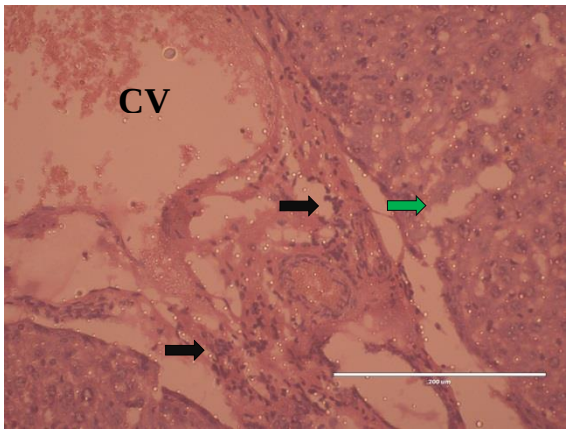
The control liver shows normal structure of hepatocytes and central vein. Enormous changes were seen in liver of model control mice. Enlarged and congested central vein, infiltration of tumor cells mixed with leukocytes and fatty degeneration of hepatic tissue was observed. After treatment with MEBM 400, normal architecture was seen. No leukocytes were observed. After treatment with EAELE 400, the central vein was found to be normal but fatty degeneration was observed. No improvement was seen after AECF 400 treatment. Tumor cells were observed. Treatment with Tamoxifen showed enlarged central vein. (Figure 5.63)

The control kidney shows normal histological features. The tumor bearing untreated animals shows congested renal vein, degenerated renal tubule and narrow glomerular space. The kidney of MEBM 400, EAELE 400 and Tamoxifen treated animals showed normal glomeruli and renal tubule. No improvement was seen after AECF 400 treatment. Tumor cells were observed. (Figure 5.64)

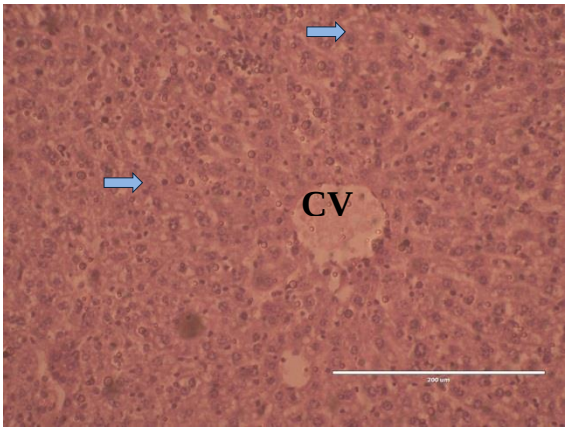
Figure 5.65 shows tumor cells and migration of tumor cells by invading cell membrane of breast.



