IV. RESULTS

The results pertaining to the study entitled **"Evaluation of the anticancer properties of *Acorus calamus* L. rhizome using *in vitro, in vivo* and *in silico* models"** are presented under the following headings:

4.1 Preliminary phytochemical studies on *A. calamus*

The results of physical screening of *A. calamus was* shown in Table I.

Table I. Preliminary phytochemical studies of MEAC

A. calamus rhizome		
Physical characters	Colour	Light brown
	Odour	Sweet aromatic
	Taste	Bitter
	Nature	Rough
Moisture content	Loss on drying	93.5% w/w
Ash values	Total ash value	6.015 w/w
	Acid insoluble ash	0.515 w/w
	Water soluble ash	3.5 w/w

The percentage yield of methanolic extract of *A. calamus* (MEAC) was found to be 5.00 % w/w (Table II).

Table II. Extractive values of *A. calamus*

Solvent	Empty weight of petri plate	Weight of petri plate + residue	Actual weight of residue	
			Extraction volume	% yield
Petroleum ether	52.3	52.67	0.37	3.7%
Chloroform	45.6	45.92	0.32	3.2%
Ethanol	53.1	53.43	0.33	3.3%
Methanol	56.0	56.5	0.5	5%
Water	46.7	46.9	0.2	2%

4.2 Phytochemical analysis of the methanolic extract of *A. calamus*

Qualitative chemical analysis of the plant extract provided the information regarding various types of phytoconstituents like alkaloids, terpenoids, steroids, carbohydrate, saponin, flavonoids etc. Table III showed the presence and absence of various phytoconstituents.

Table III. Preliminary phytochemical examination of MEAC

Phytochemicals	MEAC
Alkaloids	+
Carbohydrate	+
Steroid	-
Flavonoids	+
Phenols	+
Saponins	+
Amino acid	+
Terpenoids	+
Tannin	+

\+ Presence - Absence

Knowing the phytochemical potential of MEAC, the studies were carried out *in vitro* and *in* vivo in DAL cells.

4.3 *In vitro* cytotoxic studies of MEAC on DAL cells

Cytotoxicity studies were carried out in DAL cells by Trypan blue exclusion method showed a reduction in the viable cell count in MEAC treated groups as compared to the control group. Percentage growth of inhibition was found to be increased in a dose dependent manner. Maximum growth of inhibition was shown by 1000 μg/ml and minimum by 62.5 μg/ml. 50% growth inhibition (CTC_{50}) was found to be 440 μg/ml (Table IV and Fig.8).

Table IV. *In vitro* cytotoxicity of *A. calamus* on DAL cells

Test drug	Test concentration (μg/ml)	Viable cell (number)	% growth inhibition	CTC_{50} (μg/ml)
MEAC	1000	16	79.48	440.00
	500	57	55.46	
	250	91	32.08	
	125	100	22.48	
	62.5	66	8.33	
Control	-	-	6.93	

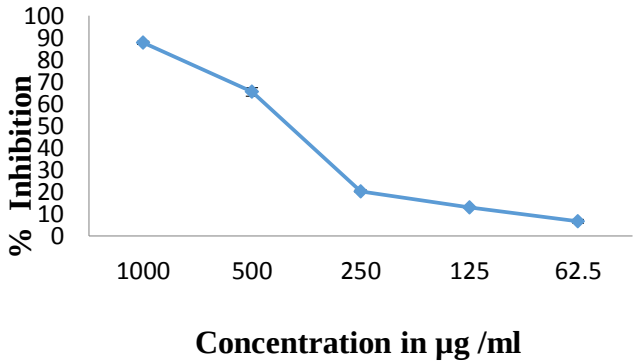


Fig. 8. Cytotoxic effect of MEAC on DAL

4.4 Acute toxicity study of MEAC

The results of acute toxicity study of MEAC are presented in Table V. No mortality or change in body weight was observed in rats at a dose level of MEAC 50 mg/kg and 500 mg/kg body weight. Some clinical signs such as tremors, pilo erection and abdominal breathing were observed after the oral dosing of 1000 and 2000 mg/kg but no mortality or change in body weight was observed.

These observations indicated that the calculated LD_{50} value (Dixons likelihood method) for the oral doses of the MEAC was found to be more than 2000 mg/kg body weight, accordingly 100 and 200 mg/kg body weight were taken as low and high dose of MEAC for the experiment.

Table V. Clinical signs of toxicity observed during acute oral toxicity study of MEAC

Dose of MEAC (mg/kg b.wt)	Latency	Symptoms
50	-	None
500	-	None
1000	-	Piloerection, abdominal breathing
1500	-	Tremor, piloerection, abdominal breathing
2000	-	Tremor, piloerection, abdominal breathing

Latency – Time of death after the dose.

4.5 Effect of MEAC on tumor growth parameters

The effect of MEAC on tumor growth parameters is presented in Table VI.

Table VI. Effect of MEAC on tumor growth parameters

Parameters	Mean survival time (Days)	Increased life span (%)	Tumor volume (ml)	Tumor PCV (ml)	Viable cell count (10^6 cells/ml)	Non-viable cell count (10^6 cells / ml)
Normal control	-	-	-	-	-	-
DAL control	22.00 ± 0.00	-	11.33 ± 0.01	6.46 ± 0.01	0.84 ± 0.01	9.77 ± 0.01
DAL+Positive Control (5-FU. 10 mg/kg)	32.65 ± 0.02[b]	48.37 ± 0.05	5.43 ± 0.05[b]	3.66 ± 0.01[b]	5.78 ± 0.01[b]	2.34 ± 0.04[b]
DAL+MEAC 100mg/kg	25.66 ± 0.02[b]	16.48 ± 0.12	7.75 ± 0.03[b]	4.66 ± 0.15[b]	4.80 ± 0.05[b]	4.42 ± 0.01[b]
DAL+MEAC 200 mg/kg	28.83 ± 0.41[b]	31.32 ± 0.53	8.85 ± 0.02[b]	4.96 ± 0.01[b]	5.25 ± 0.03[b]	2.93 ± 0.01[b]
SEd	0.106	0.142	0.017	0.038	0.016	0.006
CD (p<0.01)	0.295	0.395	0.041	0.109	0.045	0.019

Values are expressed as mean ± SD of six samples in each group; a: p<0.01 normal control vs DAL control. b: p<0.01 DAL control vs treatment groups.

4.5.1 Effect of MEAC on Mean Survival Time (MST) of control and treatedgroups of *M. musculus*

The mean survival time increased to 25.66 ± 0.02 and 28.83 ± 0.41 on administration of 100 and 200 mg/kg of MEAC respectively when compared with DAL control group (Table VI and Fig. 9).

4.5.2 Effect of MEAC on Increase in Life Span (ILS) of control and treated groups of *M. musculus*

When compared with the DAL control mice, positive control (48.37%) and treated groups MEAC 100 and 200 mg/kg showed a reduction in the percentage increase in life span (16.48% and 31.32%). Between the treated groups, 100 mg/kg dose showed lower percentage increase in life span (Table VI and Fig.9).

4.5.3 Effect of MEAC on tumor volume of control and treated groups of *M. musculus*

When compared with DAL control mice (11.33 ± 0.01 ml), all treated mice showed significant reduction in the tumor volume. Maximum reduction was found in positive control (5.43 ± 0.05; $P < 0.01$) followed by lower dose of MEAC 100 mg/kg (7.75 ±0.03 ml) (Table VI and Fig.10).

4.5.4 Effect of MEAC on tumor PCV of control and treated groups of *M. musculus*

In DAL control mice the tumor PCV was found to be 6.46 ± 0.01 ml which was reduced in positive control (3.66 ± 0.01 ml; $p < 0.01$). The data pertaining to treatment groups (MEAC 100 and 200 mg/kg) was found to be significant ($P < 0.01$) (Table VI and Fig.10).

4.5.5 Effect of MEAC on viable cell count of control and treated groups of *M. musculus*

When compared with DAL control mice, the positive control group showed a significant increase in viable cell count (5.78 ± 0.01). On administration of MEAC 200 mg/kg dose showed similar results (5.25 ± 0.03) (Table VI and Fig.11).

4.5.6 Effect of MEAC on non-viable cell count of control and treated groups of *M. musculus*

There was an increase in the level of non-viable cell count in DAL control mice when compared with MEAC treatment groups. Both the treatment groups, MEAC 100 and 200 mg/kg showed significant results when compared with DAL control mice (4.42 ± 0.01 and 2.93±0.01; $p < 0.01$). (Table VI and Fig.11).

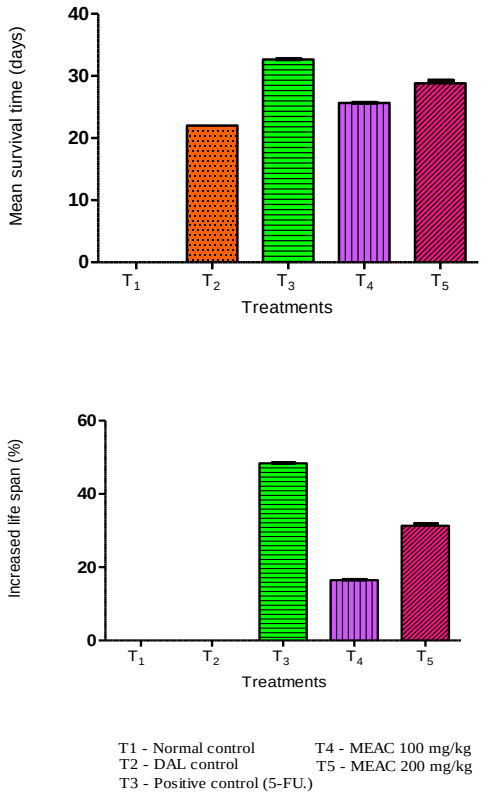


Fig.9. Effect of MEAC on mean survival time and increased life span of control and treated groups of *M. musculus*

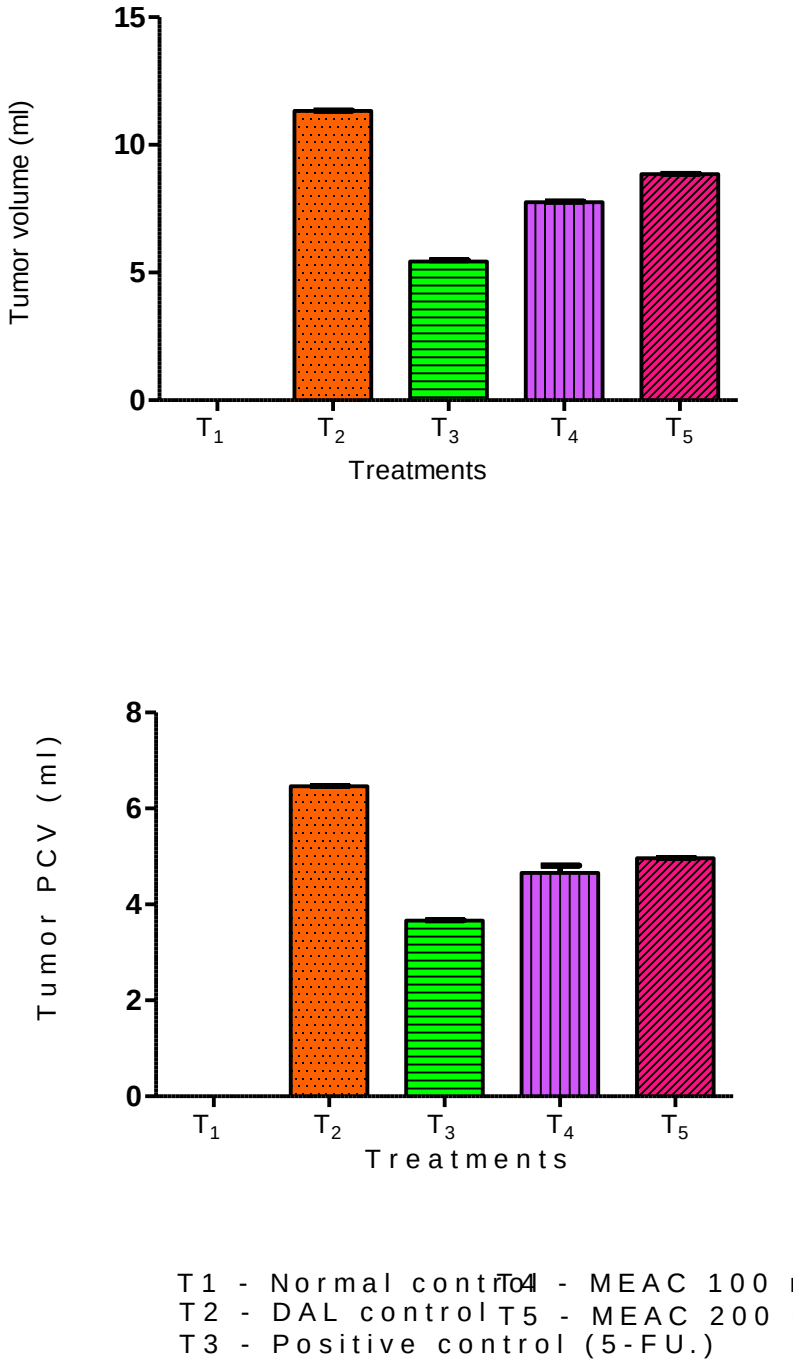


Fig.10. Effect of MEAC on tumor volume and tumor packed cell volume of control and treated groups of *M. musculus*

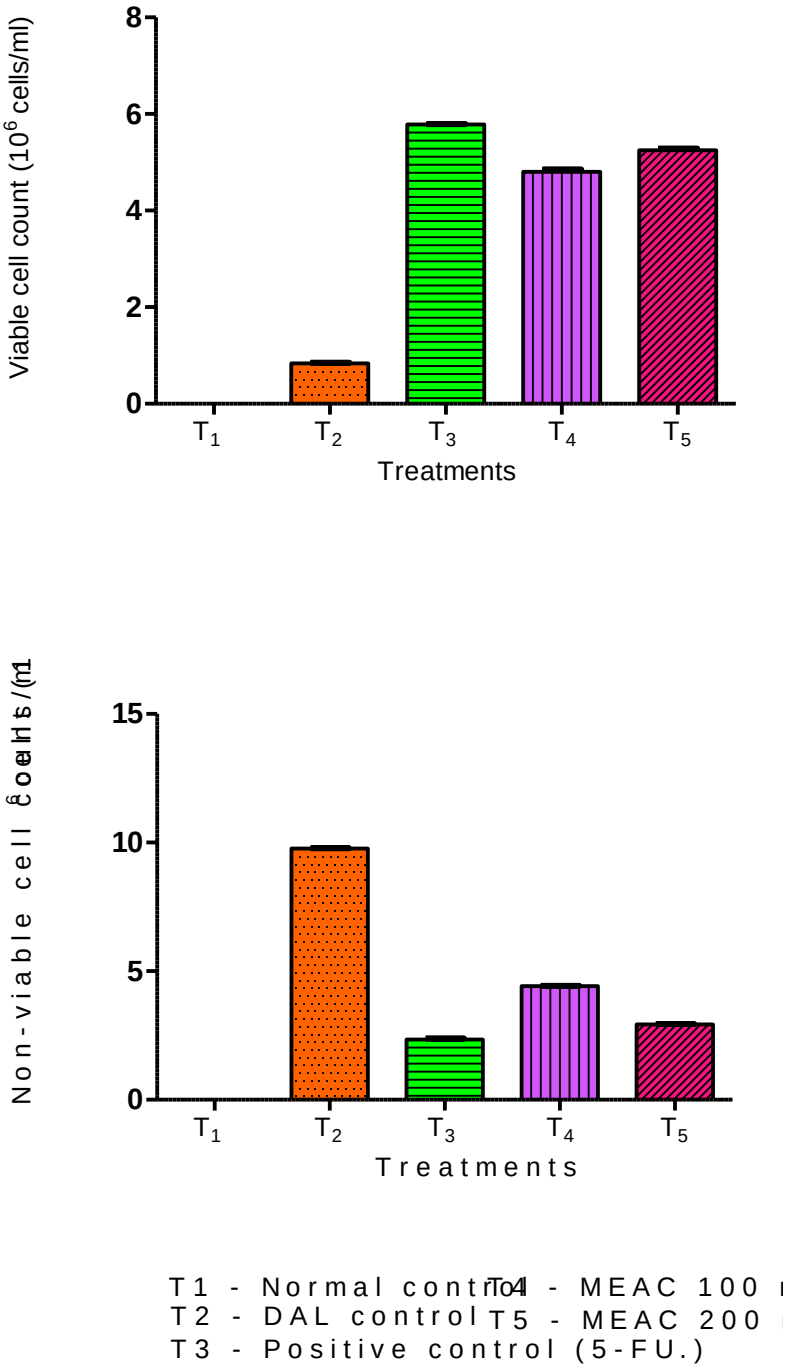


Fig.11. Effect of MEAC on viable and non-viable cell count of control and treated groups of *M. musculus*

4.6 Body weight analysis

The effect of MEAC on bodyweight analysis is presented in Table VII.

