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## Chemotherapeutic effect of Plumbagin on nitric oxide and redox status in 7, 12 dimethyl benze (a) anthrecene (DMBA) induced mammary carcinoma

Sundaramoorthy Selvaraj<sup>1</sup> \*, Kuppusamy Periyasamy<sup>1</sup>, Kalappan Vanitha<sup>1</sup>, Dhanapal Sakthisekaran<sup>1</sup>

*1.Dept.of Biochemistry, Priyadharshini dental college and hospital, Thiruvallur, Chennai, Tamilnadu, India*

### ABSTRACT

The objective of this study was to determine the efficacy of Plumbagin on attenuating malondialdehyde (MDA) and nitric oxide (NO), along with augmenting the endogenous antioxidant status in DMBA induced mammary carcinoma. Breast cancer was induced by providing 1ml of 7, 12 dimethyl benze (a) anthrecene(DMBA) 20mg/kg of body weight through orally and tissue antioxidants status along with histopathological studies were determined as per standard procedures. Western blot was also analyzed to determine the protein expression in all the experimental groups. Altered antioxidant status and upsurge free radical generation especially nitric oxide was observed in DMBA induce group when compared to control. This increase NO level was well supported by increase protein expression of iNOS and eNOS. However the altered antioxidant status was corroborated with decrease GSH protein expression. To support the detrimental effect of DMBA histopathology study showed neovasularization, presence of uniformly malignant ductal epithelial cells growing in vague cribiform pattern, necrosis formation along the tumor, and cell destructions. However, Plumbagin when administered at 4 mg/kg body weight showed attenuated free radical generation and upregulated the antioxidant activity. The increase free radical generation especially NO is also plays a pivotal causative role in breast cancer. Therapeutic efficacy targeting the antioxidant system could play a pivotal role in the treatment of breast cancer.

**Keywords:** 7, 12 dimethyl benze (a) anthrecene (DMBA), Nitric oxide (NO), Nitric oxide synthase (NOS), Plumbagin, Antioxidant and Breast cancer.

\*Corresponding Author Email: [elvamunom@gmail.com](mailto:elvamunom@gmail.com)

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## INTRODUCTION

Breast cancer is one of the most common cancers in women worldwide and it is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death. Significant risk factors includes early age at menarche, late age at menopause, family history, use of oral contraceptives, mutations in BRCA 1 and BRCA 2 etc [1]. Though, the etiology of breast cancer remains obscure and primary prevention strategies not yet available. Ample evidence supports oxidative stress being a causative role in breast cancer [2] and with free radicals generally being responsible in the genesis of many multifactorial disease, targeting the antioxidant defense system in treatment of breast cancer may provide an alternative and cost-effective treatment modality.

7,12-dimethylbenz(a)anthracene (DMBA), a class of aromatic polycyclic hydrocarbons, which are the repercussions of the ignition of tobacco and other natural substances[3]. This agent may prompt the generation of free radicals through the creation of superoxide, hydrogen peroxide and nitric oxide in cells[4]. This free radical covalently binds to the nucleophilic sites on cellular macromolecules, thereby eliciting cancerous responses [5]. Nitric oxide (NO) is a bioactive molecule that exhibits pleotropic effects within cancer cells and tumors, with concentration-dependent pro- and anti-tumor effects. NO is produced by three different nitric oxide synthase (NOS) isoforms: neuronal (nNOS/NOS1), inducible (iNOS/ NOS2), and endothelial (eNOS/NOS3) [6]. Interaction of nitric oxide (NO) with oxygen (O<sub>2</sub>) or superoxide (O<sub>2</sub><sup>-</sup>) can also result in the formation of another toxic reactive nitrogen species (RNS) like dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>) and peroxynitrite (ONOO), which can ultimately induced both nitrosative and oxidative stress[7]. In addition to the known risk factors, other factors including fatty acids are likely to play an important role in determining risk of breast cancer [8].