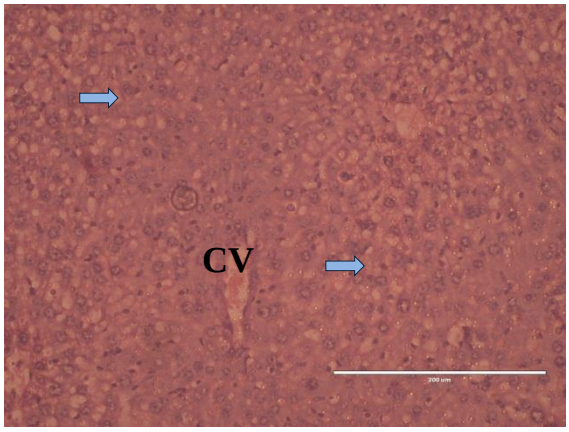
a) Normal Control- Liver



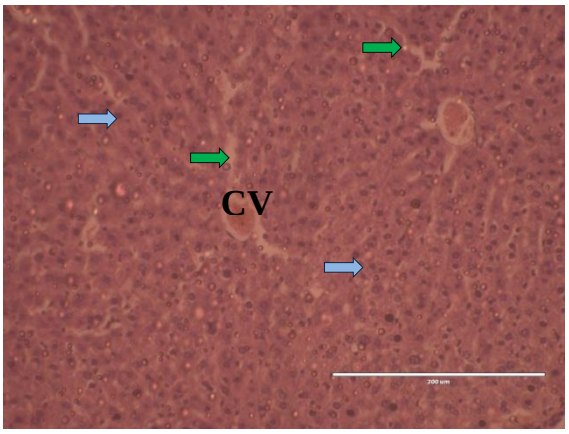
b) Model Control-Liver



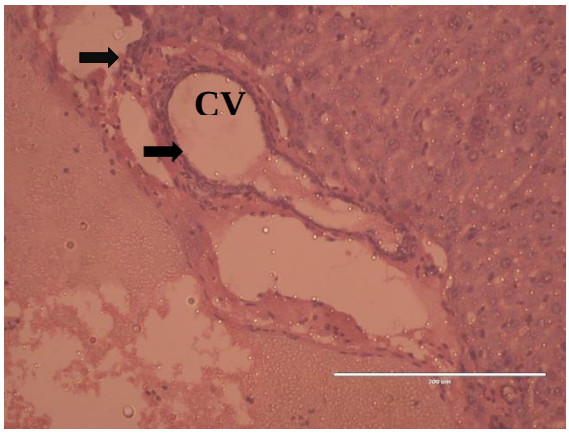
c) Tamoxifen- Liver



d) MEBM-Liver



e) EAELE 400- Liver

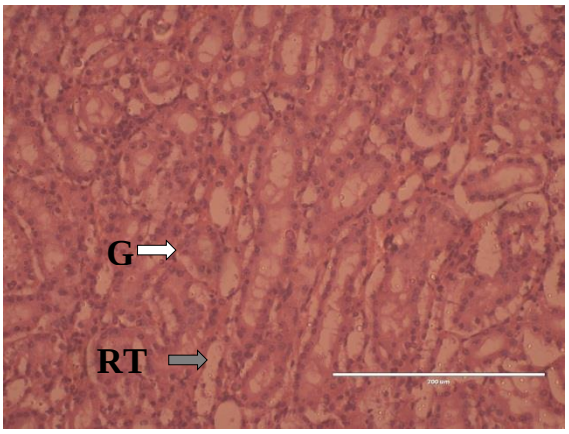


f) AECF 400- Liver

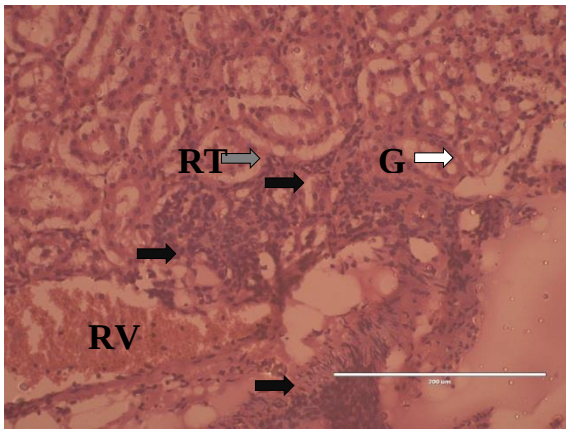
Figure 5.63: Effect of MEBM, EAELE and AECF on histopathology of liver and kidney in EAC induced solid tumors

a) normal liver: normal structure of hepatocytes and central vein; b) model control liver: Enlarged and congested central vein; fatty degeneration; tumor cells c) Tamoxifen treated liver: enlarged central vein; d) MEBM treated liver: normal heaptocytes and central vein. e) EAELE treated liver: normal architecture with fatty degeneration f) AECF treated liver: enlarged central vein; tumor cells

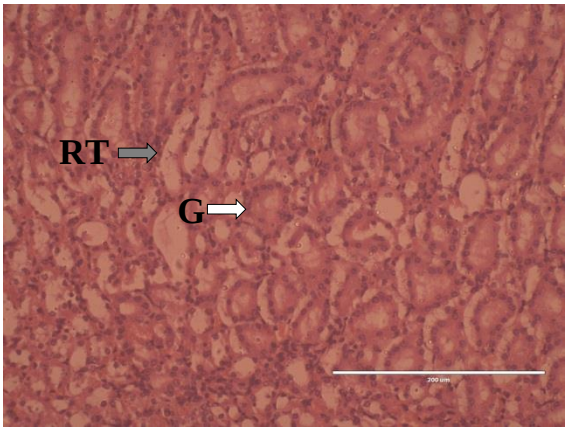
⇨: hepatocytes; ➡:fatty degeneration; ➡ :tumor cells; CV: Central vein. Magnification: 40x; Scale bar 200µm



