6.1. Introduction

Mammary carcinoma is the most common malignant neoplasm and the etiology is not yet fully understood. Various factors such as chemical carcinogens, abnormal levels of pituitary or ovarian hormones, and viruses have been proposed to play an important role in the pathogenesis. The experimental production of mammary carcinoma with oncogenic viruses or sex hormone administration has several disadvantages mainly prolonged latent period before the tumor appears. Chemical carcinogens on the other hand, can produce carcinoma of the breast in a high percentage of animals in a short period of time with a single dose. The method of Huggins (1963) for the production of mammary tumors in rats by DMBA administration is one of the most successful and widely used methods. The tumors are multifocal, locally invasive adenocarcinomas that do not metastasize. The earliest changes that are noted are alterations of the epitheliostromal junction and production of periductal fibrosis. As is noted in human breast tumors, these tumors present with varying degrees of morphological appearances and differentiation. In the current study, mammary tumors were produced in female Sprague-Dawley rats following administration of DMBA (Murad and vonHaam, 1972).

Xenograft model in nude mice

Nude mice are immune compromised and thus rule out the interference of immune defense system of the recipient. These animals lack thymus hence they are unable to produce functional T lymphocytes. Hence, human xenografts can be grown in nude mice. This model is more predictive of the clinical outcome as compared to its murine allograft counterpart and xenograft histology closely resembles that of patient’s tumor (Troiani, 2008). Hence, it is an ideal model for in vivo determination of efficacy of anti-cancer compounds.

6.2 Materials and Methods

DMBA (7,12-Dimethylbenz[a]anthracene, # D3254) was obtained from Sigma–Aldrich Co. LLC, MO, USA. The colorimetric kits for elemental analysis were purchased from Aspen Laboratories, Delhi, India. All other reagents were of analytical grade and purchased from usual sources.
6.2.1 DMBA-induced breast cancer in female SD rats

Female Sprague Dawley rats, 45 to 50 days old, inbred and maintained at the Central Animal Research Facility of Manipal University were used in the study. The animals were maintained as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The experiment was performed in accordance with the ethical norms approved by the Institutional Animal Ethics Committee (No: IAEC/KMC/53/2015).

6.2.1.1 Acute toxicity studies

Acute toxicity study was carried out in accordance with OECD 425 guidelines. NJDE and MUEA were found to be safe up to 2000 mg/kg. Animals were observed for any toxicity signs (behavioral, neurological and morphological profiles) for the first 4 h continuously and thereafter daily for 14 days. No mortality or toxicities were observed with any of the treatments.

Dose selection was done based on the MTD established from acute toxicity studies.

1/10\textsuperscript{th} dose of the limiting dose (2000 mg/kg) and a lower dose (200 and 100 mg/kg) were selected for the efficacy study.

6.2.1.2 Carcinogen treatment

A single dose of 7, 12-dimethylbenz (a) anthracene (DMBA) (30 mg/kg) was prepared in olive oil and administered intra-gastrically. The animals were palpated for the induction of tumor (tumors started developing after 13th week). Body weight was recorded weekly (Murad and vonHaam, 1972).

MUEA and NJDE were administered orally (suspended in 0.25% carboxymethyl cellulose) for 4 weeks. Treatment was started after tumor volume reached 0.5 cm. The animals were randomized into the following groups (n=5-7).
Table 6.1 Experimental groups for DMBA-induced breast cancer model

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Treatment Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal Control (0.25% CMC)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Disease Control (0.25% CMC)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NJDE 100 mg/kg p.o.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NJDE 200 mg/kg p.o.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Doxorubicin 2 mg/kg i.p. (Twice weekly)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Doxorubicin 2 mg/kg i.p. (Twice weekly) + NJDE 100 mg/kg b.w. p.o.</td>
<td>28 days</td>
</tr>
<tr>
<td>7</td>
<td>Doxorubicin 2 mg/kg i.p. (Twice weekly) + NJDE 200 mg/kg b.w. p.o.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>MUEA 100 mg/kg b.w. p.o.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>MUEA 200 mg/kg b.w. p.o.</td>
<td></td>
</tr>
</tbody>
</table>

Doxorubicin was used as standard. Two additional groups (Doxorubicin + NJDE 100 mg/kg and 200 mg/kg) were included in the study. Since, doxorubicin exerts several dose-limiting toxicities; the most important being cardiotoxicity (Nayak, 2013). Since, *N. jatamansi* is reported to have cardioprotective activity, we evaluated the cardioprotective activity in DMBA+Doxorubicin treated animals.