The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention [9]. Reduced glutathione besides its role as a scavenger of free radicals also acts in association with the detoxification enzymes glutathione peroxidase (GPx) and glutathione-s-transferase (GST). These enzymes protect cell from noxious substances by catalyzing conjugation reactions with reduced glutathione and prevents damage caused by reactive oxygen species by reducing hydrogen peroxide, lipid and phospholipid hydroperoxides [10]. Bioactive compounds from plant origin have the potential to subside biochemical redox imbalances. Thus, in search of such agents, studies have been focused on naturally occurring chemical compounds as several among them are known to possess cytotoxic

effects and have the potential for killing cancer cells [11]. Plumbagin, isolated from the roots of *Plumbago zeylanica* L. has been proven to possess anti-tumor activity both in vitro and in vivo studies [12]. Although a natural agent, plumbagin has some toxicity associated with it that has been extensively evaluated in rodents. These toxic side effects were dose related. The LD50 for these side effects in rats were 8–65 mg/kg body weight for oral (p.o.) administration and 16 mg/kg body weight for intra-peritoneal (i.p.). Yet, plumbagin has been reported to be nontoxic at 4 mg/kg body weight i.p. or 200 ppm in diet and shown to elicit therapeutic effects [13]. Though, reports of plumbagin having antitumor activity, very few studies are available on the mechanism of cell death induced by plumbagin in human cancer cells. So elucidation of the mechanisms by which plumbagin induces its anticancer therapeutic effect is necessary to provide a solid foundation for its use as an agent for prevention strategies. Thus, an understanding on the NO system and evaluating the efficacy of Plumbagin in attenuating the redox imbalance on the carcinogenic effect of DMBA-induced mammary cancer in this study, could lead to the development of new approaches and strategies for the effective treatment of breast cancer.

## MATERIALS AND METHOD

### Chemicals

Plumbagin and DMBA was purchased from Sigma Aldrich, USA and all other chemicals were of analytical grade obtained from the Sisco research Laboratory, Bombay, India.

### Animals

Experimental animals were all healthy Female Sprague Dawely rats, 6-8 weeks of age and weighing 150-180g were used. All the animals were maintained under standard laboratory conditions, housed 2 per cage (29 cm × 22 cm × 14 cm). Rats were acclimatized to laboratory condition with a 12 h light/dark cycle under constant temperature and humidity and were given libitum access to water and pellet diet (Gold Mohor rat feed, Ms. Hindustan Lever Ltd., Mumbai. Appropriate ethical clearance was obtained for this work from the Institutional Animal Ethical Committee (IAEC NO.24/028/12). All the animal experimentation involved in this work was done in accordance with national and institutional guidelines for the protection of animal welfare.

### Experimental design

The experimental animals were divided into four groups of six animals each. Group-I consist of control animals fed with standard diet and olive oil. Group-II include breast cancer bearing animals induced by providing 1ml of DMBA 20mg/kg of body weight through orally and Group-III comprise of breast cancer bearing animals treated with plumbagin (4 mg/kg B.wt.) for 4 weeks by

alternative days, whereas Group-IV consist of control animals received olive oil alone for 4 weeks. After the experimental period, the animals were sacrificed and different organs were harvested for further analysis of parameters of interest.

### **Sample collection**

All experimental animals were sacrificed by deeply anesthetized with ketamine/xylazine (90/50mg/kg.b.w) in the stipulated experimental period. The breast sample harvested from various control and experimental animals was homogenized with motor driven Teflon coated homogeniser in ice-cold Tris-HCl buffer (0.1 M, pH 7.4) to obtain 10% homogenate and subjected to various analyses (w/v).

### **Tumor weight**

Tumor weight was estimated according to the standard method. The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axis. The two short axis were measured with vernier calliper. The tumor weight was calculated by multiplying the length of the tumor with the square of the width and dividing the product by 2.

$$\text{Tumor weight (g)} = (\text{Length (cm)} \times \text{Width (cm)}^2) / 2$$

### **Biochemical analysis**

Tissue nitrite and nitrate concentrations were measured by modified micro-assay according to the method of Vodovotz[14]. The enzymatic antioxidant like SOD activity was determined by the nitroblue tetrazolium (NBT) reduction method as per Flohe[15] and catalase activity was determined according to the method Claiborne [16]. Assay of GPx activity was based on the oxidation of GSH by cumene hydroperoxide. The activity of GPx was assayed by the method of Ellman [17]. Reduced glutathione was measured by the method of [18] and malondialdehyde (MDA) was measured by thiobarbituric acid method as described by Zima[19].

