ANTIOXIDANT AND ANTITUMOR ACTIVITY OF EUPHORBIA MILII FLOWER EXTRACT AGAINST IN VIVO BREAST CANCER AND COLON CANCER IN MICE.


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ABSTRACT

Purpose: Euphorbia milii, a flowering plant belonging to spurge family Euphorbiaceae is widely used in the folk medicine for the treatment of warts, hepatitis and cancer. The aim of present study is to evaluate the antioxidant and antitumor properties of ethyl acetate extract of Euphorbia milii flower (EAEMF) against MCF-7 cell line induced breast cancer and CACO-2 cell line induced colon cancer in mice. Methods: The MCF-7 cell lines were induced subcutaneously in mammary region in female mice and CACO-2 cell lines were induced intraperitoneally. The animals were treated with ethyl acetate extract of Euphorbia milii flower at doses of 200mg/kg and 400mg/kg p.o., consecutively for 30 days. On 31st day the animal’s body weight and body circumference was noted and mice were sacrificed. The antioxidant and anticancer activity of ethyl acetate extract of Euphorbia milii flower was assessed by evaluating haematological, antioxidant and serum biochemical parameters. Results: Ethyl acetate extract of Euphorbia milii flower decreased body weight and body circumference. Haematological profile such as RBC, haemoglobin, haematocrit and WBC count reverted to normal level in ethyl acetate extract of Euphorbia milii flower treated groups of mice. The extract significantly decreased the levels of lipid peroxidation and elevated the levels of GSH, GPx and catalase. Serum biochemical parameters such as lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), alanine transaminase (ALT), and Glucose were reverted to normal levels in ethyl acetate extract of Euphorbia milii flower treated groups. The cancer specific parameters like Ferritin and Carcinob embryonic antigen (CEA) also significantly decreased in extract treated groups. Histopathology report
was in support to these results. **Conclusion:** The results revealed that Ethyl acetate extract of *Euphorbia milii* flower is effective as antioxidant and chemopreventive.

**KEYWORDS:** *Euphorbia milii*, antioxidant, breast cancer, colon cancer.

**INTRODUCTION**
Cancer is the second leading cause of death after cardiovascular diseases. An American Cancer Society estimates that about 1,660,290 new cancer cases are expected to be diagnosed in 2013. Breast cancer is one of the most serious problems in oncology. It is a leading cause of death among women in many countries. An estimated 232,340 new cases of invasive breast cancer are expected to be diagnosed among women in the US during 2013; about 2,240 new cases are expected in men. An estimated 40,030 breast cancer deaths (39,620 women, 410 men) are expected in 2013. Colon cancer is the third most common cancer in both men and women. An estimated 102,480 cases of colon cancer and 50,830 deaths from colon cancer are expected to occur in 2013, accounting for 9% [1] of all cancer deaths. Reactive oxygen species (ROS) are seemingly involved in a variety of important pathophysiological conditions including mutagenesis and carcinogenesis. [2] Free radicals play an important role in tumor promotion by direct chemical reaction or alteration of cellular metabolic processes [3], and their scavengers (SOD, CAT, GPx) represent inhibitors at different stages of cancer. [4] The enzymes are found in cytosolic and mitochondrial functions mainly involved in the biotransformation and detoxification. [5] The continuing severity and magnitude of the cancer problems make it imperative to develop chemo preventive strategies utilizing natural antioxidants to block the initiation, or arrest/reverse the progression of premalignant cells. [6] Natural products have been regarded as important sources that could produce potential cancer chemoprevention. Many chemo preventive agents are able to block or delay the promotion and/or progression of premalignant or malignant cells by modulating cell proliferation and/or differentiation. [7] Apoptosis is conceivably the most potent defence against cancer development. Activation of apoptosis in pre-cancerous cells is one of the most important mechanisms of cancer chemoprevention. [8] Antioxidants may protect against the toxicity of reactive oxygen species (ROS) by the prevention of ROS formation. [9] The analysis of antioxidant levels is considered essential as they neutralize oxygen-free radicals. [10] Development of life threatening diseases like cancer is linked to the availability of these antioxidants. [11] For all these reasons, it is important to develop a new strategy possessing anti-neoplastic and free radical scavenging properties. Therefore, it is appropriate to
investigate various phytotherapeutic origins to detect anti-tumour and free radical scavenging activities.\textsuperscript{[12]} The available semi synthetic anticancer drugs have more side effects and are cytotoxic to human beings. Since modern medicine has no effective cure for the malignant cancers and tumours, scientists are interested in finding a potent phytotherapeutic agent with non-cytotoxic properties.\textsuperscript{[13]}

The genus \textit{Euphorbia} is the largest genus of medicinal plants widely distributed in tropical countries. Different species of Euphorbia are used as a folk medicine for the treatment of various ailments such as skin diseases, intestinal parasites and warts. It has been reported that Euphorbia possesses antiarthritic, anticancer \textsuperscript{[14]}, anticonvulsant, antidiabetic, anti-eczema, anti-eczema, anti-inflammatory, antimicrobial, antioxidant, antispasmodic, antitumor, antitussive properties hormonal and myelopoiesis properties.\textsuperscript{[15]}