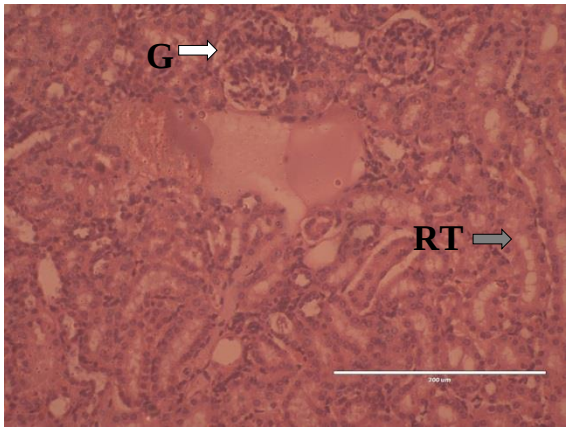
a) Normal- Kidney



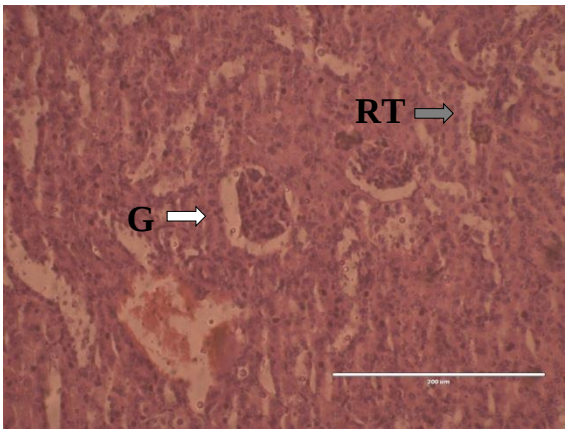
b) Model- Kidney



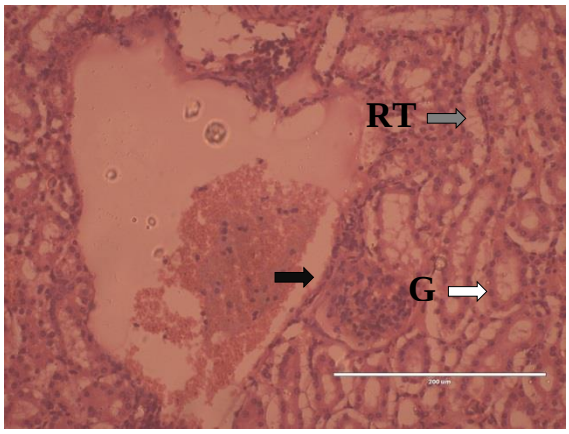
c) Tamoxifen –Kidney



d) MEBM- Kidney



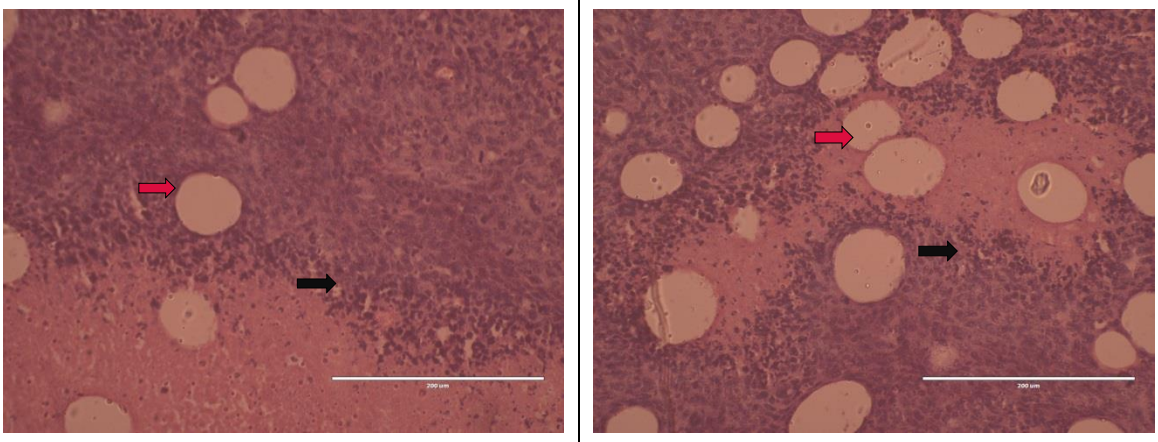
e) EAELE- Kidney



f) AECF-Kidney

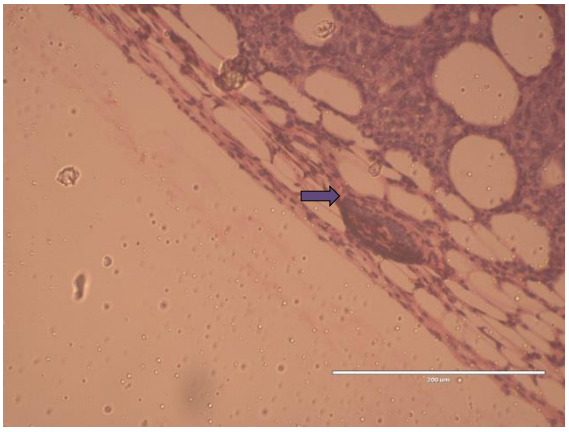
Figure 5.64: Effect of MEBM, EAELE and AECF on histopathology of kidney in EAC induced solid tumors

a) normal kidney: normal structure of glomeruli and renal tubule b) model control kidney: congested renal vein, degenerated renal tubule and narrow glomerular space; tumor cells c) Tamoxifen treated kidney: normal glomeruli and renal tubule d) MEBM treated kidney: normal glomeruli and renal tubule e) EAELE treated kidney: normal glomeruli and renal tubule f) AECF treated kidney: degenerated renal tubule; tumor cells ➡ :tumor cells; ⇨: Glomeruli (G) ➡: renal tubule (RT); RV: renal vein. Magnification: 40x; Scale bar 200µm

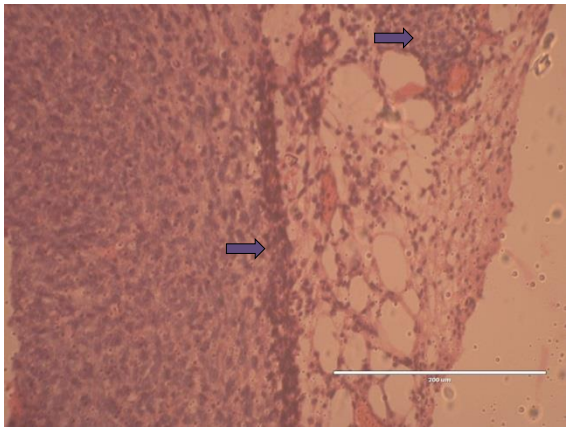


a) Tumor tissue

b) Tumor tissue



c) Tumor tissue



d) Tumor tissue

Figure 5.65: Effect of MEBM, EAELE and AECF on histopathology of liver and kidney in EAC induced solid tumors

a) Tumor tissue: milk ducts; tumor cells b) Tumor tissue: milk ducts; normal pink colored tissues surrounded by tumor cells c) Tumor tissue: Invading extracellular membrane d) Tumor tissue: Migration of tumor cells

➡ :tumor cells; ➡ : milk ducts; ➡ : migration of tumor cells. Magnification: 40x; Scale bar 200µm

SECTION 4

COMPARISON OF ANTICANCER PROFICIENCY OF SELECTED POTENT EXTRACTS OF MEDICINAL PLANTS

5.13. Preventive studies in MNU induced mammary carcinogenesis

Taking into account, the tumor parameters, receptor expressions and nucleic acid content, the anticancer potential of extracts is in following sequence: MEBM > EAELE> AECF.