### **Immunoblotting**

Tissue lysate was prepared with radio immunoassay buffer (RIPA) (Sigma) and protease inhibitor. Equal amounts of protein (50 µg) were electrophoresed on 10% SDS-PAGE. Following electrophoresis, separated proteins on SDS-PAGE gels were transferred to PVDF membrane (Millipore, USA). To block the nonspecific binding, the membranes were incubated blocking buffer with 5% skimmed milk for 2 h. Membranes were probed with primary antibodies active iNOS, eNOS and GSH protein (Biovision). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000) (Merck). The bands were developed using ECL kit (Millipore, USA) in the Chemi Doc image scanner from Bio-Rad. The band intensity was

quantified by Quantity One software (Bio-Rad, USA). The membranes were stripped and reprobed for  $\beta$ -actin (Sigma) (1:5000) as an internal control.

### Histopathological examination

Mammary tissue was fixed at 10% buffered formalin, embedded in paraffin using a conventional automated system. The blocks were cut to obtain 5-mm thick sections and stained with hematoxylin–eosin [20]. The image of serial paraffin sections of each tissue was captured by light microscopy (Olympus BX51, Hamburg, Germany).

### Statistical analysis

All the result from various groups were analyzed by SPSS statistical package version 16, for the individual parameter by one-way analysis of variance (ANOVA) when there was a significance difference, Tukey's multiple comparison was performed by fixing the significance level at  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Effect of plumbagin on total body mass and tumor incidence

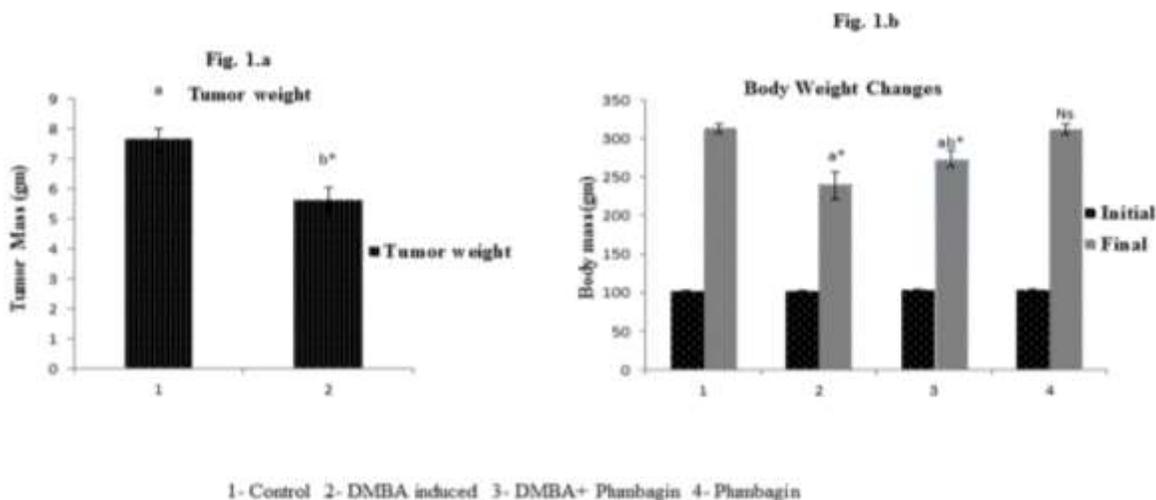


Figure 1.a and 1.b show the data related to total body mass and tumor incidence in control and experimental rats. At the beginning of the experiment there was no difference in body mass among rats.

After administration of the carcinogen (for tumorigenesis) and the therapeutic agent, total body mass was recorded periodically, once in a week, till the completion of the experimental period. There was a significant drop in the body mass in tumor-bearing (group-II) rats when compared to control(group-I) rats. In plumbagin treated rats (group-III) tumour bearing rats showed a gain in

the body mass compared to the untreated tumour-bearing (group-II) rats. Plumbagin alone treated (group-IV) rats showed no significant changes when compared to control (group-I) rats.