Table VII. Effect of MEAC on body weight analysis of treated groups of *M. musculus*

Experimental group	Before induction	After induction	
		On 11th day	On 20th day
Normal control	21.00± 0.63	-	-
DAL control	22.00 ± 0.63	26.17 ± 0.75	31.33 ± 1.03
DAL+Positive control (5-FU. 10mg/kg)	21.50 ± 0.55	24.83 ± 0.75	22.00 ± 1.26
DAL+MEAC (100mg/kg)	22.00 ± 0.63	25.83 ± 0.75	22.83 ± 0.177
DAL+MEAC (200mg/kg)	21.17 ± 0.75	26.70 ± 0.16	22.17 ± 0.75

Values are expressed as mean ± S.E.M.

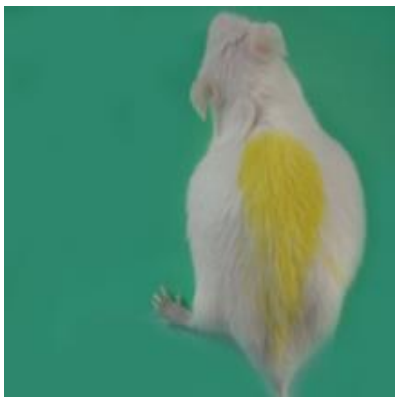
Throughout the experimental period all the animals gained body weight. After tumor induction, at the end of 11th day, when compared with tumor bearing group (26.17±0.75), the treatment groups (positive control and MEAC 100 mg/kg) showed decrease in body weight (24.83 ± 0.75 and 25.83 ± 0.75) respectively.

At the end of 20th day, when compared with DAL bearing mice (31.33± 1.03) all the treated groups showed a decrease in body weight (22.00 ± 1.26, 22.83 ± 0.177 and 22.17 ± 0.75) (Table VII and Fig.12).

DAL control

DAL+5FU.(10mg/kg)

DAL + MEAC (100 mg/kg)



DAL + MEAC (200 mg/kg)

Fig.12. Effect of MEAC on body weight analysis (20th day) of control and treated groups of *M. musculus*

4.7 Effect of MEAC on hematological parameters

The effect of MEAC on hematological parameters RBC, Hb, WBC and PCV are presented in Table VIII.

Table VIII. Effect of MEAC on hematological parameters

Parameters	RBC ($x10^6$ cells / µl)	Hemoglobin (gm/dl)	Total WBC ($x10^3$ cells / µl)	PCV (%)
Normal control	4.62 ± 0.07	14.64± 0.126	10.13 ± 0.03	20.74 ± 0.09
DAL control	2.47 ± 0.06[a]	7.68 ± 0.02[a]	23.09 ± 0.02[a]	30.27 ± 0.03[a]
DAL + Positive control (5-FU. 10 mg/kg)	3.85 ± 0.09[b]	12.61 ± 0.08[b]	11.89 ± 0.06[b]	26.55 ± 0.19[b]
DAL+MEAC 100mg/kg	3.56 ± 0.13[b]	11.43 ± 0.03[b]	13.27 ± 0.05[b]	29.20 ± 0.02[b]
DAL+MEAC 200 mg/kg	2.62 ± 0.05	9.28 ± 0.02[b]	14.80 ± 0.34[b]	26.23 ± 0.07[b]
SEd CD (p<0.01)				

Values are expressed as mean ± SD of six samples in each group; a: p<0.01 normal control vs DAL control, b: p<0.01 DAL control vs treatment groups.

4.7.1 Effect of MEAC on RBC count of control and treated groups of *M. musculus*

RBC level that generally goes down during the progression of tumor (2.47 ± 0.06) was found to improve in mice treated with MEAC 100 and 200 mg/kg (3.56 ± 0.13 and 2.62 ± 0.05). Lower dose showed more significant result (P<0.01) (Table VIII and Fig.13).

4.7.2 Effect of MEAC on hemoglobin content of control and treated groups of *M. musculus*

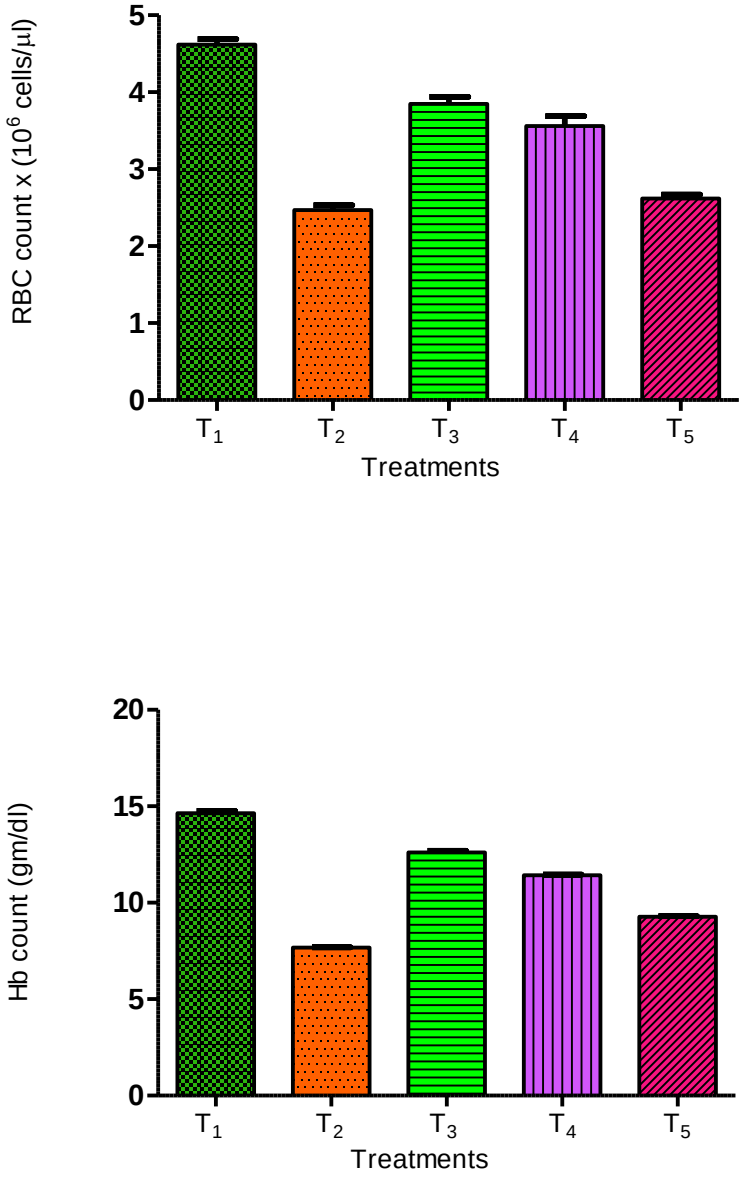
With regard to hemoglobin content all the treatment groups showed significant increase when compared to DAL control (7.68 ± 0.02). The treatment with MEAC 100 and 200 mg/kg to DAL bearing mice enhanced hemoglobin content to 11.43 ± 0.03 and 9.28 ± 0.02 respectively. The study was significant at $p < 0.01$ (Table VIII and Fig.13).

4.7.3 Effect of MEAC on total WBC count of control and treated groups of *M. musculus*

Total WBC count was found to be increased in DAL control group (23.09 ± 0.02) when compared with normal control animals (10.13 ± 0.03). Administration of MEAC at the dose of 100 and 200 mg/kg reduced the WBC count to 13.27 ± 0.05 and 14.80 ± 0.34 (Table VIII and Fig.14).

4.7.4 Effect of MEAC on PCV of control and treated groups of *M. musculus*

With regard to packed cell volume, all treatment groups showed significant ($P < 0.01$) results when compared with DAL bearing animals (30.27 ± 0.03). MEAC 200 mg/kg showed similar result like that of standard drug (Table VIII and Fig.14).



T1 - Normal control
T2 - DAL control
T3 - Positive control (5-FU.)
T4 - MEAC 100 mg/kg
T5 - MEAC 200 mg/kg

Fig.13. Effect of MEAC on RBC and Hb of control and treated groups of *M. musculus*

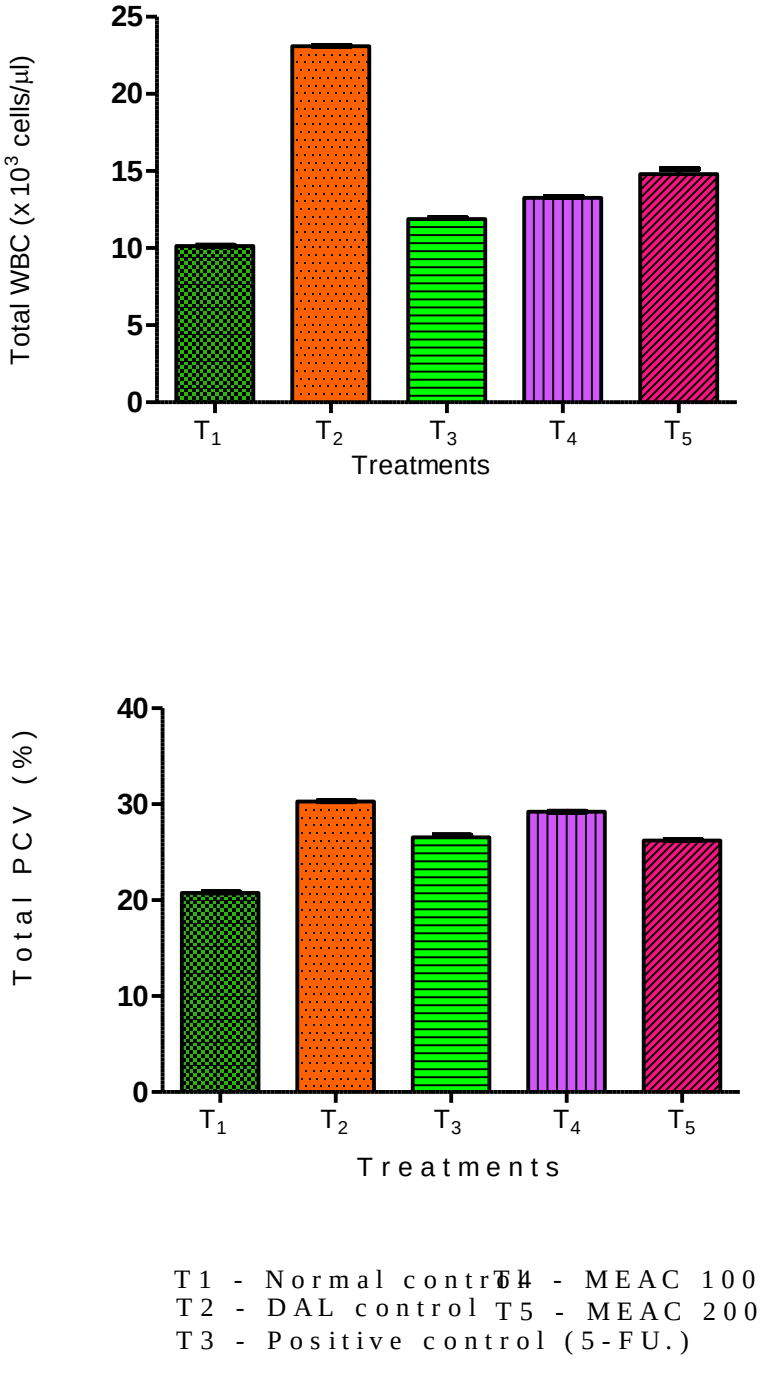


Fig.14. Effect of MEAC on WBC and total PCV of control and treated groups *M. musculus*

Table IX. Effect of MEAC on WBC differential count

Parameters	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)
Normal control	56.51 ± 0.02	1.50 ± 0.01	25.51 ± 0.01
DAL control	66.81 ± 0.01[a]	2.07 ± 0.06[a]	22.51 ± 0.01[a]
DAL+Positive Control (5-FU. 10 mg/kg)	58.53 ± 0.03[b]	0.77 ± 0.10[b]	23.83 ± 0.07[b]
DAL+MEAC 100mg/kg	59.18 ± 0.02[b]	1.21 ± 0.01[b]	23.87 ± 0.10[b]
DAL+MEAC 200mg/kg	60.52 ± 0.02[b]	1.51 ± 0.01[b]	25.00 ± 0.10[b]
SEd CD (p<0.01)	0.012 0.024	0.030 0.086	0.041 0.114

Values are expressed as mean ± SD of six samples in each group; a: p<0.01 normal control vs DAL control. b: p<0.01 DAL control vs treatment groups

4.8 Effect of MEAC on WBC differential count

In a differential count of WBC, a significant (p<0.01) increase in lymphocyte and monocyte count and a decrease in neutrophil in DAL control mice. Treatment with MEAC at different doses changed these altered parameters more or less to the normal values (Table IX, Figs.15 and 16).