The tumor burden i.e. total number of tumor in MEBM 400 was 2 which was less than Tamoxifen treated animals. The tumor burden in EAELE 400 was 5 which were same as Tamoxifen. In case of AECF 400 it was found to be 6 which were same as model control animals. The reduction in tumor weight in MEBM 400, EAELE 400 and AECF 400 was found to be 87.89%, 75.89% and 58.93%. (Figure 5.66) The latency period in MEBM 400 (82.50±1.44; $P<0.001$) and EAELE 400 (77.40±1.18; $P<0.001$) was non-significantly different from Tamoxifen. The lag period in tumor generation in AECF 400 treated groups was 72.83±2.32, which was significantly different from Tamoxifen treated animals. ($P<0.05$) (Figure 5.67)

The nucleic acid levels in MEBM 400 and EAELE 400 was non-significantly different from Tamoxifen. The significant difference was observed in AECF 400 treated animals when compared to rest of the treatment groups. (Figure 5.68)

The reduction in receptor (estrogen and progesterone) expressions by MEBM 400 treated group was non-significantly different from Tamoxifen. The outcomes of EAELE 400 and AECF 400 groups suggests less reduction in hormone expression levels as compared to Tamoxifen group suggesting less anti-estrogenic potential as compared to MEBM 400 and Tamoxifen. (Figure 5.69)

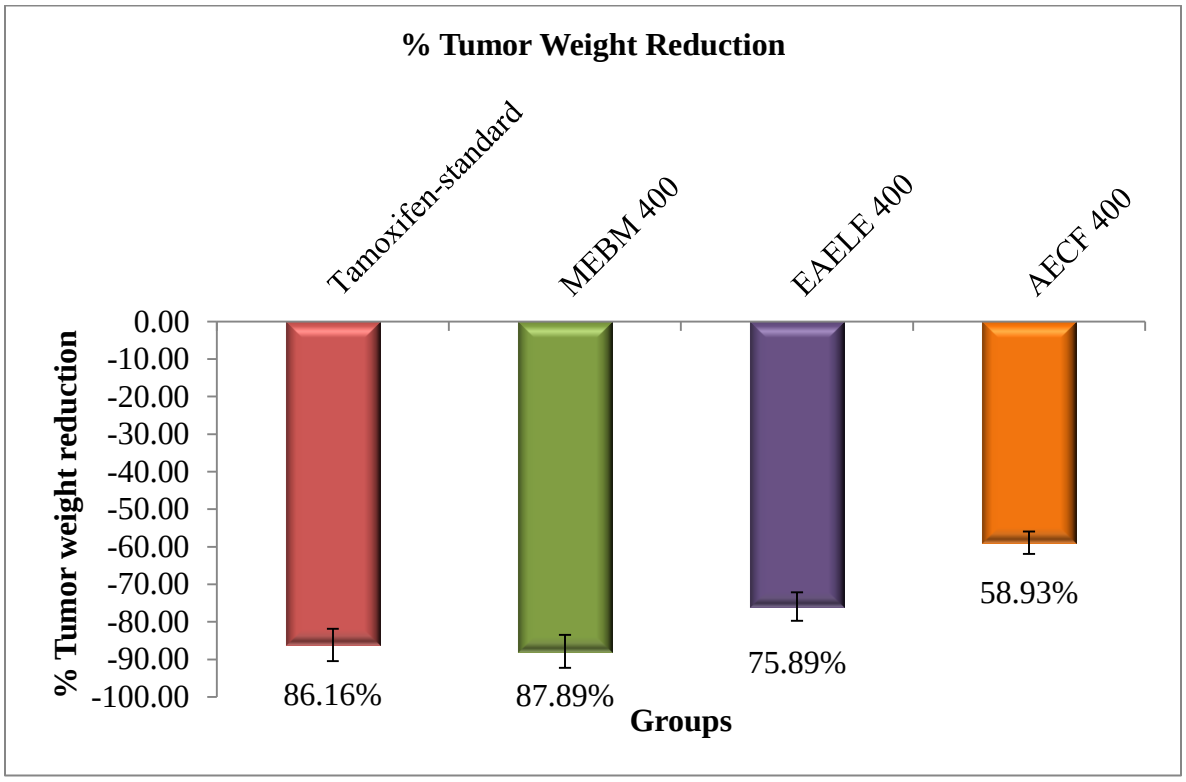


Figure 5.66: Effect of extracts on % tumor weight reduction in MNU induced mammary carcinogenesis

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using one way ANOVA analysis followed by Bonferroni's Post hoc test.