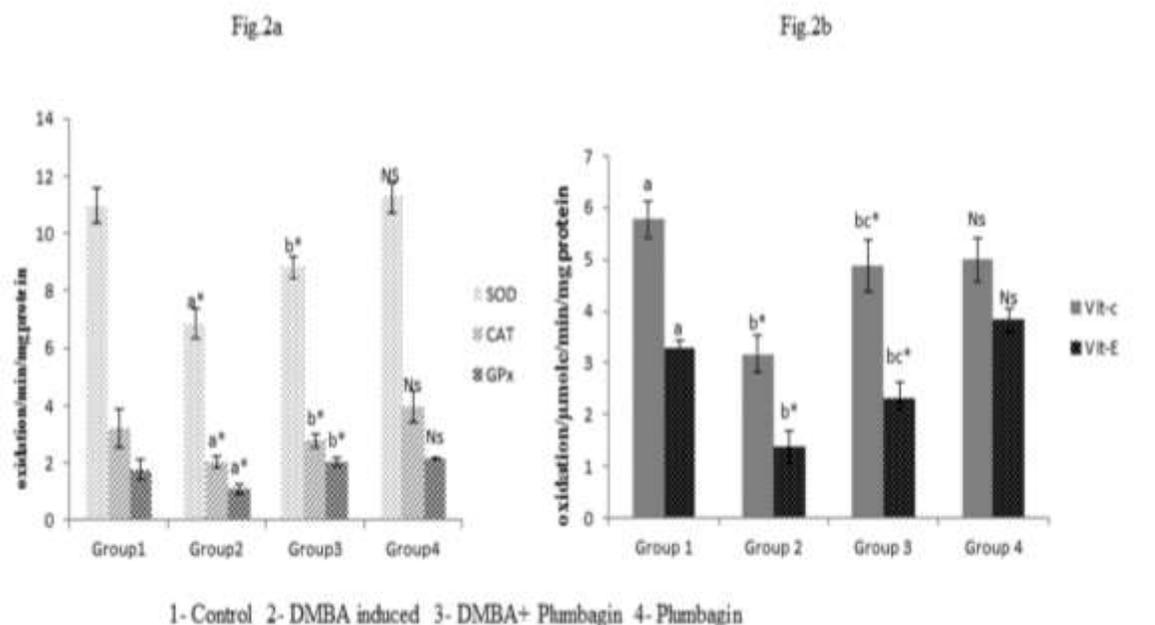
After the completion of the experimental period, the breast tissue was excised and weighed. An increase in breast tissue mass was observed in tumour-bearing (group-II) rats compared to the control rats (group-I). plumbagin administered tumour-bearing rats (group-III) showed a reduction in the tumor mass compared to the untreated tumor-bearing rats (group-II). Plumbagin alone treated rats did not show any significant change in the mammary gland when compared to control (group-I) rats. The tumour incidences were observed in carcinogen treated experimental rats. Carcinogen alone treated rat's showed 100% incidence of the, mammary carcinoma. Rats treated with plumbagin (group-III) showed marked regression in tumour development (55%) when compared to carcinogen alone treated rats (100%).

Figure 1a and 1b. Effect of plumbagin on body weight and tumor mass changes in control and experimental animals.

Breast cancer bearing animals were treated with plumbagin 4mg/kg body weight orally. Values are expressed as mean  $\pm$  SD for six animals. Group II is compared with group I; Group III and Group IV are compared with Group II; Significant levels were \*\* $p < 0.01$ , \* $p < 0.05$ .

#### **Effect of plumbagin on Antioxidants levels**

Figure 2.a and 2.b shows the level of both enzymic and non-enzymic antioxidants in control and experimental rats. The enzymic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and non-enzymic antioxidants such as reduced glutathione (GSH) were significantly ( $p < 0.05$ ) reduced in tumour-bearing (group II) rats when compared with control (group I) rats. Treatment with plumbagin (groups III) restored the level of antioxidants (both enzymic and non-enzymic) in tumour-bearing rats to near normal (control) levels. There was a significant ( $p < 0.05$ ) increase in the levels of these (enzymic and non-enzymic) antioxidants when compared with tumour-bearing (group II) rats. Notably, plumbagin alone treated (group V) rats did not show any significant change when compared with control (group I) rats.