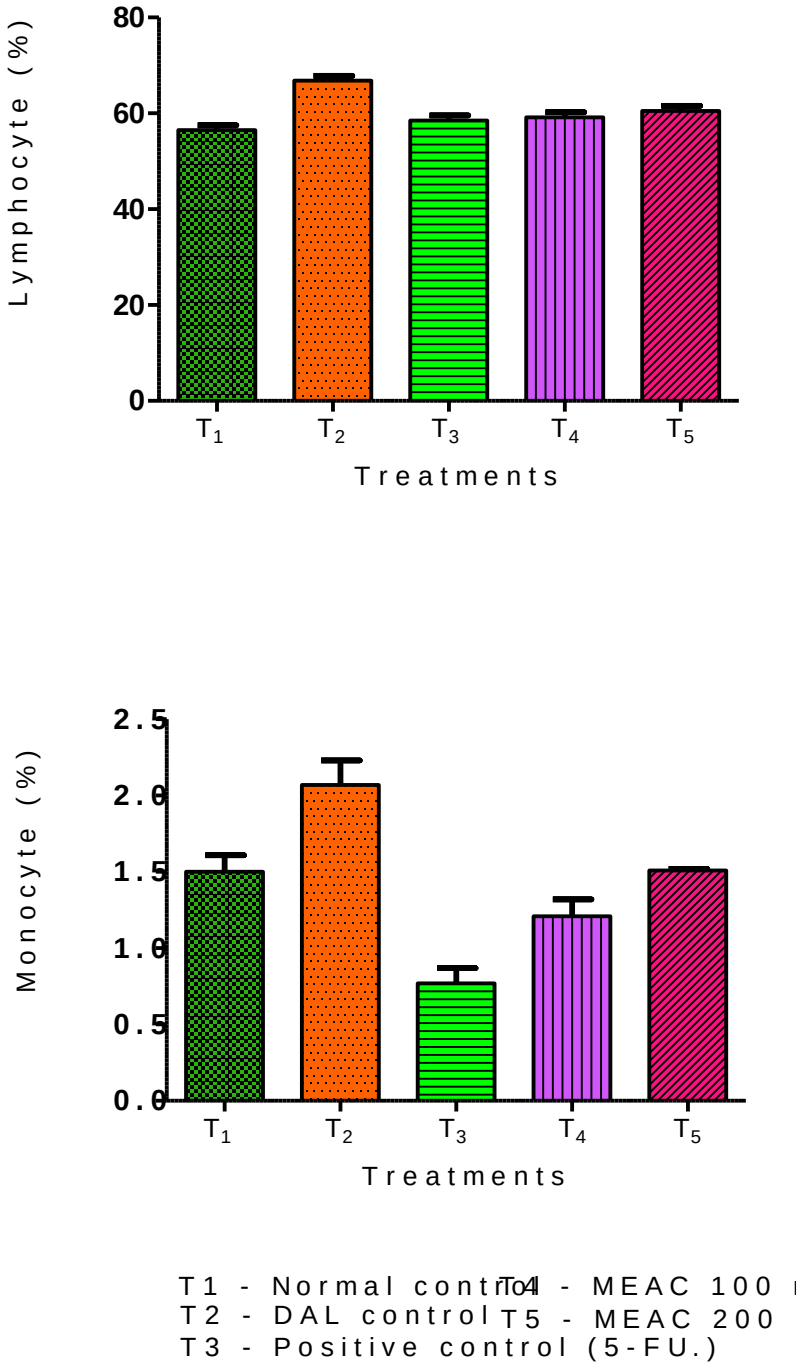


Fig.15. Effect of MEAC on lymphocyte and monocyte of control and treated groups of *M. musculus*

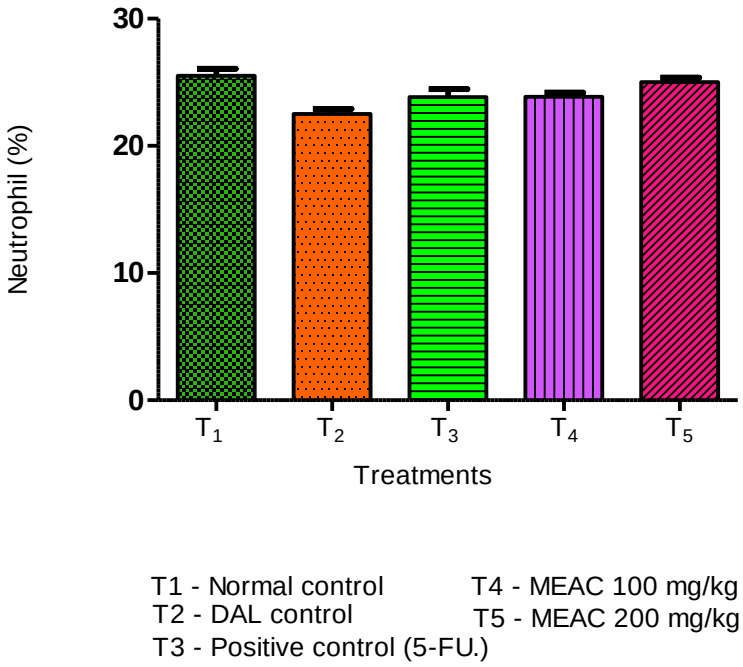


Fig.16. Effect of MEAC on neutrophil of control and treated groups of *M. musculus*

4.9 Effect of MEAC on serum biochemical parameters

The activities of ALP, SGOT and SGPT in serum of mice treated with MEAC in combination with DAL tumor cells are shown in Table X.

Table X. Effect of MEAC on serum biochemical parameters

Parameters	ALP (IU/L)	SGOT (IU/L)	SGPT (IU/L)
Normal control	90.47 ± 2.48	35.26 ± 0.65	26.17 ± 0.99
DAL control	106.25 ± 5.98[a]	70.95 ± 0.35[a]	56.50 ± 0.49[a]
DAL + Positive control (5-FU. 10 mg/kg)	91.57 ± 2.32[b]	37.45 ± 1.08[b]	28.29 ± 0.78[b]
DAL+MEAC 100mg/kg	93.96 ± 1.01[b]	40.82 ± 0.44[b]	25.90 ± 0.77[b]
DAL+ MEAC 200 mg/kg	97.61 ± 1.46[b]	42.05 ± 1.29[b]	22.90 ± 1.29[b]
SEd CD (p<0.01)	1.833 5.110	0.488 1.359	0.520 1.449

Values are expressed as mean ± SD of six samples in each group; a : p < 0.01 normal control vs DAL control b : p < 0.01 DAL control vs treatment groups

4.9.1 Effect of MEAC on ALP (Alkaline Phosphatase) of control and treated groups of *M. musculus*

The serum alkaline phosphate level was analyzed in all the experimental groups of animals. In the control group, ALP level was found to be 90.47 ± 2.48 IU/l. In the case of DAL bearing mice ALP level was found to be maximum of 106.25 ± 5.98 IU/l, which was significantly (P < 0.01) reduced in MEAC treated mice (93.96 ± 1.01and 97.61 ± 1.46) respectively (Table X and Fig.17).

4.9.2 Effect of MEAC on SGOT (Serum glutamate oxalate transaminase) of control and treated groups of *M. musculus*

SGOT level was found to be decreased significantly in all the treatment groups when compared with DAL control group (70.95 ± 0.35). The lower dose of MEAC (100 mg/kg) was found to be more significant (40.82 ± 0.44) when compared to that of the higher dose, MEAC 200 mg/kg (42.05 ± 1.29) (Table X and Fig.18).

4.9.3 Effect of MEAC on SGPT (Serum glutamate pyruvate transaminase) of control and treated groups of *M. musculus*

SGPT level shown in Table X indicated an elevated level of enzyme in DAL control (56.50 ± 0.49) when compared to the normal animals (26.17 ± 0.99). MEAC treatment groups differ significantly from DAL treated control (25.90 ± 0.77 and 22.90 ± 1.29) (Fig.18).

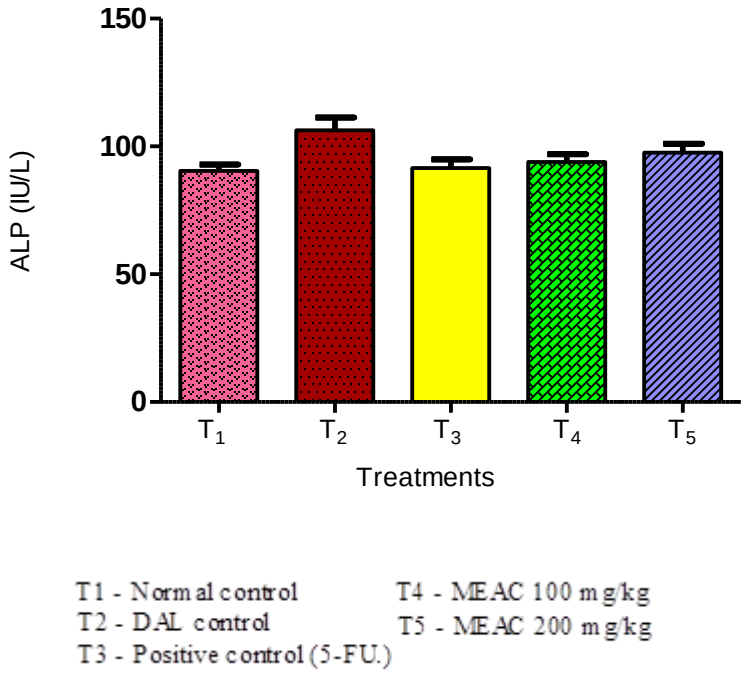


Fig.17. Effect of MEAC on ALP of control and treated groups of *M. musculus*

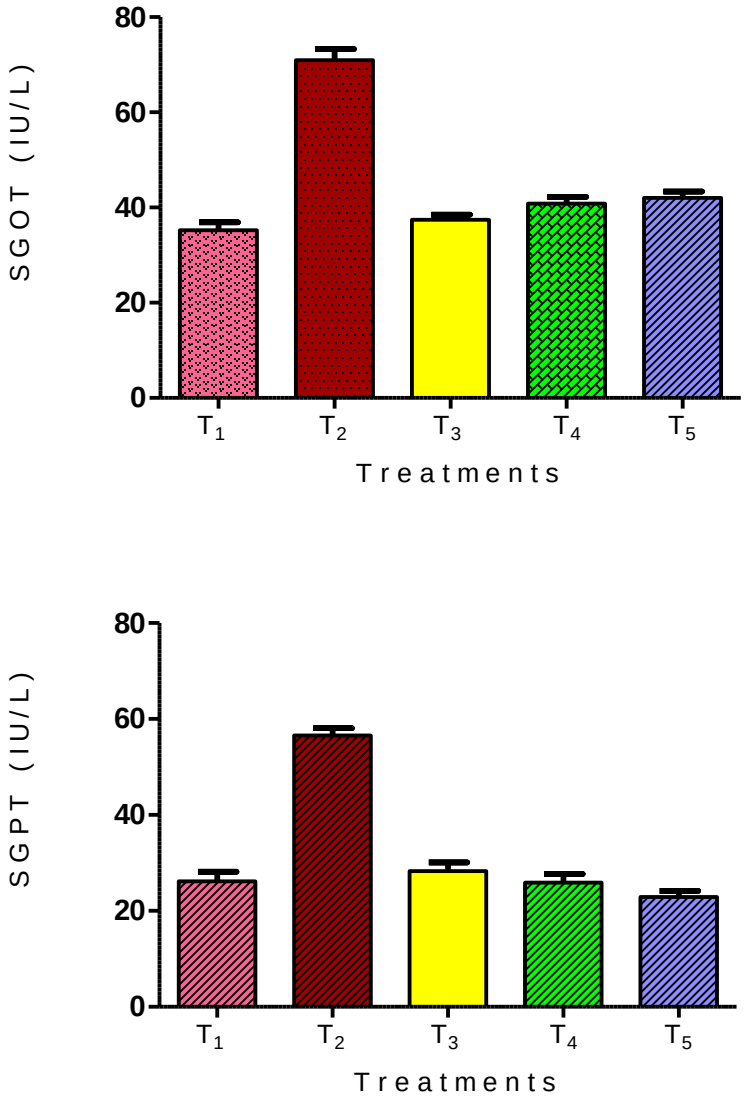


Fig.18. Effect of MEAC on SGOT and SGPT of control and treated groups of *M. musculus*

4.10 Effect of MEAC on serum protein and lipid level

The effect of MEAC on serum protein and lipid level is presented in Table XI.

Table XI. Effect of MEAC on serum protein and lipid level

Parameters	Total protein (gm/dl)	Urea (mg / dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	TGL (mg/dl)
Normal control	6.28 ± 0.56	54.45 ± 1.48	3.75 ± 0.35	1.05 ± 0.10	142.78 ± 2.92	53.38 ± 1.08	132.73 ± 11.93
DAL control	12.94 ± 0.91[a]	81.23 ± 2.93[a]	5.54 ± 0.62[a]	1.19 ± 0.18	178.90 ± 7.16[a]	25.26 ± 1.32[a]	186.89 ± 7.71[a]
DAL+Positive Control (5-FU:10 mg/kg)	6.73 ± 0.69[b]	71.13 ± 2.13[b]	4.19 ± 0.45[b]	1.03 ± 0.09	149.68 ± 8.93[b]	32.02 ± 0.73[b]	141.07 ± 0.86[b]
DAL+MEAC 100 mg/kg	6.82 ± 0.93[b]	72.23 ± 4.15[b]	5.01 ± 0.41[b]	1.05 ± 0.04	175.83 ± 13.11	46.88 ± 0.83[b]	146.53 ± 1.42[b]
DAL+MEAC 200 mg/kg	6.10 ± 0.64[b]	70.09 ± 3.93[b]	4.24 ± 0.62[b]	1.02 ± 0.04	167.92 ± 9.47[b]	41..28 ± 0.64[b]	162.14 ± 0.73[b]
SEd CD (p<0.01)	0.439 1.223	1.787 4.983	0.290 0.809	0.059 0.166	5.170 14.413	0.550 1.553	3.697 10.307

Values are expressed as mean ± SD of six samples in each group; a: p<0.01 normal control vs DAL control. b: p<0.01 DAL control vs treatment groups

4.10.1 Effect of MEAC on total protein of control and treated groups of *M. musculus*

With regard to total protein content all the treatment groups were found to be significantly reduced when compared with DAL control group (12.94 ± 0.91). MEAC treatment with higher dose (200 mg/kg) showed near normal values (6.10 ± 0.64) (Table XI and Fig.19).

4.10.2 Effect of MEAC on urea and uric acid of control and treated groups of *M. musculus*

When compared with the DAL control animals the level of urea and uric acid were found to be significantly reduced in both the MEAC treated groups (Table XI and Fig. 20).