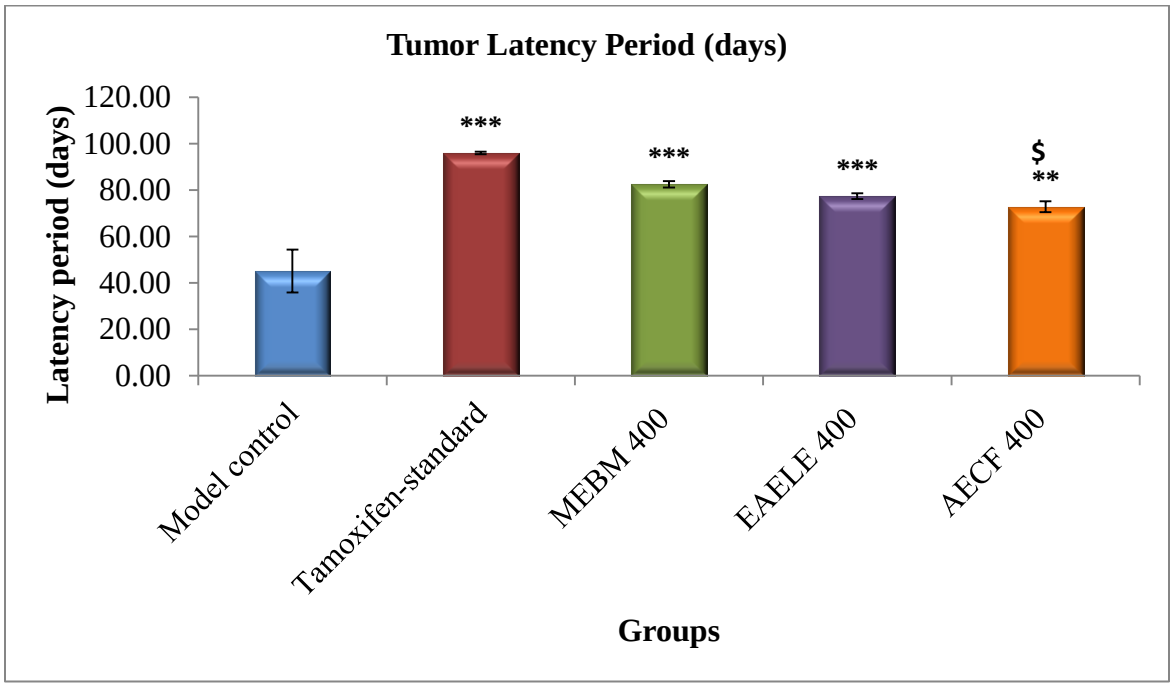


Figure 5.67: Effect of extracts on latency period in MNU induced mammary carcinogenesis

Values are expressed as 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 model control vs. all other groups (***P<0.001); extract treated groups with Tamoxifen ($: P<0.05).

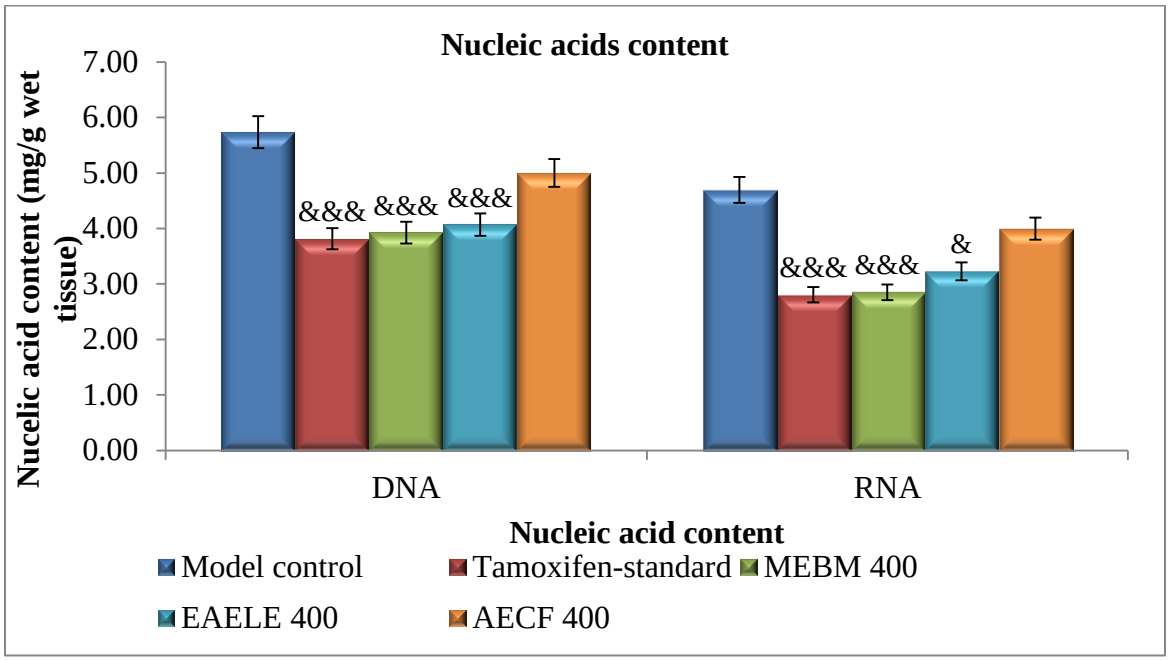


Figure 5.68: Effect of extracts on nucleic acid content in MNU induced mammary carcinogenesis

Values are expressed as 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 AECF vs. other treatment groups (& P<0.05, &&& P<0.001).