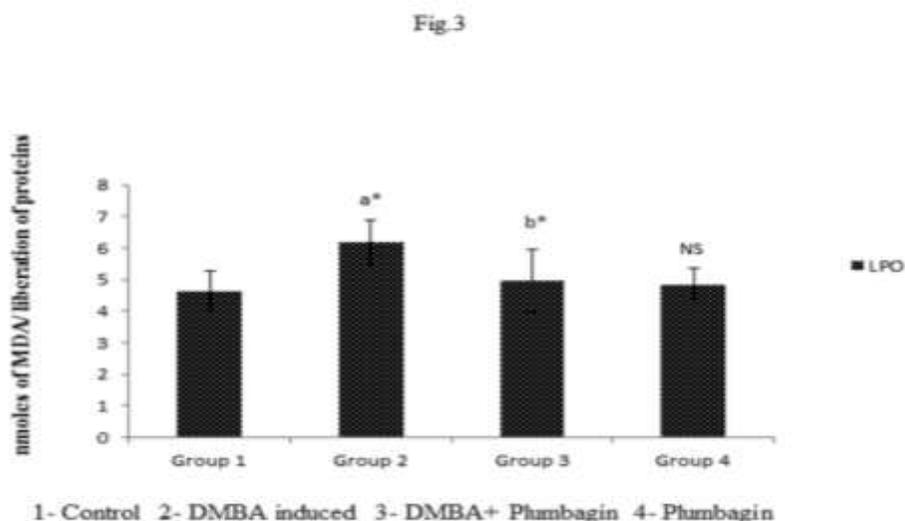


**Figure 2a and 2b. Effect of plumbagin on enzymic and non-enzymic Antioxidant activities in mammary tissue of control and experimental animals.**

Breast cancer bearing animals was treated with plumbagin 4mg/kg body weight orally. Values are expressed as mean  $\pm$  SD for six animals. Group II is compared with group I; Group III and Group IV are compared with Group II; Significant levels were  $**p < 0.01$ ,  $*p < 0.05$  and NS  $p > 0.05$ .

#### **Effect of plumbagin on macromolecular damage**

Figure 3 depicts the level of lipid peroxidation (LPO) in control and experimental rats. It was found that tumour-bearing rats (group II) showed a significant ( $p < 0.05$ ) increase in LPO when compared with control (group I) rats. Plumbagin treated (groups III) rats showed a significant ( $p < 0.05$ ) decrease in the levels of LPO when compared with tumour-bearing (group II) rats. There was no significant difference in the LPO between plumbagin alone treated (group IV) and control (group I) rats.