\textit{Euphorbia milii} (Euphorbiaceae), a flowering plant commonly known as ‘Christ plant’ or ‘Christ thrown’. It is ornamental shrub native to Madagascar and Philippines, widely distributed in India. \textit{Euphorbia milii} widely used in folk medicine for the treatment of warts (South Brazil), cancer and hepatitis (china). It has been reported that \textit{Euphorbia milii} possesses antifungal and antinociceptive property, acts as natural molluscicide, can curb the spread of schistosomiasis. Phytochemical studies of \textit{Euphorbia milii} revealed the presence of \(\beta\)-sitosterol, cycloartenol, \(\beta\)-amyrin acetate, lupeol, euphol, triterpenes, phenols and flavonoids \textsuperscript{[16]} some of the latter diterpene esters of ingenol are potent skin irritants but, in contrast with other closely-related ingenol and phorbol derivatives, they showed no tumour promoting activity \textsuperscript{[17]}, Milliamines isolated from \textit{E. milii} latex exhibited potent molluscidical activity.\textsuperscript{[18]} Therefore the present study has been planned to evaluate the antioxidant and antitumor properties of \textit{Euphorbia milii} flower extract against \textit{in vivo} breast cancer and colon cancer in mice.

**MATERIALS AND METHODS**

**Chemicals**

**Collection And Authentication Of Plant**

The flowers of \textit{Euphorbia milii} were collected from in and around Acharya N.G. Ranga Agriculture university and authenticated from Department of Botany, Osmania University, with voucher No.0279, then they were shade dried, grounded coarsely and stored in an air tight container.
Extraction Of Flower
Coarsely powdered flowers were packed in a soxhlet apparatus and subjected to extraction using ethyl acetate as solvent. After extraction, solvent was distilled off and excess solvent was completely removed by using a rotary flash evaporator and then concentrated extract obtained was completely dried and stored at 4°C in a refrigerator.

Preliminary Phytochemical Screening
Preliminary phytochemical screening was done for the presence of carbohydrates, proteins, saponins, alkaloids, flavonoids, tannins, tri-terpinoids and phenolic compounds according to the procedure described in “Textbook of Practical Pharmacognosy” by C.K. KOKATE.¹⁹

Animals
Thirty female Swiss albino mice for breast cancer weighing 18-22gm and thirty Swiss albino mice of either sex for colon cancer weighing 25-30gm were obtained from National Institute of Nutrition (NIN), Hyderabad, India. All the animals were kept under constant environmental conditions with a 12/12 light-dark cycle and temperature of 23±2°C, fed with standard granulated chow, and given drinking water ad libitum. The animal experiments were carried out in accordance with the Institutional Protocols of Animal Care. The experimental protocol (Reg no: MRCP/CPCSEA/IAEC/2011-12/MPCOL/16) was approved by Institutional Animals Ethic Committee (IAEC).

Acute Toxicity Studies
Acute toxicity study of ethyl acetate extract of Euphorbia milii flower was carried out by adopting fixed dose method of CPCSEA, OECD guideline no.423. Swiss albino mice of either sex weighing 20-25gm were used for the study.

Experimental Design
cell lines: Cell lines for Breast cancer (MCF-7) and Colon cancer (CACO-2) were obtained from National Institute of Nutrition (NIN), Hyderabad. The cells were supplemented with a medium containing foetal bovine serum (FBS) and Calf serum with 10% glycerol and maintained at -4°C.

Tumour Transplantation & Treatment Schedule
For the induction of breast cancer, female mice were transplanted with MCF-7 cell lines [(0.2ml) 2× 10⁶ cells/mouse] subcutaneously under mammary fat pads. For the induction of
colon cancer, mice were transplanted with CACO-2 cell lines [(0.2ml) 2× 10^6 cells/mouse] intraperitoneally.

Animals are divided into five groups of six animals each. (N=6). Animals from groups 2, 3, 4 and 5 were transplanted with breast cancer cell lines. They are divided as follows:

**Breast cancer**

Group-I: Control group (1% tween 80-1ml/100g)
Group-II: MCF-7 induced ((0.2ml) 2× 10^6 cells/mouse)
Group-III: MCF-7 induced + 5-fluorouracil (20mg/kg)
Group-IV: MCF-7 induced + *Euphorbia milii* flower extract (200mg/kg)
Group-V: MCF-7 induced + *Euphorbia milii* flower extract (400mg/kg).

**Colon cancer**

Group-I: Control group (1% tween 80-1ml/100gm)
Group-II: CACO-2 induced ((0.2ml) 2× 10^6 cells/mouse)
Group-III: CACO-2 induced + 5-fluorouracil (20mg/kg)
Group-IV: CACO-2 induced + *Euphorbia milii* flower extract (200mg/kg)
Group-V: CACO-2 induced + *Euphorbia milii* flower extract (400mg/kg)

Animals were divided into five groups as said above. The control group animals were given 1% tween80 1ml/100g for 30 days. Group 2 animals were transplanted with cancer cell line on the 1st day. Group 3 animals were transplanted with cancer cell line on the 1st day and the standard drug 5 fluorouracil 20mg/kg from the 3rd day intra peritoneally until 30th day. Group 4 & 5 animals were transplanted with cancer cell line on the 1st day and *Euphorbia milii* extract 200mg/kg & 400mg/kg p.o., from the 3rd day until 30th day.