4.10.3 Effect of MEAC on creatinine of control and treated groups of *M. musculus*

Data pertaining to creatinine revealed that treatment with MEAC (100 and 200 mg/kg) did not differ significantly from DAL treated control (Table XI and Fig.21).

4.10.4 Effect of MEAC on cholesterol of control and treated groups of *M. musculus*

In DAL bearing mice there was an increase in cholesterol level (178.90 ± 7.16) when compared with normal control (142.78 ± 2.92). MEAC 200 mg/kg (167.92 ± 9.47) was found to be significant ($P<0.01$) when compared to that of DAL control animals (Table XI and Fig.21).

4.10.5 Effect of MEAC on HDL of control and treated groups of *M. musculus*

With administration of MEAC (100 and 200 mg/kg) to the DAL induced mice the level of HDL was found to be significantly ($P< 0.01$) increased (46.88 ± 0.83 and 41.28 ± 0.64) compared to that of the DAL control mice (25.26 ± 1.32). Lower dose showed more significant result (Table XI and Fig.22).

4.10.6 Effect of MEAC on TGL of control and treated groups of *M. musculus*

The administration of MEAC significantly reduced the triglyceride level in 100 and 200 mg/kg treated groups (146.53± 1.42 and 162.14 ± 0.73) when compared with DAL control animals (186.89 ± 7.71).The lower dose of MEAC was found to be more significant (Table XI and Fig.22).

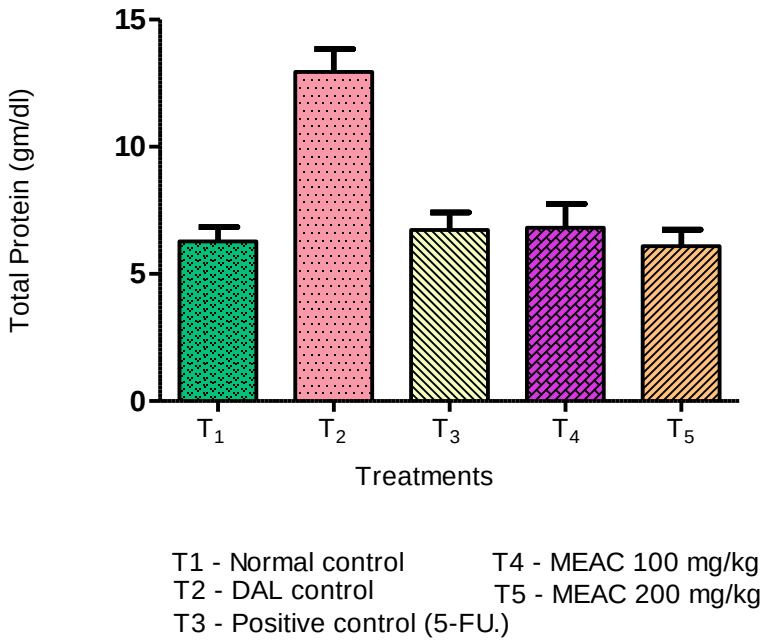


Fig.19. Effect of MEAC on total protein of control and treated groups of *M. musculus*

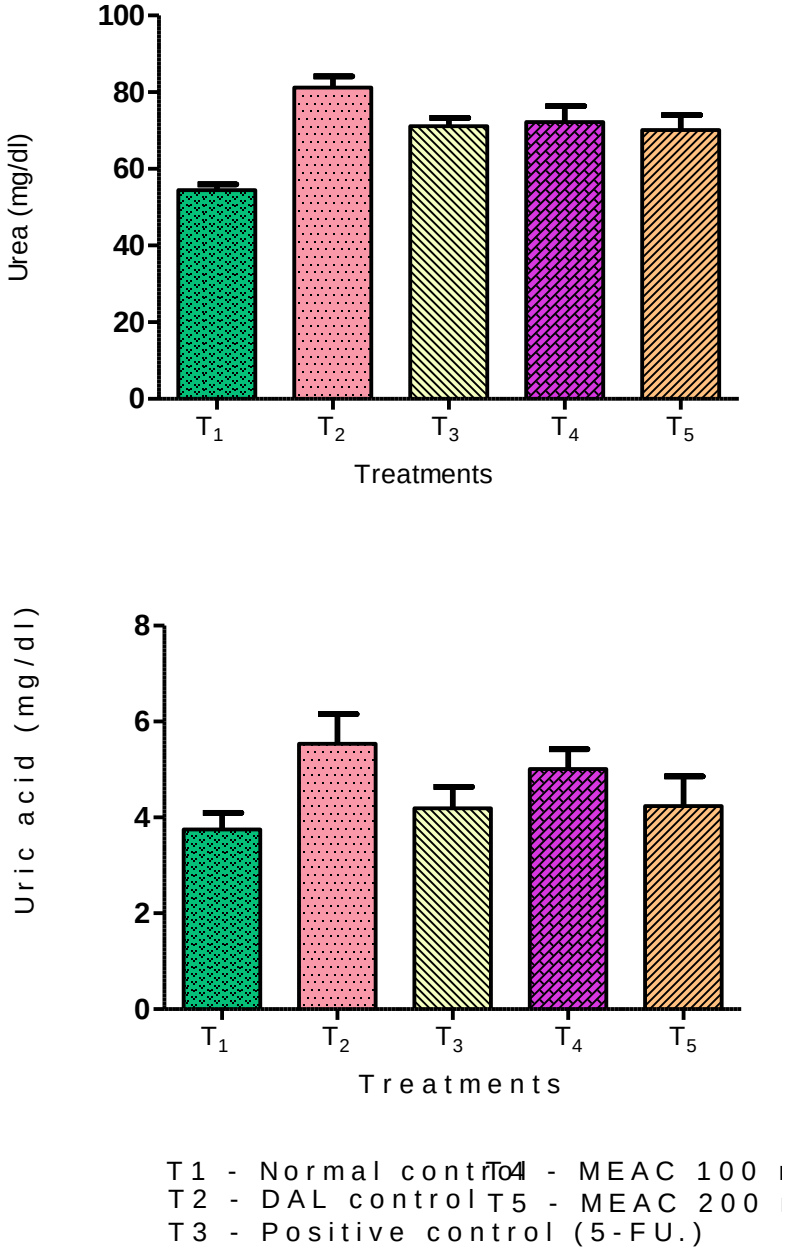
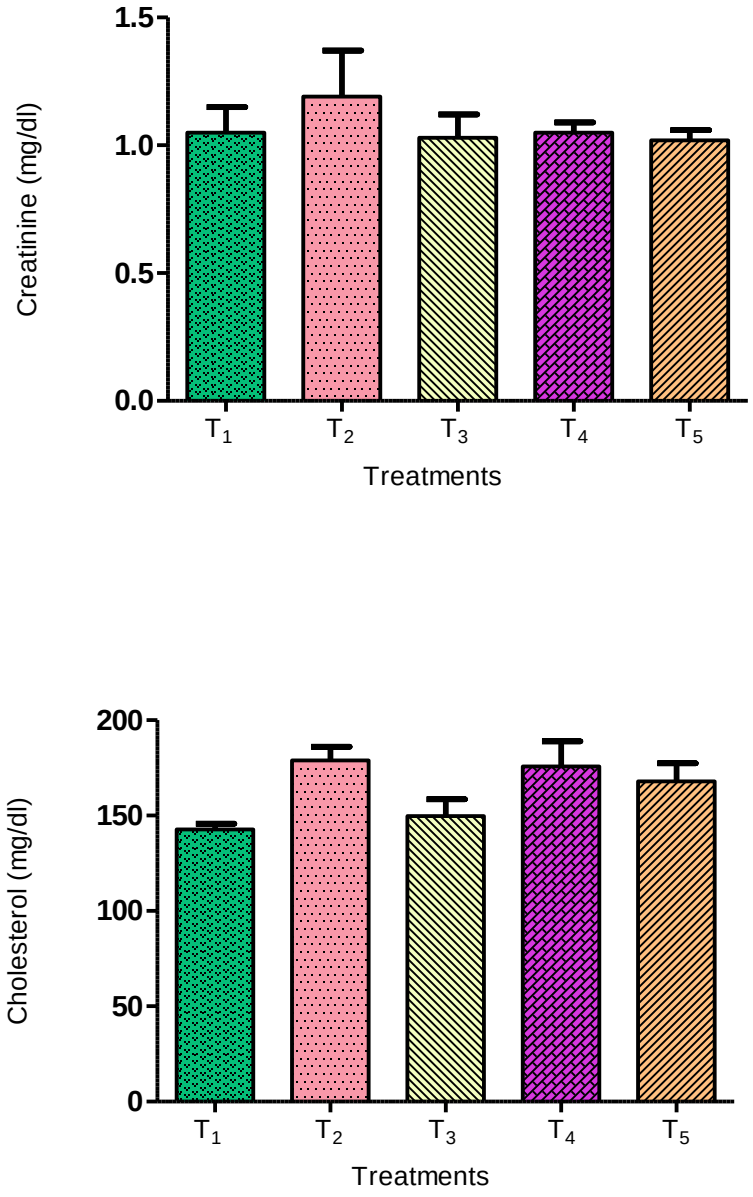


Fig.20. Effect of MEAC on urea and uric acid of control and treated groups of *M. musculus*



T1 - Normal control
T2 - DAL control
T3 - Positive control (5-FU.)
T4 - MEAC 100 mg/kg
T5 - MEAC 200 mg/kg

Fig.21. Effect of MEAC on creatinine and cholesterol of control and treated groups of *M. musculus*

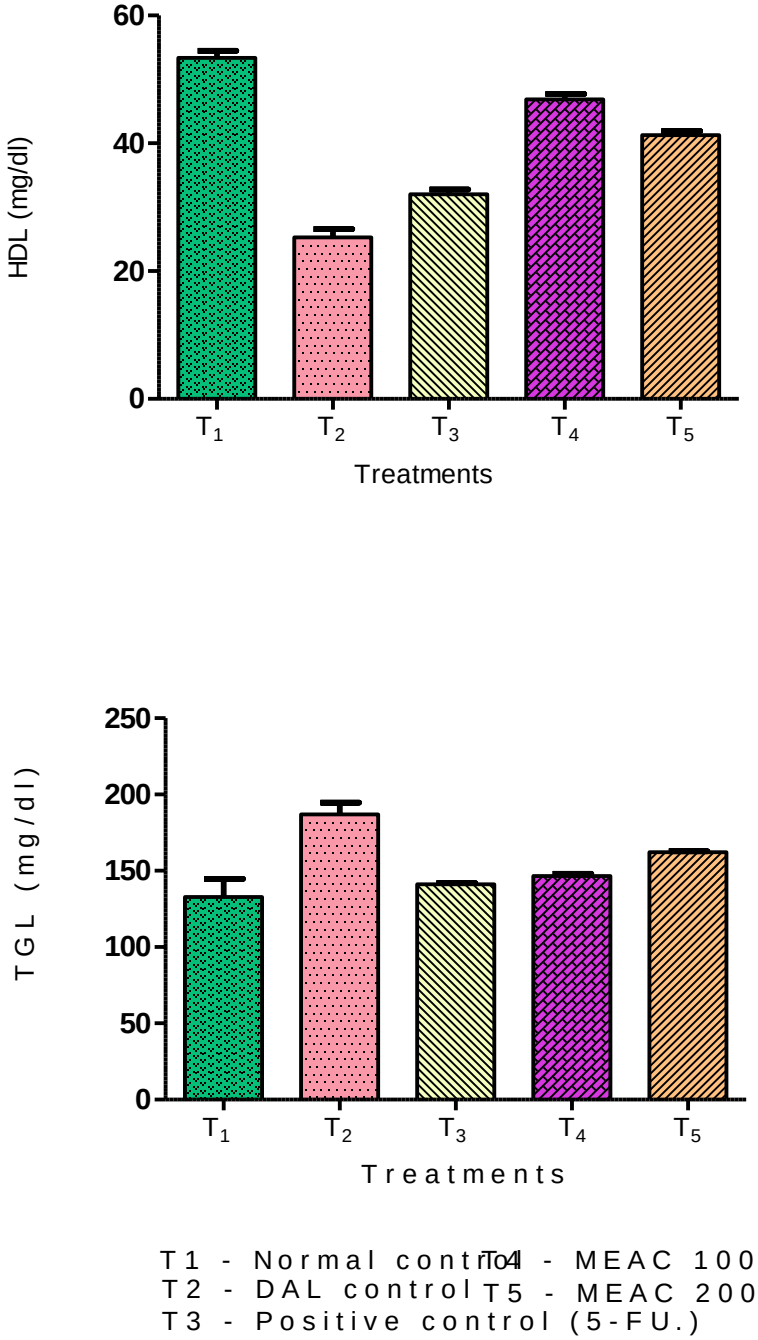
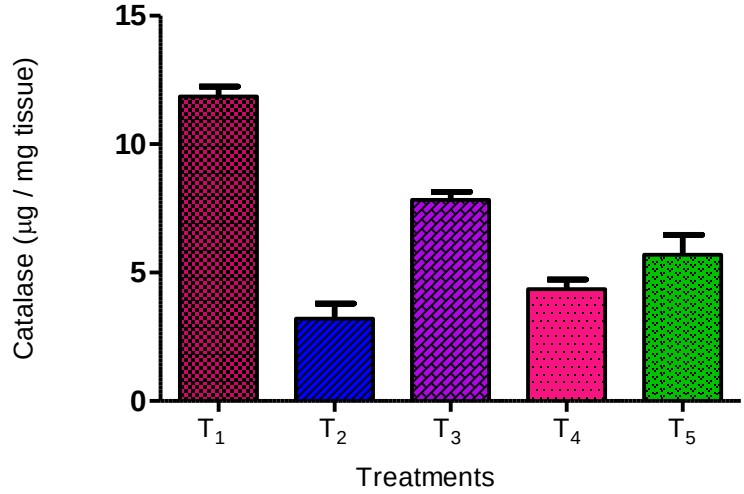
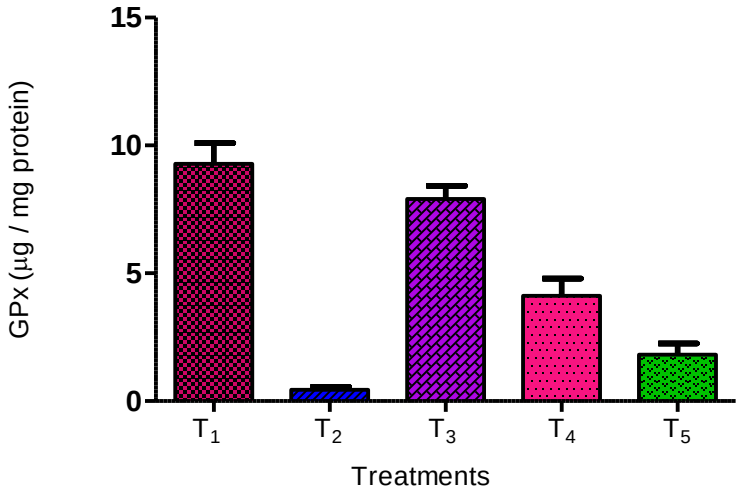