At the end of the study, animals were fasted overnight and sacrificed. Blood was collected and the serum separated for biochemical estimations. The tumor was excised and stored in 10% neutral-buffered formalin (NBF) for histopathology.

6.2.1.3 General observations

The total body weight of the animals was recorded every week throughout the duration of the study. At the end of the study, animals were sacrificed and different organs: liver, spleen, and heart were dissected out and weighed.

6.2.1.4 Tumor weight and volume

The tumors were measured using Vernier Calipers (Arivazhagan and Pillai, 2014) and tumor volume was measured using the formula $V = \frac{(L \times W^2)}{2}$ (Tabaczar, 2015; Marquesa, 2015).
Tumor volume = (length \times width^2) / 2, where length and width are in centimeters.

The tumors were excised and weighed at the termination of the study.

### 6.2.1.5 Hematology parameters

Blood was collected from the retro-orbital plexus of animals into di-potassium EDTA-coated vacutainers and analyzed using a veterinary blood cell counter (PCE-210 VET, ERMA Inc., Tokyo, Japan).

### 6.2.1.6 Estimation of biochemical and oxidative-stress related biomarkers

Mammary tissues and livers were excised from the animals and blood was removed by washing with ice-cold PBS, blotted and weighed. 10% w/v homogenate of the tissues was prepared in cold 1.15% w/v potassium chloride solution. This homogenate was centrifuged at 10000 rpm for 10 min at 4 °C to obtain a clear supernatant which was used for the estimation of anti-oxidant and biochemical parameters.

#### 6.2.1.6.1 Lipid peroxidation

To 200 μl of 10% w/v tissue homogenate, 200 μl of sodium dodecyl sulphate (8.1%), 1.5 ml of 20% acetic acid solution (pH 3.5, adjusted with sodium hydroxide) and 1.5 ml of aqueous solution of thiobarbituric acid (0.8%) were added. The total volume was made up to 4 ml with distilled water and heated at 95 °C in a water bath for 1 h. The mixture was then cooled, and 1 ml of distilled water and 5 ml of mixture of n-butanol and pyridine (15:1) were added. The mixture was shaken vigorously and centrifuged at 5000 rpm for 5 min. The upper layer was removed and absorbance was measured at 532 nm using UV/Vis spectrophotometer (Ohkawa et al., 1979).

#### 6.2.1.6.2 Estimation of catalase

50 μl of tissue homogenate was added to 3 ml of freshly prepared 0.036% w/w of hydrogen peroxide solution (prepared in phosphate buffer using 30% w/w H₂O₂) and the absorbance was recorded at 240 nm for 0 to 30 s (Cohen et al., 1970).

#### 6.2.1.6.3 Estimation of SOD

The SOD estimation assay was based on the ability of SOD to inhibit the spontaneous oxidation of adrenaline to adrenochrome. 50 μl of tissue homogenate was added to the
reaction mixture consisting of $3 \times 10^{-4}$ M adrenaline and 0.05 M carbonate buffer (pH 10.2). The decrease in absorbance of adrenochrome was recorded at 480 nm. 1 unit of SOD activity is equal to the amount of enzyme, needed to inhibit 50% autooxidation of adrenaline (Mishra, 1972).

6.2.1.6.4 Estimation of GSH

To the tissue homogenate (in 0.1 M phosphate buffer pH 7.4), equal volume of 20% TCA containing 1 mM EDTA was added to precipitate the proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 5000 rpm. The supernatant was added to 1.8 ml of Ellman's reagent (DTNB), 0.1 mM), prepared in 0.3 M phosphate buffer with 1% sodium citrate solution. The volume was made up to 2 ml in all the tubes and absorbance recorded at 412 nm against blank. The GSH concentration was calculated from the standard curve for reduced glutathione (concentration range 0–0.1 mM) and results were expressed as nmol/mg protein (Ellman, 1959).