**Antioxidant Parameters**

The following biochemical measurements were carried out in the liver tissues. The organs were excised and quickly removed from the experimental groups for the estimation of antioxidant enzymes. Liver Tissues was washed thoroughly with ice-cold normal phosphate buffer saline, pH 7.2 (PBS, 0.9%) and cut into small pieces with a heavy-duty blade. Tissues were homogenized by a glass homogenizer tube in cold PBS, centrifuged using Remi cool centrifuge at 8000 rpm for 30 min, and the supernatant was diluted with PBS up to a final protein concentration. The supernatant was used for estimation of biochemical parameters.
such as Malondialdehyde (MDA), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH) in all the tissues of breast cancer and colon cancer.

**Lipid Peroxidation: [Melonaldialdehyde (MDA)]**
The method of Ohkawa et al., (1979) was used for the estimation. All the reagents were freshly prepared and to the 0.2 ml of experimental sample, 0.2ml of 8.1 % SLS, 1.5 ml of 20 % Acetic acid (pH 3.5) and 1.5 ml of 0.8 % aqueous solution of TBA was added and made the volume up to 4 ml with double distilled water. Then heat the mixture at 95ºC for 60 min in water bath on hot plate to develop light pink colour. The mixture was allowed to cool and measure the absorbance spectrophotometrically at 532 nm using U.V – Visible spectrophotometer and the MDA content with extinction coefficient of 156 nM⁻¹·cm⁻¹ and was expressed as nmol/g wet tissue. Finally MDA content was calculated by following formula.

\[
\text{lipid peroxides (nmol MDA/g Tissue)} = (\text{Abs} / 156) \times \left[ \frac{\text{total volume (4 ml)} \times \text{sample volume (0.2 ml)}}{\text{dilution factor (10)}} \right] \times 1000
\]

**Reduced Glutathione (GSH)**
Tissue GSH concentration was measured by method described by (Ellman et al., 1959).

Reduced glutathione is taken as a reference standard for preparation of standard graph. To 2ml of 0.1M potassium phosphate pH 8.4, 0.1ml of standard or experimental sample, 0.5ml of DTNB were added and made the volume up to 3 ml with DDW. Then the mixture was incubated for 10 min at room temperature and measured the absorbance at 412 in U.V – Visible spectrophotometer and calculated the GSH content from standard graph.

\[
\text{n moles GSH per ml of sample} = \frac{\text{DA412/min(sample)}}{\text{DA412/min(1 nmole)}} \times \text{dil} \times \text{vol}
\]

**Glutathione Peroxidase (GPx)**
Total glutathione peroxidase was determined by measuring the rate of oxidation of glutathione at 420 nm, according to the method given by Rotruck. Continuous Spectrophotometric Rate Determination was done. The reaction mixture consisted of 0.02ml
of 0.8mM EDTA, 0.1ml of 10mM Sodium azide, 0.1 ml of 2.5Mm H₂O₂, 0.2ml of homogenate and was arrested by adding 0.5ml of 10% of TCA and the tubes were centrifuged at 2000 rpm for 15 min. 3 ml of 0.3Mm of Disodium hydrogen phosphate and 1 ml of 0.04% DTNB were added to the supernatant and develops colour was detected at 420 nm immediately. Glutathione peroxidase (GPₓ) activity was expressed as µ mole of the oxidized glutathione / min / mg protein and calculated as:

\[ \text{GPₓ} = \frac{\text{Test O.D} \times 20 \times \text{Total volume}}{0.11 \times \text{Sample volume} \times \text{mg protein per ml}} \]

**Catalase**

Catalase activity in tissue was determined by measuring the rate of decomposition of hydrogen peroxide at 240 nm, according to the method given by Aebi et al (Aebi et al., 1974).[E]

Reaction mixture was prepared by adding 1.95 ml phosphate buffer (50 mM, pH 7) to 50 µL of sample and 1 ml of hydrogen peroxide (30mM). Changes in absorbance were recorded at 240nm for 1 min at 15 sec interval by U.V-Visible spectrophotometer and then the activity was calculated in terms of K/min by following formula.

**catalase activity (K/min) = \left( \frac{1}{\Delta t} \right) \times \ln \left( \frac{s1}{s2} \right) = \left( \frac{2.3}{\Delta t} \right) \times \log \left( \frac{s1}{s2} \right)\)**

Where, \( \Delta t = t2-t1 \) (time interval)

\( s1 \) and \( s2 = H₂O₂ \) concentrations at times \( t1 \) and \( t2 \).

**serum parameters**

On 31st day the body weight and body circumference was noted and animals were sacrificed using light ether anesthesia. Blood was collected by carotid bleeding method. Blood was centrifuged using Remi cool centrifuge at 4000 rpm for 15 min. Serum was separated for the estimation of various biochemical parameters like alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyl transpeptidase (GGT) and glucose, ferritin and Carcino embryonic antigen (CEA).

**Histopathological Estimation**

At the end of the experiment (day 31), all the animals were anesthetized by light ether and the breast tissue (breast cancer) and colon (colon cancer) were excised out and fixed in buffered
formalin (10%). Five micron thick sections were prepared by using microtome and these sections were stained with haematoxylin and eosin. For histological alterations these slides were observed under light microscope with 40x magnification.

**Statistical Analysis**
Statistical analysis was performed using Graph Pad Prism 5 (version 5.00 for Windows 98, Graph Pad Software, San Diego, California) software. All data are presented as the mean± standard error of mean (SEM). The results were analysed for statistical significance by unpaired t-test followed by Dunnet’s posthoc test of significance. P value less than 0.05 were considered as statistically significant.