Fig.22. Effect of MEAC on HDL and triglycerides of control and treated groups of *M.musculus*





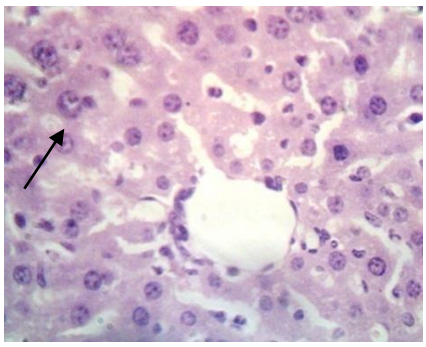
T1 - Normal control
T2 - DAL control
T3 - Positive control (5-FU.)
T4 - MEAC 100 mg/kg
T5 - MEAC 200 mg/kg

Fig.28. Effect of MEAC on kidney CAT and GPx of control and treated groups of *M. musculus*

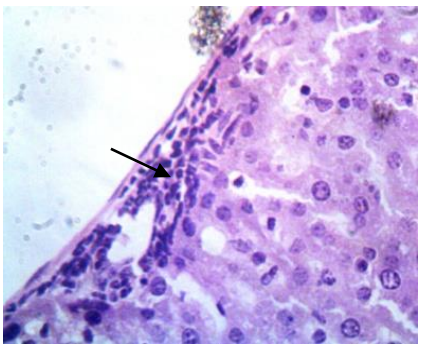
4.12 Effect of MEAC on histopathological studies

Histological examination of the liver and kidney tissue under a light microscope was done to observe the effects of MEAC on the structural integrity of the cells. The liver of normal mice showed normal histological appearance with central vein and unremarkable sinusoids and portal tracts. Parenchyma shows normal hepatocytes (Fig.31.a). Haematoxylin and Eosin stained section of liver tissue of DAL treated mice showed loss of normal architecture. Dissolution of hepatic cords, congested branches of portal vein, destruction of bile, ductless epithelium with inflammatory cells at portal area was observed. Lobules showed neutrophilic satellitosis with apoptosis. Standard treated groups showed neutrophilic satellitosis and confluent necrosis. No fibrosis, cirrhosis or malignancy seen in MEAC treated sections of liver tissue revealed manifestation of mild hepatic damage with preserved architecture. There is no fibrosis, submassive necrosis and carcinoma (Fig.31.d and 31.e).

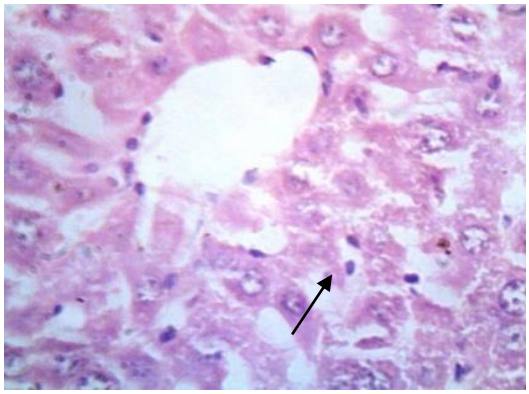
The kidney of normal animals showed normal zonal variation extending from center to medulla with normal glomeruli. Tubules were normal. Medulla showed collecting duct whereas in DAL control mice, alterations in glomerular region was observed and suppuration with collection of foamy macrophages (Fig.32.b). The animals treated with MEAC 100 and 200 mg/kg exhibited regeneration of glomerular region with slightly dilated tubules (Fig.32.d and 32.e). No acute tubular necrosis, malignancy or kidney infarct was seen. Renal medulla was found to be normal.



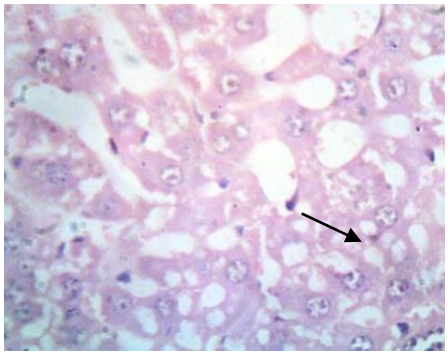
a. Normal control



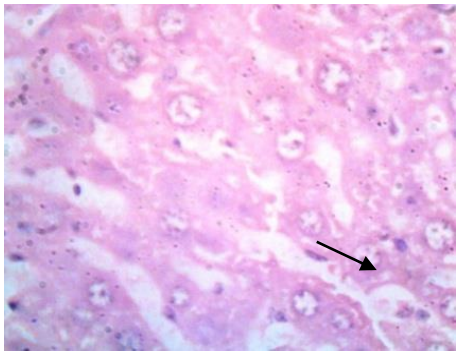
b. DAL control



c. DAL + Positive control

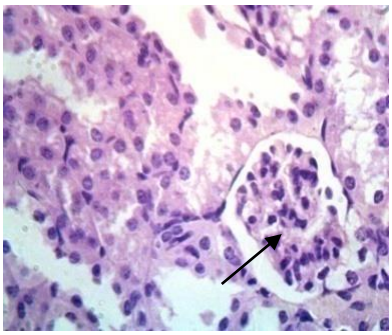


d. DAL+MEAC 100

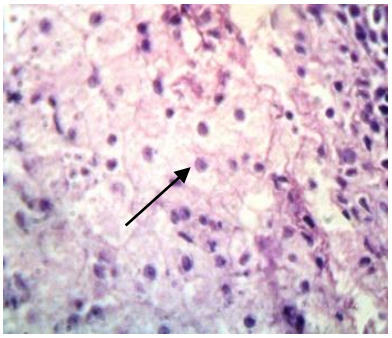


e. DAL+MEAC 200

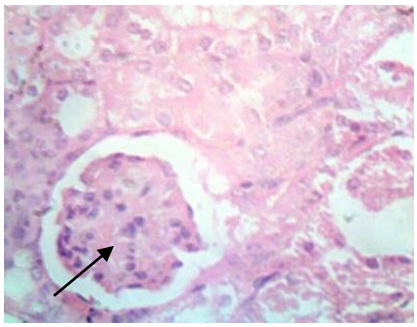
Fig. 31. Histology of liver stained with haematoxylin–eosin of mice from different groups.



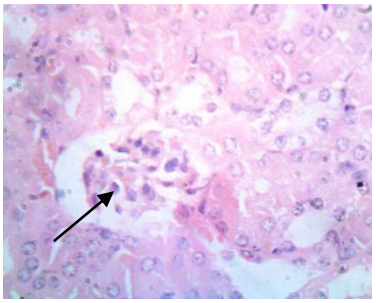
a. Normal control



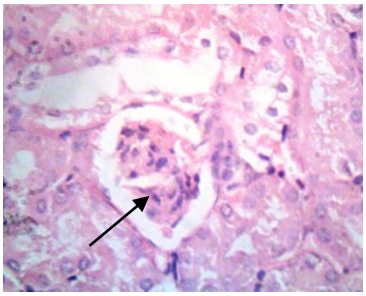
b. DAL control



c. DAL+ Positive control



d. DAL+ MEAC 100 mg/kg



e. DAL+MEAC 200 mg/kg

Fig.32. Histology of kidney stained with haematoxylin-eosin of mice from different groups.

4.13 Cell proliferation assay (MTT) in MCF-7 cells

In the present study MCF-7 cells showed growth inhibition in a dose dependent manner when treated with MEAC at concentrations ranging from 18.75 - 300 µg/ml. The percentage of dead cells for each concentration was found to be 17.04, 45.23, 58.64, 77.86 and 94.50. The IC_{50} value of MEAC was found to be 52.07 µg/ml (Table XIV and Figs.33 and 34).

Table XIV. Cytotoxicity of MEAC on MCF - 7 cells

Test conc. (µg/ml)	Growth inhibition (%)	IC_{50} (µg/ml)	R^2
18.75	17.04	52.07	0.97
37.5	45.23		
75	58.64		
150	77.86		
300	94.50		

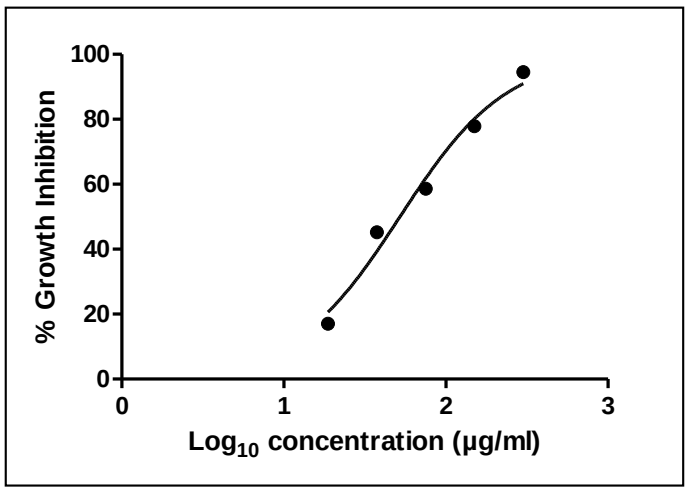


Fig. 33. Percentage growth inhibition of MEAC on MCF-7 cells

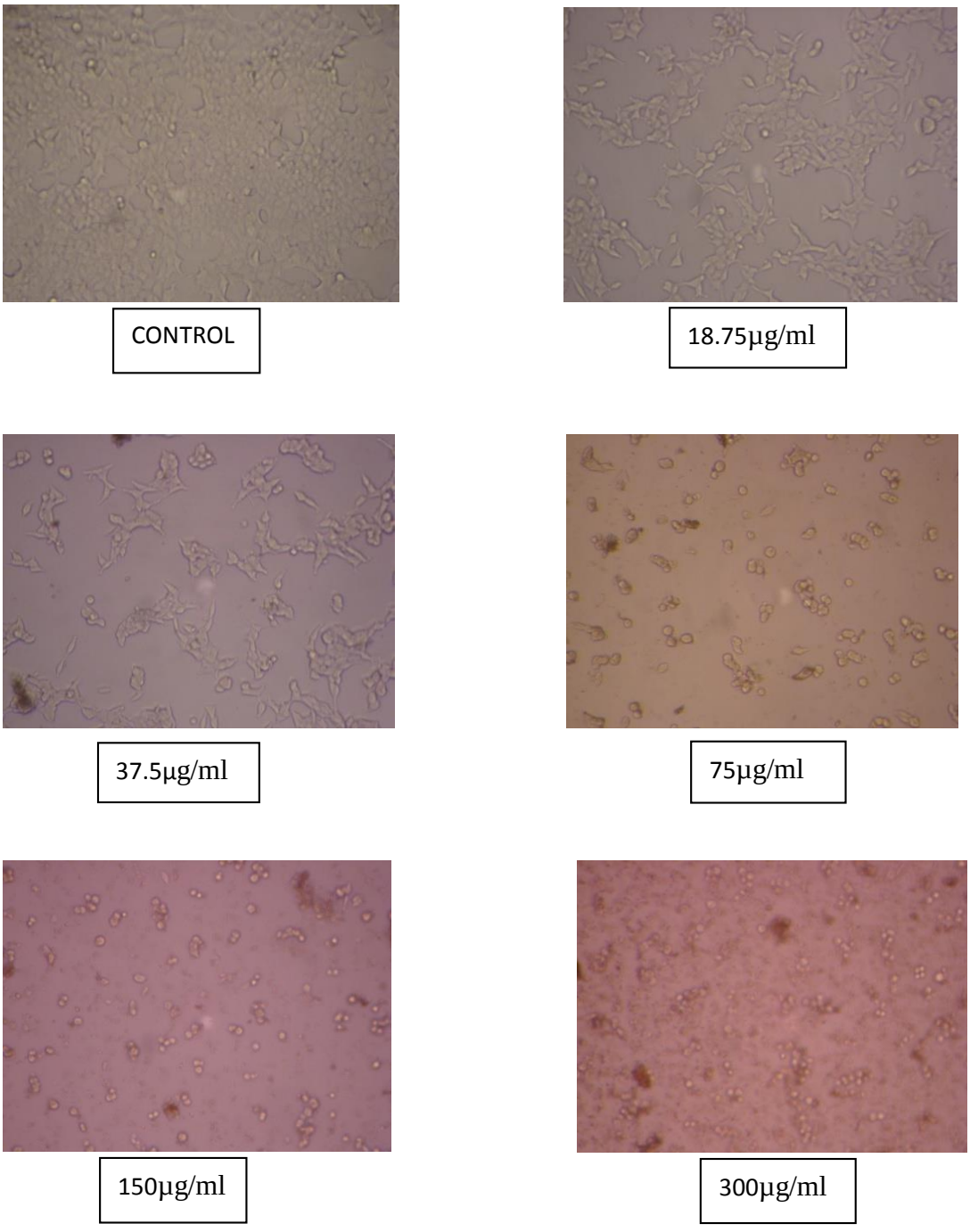


Fig. 34. Growth inhibition of MEAC against MCF -7 cell line

4.14 Cytokinesis block micronucleus assay

4.14.1 Cytotoxicity of MEAC in human breast cancer lymphocyte

A concentration dependent decline was seen in the survival of cells exposed to MEAC for 24 h. (Table XV and Fig.35). The concentration MEAC up to 40 µg/ml did not affect the viability of lymphocyte cells during the 24 hours exposure, but at the concentration of 60 µg/ml, the cell viability decreased below 50%.

Table XV. Cytotoxicity of MEAC in human breast cancer lymphocyte

Concentration of MEAC (µg/ml)	viable cells (%)
10	74
20	75
40	78
60	48
80	45
100	44

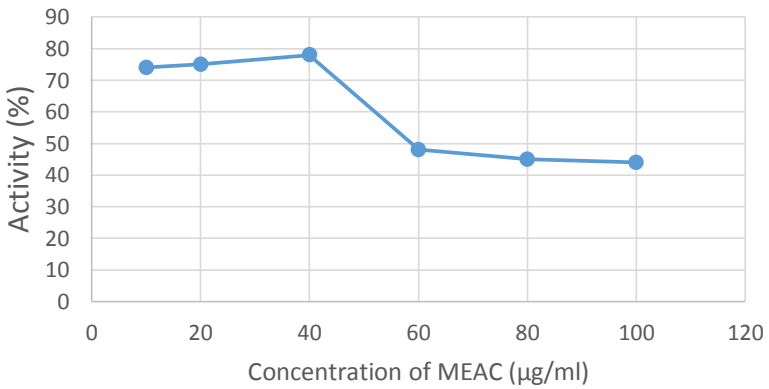


Fig.35. Cytotoxicity of MEAC on human breast cancer lymphocyte cells

4.14.2 Determination of micronuclei formation in human breast cancer lymphocyte

The result of CBMN test is displayed in Table XVI and Figs.36, 37 and 38.

When MN formation was analyzed after treatment with different concentrations of methanol extracts of *A. calamus*, significant changes in the frequency of MN were detected for 20 µl and 40 µl ($p < 0.01$). The lower extract concentration (10 µl) did not induce any change in MN frequencies compared to control.

