ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

PHARMACOLOGY

# CHEMOTHERAPEUTIC POTENTIAL OF D-PINITOL AGAINST 7, 12 DIMETHYLBENZ (A) ANTHRACENE (DMBA) INDUCED MAMMARY CARCINOMA IN SPRAGUE DAWLEY RATS.

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## ABSTRACT

7, 12-dimethylbenz (a)anthracene(DMBA) acts as a potent site and organ specific carcinogen by generating various reactive metabolic intermediates leading to oxidative stress. Female Sprague Dawley rats were divided into four groups and each group consisting of six animals. Group I and group IV were vector and drug control. The group II and group III animals were treated with DMBA 20 mg/kg bodyweight to induce mammary carcinoma. Rats received cancer bearing Group III animals were treated with D-Pinitol at the concentration of 30 mg/kg bodyweight for 45 days orally. At the end of the experimental period all the rats were sacrificed. The breast and liver tissues levels of the enzymic and non-enzymic antioxidants were significantly decreased in cancer bearing animals when compared to the control animals. Phase I and II xenobiotic metabolizing enzymes and Lipid peroxide levels (LPO) were estimated. Western blotting analysis showed over expression of Bcl-2 in the mammary tissue of DMBA induced group II rats. From our results, we conclude that D-Pinitol is a potent antioxidant and play a protective role against DMBA induced breast cancer.

ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

## KEYWORDS

Breast cancer, 7, 12-dimethyl-benz(*a*)anthracene, D-Pinitol, antioxidants

## INTRODUCTION

Breast cancer is the most common cancer among women and is the second leading cause of cancer related death. According to the American Cancer Society, about 2,49,100 women will be diagnosed with breast cancer and more than 39,840 women are expected to die from breast cancer in 2011[1]. The average women's lifetime risk of developing breast cancer is approximately 12%. The risk factors involved in the development of breast cancer include: older age, environmental factors, early menarche, late menopause, family history, prolonged hormone replacement therapy and alcohol consumption. A number of genes including BRCA1, BRCA2, HER-2/neu and p53 have been linked to breast cancer susceptibility and developmant[2]. 7,12 Dimethylbenz(a)anthracene (DMBA) is a polycyclic aromatic hydrocarbon (PAH) which is commonly found in our environment and they can be isolated from diesel exhaust, barbequed meat, tobacco smoke and overheated cooking oil etc., DMBA induced rat mammary carcinoma model has found a broad application as a tool for assessing the efficacy of chemotherapy agents in inhibiting the formation of mammary tumors in preclinical studies[3]. In mammary epithelial cells, DMBA undergo metabolic activation to form its active metabolite, dihydrodiolepoxides which can damage DNA and form DMBA-DNA adducts contributing to carcinogenesis. Reactive oxygen species such as superoxide anion ($O_2^{\bullet-}$) hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\bullet}$) are highly produced during metabolic activation of DMBA which may lead to oxidative damage to cells and thereby decreasing the efficiency of antioxidant defense mechanism. Chemotherapy is a novel and promising approach to control, inhibit or to suppress the tumor cell proliferation by using natural or synthetic entities. D-Pinitol (3-O-methyl-chiro-inositol) is a naturally occurring compound found in soya bean seeds, pine wood, alfalfa and legumes. It has been found to possess multi functional properties such as inhibition of the T-helpercell-1 response[4], antiviral[5], larvicidal[6], anti-inflammatory[7], antihyperlipidemic[8], cardioprotective[9], and inhibition of ovalbumin-induced airway inflammation[10]. Further, D-Pinitol is an active ingredient of *Talisapatra*, a traditional Ayurvedic medicine and has been shown to exhibit antidiabetic activities[11]. Even though D-Pinitol showed its various excellent therapeutic uses against various diseases and there is a paucity of information on the usage of D-Pinitol especially on DMBA induced breast carcinoma in experimental rats. Therefore, it is of interest to investigate the anticancer property of D-Pinitol on DMBA induced breast cancer and to provide the scientific rationale for use the drug D-Pinitol as an effective chemotherapeutic agent against breast cancer.

## MATERIALS AND METHODS

***(i) Chemicals:***

D-Pinitol and DMBA were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All the other chemicals used in this study were of analytical grade available commercially.

***(ii) Animals:***

Female Sprague–Dawley rats at the age group of 45-48 days were procured from the Central Animal House Facility, Dr.ALM PG

IBMS, University of Madras, Taramani. The animals were housed in well ventilated large spacious polypropylene cages and had 12 h light and dark cycle throughout the experimental period. The animals received a balanced diet of commercially available pellet rat feed and water *ad libitum*. The Guidelines for Breeding and Experiments on Animals, 1998 defined by the Ministry of Social Justice and Empowerment of India were followed and the protocol was approved by the Institutional Animal Ethics Committee (IAEC No. 01/024/2010).

***(iii) Experimental design:***

The rats were divided into four groups and each group consisting six animals. Group I rats received single dose of 1 ml of emulsion of corn oil given orally throughout the experimental period, served as vehicle treated control. Rats in Groups II and III were induced mammary carcinogenesis by providing single dose of 20 mg/kg body weight of DMBA in 1ml emulsion of corn oil intragastrically. Group II rats received no other treatment. After 60 days the cancer bearing Group III rats received D-Pinitol at the concentration of 30 mg/kg bodyweight for 45 days orally. Group IV rats were treated with D-Pinitol alone at the concentration of 30 mg/kg body weight for 45 days orally. At the end of the experimental period all the rats were sacrificed by cervical dislocation. Breast and liver tissues were dissected out and tissue homogenates were prepared in 0.1 M Tris-HCl buffer pH 7.4 which was stored at -80° C, until its use for further analysis.