6.2.1.7 Estimation of serum biomarkers

Aspartate aminotransferase (AST), alanine transaminase (ALT) were determined using appropriate kits in an autoanalyzer (Roche Diagnostics, Indianapolis, IN, USA).

6.2.1.8 Elemental analysis in serum

Elemental analysis was carried out for copper, zinc, calcium, and magnesium in serum by colorimetric assay kits (Aspen colorimetric assay kits).

6.2.1.9 DNA Fragmentation in tumor tissue samples

Quantitation of DNA fragmentation was done by the colorimetric diphenylamine assay (Burton, 1956). The tumor homogenates were mixed with equal volume of buffer containing 20 mM Tris-HCl, 20 mM ethylenediaminetetraacetate (EDTA), 0.5% Triton X-100, pH 7.5, and centrifuged at 15,000 rpm for 15 min at 4°C to separate intact DNA in the pellet from fragmented/damaged DNA in the supernatant fraction. Perchloric acid (final concentration 0.5 M) was added to the pellet and supernatant samples which were heated at 90°C for 15 min and then centrifuged to remove precipitated proteins. The resulting supernatants, whether containing whole or fragmented DNA, were treated with 58.7 mM diphenylamine for 16–20 h at room temperature in dark and the absorbance
was recorded at 600 nm. DNA fragmentation was expressed as the percentage of fragmented DNA to total DNA.

6.2.1.10 Histopathology

6.2.1.10.1 Hematoxylin and eosin staining

Mammary and heart tissues were fixed in 10% neutral buffered formalin (NBF), dehydrated with alcohol and cleared thoroughly using xylene. The tissues were impregnated with paraffin wax and 5 μm sections were cut using a rotary microtome (RM2245, Leica Microsystems GmbH, Wetzlar, Germany), mounted on slides, de-waxed with xylene followed by rehydration through graded series of alcohol. The tissues were then stained with hematoxylin and eosin. The slides were observed under a microscope, analyzed by a pathologist who was blinded to the samples, and photographs taken. The slides were observed for the change in the tubular–alveolar pattern of the mammary gland for identification of the type of carcinoma, infiltration of immune cells; necrosis and hemorrhage.

6.2.1.10.2 Carmine alum staining

Whole mammary gland mounts of the animals were prepared. The mammary glands were dissected at the time of sacrifice, spread evenly on the slides, and placed in NBF overnight. The glands were defatted in acetone for 4 h or overnight, placed in 70% alcohol for 30 min, and hydrated in water for 10 min, followed by staining in alum carmine overnight. After the staining, glands were run through a series of graded alcohols (30–100% ethanol) and placed in xylene to clear the tissue. Glands were then compressed between two glass slides for 24 h, released and allowed to expand for 8 h, and mounted using a glass cover slip and glycerine (Mishra, 2011).

6.2.1.11 Statistical analysis

Statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Tukey’s post hoc test using Prism 6.05 Version (GraphPad Software Inc., La Jolla, California, USA). The effect on tumor growth inhibition was analyzed by two-way ANOVA followed by Bonferroni’s multiple comparison test. Results were expressed as Mean ± SEM and p < 0.05 was considered significant.
6.2.2 MDA-MB-231Br xenograft model in nude mice

6.2.2.1 Maximum tolerated dose (MTD)

MTD was established in athymic nude mice to determine the dose for efficacy study. Generally, while determination the MTD, the practice is to conserve the compound and minimize the number of animals sacrificed. Therefore, a single animal is given a single injection of 400 mg/kg (or lower if the compound is anticipated to be extremely potent, e.g. natural products); a second mouse receives a dose of 200 mg/kg and a third mouse receives a single dose of 100 mg/kg following which the mice kept under observation for a period of two weeks. The mice are sacrificed if they are found to lose more than 20% of their body weight or if there are other signs of significant toxicity. If all of the three mice need to be sacrificed, the next 3 dose levels (50, 35 and 12.5 mg/kg) are tested in a similar manner. This process is repeated until a tolerated dose is found that is designated as the MTD and is used to calculate the amount of material administered to mice during efficacy testing. The mice are allowed *ad libitum* feed and water. Dose volumes are generally 0.1 mL/10 grams body weight but may be up to 0.2 mL/10 grams of body weight for IP, IV, SC and PO routes.