Table XVI. Micronucleus frequency in human lymphocyte with MEAC

MEAC (mg / µl)	Frequency of MN (MN ± SEM)
Control	12.8 ± 0.37
10.00	11.4 ± 0.6
20.00	9.6 ± 0.24 *
40.00	9.4 ± 0.50 *

ANOVA: * significantly different from control ($p < 0.01$) MN: Micronucleus; SEM: Standard error mean.

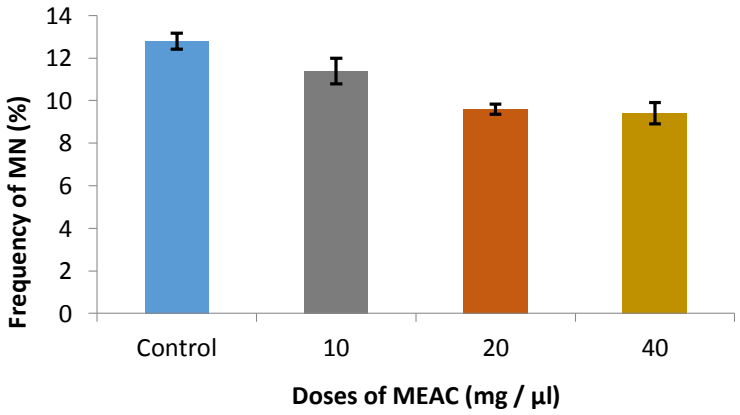


Fig.36. Micronucleus frequency in human lymphocyte treated with MEAC

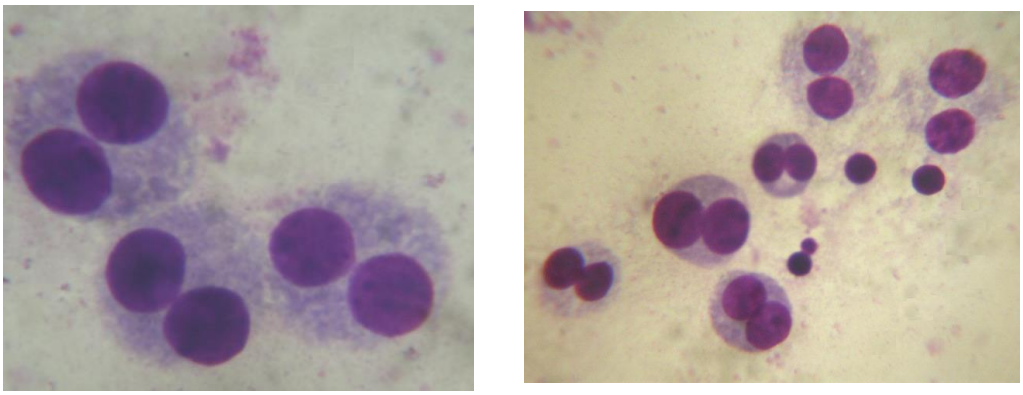
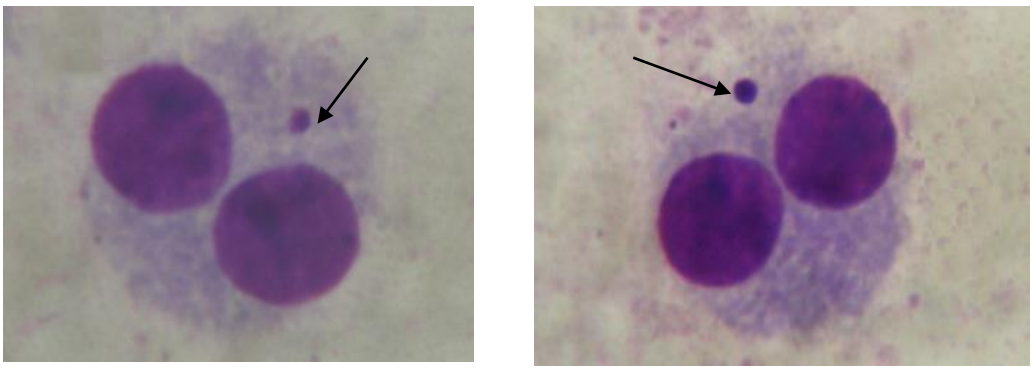


Fig.37. Cytokinesis block with normal binucleated lymphocytes



MN - Micronuclei

Fig. 38. Human breast cancer lymphocyte with micronuclei

4.14.3 DNA repair proficiency by chromosome sensitivity analysis

For chromosome sensitivity analysis the mean number of breaks/cell (b/c) were calculated. Only frank chromatid breaks were scored. The frequency of breaks was expressed as b/c for comparison. Any individual expression < 0.8 b/c was considered hyposensitive, 0.8-1.0 was considered sensitive and those >1.0b/c was considered hypersensitive. A minimum of 100 metaphases per culture was scored and data were analyzed (Table XVII and Fig.39).

With regard to mutagen sensitivity, there was a significant variation within the subjects. Among the breast cancer patients all the treatment groups were found to be sensitive whereas the healthy subjects were hyposensitive. The lowest range of mean break / per cell value was observed in the dose range of 40 mg/µl in both breast cancer and healthy individuals (0.8102 and 0.7105). Between the four treatment groups of both the breast cancer and healthy animals, dose dependent decrease in the mean break / per cell value was observed.

Table XVII. Comparison of bleomycin induced chromatid breaks in breast cancer patients and in normal controls

Doses of MEAC (mg / µl)	Mean b/c value	
	Breast cancer	Healthy individual
Control	0.8311	0.769
10.00	0.8228	0.7415
20.00	0.8254	0.7275
40.00	0.8102	0.7105

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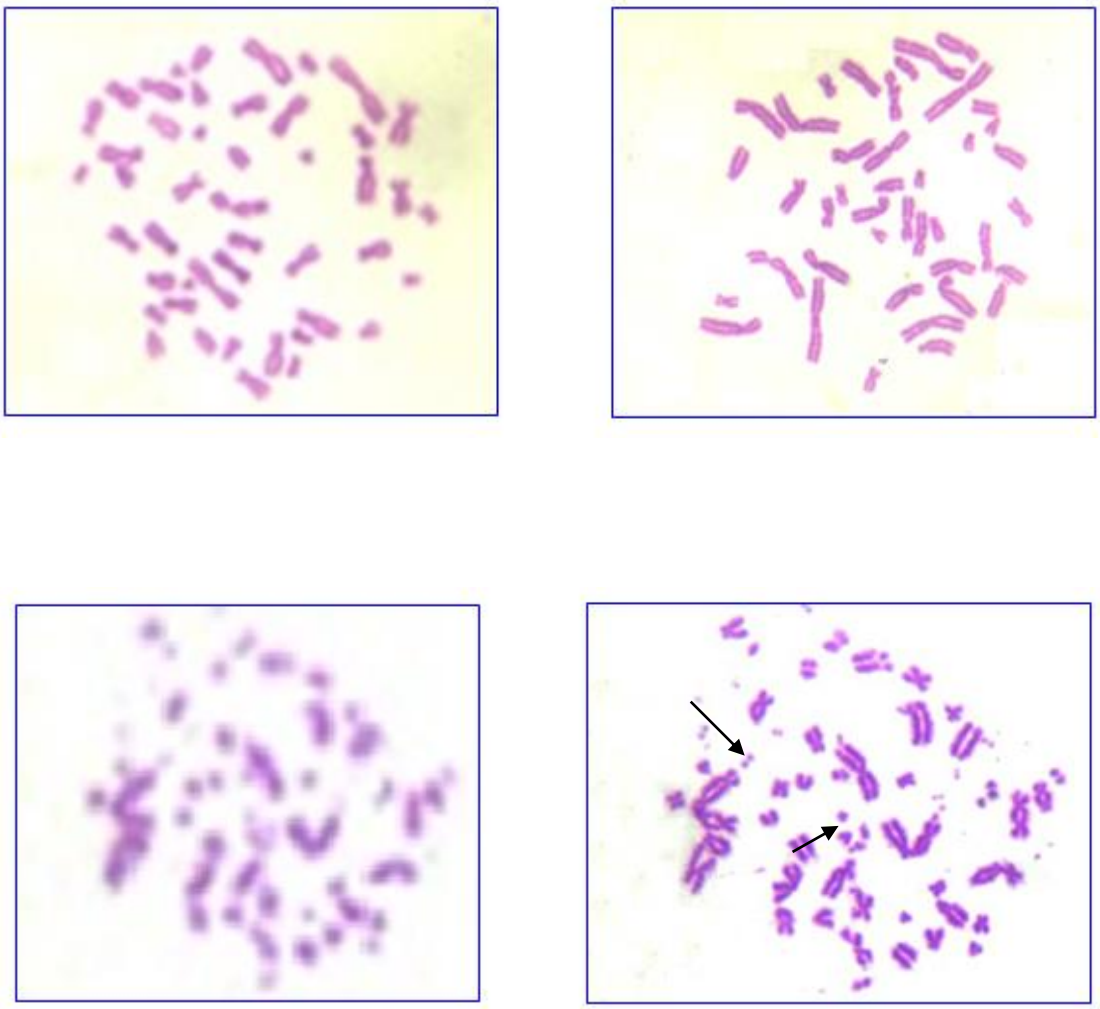


Fig. 39. Metaphases showing mutagen induced chromatid breaks in breast cancer cells

4.15 Chromatographic studies of *A. calamus*

The GC-MS analysis of *A. calamus* showed the presence of 14 compounds such as α-asarone, tetradecanoic acid and linoleic acid (Table XVIII and Fig.40).

Molecular interaction observation of proteins and isolated compounds were also studied (Table XIX).

Table XVIII. Volatile organic compounds from *A. calamus* in GC-MS

RT	Compound Name	Formula	Mol.Wt
8.637	3-Hydroxy-2-methyl-4-pyrone	$C_6H_6O_3$	126.11
10.445	Azulene	$C_{10}H_8$	128.17
14.211	Methyleugenol	$C_{11}H_{14}O_2$	178.22
15.268	Cedranone	$C_{15}H_{24}O$	220.35
16.916	alpha-Asarone	$C_{12}H_{16}O_3$	208.25
17.059	Beta.-Copaen-4 .Alpha.-Ol	$C_{15}H_{24}O$	220.35
17.743	Tetradecanoic Acid	$C_{14}H_{28}O_2$	228.37
17.862	Isocalamendiol	C15H26O2	238.00
19.571	Diethyl2-hydroxy-2-(2-hydroxyphenyl) propanedioate	$C_{13}H_{16}O_6$	268.26
19.635	2,2-Dimethyldecahydronaphthalene	$C_{12}H_{22}$	166.30
19.736	2-hydroxy-6-undecylbenzoic acid	$C_{18}H_{28}O_3$	292.41
19.866	[(2R)-2-[(1S)-1-hexadecanoyloxy-2-hydroxyethyl]-4-hydroxy-5-oxo-2H-furan-3-yl] hexadecanoate	$C_{38}H_{68}O_8$	652.94
21.141	Linoleic acid	$C_{18}H_{32}O_2$	280.44
26.492	Methyl Cinnamate	$C_{10}H_{10}O_2$	162.18

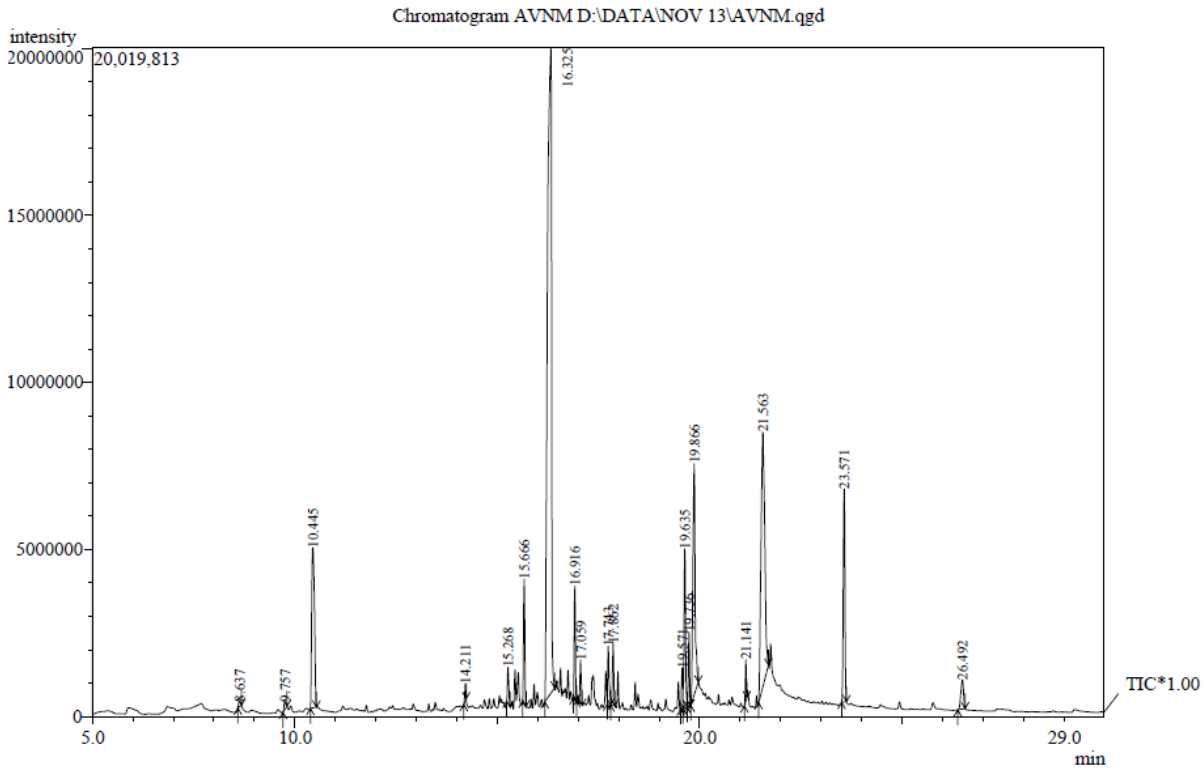


Fig. 40. GC-MS chromatogram of volatile organic compounds from *A. calamus*

4.16 *In silico* assay

Pharmacophore elucidation and docking studies on volatile compounds of MEAC for breast cancer

In this study the ligand-protein molecular docking simulation was used to investigate and to confirm the potential molecular target for breast cancer proteins. The analysis of the best docked ligands against the selected anticancer drug target revealed the binding mode of compounds involved in this study and confirm the role of the phytocomponents present in MEAC. Binding energies of drug-enzyme interactions are important to describe how fit the drug binds to the target macro molecule.