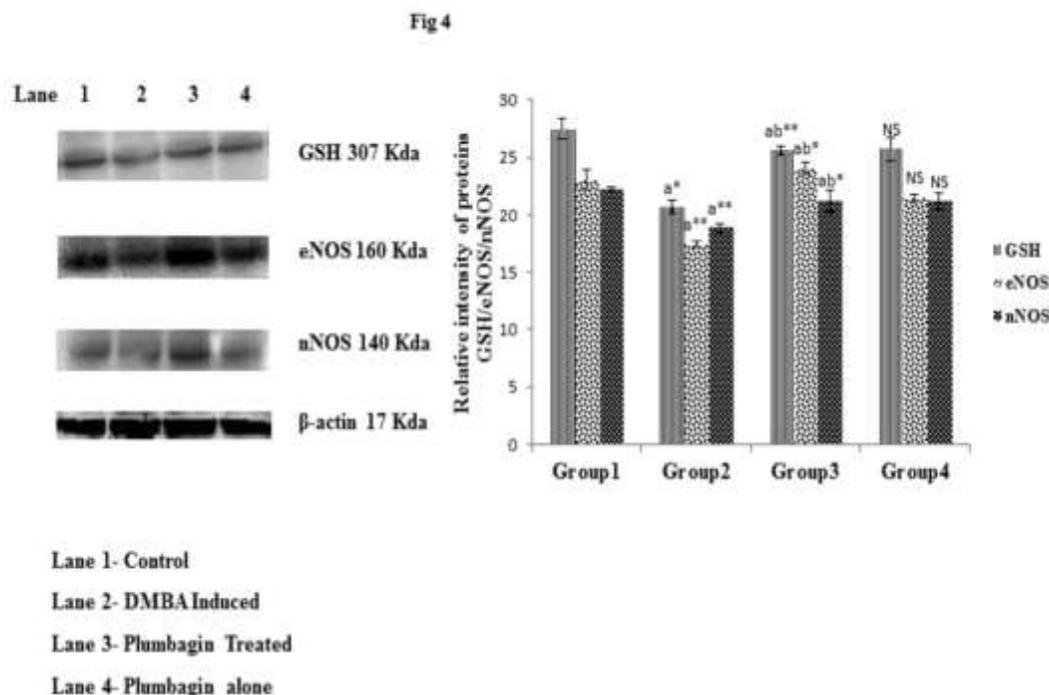


**Figure 3. Effect of plumbagin on the levels of lipid peroxidation in mammary tissue of control and experimental animals.**

Breast cancer bearing animals were treated with plumbagin 4mg/kg body weight orally. Values are expressed as mean  $\pm$  SD for six animals. Units: LPO is nmoles of MDA per milligram protein. Group II is compared with group I; Group III and Group IV is compared with Group II; Significant levels were \*\* $p < 0.01$ , \* $p < 0.05$  and NS  $p > 0.05$ .

#### **Effect of plumbagin on nitric oxide synthase (NOS) enzymes and reduced glutathione protein expression**

Figure 4. depicts the protein expression in control and experimental rats. The increase NO level It was found that tumour-bearing rats (group II) showed a significant ( $p < 0.05$ ) upregulated protein expression of both iNOS and nNOS when compared with control (group I) rats. Plumbagin treated (groups III) rats showed a significant ( $p < 0.05$ ) decrease in the levels of both NOS expression when compared with tumour-bearing (group II) rats. The altered redox status was depicted with a significant decrease in GSH expression in tumour-bearing rats (group II) showed a ( $p < 0.05$ ) when compared with control (group I). Plumbagin treated (groups III) rats showed a significant ( $p < 0.05$ ) increase in GSH expression when compared with tumour-bearing (group II) rats. There was no significant difference in the protein expression between plumbagin alone treated (group IV) and control (group I) rats in this experimental study.



Breast cancer bearing animals were treated with plumbagin 4mg/kg body weight orally. Values are expressed as mean  $\pm$  SD for six animals. Group II is compared with group I; Group III and Group IV is compared with Group II; Significant levels were  $**p < 0.01$ ,  $*p < 0.05$  and NS  $p > 0.05$ .

#### Effect of plumbagin on nitric oxide levels

Table.1.shows the level of NO in control and experimental rats. NO levels were significantly ( $p < 0.05$ ) increased in tumour-bearing (group II) rats when compared with control (group I) rats. Treatment with plumbagin (groups III) restored the NO level in tumour-bearing rats to near normal (control) levels. The plumbagin alone treated (group V) rats did not show any significant change when compared with control (group I) rats.

**Table 1 Levels of nitric oxide (NO) in control and experimental mammary tissue.**

Groups (Parameter)	Group1 (Control)	Group2 (DMBA)	Group3 (DMBA+ plumbagin)	Group4 (Plumbagin)
NO (nmol/mg protein)	8.97 $\pm$ 0.27	14.23 $\pm$ 0.73ac**	9.70 $\pm$ 0.59abd*	8.38 $\pm$ 0.32aNS

DMBA: 7,12-dimethylbenz[a]anthracene, Plumbagin, NS: non significant.

a.Groups II, III, and IV compared with control group I.