The vehicle used is 10% DMSO in saline/0.05% Tween 80. (U.S. National Cancer Institute/National Institute of Health. Developmental therapeutics program, 2013).

6.2.2.2 Xenograft solid tumor growth delay study

Athymic nude mice were inoculated s.c. in the dorsal flank with triple negative brain-seeking metastatic MDA-MB-231Br cells and allowed to grow till the volume of tumor nodule reaches about 100 mm³. Animals were randomized based on tumor volume followed by administration of test fractions orally. Treatment with fractions/sub-fractions was done on 5 consecutive days followed by 2 days of rest until Day 25. The effect on tumor growth was monitored on Day 1, 5, 10, 14, 18, 21, and 25 days by assessing the tumor volume by Vernier calipers. After 25 days, the animals were sacrificed under anesthesia.

The fractions that were tested in xenograft model had demonstrated significant activity against MBA-MB-231Br cells in the *in vitro* studies. The doses selected were determined based on the MTD study performed in nude mice.
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Table 6.2 Experimental groups for Xenograft model

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Treatment</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Disease Control (Saline)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MUEA 150mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PA-rich oil 50 mg/kg</td>
<td></td>
<td>25 days</td>
</tr>
<tr>
<td>4</td>
<td>NJDE/F2/91/9 50 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NJDE/F4 25 mg/kg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.2.2.3 Statistical analysis

Results were expressed as Mean ± SEM and data was subjected to appropriate statistical analysis using one-way Analysis of Variance (ANOVA) followed by Dunnett’s post-hoc test or two-way ANOVA followed by Bonferroni’s multiple comparison test. p<0.05 will be considered as significant.

6.3 Results and Discussion

The in vitro studies showed that NJDE fraction (diethyl ether fraction from Nardostachys jatamansi) and MUEA (ethyl acetate fraction of Memecylon umbellatum) were highly effective in vitro against breast cancer cell lines (MCF-7 and MDA-MB-231 cells). Therefore, the fractions were evaluated for the in vivo activity in DMBA-induced mammary tumor model and MDA-MB-231 xenograft model for their potential anti-tumor activity.

6.3.1 DMBA-induced cancer in Sprague Dawley rats

Environmental pollutants like polycyclic aromatic hydrocarbons are recognized as carcinogens. DMBA is a potent carcinogenic and mutagenic polycyclic aromatic hydrocarbon that acts as a key contributor of oxidative stress – induced cancer, leading to free radicals production that is very toxic to cell organelles. DMBA-induced breast cancer induced mammary tumor in rodents that is similar to human breast cancer. DMBA administration leads to up-regulation of its cytosolic aryl hydrocarbon receptor (AhR) which translocates into the nucleus and forms a complex with the receptor nuclear translocator leading to up-regulation of cytochrome P450 which metabolizes DMBA into epoxide that readily form DNA adducts leading to malignancy (Rajakumar et al., 2015).
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Being lipid-soluble, DMBA can accumulate in the mammary fat pads resulting in high risk of breast cancer (Rengarajan et al., 2015).

6.3.1.1 Tumor growth inhibition

The effect of treatments on the tumor growth was evaluated by measuring the tumor volume every week. After the animals were sacrificed, the mean tumor weight was also determined. All treatments were found to significantly reduce the mean tumor volume and tumor weight compared to the DMBA control animals (Figure 6.1 and Figure 6.2). MUEA at 200 mg/kg significantly reduced the tumor volume after 4 weeks of treatment. However, MUEA at the lower dose, 100 mg/kg did not reduce tumor growth significantly as compared to the DMBA control animals. A dose-dependent decrease in tumor volume was observed with MUEA. Polyphenolic-rich fraction MUEA, which was found to be very effective in the in vitro studies, was unable to significantly reduce the tumor weight and volume. This could be attributed to the poor absorption or other pharmacokinetic properties of the fraction.

NJDE at 100 mg/kg significantly reduced the tumor volume after 3 weeks of treatment while at 200 mg/kg significantly reduced tumor growth after 2 weeks of treatment. Doxorubicin significantly (p<0.05) reduced the tumor volume after one week of treatment. Treatment with the combination, doxorubicin and NJDE at 100 mg/kg and 200 mg/kg both, significantly reduced the tumor volume after one week of treatment as compared to the DMBA control animals.