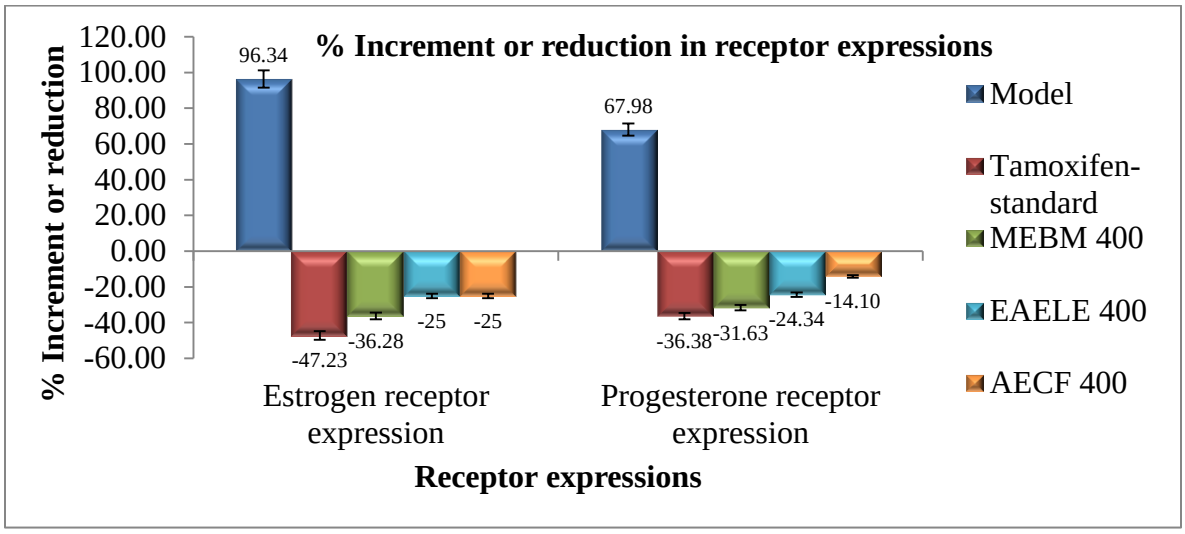


Figure 5.69: Effect of extracts on % increment or reduction in estrogen and progesterone in MNU induced mammary carcinogenesis

The % increment in model control is with respect to normal control animals. The % reduction in treated group is with respect to model control animals.

5.14. *In-vitro* mechanistic assays

In Chick Chorioallantoic Membrane assay, the extracts (MEBM, EAELE and AECF) were found to be potent anti-angiogenic. The 3 ± 0.18 mm and 2.5± 0.45 mm zone of inhibition was seen in MEBM and EAELE treated membrane at concentration 20µg/ml. The 1± 0.75 mm zone of inhibition was observed in AECF at concentration 30µg/ml.

In Annexin V- FITC binding assay, MEBM, EAELE and AECF were perceived to be highly apoptotic (16.3%, 13.7% and 10.5% respectively). (Figure 5.70)

In scratch motility assay, MEBM drastically decreased cell motility. The % inhibition in motility was found to be 69.26± 1.76%. In EAELE treated wells, the % inhibition in wound closure was 67.04± 2.03%. The lowest inhibition in MCF-7 cell migration was shown by AECF (61.11± 1.15%) which was significantly different ($P<0.05$) from MEBM. (Figure 5.71)

These results are a further indication that MEBM possess properties which can halt cancer cells to proliferate via holding back angiogenesis, increasing apoptosis and hindering their motility. The EAELE also own anti-angiogenic potential with apoptosis instigating quality and anti-migratory capacity in MCF-7 breast cancer cells. The AECF also claim anti-angiogenic and apoptotic potential but lack migratory potential as compared to MEBM.

As no extract induced oxidative stress in DCFH assay, the anti-proliferative and apoptotic effect is not due to generation of reactive oxygen species.

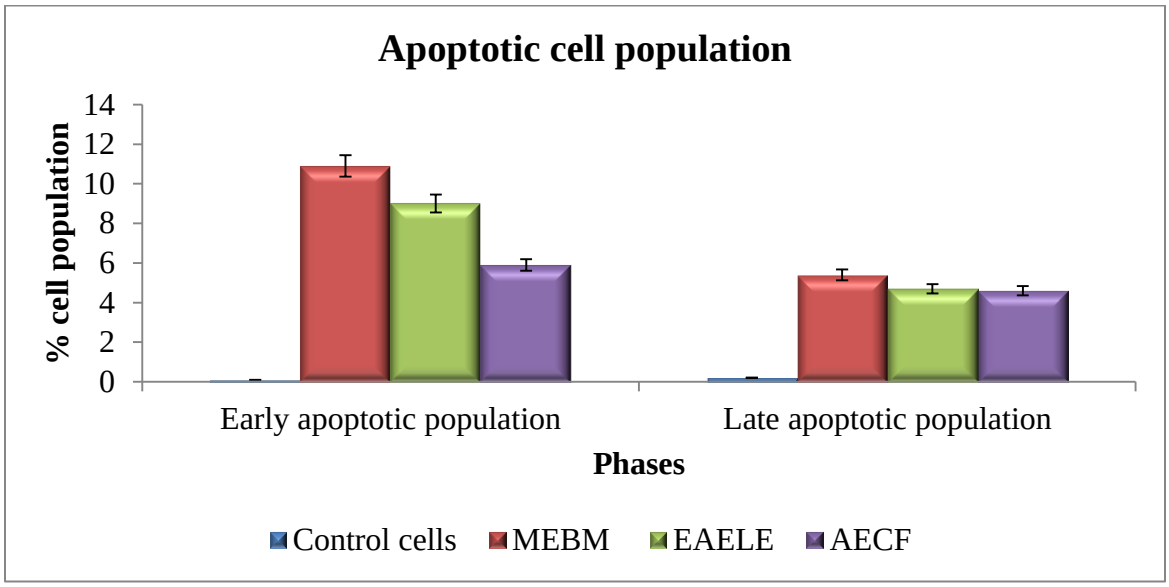


Figure 5.70: Effect of extracts on the apoptosis on MCF-7 human breast cancer cell line