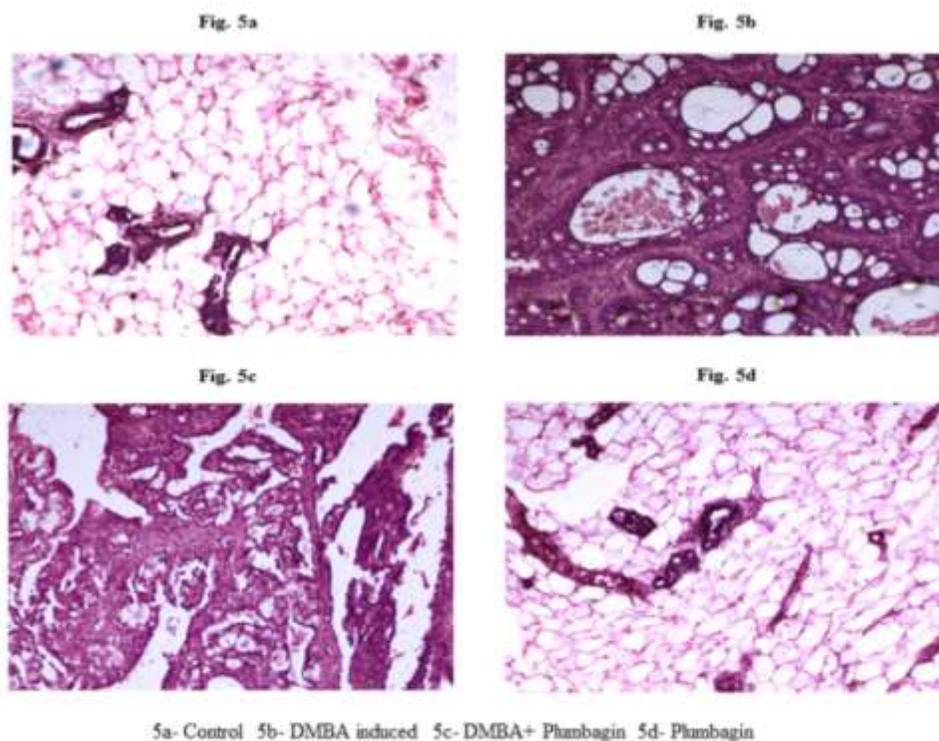
b.Groups III and IV compared with control group II.

C\*\* $p < 0.01$ .

d\* $p < 0.05$ .

### Histological examination

Figure 5. Photomicrographs showing the morphology of mammary gland tissues in control and DMBA induced rats. Figure 5a (Control): The mammary gland tissues showing normal histo-architecture of admixture of fibrous fatty tissues and ductal structures. Figure 5b (Induced): Histological section of DMBA induced group showed the development of carcinoma as evidence by neovasularization, presence of uniformed malignant ductal epithelial cells growing in vague cribriform pattern, necrosis formation along the tumor, and cell destructions. Figure 5c (Treated): Histological section of Plumbagin treated group shows the absence of malignant appearance of ductal tissues, and resulted in complete disappearance of abnormal changes caused by DMBA. Figure 5d (Drug alone): Histological section shows normal morphology which resembles that of the control.



### Figure 5. Photomicrographs showing the morphology of mammary gland tissues in control and DMBA induced rats.

Figure 5a (Control): The mammary gland tissues showing normal histo-architecture of admixture of fibro fatty tissues and ductal structures. Figure 5b (Induced): Histological section of DMBA induced group shows the development of carcinoma as evidence by neovasularization, presence of uniformed malignant ductal epithelial cells growing in vague cribriform pattern, necrosis formation along the tumor, and cell destructions. Figure 5c (Treated): Histological section of Plumbagin

treated group shows the absence of malignant appearance of ductal tissues, and resulted in complete disappearance of abnormal changes caused by DMBA. Figure 5d (Drug alone): Histological section shows normal morphology, which resembles that of the control.

### **Discussion**

Animal experimental systems are particularly useful for the study of human mammary carcinogenesis. Since the rat mammary gland shows a high susceptibility to developing neoplasms which closely mimic human breast cancer, they have been selected in comparison to other animal models [15]. In the present study, treatment with plumbagin exhibited potential anticancer activity on DMBA-induced mammary tumors in rats. As a result, in plumbagin treated group the body weight had also slightly increased, the tumor volume decreased, and the percentage of tumor inhibition was statistically significant ( $P < 0.05$ ).