In vitro studies of the fractions in MDA-MB-231 breast cancer cells showed upregulation of important proapoptotic markers like caspase 3/7. The ability of the fractions to modulate these marker genes could be correlated with the reduction of tumor growth observed in SD rats in vivo. However, this should be read with caution, since the in vitro and in vivo tumor microenvironments are highly variable, and further in vivo studies are needed to confirm the effect of the fractions on these apoptotic markers.

The reference chemotherapeutic agent, doxorubicin has a very poor oral bioavailability (less than 5%) (Kim et al., 2013). Oral bioavailability is a highly desirable attribute in drug discovery. The fractions were administered orally to the animals and the efficacy showed by them has pointed on their better oral bioavailability compared to doxorubicin.
Figure 6.1 Effect of treatments on tumor growth

![Graph showing tumor growth vs. days with different treatments]

All values are expressed as Mean ± SEM. Statistical analysis was carried out using Two way ANOVA followed by post-hoc Bonferroni’s test. (n=5-7)

Figure 6.2 Effect of treatments on tumor weight

![Bar graph showing tumor weight with different treatments]

All values are expressed as Mean ± SEM. Statistical analysis was carried out using Two way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

6.3.1.2 Hematology parameters

Treatment with DMBA did not have any significant effect on the hematology parameters. The WBC levels were found to decrease significantly in all doxorubicin treated groups as compared to normal control animals. This could be due to the bone
marrow toxicity of doxorubicin. All other changes in blood parameters with treatments were not significant (Figure 6.3).

**Figure 6.3 Effect of treatments on hematological parameters in Sprague Dawley rats**

All values are expressed as Mean ± SEM. Statistical analysis was carried out using One way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

### 6.3.1.4 Effect on biochemical parameters

The ALT levels were found to increase in DMBA control animals as compared to normal control, however, the increase was not significant. Doxorubicin treated animals had a significant (p<0.05) increase in ALT levels as compared to DMBA treated animals. Of note, animals treated with the combination doxorubicin + NJDE (at both doses) did not have elevated ALT levels as compared to DMBA treated animals. This could be due to the protective effect of NJDE fractions on the liver and could be explored further.

There were no significant changes in the levels of AST and ALP in any of the treatments groups. The results are presented in Figure 6.4.
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Figure 6.4 Effect of treatments on serum biochemical parameter

All values are expressed as Mean ± SEM. Statistical analysis was carried out using One way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

6.3.1.5 Elemental analysis in serum

Elemental analysis revealed significant (p<0.05) hypercalcemia in DMBA treated animals while treatment groups showed significant reduction (p < 0.05) in serum calcium levels. No significant changes were observed in the levels of magnesium, zinc, and copper upon treatment with DMBA. However, significant (p<0.05) increase in copper was observed in the doxorubicin treated animals as compared to the DMBA control animals.

Hypercalcemia has been reported to occur in up to 20 to 30 percent of patients with cancer at some time during the course of their disease (Stewart 2005). While an association between exposure to metals and the risk of the lung, breast, colorectum, cancers was discussed, it was demonstrated that breast cancer patients have abnormal levels of copper, zinc among other trace metals (Saleh et al., 2010).

Significant (p<0.05) increase in serum calcium levels was observed in DMBA control animals that has been reported previously (Bobrowska-Korczak, 2012). Treatment with NJDE and MUEA at both doses resulted in normalization of serum calcium levels with a
significant (p<0.05) decrease in the serum calcium levels as compared to the disease control animals. Of note, doxorubicin treated animals, did not show reduction in calcium levels as compared to disease control (Figure 6.5).

**Figure 6.5 Effect of treatment on serum elements**

![Graph showing effect of treatment on serum elements](image)

All values are expressed as Mean ± SEM. Statistical analysis was carried out using One way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

### 6.3.1.6 Organ Index

There was no significant difference in the organ weights of liver, spleen and heart between the normal control and DMBA-treated animals. Doxorubicin showed slight increase in the heart weight/body weight (HW/BW) ratio however in doxorubicin-treated rats, the body weight gain of rats was significantly decreased in all groups of rats treated with doxorubicin alone. Doxorubicin causes reduction in body weight and one of the most concerning side effects is loss of body weight. Of note, the animals treated with doxorubicin + NJDE at both doses prevented any change in heart weight with no significant change in comparison to the normal and disease control animals. Hence, this could be indicative of NJDE as a promising source of cytoprotective agents for the treatment of doxorubicin-induced cardiac toxicity.