**RESULTS**

**Preliminary Phytochemical Screening**
The main chemical constituents that are found in the flower extract of *Euphorbia milii* include flavonoids, phenols, triterpenoids and tannins.

**Acute Toxicity Studies**
Acute toxicity studies were performed according to the OECD Guideline no. 423. No mortality was observed at the dose of 2000mg/kg. Hence 1/5th and 1/10th dose of 2000mg/kg i.e.; 200mg/kg and 400mg/kg have been used.

**Fig.1. Effect of (EAEEMF) on body weight and body circumference in breast cancer.**
Values are expressed as mean percentage, (n=6). Data was analyzed by percentage difference in body circumference from initial to final, 5-FU= 5 Flouroacil, EAEEMF= Ethyl Acetate Extract of *Euphorbia milii* flower.
Effect Of *Euphorbia Milii* On Body Weight And Body Circumference

The body weight and body circumference of animals in cell line induced groups were increased when compared to normal control groups; whereas the animals treated with *Euphorbia milii* & standard showed decrease in body weight and body circumference when compared to cell line induced groups. (Fig. 1 & 2).

![Graph showing effect of EAEEMF on body weight and body circumference in colon cancer](image)

**Fig.2. Effect of (EAEEMF) on body weight and body circumference in colon cancer**

Values are expressed as mean percentage, (n=6). Data was analyzed by percentage difference in body circumference from initial to final, 5-FU= 5 Fluourouracil, EAEEMF= Ethyl Acetate Extract of *Euphorbia milii* flower.

effect of *Euphorbia milii* on haematological parameters

Haematological parameters in cell line induced groups of both breast cancer and colon cancer were found to be significantly altered compared to those of the normal control group. The effect of *Euphorbia milii* was comparable to those of 5-fluorouracil treated animals. In case of Breast and colon cancer induced groups, the WBC count was found to be increased in cell line induced animals when compared to normal control; whereas RBC count, Haematocrit and haemoglobin levels were significantly decreased in cell line induced animals when compared to the normal control group. Treatment with *Euphorbia milii* showed significant decrease in WBC count compared to cell line induced animals whereas *Euphorbia milii* showed significant increase in RBC count, haematocrit and Hb level when compared to cell line induced groups. (As shown in Table 1 & 2).
Table 1. Effect of *Euphorbia milii* on haematological parameters in breast cancer.

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>GROUP I</th>
<th>GROUP II</th>
<th>GROUP III</th>
<th>GROUP IV</th>
<th>GROUP V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g %)</td>
<td>14.57±0.654</td>
<td>12.15±0.366b</td>
<td>14±0.543</td>
<td>13.9±0.25***</td>
<td>14.7±0.50**</td>
</tr>
<tr>
<td>Haemotocrit (vol %)</td>
<td>50.25±1.31</td>
<td>31±1.58b</td>
<td>37.5±2.47</td>
<td>35.3±1.98</td>
<td>37±1.52*</td>
</tr>
<tr>
<td>RBC (M/Cmm)</td>
<td>8±0.33</td>
<td>6.3±0.214</td>
<td>7.07±0.11</td>
<td>6.8±0.19</td>
<td>7.4±0.229*</td>
</tr>
<tr>
<td>Platelet count (Lakhs/Cmm)</td>
<td>8.12±0.332</td>
<td>7.2±0.273</td>
<td>6.32±0.258*</td>
<td>5.73±0.20</td>
<td>6.55±0.487</td>
</tr>
<tr>
<td>WBC(cells/Cmm)</td>
<td>887.5±42.7</td>
<td>6875±426.9c</td>
<td>2025±438.5*</td>
<td>4540±324*</td>
<td>3200±294.3**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group. 5-FU= 5 Fluorouracil, EAEEM= Ethyl Acetate Extract of *Euphorbia milii*.

Table 2. Effect of *Euphorbia milii* on haematological parameters in colon cancer.

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>GROUP I</th>
<th>GROUP II</th>
<th>GROUP III</th>
<th>GROUP</th>
<th>GROUP V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g %)</td>
<td>14.175±1.24</td>
<td>11.80±0.50a</td>
<td>2.82±0.708</td>
<td>13.34±0.63</td>
<td>13.95±0.54*</td>
</tr>
<tr>
<td>Haemotocrit (vol %)</td>
<td>48.34±2.3</td>
<td>35.2±2.54b</td>
<td>39.9±1.34</td>
<td>41.5±1.0</td>
<td>43.2±1.23*</td>
</tr>
<tr>
<td>RBC (M/Cmm)</td>
<td>8.8±0.3</td>
<td>6.48±0.42a</td>
<td>7.17±0.19</td>
<td>6.9±0.25</td>
<td>7.9±0.22*</td>
</tr>
<tr>
<td>Platelet count (Lakhs/Cmm)</td>
<td>8.39±0.22</td>
<td>6.3±0.33c</td>
<td>5.47±0.325</td>
<td>6.21±0.28</td>
<td>6.82±0.17</td>
</tr>
<tr>
<td>WBC(cells/Cmm)</td>
<td>862±55.4</td>
<td>2900±443.4c</td>
<td>525±342.4*</td>
<td>1265±289*</td>
<td>1920±316.23</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group. 5-FU= 5 Fluorouracil, EAEEMF= Ethyl Acetate Extract of *Euphorbia milii* flower.