The enzymatic and non-enzymatic antioxidant defense systems of the cell prevent oxidant mediated damage to different biomolecules such as lipids, protein and DNA by neutralizing free radical generation [21, 22]. This present study revealed a dynamic imbalance between the amount of free radicals generated in particularly nitric oxide and antioxidant defense system in the body. Decreased enzymatic (SOD, CAT, GPx) and non-enzymatic antioxidant (GSH) activity found in DMBA-induced rats could have caused and upsurge generation of both ROS and RNS level. So, naturally the ability of the endogenous antioxidant to quench or scavenge the free radicals against their deleterious effects of free radicals has been severely compromised. The altered antioxidant status was corroborated with decrease GSH protein expression in DMBA-induced rats. Depletion of reduced glutathione is an index of oxidative stress and this could have led to increase NO level production in DMBA-induced rats. To support this finding we also observed and increase protein expression nNOS and eNOS enzymes. Thus the detrimental effect of NOS enzyme in DMBA-induced rats is obvious. Increased iNOS expression has recently been postulated as a prognostic factor for reduced survival in patients with basal-like ER $\alpha$ -negative breast cancer through the induction of interleukin-8 (IL-8), CD44, c-Myc.

The higher level of nitric oxide, which may increase production of peroxynitrite and, further oxidation of GSH or increased utilization of GSH for detoxification of lipid hydroperoxides, formed due to high oxidative stress in post-operative breast cancer patients, as described by [23]. Tissue MDA levels increased significantly in the rats with breast cancer, were as treated with plumbagin significant reduction in the levels and near to the normal in control group. Eventually justifying the increased levels TBARS levels in tissues, which upon treatment with plumbagin significantly altered these levels to near normal when compared to normal control rats.

Lipid peroxidation products are considered as oxidative stress promoting agent, which possess a strong inhibiting effect on the cellular antioxidant system[24].For instance, the end-product malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts to them [25].

Our finding clearly indicates that increase free radicals generation could have been a prime cause in the genesis of DMBA-induced mammary carcinoma and the efficacy of plumbagin as a potent free radical scavenger. Control administration of these potential free radicals scavenger is necessary because these antioxidants may inhibit apoptosis in cancerous cells induced by oxidative stress following chemotherapy by scavenging free radicals and may exert antiapoptotic and cancer promoting effects in cancer patients [26,27]. However, recent studies indicate that plumbagin can down-regulate the expression of nuclear factor kappa B (NF-kB) regulated gene products involved in the cell proliferation and anti-apoptosis. Inhibition of the NF-kB activation pathway by plumbagin was found to increase the apoptotic activity of tumor necrosis factor and paclitaxel [13]. Several histopathological findings of previous studies showed different degrees of differentiation. In some respects, the morphological appearance of the tumor is similar to the microscopic patterns of human breast cancer [28]. In our study the histopathological changes caused by 7,12 dimethyl benz(a)anthracene induction showed abnormal proliferation of closely packed ducts, hyperplasia and rupture of epithelial cells.The generation of ROS and the peroxidation of membrane lipids are well associated with the initiation of carcinogenesis affecting the normal biochemical process, which further leads to the reduction in body weight[29].This abnormal proliferation could also have been responsible due to increase iNOS expression. To support this finding [30] reported that iNOS expression has been correlated with increased tumor grade and aggressiveness of breast cancer cells. Treatment with plumbagin showed mere normal architecture of fibrofatty tissue with few ducts. The mechanism of how plumbagin act as chemopreventive agent in this study is still uncertain, but it is believed that the therapeutic could have acted through regulating the altered redox status and NOS system. Interestingly, in a very recent report, plumbagin was shown to induce cell cycle arrest and apoptosis through reactive oxygen species (ROS) in human melanoma A375.S2 cells [31].

In conclusion, from the results obtained in this study it can be inferred that plumbagin positively modulated the antioxidant activity by quenching and detoxifying the free radicals induced by 7,12 dimethyl benz(a)anthracene. Hence, plumbagin as a chemopreventive agent might have a promising role in reducing the toxicity of DMBA-induced mammary carcinogenesis.

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## CONFLICT OF INTEREST

All the authors declare to have no conflict of interest.

All the authors have contributed to this work to do the statistical analysis and histopathological studies. They have given many suggestions and guided for consecrating the data.

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