The spleen weight was significantly increased in doxorubicin treated animals as compared to normal and DMBA control groups. Increase in spleen weight, as reported earlier (Singh et al., 2015), could be because of gross lesions and increase in
hematopoietic cell proliferation in the tissue during the doxorubicin therapy. Treatment with NJDE fraction prevented the increase in spleen weight (Figure 6.6).

**Figure 6.6 Organ Index**

![Organ Index Graph](image)

All values are expressed as Mean ± SEM. Statistical analysis was carried out using One way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

**6.3.1.7 Antioxidant parameters in liver**

Depletion of GSH was observed in the liver of DMBA-treated rats compared to normal control animals however, it was not statistically significant. Treatment with the fractions was able to restore the GSH content. The changes in other treatment groups were non-significant compared to the normal control. No significant differences were observed in the levels of antioxidant enzymes, catalase and SOD in liver tissue (Figure 6.7).
Figure 6.7  Effect of treatments on liver antioxidant enzymes

All values are expressed as Mean ± SEM. Statistical analysis was carried out using One way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

6.3.1.8 Antioxidant parameters in mammary tissue

Significant (p<0.05) depletion of GSH was observed in the mammary tissue of DMBA-treated rats as compared to normal control animals. Treatment with the NJDE fraction at both doses was able to normalize the GSH content with no significant difference with normal control. However, other treatment groups had significant reduction in GSH levels as compared to the normal control.

Lipid peroxidation levels are an excellent indicator for the extent of oxidative damage in tissue (Janero, 1990). MDA (Malondialdehyde), the end product of lipid peroxidation, was estimated in the excised breast tissues. The level of MDA was significantly (p<0.05) increased in the DMBA control as compared to the normal control indicating oxidative stress in the breast tissue by DMBA. Treatment with polyphenolic rich-MUEA fraction at both doses, was found to significantly (p<0.05) reduce MDA levels in the breast tissue. Similarly, treatment with NJDE was also found to significantly (p<0.05) reduce lipid peroxidation in breast tissue.
Notably, animals treated with doxorubicin exhibited oxidative stress that was comparable to DMBA-treated animals. Doxorubicin in combination with NJDE fraction, at both doses reduced the levels of MDA significantly (p<0.05) as compared to DMBA-treated animals. Hence, it may be considered that NJDE fraction ameliorates the oxidative stress via lipid peroxidation induced by DMBA and doxorubicin.

No significant changes were observed in the catalase and SOD levels after treatment with the fractions (Figure 6.8).

**Figure 6.8 Effect of treatments on mammary tissue antioxidant enzymes**

![Graph showing the effect of treatments on mammary tissue antioxidant enzymes.](image)

All values are expressed as Mean ± SEM. Statistical analysis was carried out using One way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

**6.3.1.9 DNA Fragmentation in tumor tissue samples**

Oral administration of DMBA, led to significant induction of DNA fragmentation in tumor tissue (Table 8). DNA fragmentation (*2-fold) was observed with the release of nucleotides in the supernatant as estimated by the diphenylamine assay.

Treatment with MUEA fraction also enhanced DNA fragmentation significantly (p<0.05) in tumor tissues at 200 mg/kg however, the effect was not observed at 100 mg/kg.
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Treatment with NJDE fraction at both doses was found to significantly (p<0.05) increase DNA fragmentation in the tumor tissues as compared to the DMBA-treated animals in a dose-dependent manner.

The in vivo results were in conjunction with the in vitro results wherein, MUEA and NJDE fractions were found to exhibit apoptosis in cancer cells. The pre-treatment of animals with fractions enhanced DNA fragmentation, a marker of apoptosis thereby enhancing tumor cell death in vivo (Figure 6.9).