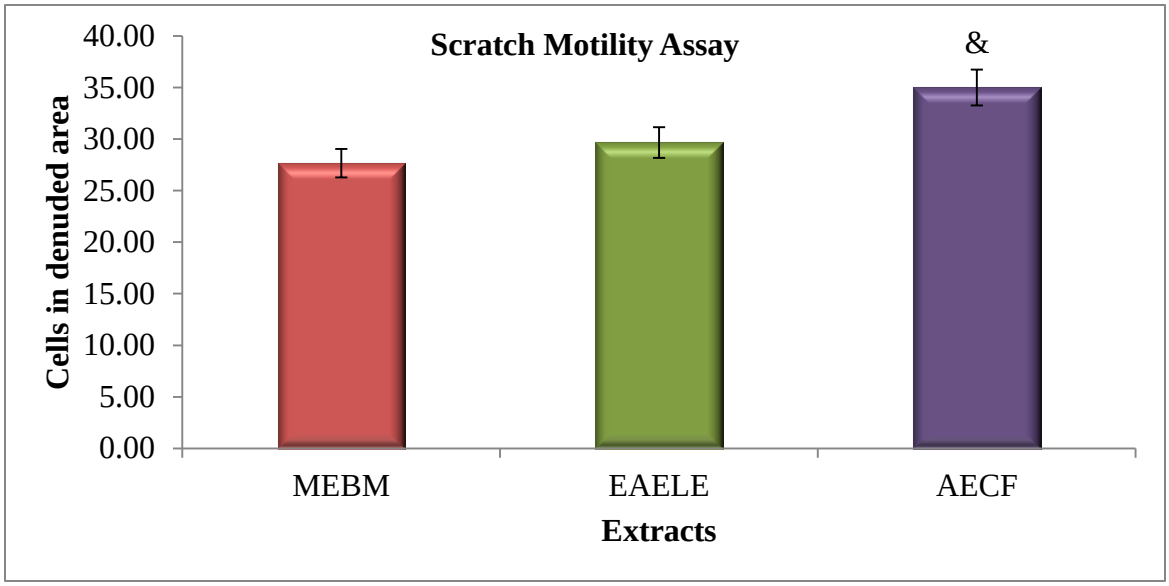


Figure 5.71: Effect of extracts on the cell migration (metastasis) on MCF-7 human breast cancer cell line.

Values are expressed as Mean ± SEM of 3 animals. Values are statistically evaluated using one way ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with AECF vs. other treatment groups (& $P<0.05$).

5.15. Curative studies in EAC induced solid mammary cancer

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 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. (Figure 5.60)

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. (Figure 5.62)

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. However, no difference was observed with EAELE and Tamoxifen. No significant difference was observed in AECF treated animals and model control. (Figure 5.72) 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. (Figure 5.73) The lysosomal enzyme level was significantly ($P<0.01$) higher in AECF as compared to MEBM. (Figure 5.74) 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.

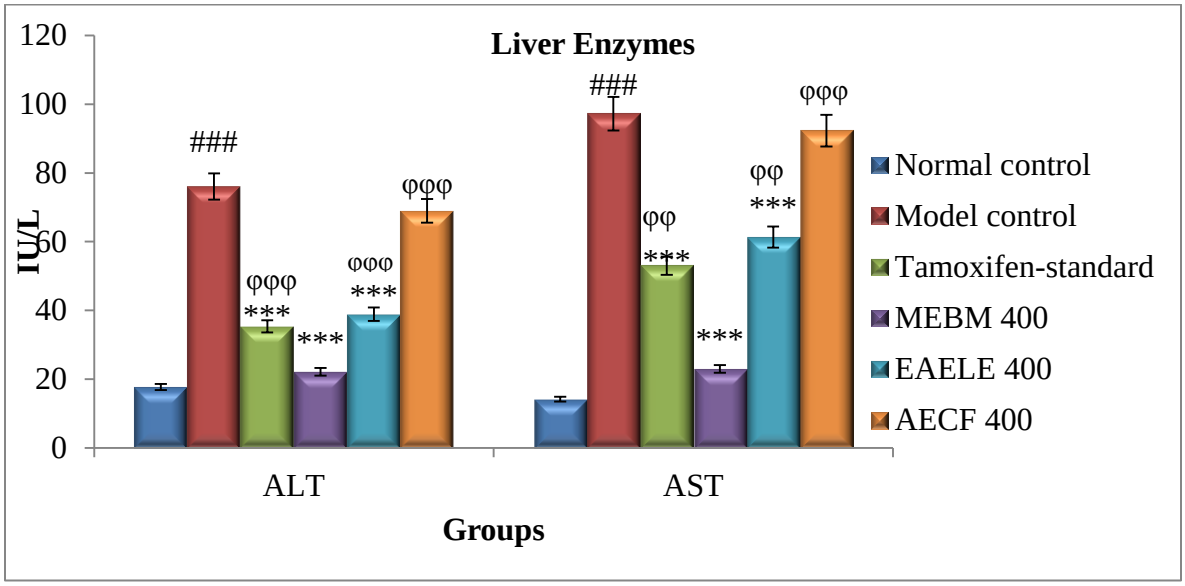


Figure 5.72: Effect of extracts on liver enzyme levels in EAC induced solid tumors