Effect Of *Euphorbia Milii* On Serum Parameters

Treatment with *Euphorbia milii* had significant influence on liver enzymes. The effect of *Euphorbia milii* was comparable to those of 5-fluorouracil treated animals.

There was a significant (p<0.001) increase in SGPT, GGT and ALP level in cell line induced animals, when compared to the normal control animals. Treatment with *Euphorbia milii* high dose showed significant decrease in the levels of SGPT (p<0.01), GGT (p<0.01) and ALP (p<0.001) similar to the levels in standard 5-Fluorouracil treated group in breast cancer induced groups (As shown in Table 3 & 4). The animals treated with *Euphorbia milii* showed significant (p<0.001) decrease in levels of SGPT, GGT and ALP similar to the levels of animals treated with standard 5-fluorouracil in colon cancer groups (As shown in Table 3 & 4). There was a significant decrease in LDH (p<0.001) and Glucose (p<0.05) levels in cell
line induced animals when compared to the normal control animals. Treatment with the *Euphorbia milii* and the standard drug 5-Fluorouracil showed a significant increase in LDH (p<0.01) and Glucose (p<0.01) levels in breast and colon cancer induced animals (As shown in Table 3 & 4).

**Table 3. Effect of Euphorbia milii on serum parameters in breast cancer**

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (IU/L)</th>
<th>LDH(IU/L)</th>
<th>GGT(IU/L)</th>
<th>ALP(IU/L)</th>
<th>Glucose(mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>19.8±1.66</td>
<td>33.52±0.94</td>
<td>14.42±0.853</td>
<td>14.42±0.4</td>
<td>103±6.18</td>
</tr>
<tr>
<td>Group II</td>
<td>33.6±1.6c</td>
<td>25.75±0.654c</td>
<td>26.87±1.23c</td>
<td>18.38±0.39c</td>
<td>84±5.4a</td>
</tr>
<tr>
<td>Group III</td>
<td>23.7±1.895**</td>
<td>43.5±1.03***</td>
<td>16.92±1.76**</td>
<td>12±0.48*</td>
<td>117.25±5.85**</td>
</tr>
<tr>
<td>Group IV</td>
<td>28.34±1.45*</td>
<td>52.3±0.85*</td>
<td>18.83±1.03**</td>
<td>15±0.53**</td>
<td>89±5.32</td>
</tr>
<tr>
<td>Group V</td>
<td>24.9±1.36**</td>
<td>50.72±0.904***</td>
<td>17.91±0.92**</td>
<td>13±0.45***</td>
<td>108±4.45**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group. 5-FU= 5 Fluorouracil, EAEEMF= Ethyl Acetate Extract of Euphorbia milii flower.

**Table 4. Effect of Euphorbia milii on serum parameters in colon cancer**

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (IU/L)</th>
<th>LDH(IU/L)</th>
<th>GGT(IU/L)</th>
<th>ALP(IU/L)</th>
<th>Glucose(mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>22.6±0.967</td>
<td>41.9±0.94</td>
<td>14.35±0.78</td>
<td>13.72±0.525</td>
<td>93.7±6.03</td>
</tr>
<tr>
<td>Group II</td>
<td>37±1.03c</td>
<td>32.82±0.743c</td>
<td>24.2±0.834c</td>
<td>16.75±0.556c</td>
<td>73.5±3.88a</td>
</tr>
<tr>
<td>Group III</td>
<td>24±1.42***</td>
<td>64.8±1.15***</td>
<td>16.3±0.88*</td>
<td>10±0.81**</td>
<td>130±4.56***</td>
</tr>
<tr>
<td>Group IV</td>
<td>27.7±1.01**</td>
<td>54.3±1.357***</td>
<td>13.81±0.62***</td>
<td>12.2±0.932**</td>
<td>132±3.88***</td>
</tr>
<tr>
<td>Group V</td>
<td>25.2±1.15***</td>
<td>55.9±2.01***</td>
<td>14.85±0.57***</td>
<td>11.67±0.85***</td>
<td>133.9±2.98**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group. 5-FU= 5 Fluorouracil, EAEEMF= Ethyl Acetate Extract of Euphorbia milii flower.

**Effect of Euphorbia milii on Ferritin and Carcinoembryonic antigen (CEA)**

Increased levels of Ferritin and CEA indicates the propagation of the cancer. Animals in cell line induced groups showed significant(p<0.001) increase in levels of ferritin and CEA when compared to normal control. Treatment with *Euphorbia milii* and standard 5-fluorouracil showed significant(p<0.001)decrease in the levels of ferritin and CEA (As shown in Table 5 & 6).
Table 5. Effect of *Euphorbia milii* on ferritin and CEA in breast cancer.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ferritin (ng/ml)</th>
<th>CEA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>81.25±1.65</td>
<td>12.7±1.1</td>
</tr>
<tr>
<td>Group II</td>
<td>203.2±4.63</td>
<td>45.5±2.4</td>
</tr>
<tr>
<td>Group III</td>
<td>134.5±2.1***</td>
<td>13.7±1.75***</td>
</tr>
<tr>
<td>Group IV</td>
<td>182.4±5.3**</td>
<td>34.5±2.8*</td>
</tr>
<tr>
<td>Group IV</td>
<td>167.7±6.2***</td>
<td>26.2±2.53***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group, 5-FU= 5 Fluorouracil, EAEEMF= Ethyl Acetate Extract of *Euphorbia milii* flower.