Figure 6.9 Effect of treatments on DNA fragmentation in breast tumor

All values are expressed as Mean ± SEM. Statistical analysis was carried out using One way ANOVA followed by post-hoc Dunnett’s test. (n=5-7)

6.3.1.10 Histopathology of tumor samples

Disease Control (DMBA-treated) sections showed mammary tissue with tumor composed of malignant cells arranged in tubular and small glandular pattern seen infiltrating the stroma. At places, ducts showed varying degree of intra-ductal proliferation and features of ductal carcinoma in situ features. Mild peritumoral lymphocytic infiltration was seen. Findings are of tubular carcinoma of breast.

Mammary histology sections of NJDE and MUEA treated animals at both doses showed breast tissue with large area of fibrosis, hyalinization, patchy necrosis and atrophic lobules. No evidence of malignancy was seen. Doxorubicin treated animals had breast
tissue with large area of fibrosis, hyalinization, patchy necrosis and atrophic lobules. No evidence of malignancy was observed (Figure 6.10).

**Figure 6.10 Histopathology (hematoxylin and eosin staining) of breast tumor (40X)**

![Histopathology images](image)

A, B: Disease Control C: NJDE 100 D: NJDE 200 E: Doxorubicin F: Doxorubicin + NJDE 100 G: Doxorubicin + NJDE 200 H: MUEA 100 I: MUEA 200

**6.3.1.11 Whole mount of breast tissue (carmine alum staining)**

In whole mounts of mammary glands, an increase in the count of terminal end buds (TEBs), terminal ducts (TDs), and lobules was observed seen in the DMBA-treated rat mammary glands as compared to normal control animals demonstrating proliferation of breast cells. Upon treatment with doxorubicin, the branching was found to be less as compared to DMBA treated animals. Similarly, treatment with Doxorubicin + NJDE demonstrated lesser branching in the breast tissue. Treatment with NJDE at 100 and 200 mg/kg also showed lesser branching as compared to DMBA-treated animals. MUEA at both doses showed slightly less branching in the count of TEBs, TDs and lobules as compared to the DMBA-treated animals (Figure 6.11).
A: Normal Control  B: Disease Control  C: Doxorubicin D: NJDE 100  E: NJDE 200
F: Doxorubicin + NJDE 100  G: Doxorubicin + NJDE 200  H: MUEA 100
I: MUEA 200

6.3.1.12 Cardioprotective activity of NJDE fraction

Doxorubicin is one of the most commonly systemic treatments to improve several adult and also pediatric cancers, including both hematological and solid tumors. Unfortunately, it is associated with dose-related toxicities, such as hematopoietic suppression and hepatotoxicity; although the most serious concern is the life-threatening cardiomyopathy. Multiple cytotoxic mechanisms are involved in the pathogenesis of doxorubicin-induced cardiotoxicity of which doxorubicin-induced oxidative stress is the cornerstone. Presently, there are no effective therapeutic agents for doxorubicin-associated cardiotoxicity. Therefore, the investigation of cytoprotective agents for healthy tissues
without affecting the anti-cancer effects is of urgent need. There are previous reports that suggest that *N. jatamansi* ameliorates doxorubicin-induced cardiotoxicity. Our results are in line with the cardioprotective effects of *N. jatamansi* which exhibits (Singh et al., 2015).

Heart sections of doxorubicin-treated animals showed mild congestion, occasional myofibrils with vacuolation, hyperplasia and hypertrophy of cardiac myocytes and infiltration of few lymphocytes. Animals treated with NJDE + Doxorubicin showed mild hyperplasia and hypertrophy. In addition to the reduction of heart index as mentioned above in Figure 6.7, NJDE exhibited cardioprotective effect in doxorubicin treated animals (Figure 6.12).

**Figure 6.12 Effect of treatments on heart tissue (40X)**

![Effect of treatments on heart tissue](image)

6.3.2. MDA-MB-231Br xenograft model in nude mice

The fractions were selected to be tested for anti-tumor activity in MDA-MB-231Br (brain seeking metastatic breast cancer) xenograft based on their significant *in vitro* anticancer activity in MDA-MB-231Br cells (Figure 7.10).