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001); MEBM vs. all other treatment groups (φφφ P<0.001)

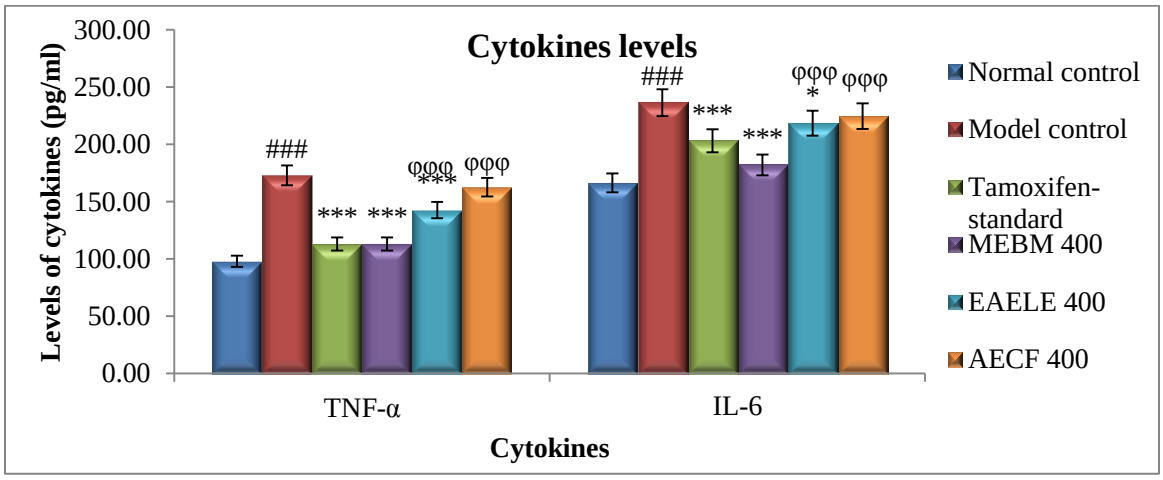


Figure 5.73: Effect of extracts on cytokine levels in EAC induced solid tumors

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 normal control vs. model control (###P<0.001); model control vs. all other groups (***P<0.001); MEBM vs. all other treatment groups (φφφ P<0.001)

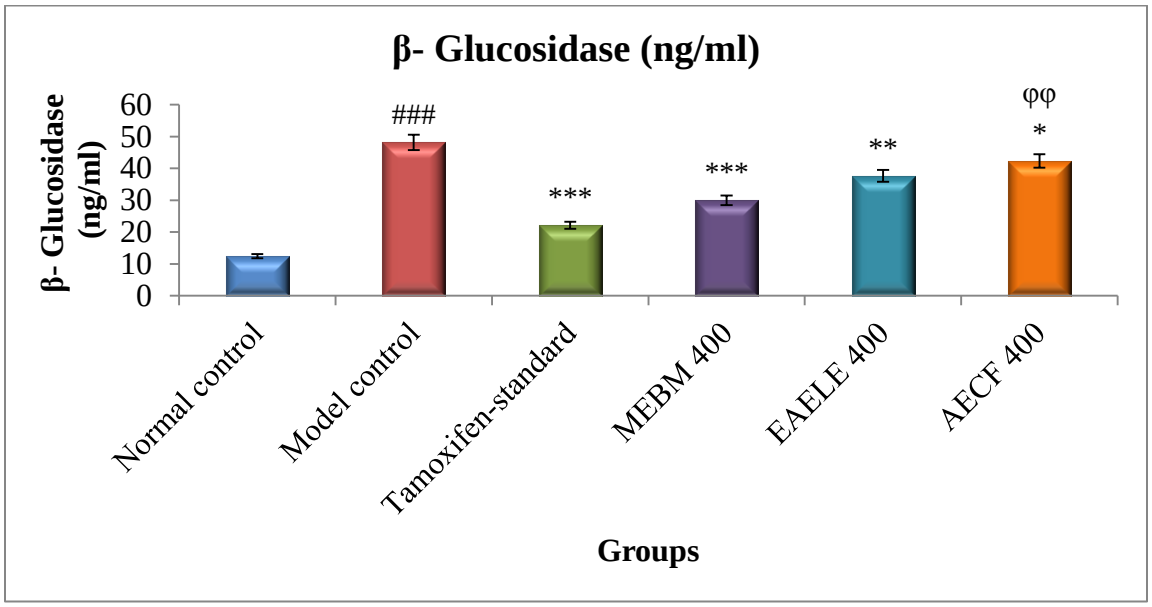


Figure 5.74: Effect of extracts on β- Glucosidase levels in EAC induced solid tumors

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 normal control vs. model control (###P<0.001); model control vs. all other groups (*P<0.05, **P<0.01, ***P<0.001); MEBM vs. all other treatment groups (φφ P<0.01).