Table 6. Effect of *Euphorbia milii* on Ferritin and CEA in colon cancer

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ferritin (ng/ml)</th>
<th>CEA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>73.75±1.44</td>
<td>13±1.29</td>
</tr>
<tr>
<td>Group II</td>
<td>157.5±6.8b</td>
<td>81.5±1.32</td>
</tr>
<tr>
<td>Group III</td>
<td>92.25±1.49***</td>
<td>24.2±1.89***</td>
</tr>
<tr>
<td>Group IV</td>
<td>115±4.56***</td>
<td>46.5±2.60***</td>
</tr>
<tr>
<td>Group V</td>
<td>100.3±3.78***</td>
<td>37.5±2.83***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group, 5-FU= 5 Fluorouracil, EAEEMF= Ethyl Acetate Extract of *Euphorbia milii* flower.

**Effect of *Euphorbia milii* extract on antioxidant parameters**

The levels of MDA were significantly increased in cell line induced animals indicating lipid peroxidation. Treatment with *Euphorbia milii* significantly reduced the MDA levels when compared to the cell line induced animals. (As shown in Table 9 & graph 12).

The level of antioxidant enzymes like catalase, GSH and GPx were almost depleted in cell line induced groups. The treatment with *Euphorbia milii* showed significant increase in antioxidant levels of catalase, GSH and GPx. (As shown in Table 7 & 8).

Table 7. Effects of *Euphorbia milii* extract on antioxidant parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase (µmole/min/mg)</th>
<th>GSH (µmole/min/mg)</th>
<th>GPX (µmole/min/mg)</th>
<th>MDA (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.44±0.03</td>
<td>14.4±0.256</td>
<td>9±0.317</td>
<td>65.7±2.6</td>
</tr>
<tr>
<td>II</td>
<td>0.21±0.03</td>
<td>8.3±0.23</td>
<td>5.2±0.24</td>
<td>100.2±3.9</td>
</tr>
<tr>
<td>III</td>
<td>0.41±0.03</td>
<td>11.9±0.16**</td>
<td>7.8±0.25**</td>
<td>72.2±2.3**</td>
</tr>
<tr>
<td>IV</td>
<td>0.28±0.028</td>
<td>10.8±0.18**</td>
<td>6.3±0.214**</td>
<td>86.7±1.78**</td>
</tr>
<tr>
<td>V</td>
<td>0.35±0.025</td>
<td>11±0.194***</td>
<td>7.3±0.235***</td>
<td>69.5±1.34***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group, 5-FU= 5 Fluorouracil, EAEEMF= Ethyl Acetate Extract of *Euphorbia milii* flower.
Table 8. Effects of *Euphorbia milii* extract on antioxidant parameters:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase (µmole/min/mg)</th>
<th>GSH (µmole/min/mg)</th>
<th>GPx (µmole/min/mg)</th>
<th>MDA (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.47±0.02</td>
<td>16.6±0.297</td>
<td>9.9±0.375</td>
<td>72.9±1.54</td>
</tr>
<tr>
<td>II</td>
<td>0.24±0.067</td>
<td>5.6±0.24</td>
<td>4.82±0.193</td>
<td>95.8±2.67</td>
</tr>
<tr>
<td>III</td>
<td>0.4±0.027</td>
<td>13.75±0.39***</td>
<td>7.4±0.2**</td>
<td>73.4±1.8***</td>
</tr>
<tr>
<td>IV</td>
<td>0.31±0.024*</td>
<td>11.2±0.28**</td>
<td>6.1±0.147***</td>
<td>84.4±2.2***</td>
</tr>
<tr>
<td>V</td>
<td>0.38±0.043</td>
<td>11.98±0.273***</td>
<td>6.85±0.165***</td>
<td>76.2±2.3***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM., Data was analyzed by unpaired t test. cp<0.001, bp<0.01 and ap<0.05 as compared with the normal control; ***p<0.001 **p<0.01 and *p<0.05 as compared with cell line induced group, 5-FU= 5 Fluorouracil, EAEEMF= Ethyl Acetate Extract of *Euphorbia milii* flower.

Similarly the above results were supported by histopathological studies (As shown in Fig.3 & 4).

3.(a)Normal breast tissue: Epidermis, dermis and connective tissue and hair follicles appeared normal.

(b) MCF-7 Cell line induced breast tissue: Severe sub mucosal inflammation and fibrosis noticed. Infiltration of inflammatory cells [lymphocytes, neutrophils] and fibrosis noticed.
(c) MCF-7 Cell line induced+ 5-FU treated: Hyperplasia of epidermal layer. Particularly stratum basal epithelial layer showed continuous proliferation and hyperplastic changes. Dermis, hair follicle and sebaceous gland appeared normal.

(d) MCF-7 Cell line induced+ EAFEMF-I (200mg/kg) treated: Epidermis, dermis, subcutaneous, sebaceous glands and hair follicles appeared normal.

(e) MCF-7 Cell line induced+ EAEEMF-II (400mg/kg) treated: Epidermis, dermis, subcutaneous, sebaceous glands and hair follicle appeared normal.

Fig.3. Histopathology of breast tissue
4. (a) Normal colon

Mucosal, sub mucosal region contain mucosal glands and muscular region appeared normal.