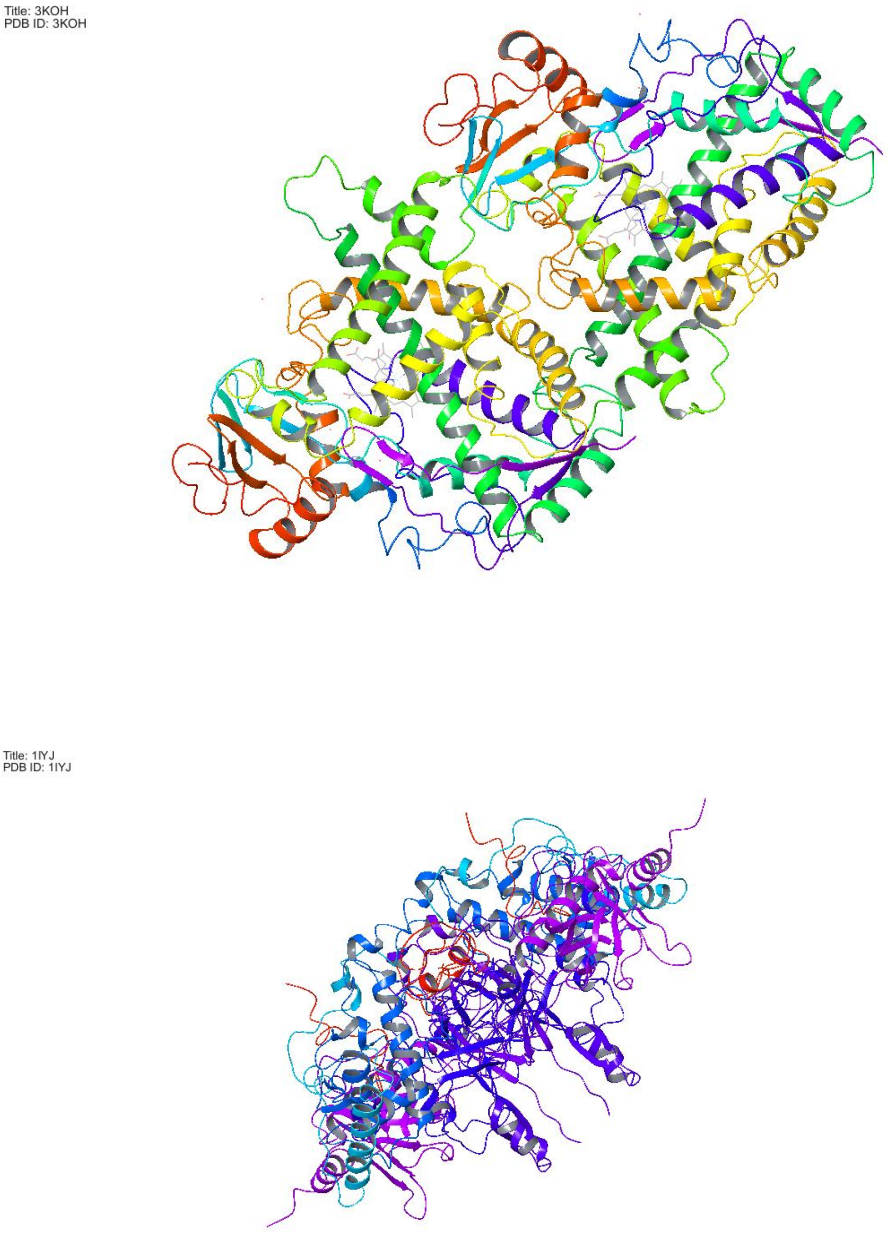
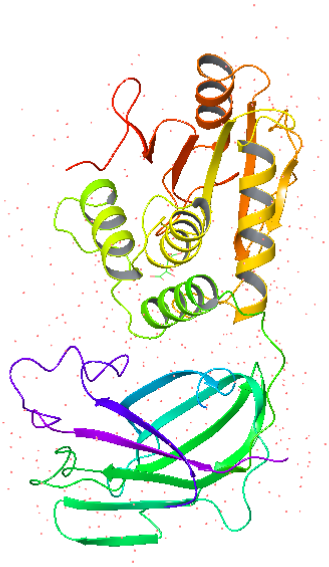


Fig.41. 3D Structure of target proteins - BRCA1 [PDB ID: 3KOH] and BRCA2 [PDB ID: 1IYJ] protein

Title: 1D5R
PDB ID: 1D5R



Title: 4HDO
PDB ID: 4HDO

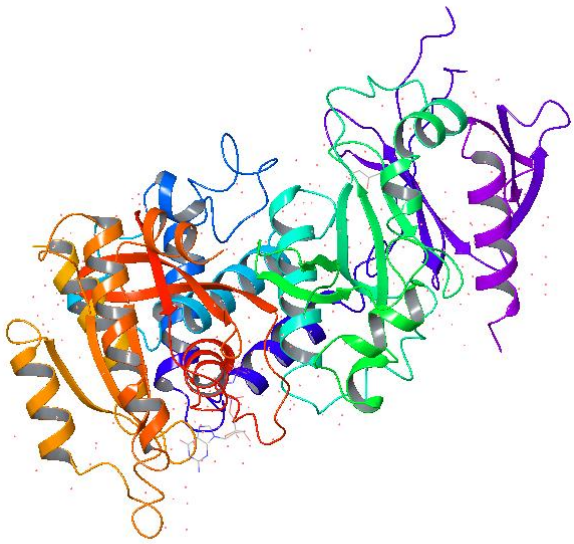
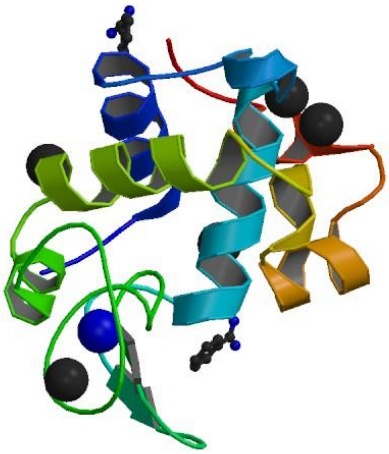
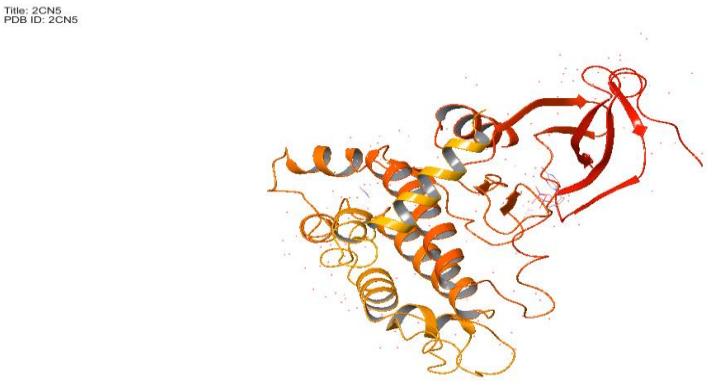


Fig. 42. 3D Structure of target protein PTEN [PDB ID: 1D5R] and ATM [PDB ID: 4HDO] protein





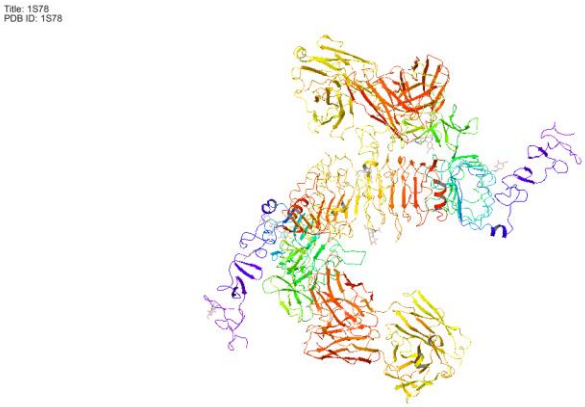


Fig.43. 3D Structure of target proteins HER2 [PDB ID: IN8Z], CHEK2 [PDB ID: 2CN5] and ERBb2 [PDB ID:1S78]

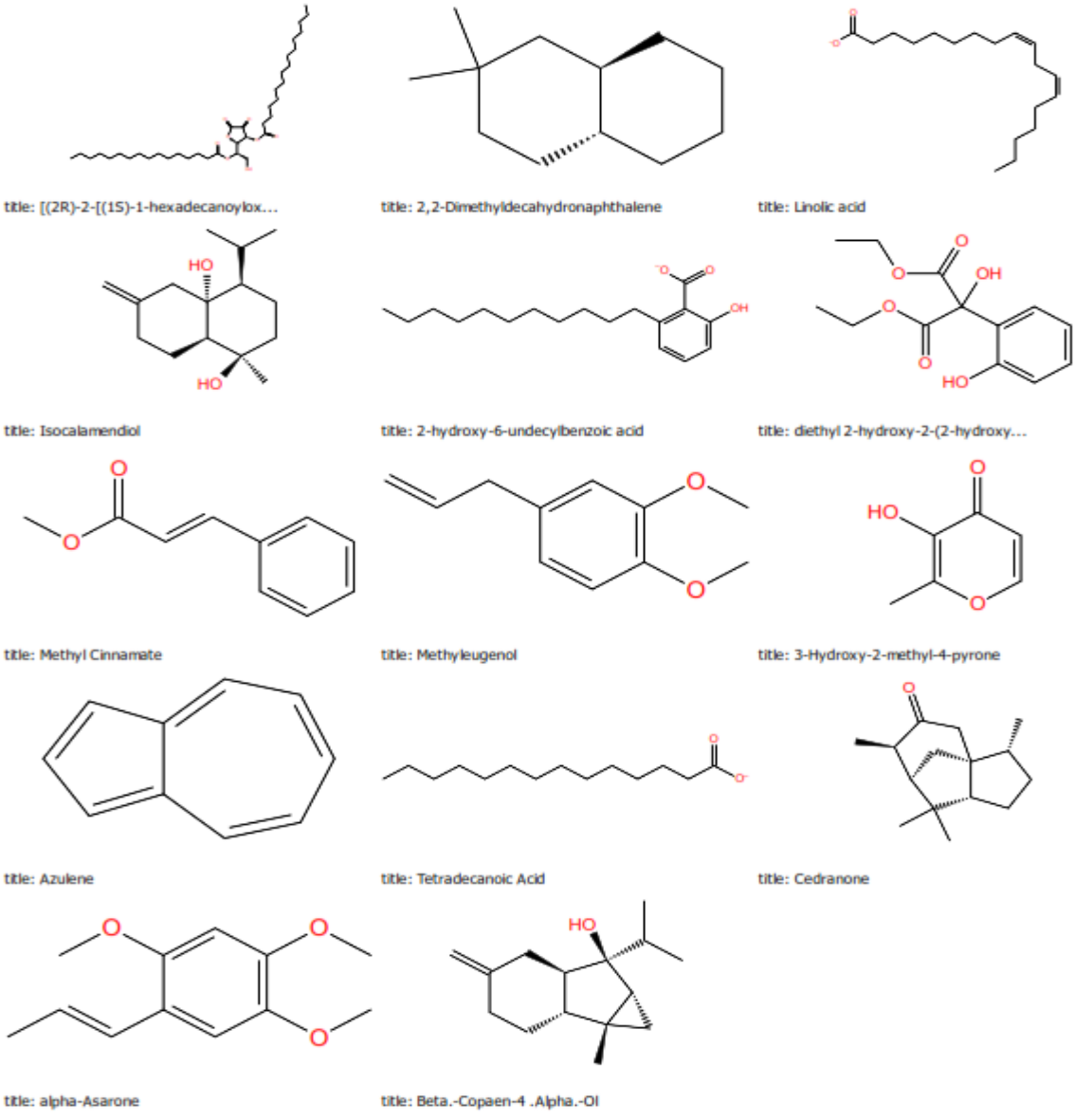


Fig.44. Structures of the ligands isolated from MEAC

4.16.1 Molecular interaction observation of proteins and isolated compounds

Docking results of 14 isolated compounds from *A. calamus* was shown in table XIX.

Table XIX. G score results

1S78		1D5R		2CN5		4HDO	
Ligand ID	G score	Ligand ID	G score	Ligand ID	G score	Ligand ID	G score
147670	-7.99	11005	-5.98	9121499	-5.27	11005	-4.26
10408116	-5.65	4444105	-5.42	10408116	-4.49	10408116	-4.13
8369	-5.3	9121499	-5.19	4444105	-4.28	4477072	-3.8
6431225	-5.02	7127	-4.69	147670	-3.56	8369	-3.64
636822	-4.87	10408116	-4.36	8369	-3.06	6431225	-3.24
4477072	-4.79	147670	-3.99	7127	-2.84	636822	-3.11
21105944	-4.39	8369	-3.49	636822	-2.08	21105944	-2.59
9121499	-4.38	4477072	-3.14			7127	-2.2
7127	-4.17	6431225	-2.97			9121499	-2.16
514616	-3.92	9231	-2.57			9231	-2.01
9231	-3.29	21105944	-2.57			514616	-1.99
11005	-2.23	636822	-2.53			111402	-1.94
4444105	-0.25	111402	-2.26			4444105	-0.94
		514616	-2.19			147670	-0.73

4.16.2 Protein and ligand interaction

The ligand was docked with target protein, and the best docking poses were identified. Intermolecular flexible docking simulations were performed and the energy values were calculated from the docked conformations of ligand and target molecules. The ligand molecules had a greater binding affinity with the target breast cancer proteins. The binding pose and their energy scores were listed in Table XX. Figs.45, 46, 47 and 48 showed how the phytocomponents of *A. calamus* fits into the binding regions of the target breast cancer proteins.

Table XX. Showing protein-ligand interaction

Protein name	Interaction amino acids	Bond length(A)	Lipophilic interaction score	Lipophilic pair term with ligand atom score	Rotatable bond penalty
1S78	VAL 3	2.01A			
	THR 5	2.10A			
	ASN 466	2.09A			
	GLY 442	2.12A	-5.43	-3.18	0.62
1D5R	Arg 130	2.29A, 1.85A			
	Cys 124	2.39A	-2.43	-3.65	0.1
2CN5	LYS 209	1.63A			
	ARG 183	1.85A	-1.43	-3.65	0.81
4HDO	ARG 474	1.79A, 2.03A			
	VAL 469	1.62A	-1.43	-2.94	0.11

4.16.2.1 G score ranked 1 complex - ERBb2 target protein

Canonical ranking indicated [(2R)-2-[(1S)-1- hexadecanoyloxy-2-hydroxyethyl]-4-hydroxy-5-oxo-2H- furan-3-yl] complexed to ERBb2 protein with G score of -7.99. The protein residues such as VAL 3, THR 5, ASN 466, GLY 442 interacted in the bond distance of 2.01A, 2.10A, 2.09A and 2.12A correspondingly with a lipophilic interaction score of -5.43, Lipophilic pair term with ligand atom score of -3.18 and 0.62 score for rotatable bond penality from ligand atom (Fig.45).