6.3.2.1 Determination of MTD in nude mice

The MTD was determined for the selected fractions MUEA, NJDE and column sub-fractions PA-rich oil and NJDE/F2/91/9.
Table 6.3 MTD for the selected fractions/sub-fractions to be tested in MDA-MB-231Br xenograft model

<table>
<thead>
<tr>
<th>Selected fraction</th>
<th>MTD (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUEA</td>
<td>150</td>
</tr>
<tr>
<td>PA-rich oil</td>
<td>50</td>
</tr>
<tr>
<td>NJDE/F2/91/9</td>
<td>50</td>
</tr>
<tr>
<td>NJDE/F4</td>
<td>25</td>
</tr>
</tbody>
</table>

6.3.2.2 Tumor growth inhibition in MDA-MB-231Br xenograft

The results demonstrated in vivo efficacy of all tested fractions and sub-fractions against MDA-MB-231Br xenografts. MUEA at 150 mg/kg showed significant reduction of tumor growth as compared to disease control mice starting from Day 5 onwards. NJDE fractions showed better tumor growth inhibition as compared to MUEA fraction. NJDE/C1/F4/85/15 fraction at 25 mg/kg exhibited significant (p<0.05) inhibition of MDA-MB-231Br tumor growth in vivo from Day 5 onwards. The column sub-fractions PA-rich oil and NJDE/C2/F2/91/9 also demonstrated significant (p<0.05) tumor growth inhibition of MDA-MB-231Br xenografts at 50 mg/kg dose. The PA-rich oil fraction had demonstrated excellent in vitro inhibitory activity against a number of cell lines. Similarly, NJDE/C2/F2/91/9 also demonstrated excellent in vitro inhibitory activity against MDA-MB-231Br xenografts. The significant antitumor activity by the fractions/sub-fractions warrant further investigation for their mechanistic evaluation in vivo (Figure 6.13).
All values are expressed as Mean ± SEM. Statistical analysis was carried out using Two way ANOVA followed by post-hoc Bonferroni’s test. (n=3).

6.4 Summary of in vivo activity

Both the plant fractions demonstrated significant ant-tumor activity in the in vivo models: DMBA induced breast cancer in SD rats and MDA-MB-231 xenograft in nude mice.

The MUEA fraction demonstrated significant (p < 0.05) reduction in tumor growth volume and tumor weight at 200 mg/kg in the DMBA-induced breast cancer in SD rats. Elemental analysis revealed significant hypercalcemia in DMBA treated animals while MUEA fraction treated animals showed significant (p < 0.05) reduction in serum calcium levels. Antioxidant parameters in mammary gland showed diminished lipid peroxidation (p < 0.05) in MUEA treated groups at both doses. MUEA was more effective at the higher dose i.e. 200 mg/kg. In MDA-MB-231Br xenograft model, MUEA at 150 mg/kg showed significant (p<0.05) reduction of tumor growth.

NJDE fraction at both doses was found to exhibit significant (p < 0.05) reduction in tumor growth volume and tumor weight in the DMBA-induced breast cancer in SD rats. In combination with doxorubicin, NJDE was found to demonstrate cardioprotective activity and better overall health of the animals as shown by maintenance of body weight, lesser reduction in WBC count and serum AST levels as compared to doxorubicin treated animals. Oxidative stress and trace elements have been implicated in
the development of breast cancer. Elemental analysis revealed significant hypercalcemia in DMBA treated animals while treatment groups showed significant reduction (p < 0.05) in serum calcium levels. Of note, antioxidant parameters in mammary gland showed diminished lipid peroxidation (p < 0.05) in NJDE treated groups. Overall, NJDE was more effective at the higher dose i.e. 200 mg/kg. In MDA-MB-231Br xenograft model, NJDE/C1/F4/85/15 fraction at 25 mg/kg exhibited significant (p<0.05) inhibition of MDA-MB-231Br tumor in vivo from Day 5 onwards. The column sub-fractions PA-rich oil and NJDE/F2/91/9 also demonstrated significant (p<0.05) tumor growth inhibition of MDA-MB-231Br xenografts at the 50 mg/kg dose.

Therefore, to conclude, NJDE and MUEA exhibit significant antitumor potential in DMBA-induced breast cancer in SD rats. NJDE is cardioprotective in nature as it was found to alleviate the symptoms of myocardial damage/cardiomyopathy associated with doxorubicin treatment. NJDE and MUEA are promising candidates for the development of anticancer therapeutics.

6.5 References


Janero, DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free radical biology & medicine 1990;
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