(b) CACO-2Cell line induced colon

Mucosal degeneration of intestinal epithelial cells in the mucosal layer, sub mucosal glands and muscular region appeared normal.

(c) CACO-2Cell line induced+5-FU treated: Goblet cells proliferation observed in the mucosal and sub mucosal glandular region.
(d) CACO-2 Cell line induced+ EAEEMF-I
(200mg/kg) treated: Sub mucosal gland proliferation noticed in the colon, glandular mucosa and goblet cells in the mucosal glands are normal. Mucosal region appeared normal.

(e) CACO-2 Cell line induced+ EAFEMF-II
(400mg/kg) treated: Moderate goblet cells proliferation in the mucosal and glandular region of colon. Muscular region appeared normal.

**Fig. 4. Histopathology of colon**

**DISCUSSION**

Natural products have been regarded as important sources that could produce potential chemoprevention. Cancer chemoprevention can be achieved by the use of natural, synthetic or biologic compounds that reverse, suppress or prevent the development of epithelial malignancies. Many chemopreventive agents are able to block or delay the promotion and/or progression of premalignant or malignant cells by modulating cell proliferation and/or differentiation. Apoptosis is conceivably the most potent defence against cancer development. Activation of apoptosis in pre-cancerous cells is one of the most important mechanisms of cancer chemoprevention.
Historically, natural products have played an important role in antitumor drug discovery.\textsuperscript{[21]} In this context, plant families presenting bioactive compounds, in particular terpenes have shown anti-tumoural properties. Several studies revealed the cytotoxic activity of Euphorbiaceae species against different tumour cell lines, including carcinoma cells. The present study was carried out to evaluate antioxidant & anticancer activity of ethyl acetate extract of \textit{Euphorbia milii} flowers on cell line induced in vivo breast cancer and colon cancer. Chemoprevention involves the use of either Natural or synthetic compounds to delay inhibit or reverse the development of cancer in normal or pre-neoplastic conditions.\textsuperscript{[22]}

The high dose of \textit{Euphorbia milii} elicited a significant decrease in the body weight and body circumference of the animals when compared to cell line induced group animals. One of the reliable criteria for evaluating anticancer activity is change in WBC count.\textsuperscript{[23]} White blood cells main function is to fight infection, defend the body by phagocytosis against invasion by foreign organisms, and to produce, or at least transport and distribute, antibodies in the immune response. The animals treated with both the doses of \textit{Euphorbia milii} elicited a significant decrease in WBC count when compared to cell line induced animals in both the cancers.

Usually, in cancer chemotherapy the major problems that are being encountered are of myelosupression and anemia.\textsuperscript{[24]} The anemia encountered in tumor bearing mice is mainly due to reduction in RBC, hemoglobin percentage or hematocrit, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions.\textsuperscript{[25]} In the animals treated with \textit{Euphorbia milii} increased the levels of RBC, hemoglobin and hematocrit which were decreased in case tumor bearing animals.

Serum biochemical parameters play important role in diagnosis of the diseases. Alanine amino transferase (ALT) is an enzyme produced in hepatocytes, the major cell type in the liver. Alanine amino transferase (ALT) catalyzes the transamination of L-alanine and α - ketoglutarate (α - KG) to form Pyruvate and L-glutamate. The level of ALT in the blood is increased in conditions in which hepatocytes are damaged. The \textit{Euphorbia milii} extract treated groups showed significant decrease in ALT levels in both cancers when compared to cell line induced groups. Alkaline phosphatase (ALP) is an enzyme, or more precisely a family of related enzymes, produced in the bile ducts, intestine, kidney, placenta and bone. Serum ALP levels are frequently elevated in patients with metastatic breast and colorectal cancer. In the present study cell line induced group showed significant increase in the ALP
levels\(^{[23]}\) whereas the *Euphorbia milii* extract treated groups showed significant decrease in ALP levels. Gamma-glutamyl transferase (GGT) is a membrane-bound enzyme involved in the metabolism of glutathione (gamma glutamyl-cysteinyI-glycine; GSH) and is expressed by a wide number of cell types.\(^{[26]}\) GGT has been traditionally regarded as a component of the cell protection system against oxidative stress. A dysregulated expression of GGT has been detected in several tumour types like colon, liver, breast.\(^{[27]}\) In the present study the cell line induced mice in breast cancer and colon cancer showed significant increase in levels of GGT when compared to normal control group. The *Euphorbia milii* extracted treated group in both the cancers maintained significantly levels of GGT. Increases in LDH levels are usually found in cellular death and/or cell membrane damage. This cell membrane damage or cell death is due to increase in ROS (reactive oxygen species). In cell line induced groups of breast cancer and colon cancer the increase in LDH was observed and The *Euphorbia milii* extract treated groups showed significant decrease in LDH levels. Glucose is the main source of energy for the cells to grow. The breakdown of glucose to provide energy is called glycolysis.\(^{[28]}\) Healthy cells can use other forms of ‘food’ like fats, as precursors but Cancer cells cannot. Cancer cells need supplies of common glucose to grow. They can only derive energy from glycolysis in the cytoplasm for which they use stored glucose in the body thereby decreasing levels of glucose in the serum.\(^{[29]}\) In the present study the decreased levels of serum glucose were observed in cell line induced mice in breast cancer and colon cancer when compared to normal control group. The animals treated with *Euphorbia milii* extract restored or maintained the glucose levels in the serum as normal when compared to cell line induced groups.