4.16.2.2 G score ranked 2 complex - PTEN target protein

Docking analysis revealed tetradecanoic acid complexed to PTEN protein with G score of -5.98. Interactions with the protein residues of Arg 130, Cys 124 were observed at the bond distance of 2.29A [couple of H Bond] and 1.85A correspondingly. Lipophilic interaction score of -2.43 and lipophilic pair term with ligand atom score of -3.65 and 0.10 score for rotatable bond penalty from ligand atom was observed (Fig.46).

4.16.2.3 G score ranked 3 complex - CHEK2 target protein

2-hydroxy-6-undecylbenzoic acid when complexed to Check Point kinase 2 indicated a G score of -5.27. In the protein residues such as LYS 209, ARG 183 interacted in the bond distance of 1.63A, 1.85A correspondingly with a lipophilic interaction score of -1.43, lipophilic pair term with ligand atom score is -3.65 and 0.81 score for rotatable bond penalty from ligand atom (Fig.47).

4.16.2.4 G score ranked 4 complex - ATM target protein

Linolic acid complexed to ATM was observed with G score of -4.26. The protein residues such as ARG 474, VAL 469 interacted in the bond distance of 1.79A, 2.03A [Couple of H Bond] and 1.62A correspondingly. Lipophilic interaction score is -1.43, lipophilic pair term with ligand atom score is -2.94 and 0.11 score for rotatable bond penalty from ligand atom (Fig.48).

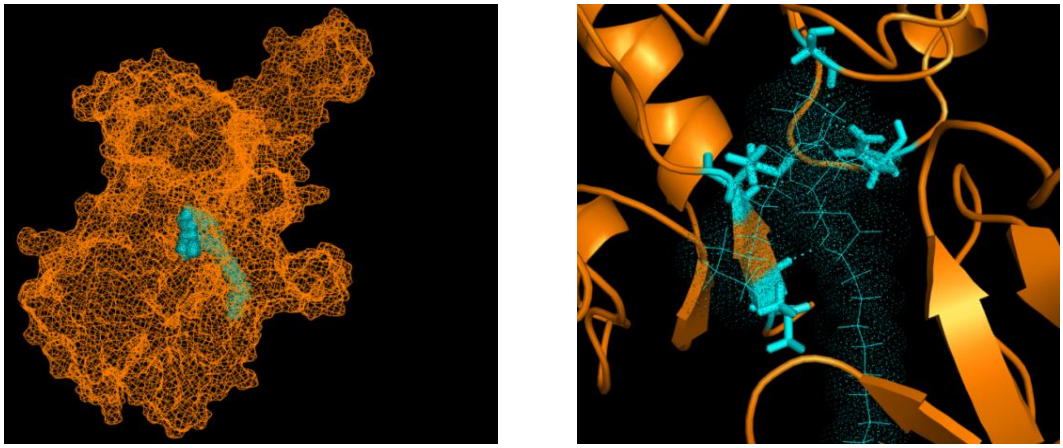


Fig. 45. Interaction profile of [(2R)-2-[(1S)-1- hexadecanoyloxy-2-hydroxyethyl]-4-hydroxy-5-oxo-2H- furan-3-yl] complexed to ERBb2 protein

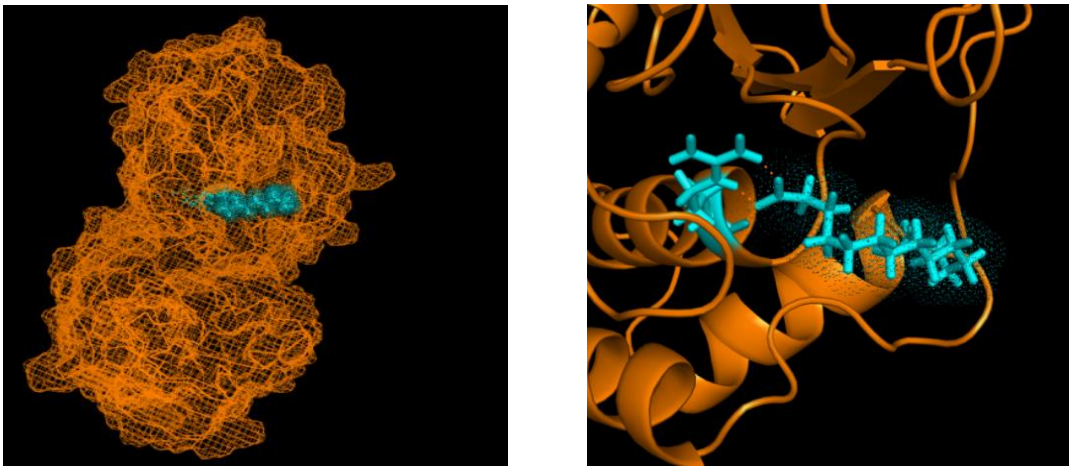


Fig. 46. Interaction profile of Tetradecanoic acid complexed to PTEN protein

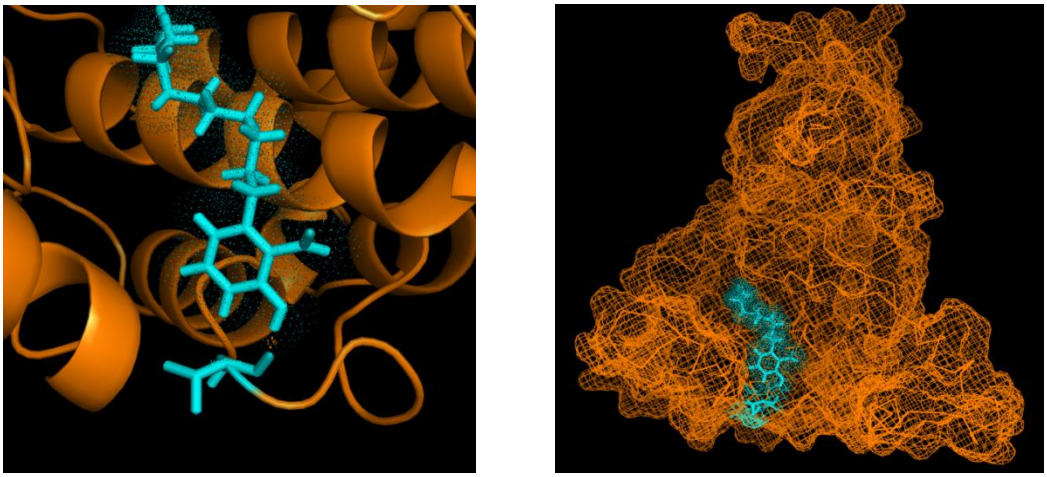


Fig.47. Interaction profile of 2-hydroxy-6-undecylbenzoic acid complexed to Check Point kinase 2

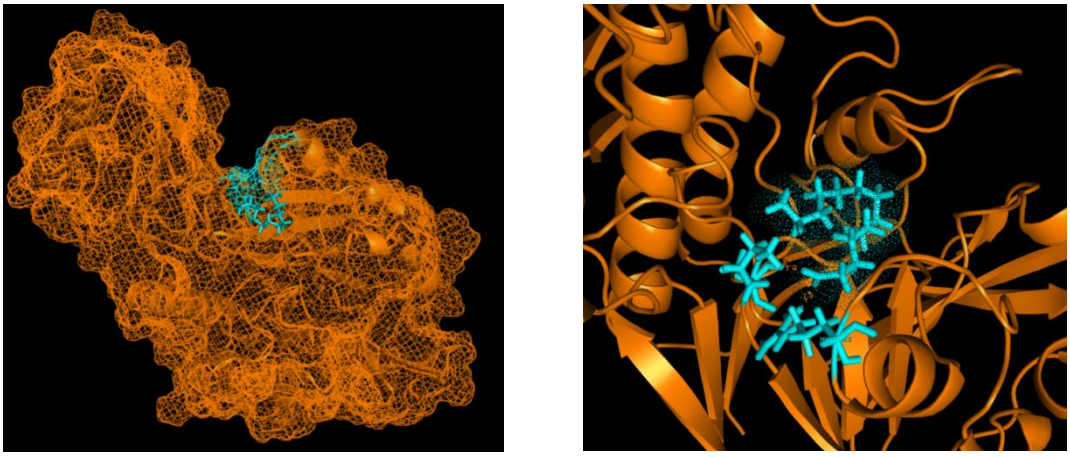


Fig.48. Interaction profile of Linoleic acid complexed to mutated Ataxia telangiectasia protein-co-crystal ligand complex

4.16.3 Molecular dynamics study of [(2R) - 2 - [(1S) - 1 - Hexadecanoyloxy - 2 - Hydroxyethyl] – 4 – Hydroxy – 5 – Oxo - 2H – Furan – 3 – Yl] complexed to ERBb2 protein

Stability protein ligand complex

Molecular dynamics was performed to look into the stability of protein and ligand complex in water solvent. After 1000 Picosecond equilibration a trajectory for 100 Picoseconds was generated for 1000 samples. Root Mean Square Deviation (RMSD) was used to measure the scalar distance between atoms of the conformational structures. RMSD is given as;

$$RMSD = \sqrt{\frac{1}{M}\sum_{i} m_i \| r_{i,1} - r_{i,2} \|}$$

The RMSD analysis predicts stability of protein and its structural variation while evolving with time. The RMSD of protein-ligand complex for the trajectories written for 1 ns production run was analyzed to identify the stability of the system at each time interval. It was observed that the complex is stable within early 500 ps production run (Fig.49). Between 750 to 900 ps, the complex was observed to fluctuate from equilibrium with minor deviation of 0.1 nm, thereafter i.e., from 140.1 ps to 200 ps the system again attained equilibrium. On an average, system was observed to be stable with RMSD of 3.3 nm. The system achieves equilibrium early at 10-15 ps and remains stable thereafter for 1 ns simulation period. The complex stability shows that there is no major structural variation in protein after binding with ligand.

This state of observation of protein structure stability in the TIP3P (water) environment observed through gyration of complexity of each trajectories in super imposed manner and the relational difference of each complex was notified for protein structure stability (Fig.50).

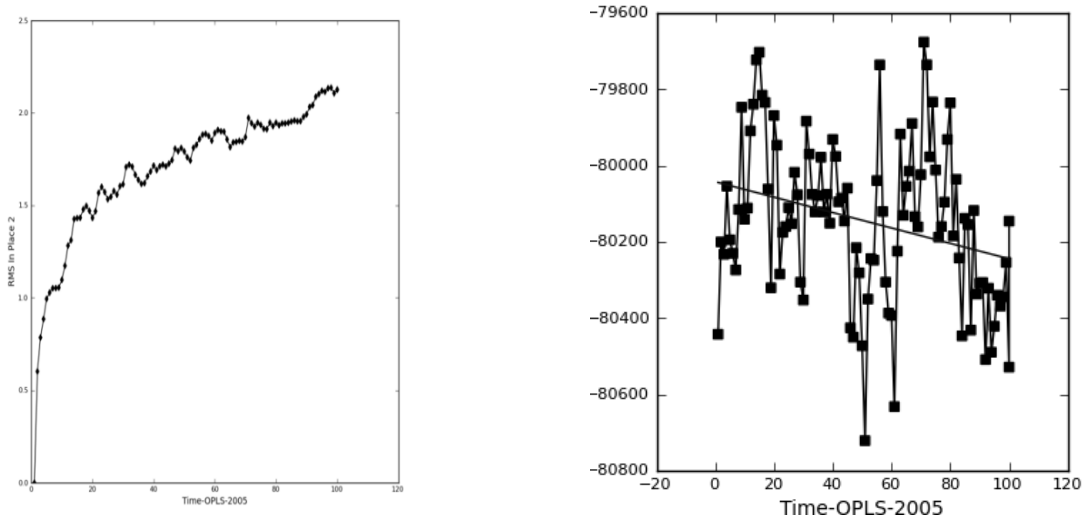


Fig. 49. RMSD graph and potential energy graph

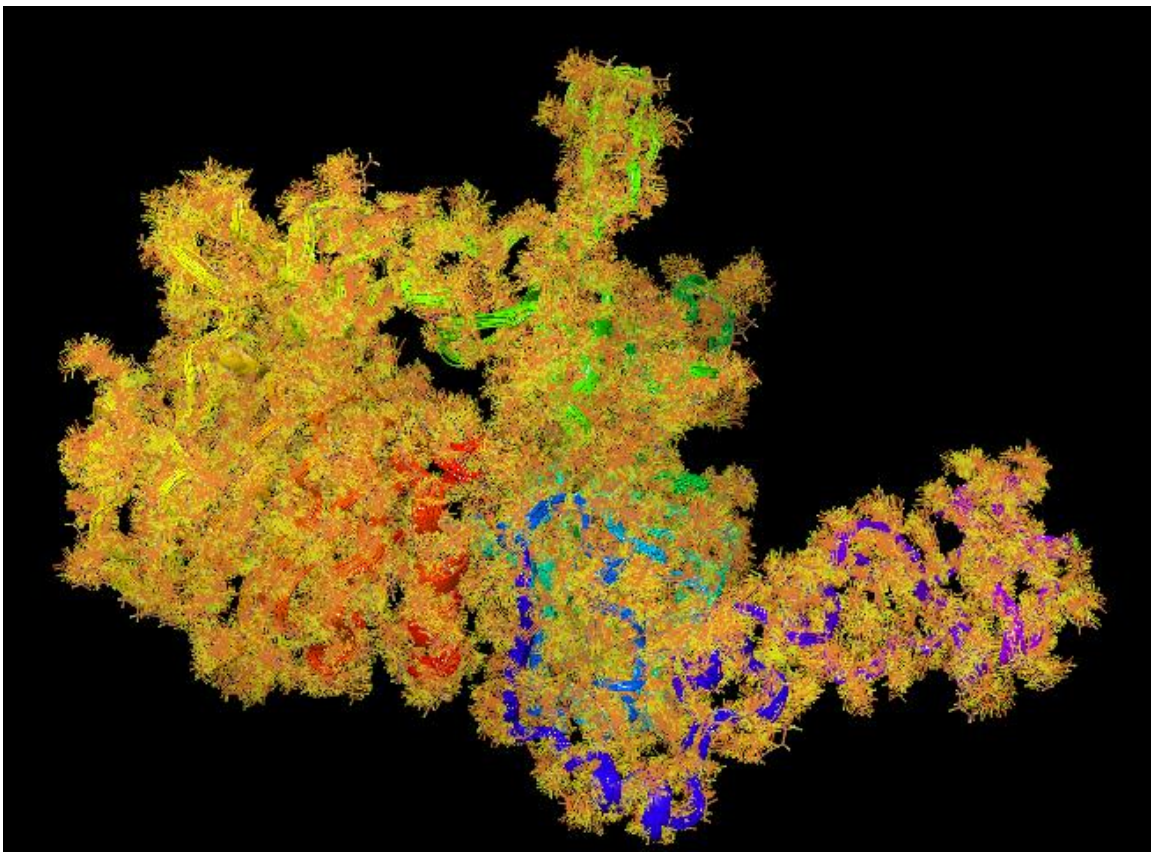


Fig. 50. 1 to 1000 sample superimposed structure