Ferritin is the major intracellular iron storage protein in all organisms. Iron serves as a nutrient for cell growth. Iron is an absolute requirement for cell proliferation, as iron-containing proteins catalyse key reactions involved in oxygen sensing, energy metabolism, respiration and DNA synthesis. Without iron, cells are unable to proceed from G1 to the S phase of cell cycle. Ferritin iron represents approximately 20% of total iron in the body.\(^{[30]}\) Iron stored in ferritin can be mobilized prior to ferritin degradation. Ferritin’s iron scavenging function is also thought to prevent iron-mediated catalysis of reactive oxygen species (ROS) \(^{[31]}\), which provoke tissue damage and may cause cancer and neuro degeneration A study on ferritin showed a positive association between dietary and body iron stores with colorectal cancer risk. Serum ferritin in cancer showed statistically-significant increases in both localized and metastatic diseases. The cancer-associated elevation in serum ferritin is most
likely induced by an inflammatory state. The body iron stores were positively associated with the development of precancerous lesions in the colon, colonic adenoma or polyps. Increasing iron concentration in human intestinal CACO-2 cells resulted in increased protein and DNA oxidative damage.\(^{32}\) In the present study increased levels of ferritin were observed in cell induced mice in both breast cancer and colon cancer when compared to normal control mice. The animals treated with *Euphorbia milii* extract showed decreased levels of ferritin in the serum when compared to cell line induced groups.

Carcinoembryonic antigen (CEA) an oncofetal antigen \(^{33}\) is a member of the immunoglobulin supergene family and may participate in intercellular recognition and attachment. CEA promotes homotypic aggregation of carcinoma cells to each other. CEA modulates intercellular adhesion, functions as a promoter of cellular aggregation, regulates the innate immune system, and mediates signal transduction. Accordingly, it is hypothesized that CEA plays an important role in tumour invasion and metastasis. CEA has become the one of the most widely known tumour markers for gastrointestinal tract diseases, especially for colon cancer. An increased level of CEA was observed in colorectal cancer, breast cancer, gastric cancer, lung cancer. CEA measurement is mainly used as a tumour marker to monitor cancer treatment. In the present study increased levels of CEA were observed in cell line induced mice in both breast cancer and colon cancer when compared to normal control mice.\(^{34}\) The animals treated with *Euphorbia milii* extract showed significant decrease in levels of CEA in the serum when compared to cell line induced groups.

Oxidative stress plays an important role in carcinogenesis because of induction of DNA damage and its effects on intracellular signal transduction pathways. Antioxidant enzymes play a major role to combat the free radical induced damage due to oxidative stress. Antioxidants are the first source of protection of the body against free radicals and other oxidants, being the compounds that halt the attack and the formation of radical species within cells.\(^{35}\) Higher levels of antioxidant enzymes like Glutathione, Catalase, and GPx were observed in the liver. The levels of the antioxidant enzymes decrease due to oxidative stress and cancer.\(^{36}\) Glutathione is also an important antioxidant compound responsible for maintaining intracellular redox homeostasis. It is found in particularly high concentration in the liver and is known to have a key function in the protective process\(^ {37}\) Glutathione exists in reduced (GSH) and oxidized glutathione disulphide (GSSG) states. GSH is an essential cofactor for antioxidant cells known as GSH peroxidases, including GPx, which are used to
detoxify peroxides, including the H$_2$O$_2$ generated in cell membranes that react with GSH. This redox balance is altered under hypoxia conditions.$^{[38]}$ In the case of oxidative stress and tumors, the levels of GSH and GPx were depleted with the production of ROS and NO•. The animals treated with *Euphorbia milii* extract maintained or restored the levels of antioxidant enzymes Glutathione and GPx significantly when compared to cell line induced mice in both breast cancer and colon cancer. Catalase (CAT) is a very important enzyme of all living organisms which catalyses the decomposition of hydrogen peroxide to water and oxygen. It was reported that 22% lower CAT activity was observed in epidermis of liver in malignant diseases like cancer.$^{[39]}$ The decreased CAT activity was due to the inflammation which leads to increase hydrogen peroxide intracellularly and create an intracellular environment favorable to DNA damage and the promotion of cancer.$^{[40]}$ The animals treated with *Euphorbia milii* extract maintained or restored the levels of Catalase significantly when compared to cell line induced mice in both breast cancer and colon cancer.

Malondialdehyde (MDA) is formed during oxidative degeneration as a product of reactive oxygen species, which acts as an indicator for lipid peroxidation. MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues.$^{[41]}$ The animals treated with *Euphorbia milii* extract showed significant decreased levels of MDA when compared to cell line induced mice in both breast cancer and colon cancer supporting antioxidant activity.

Taken together our results showed that *Euphorbia milii* restored/ maintained the levels of serum enzymes, increased the levels of antioxidant enzymes and decreased the levels of tumour marker’s like ferritin and CEA. Based on our data we can assume that *Euphorbia milii* showed the antioxidant and antitumor properties by inducing apoptosis and reducing the levels of reactive oxygen species, thereby indicating the chemopreventive nature of natural products.

In conclusion, anti-oxidant and anti-tumour activity of ethyl acetate flower extract of *Euphorbia milii* may be due to the presence of triterpenes, phenols and flavonoids.

**REFERENCES**