***(iv)Biochemical Estimation:***

The breast and liver tissue homogenates were used for estimation of enzymic and non-enzymic antioxidants such as Superoxide dismutase (SOD)[12],Catalase(CAT)[13],Glutathione Peroxidase (GPx)[14],Reduced glutathione (GSH)[15],Vitamin-E (α-tocopherol)[16]and Vitamin-C (ascorbic acid)[17]. Lipid peroxidation (LPO) [18] levels in breast tissue homogenate were estimated. The levels of phase I enzymes[19] (Cytochrome$P_{450}$, Cytochrome $b_5$, NADPH Cytochrome 'C' reductase) and phase II enzymes[20] (Glutathione-S-Transferase (GST) and UDP-Glucuronyl transferase) in liver tissue homogenate were also determined.

***(v) Western blotting:***

Tumors were disaggregated by treatment with an enzyme mixture containing 2.0 g/l collagenase, 0.5 g/l proteases and 2.0 g/l DNase for 90 min at 37°C. The resulting cell suspensions were filtered through a 30µm nylon mesh. Centrifuged cells were washed in phosphate-buffered saline and boiled in Laemmli lysis buffer for 5 min. Breast tissue proteins (50µg/lane) were separated on 12% SDS-PAGE and transferred to PVDF membrane. The membrane was probed with Bcl-2 specific antibody (1:2000 dilution) to determine their levels, which were then visualized under Enhanced Chemiluminescent System (ECL) and the signals were captured by Chemi Doc XPS system. β-actin was used as an internal.

***STATISTICAL ANALYSIS:***

The values are expressed as Mean±S.D for six rats in each group. Statistically, significance differences between the groups were calculated using One-way Analysis of Variance (ANOVA) followed by the Student's Turkey's for multiple comparisons using Statistical Package for Social Sciences (SPSS) computer package. Values of $p < 0.05$ were considered to be significant.

# RESULTS

***(i)Antioxidants:***

**Table 1**

***Effect of D-Pinitol on enzymic and non-enzymic antioxidants in the liver of control and experimental animals.***

| Parameters | Group I (control) | Group II (DMBA) | Group III (DMBA+ D-Pinitol) | Group IV (D-Pinitol) |
|---|---|---|---|---|
| Superoxide dismutase | 7.89±0.28 | 3.59±0.27$^{a*}$ | 6.47±0.28 $^{a* b*}$ | 7.58±0.56 $^{a NS}$ |
| Catalase | 64.85±4.26 | 43.62±3.98$^{a*}$ | 59.89±4.01$^{a* b*}$ | 63.91±3.63 $^{a NS}$ |
| Glutathione peroxidase | 4.91±0.34 | 2.10±0.19 $^{a*}$ | 3.32±0.22 $^{a* b*}$ | 4.52±0.39 $^{a NS}$ |
| Reduced glutathione | 8.65±0.21 | 5.43±0.10 $^{a*}$ | 6.33±0.24 $^{a* b*}$ | 8.27±0.39 $^{a NS}$ |
| VIT -E | 6.42±0.56 | 3.88±0.38$^{a*}$ | 4.04±0.27 $^{a*b*}$ | 6.32±0.31 $^{a NS}$ |
| VIT - C | 0.91±0.06 | 0.12±0.05$^{a*}$ | 0.59±0.07 $^{a* b*}$ | 0.87±0.07 $^{a NS}$ |

Unit are expressed as: SOD=units /mg protein: CAT=µmoles of $H_2O_2$ consumed /mg protein/min: GPx=µ g of GSH utilized/mg protein/min: GSH= µg/mg protein/min; VIT-E and VIT-C=mg/g of wet tissue.
Each value represents mean ± SD of six animals; a – Group II,III &IV compared with Group I; b – Group III compared with Group II. Statistical significance-$^{*}$p<0.001; $^{\#}$p<0.01; $^{@}$p<0.05; $^{NS}$ – No significant.

**Table 2**

***Effect of D-Pinitol on enzymic and non-enzymic antioxidants in the breast of control and experimental animals.***

| Parameters | Group I (control) | Group II (DMBA) | Group III (DMBA+ *D-Pinitol*) | Group IV (*D-Pinitol*) |
|---|---|---|---|---|
| Superoxide dismutase | 15.44±0.10 | 6.63±0.61$^{a*}$ | 12.21±0.94 $^{a* b*}$ | 15.01±0.25 $^{a NS}$ |
| Catalase | 68.08±2.19 | 42.78±4.62$^{a*}$ | 51.26±3.98$^{a* b*}$ | 67.85±2.74 $^{a NS}$ |
| Glutathione peroxidase | 12.44±0.74 | 6.56±0.41 $^{a*}$ | 8.47±0.83 $^{a* b*}$ | 12.28±0.16 $^{a NS}$ |
| Reduced glutathione | 14.18±0.61 | 7.21±0.49 $^{a*}$ | 9.34±0.26 $^{a* b*}$ | 14.08±0.92 $^{a NS}$ |
| VIT - E | 5.11±0.42 | 3.54±0.73$^{a*}$ | 4.39±0.14 $^{a*b*}$ | 5.03±0.38 $^{a NS}$ |
| VIT -C | 3.49±0.79 | 1.69±0.06$^{a*}$ | 2.92±0.47 $^{a* b*}$ | 3.37±0.16 $^{a NS}$ |

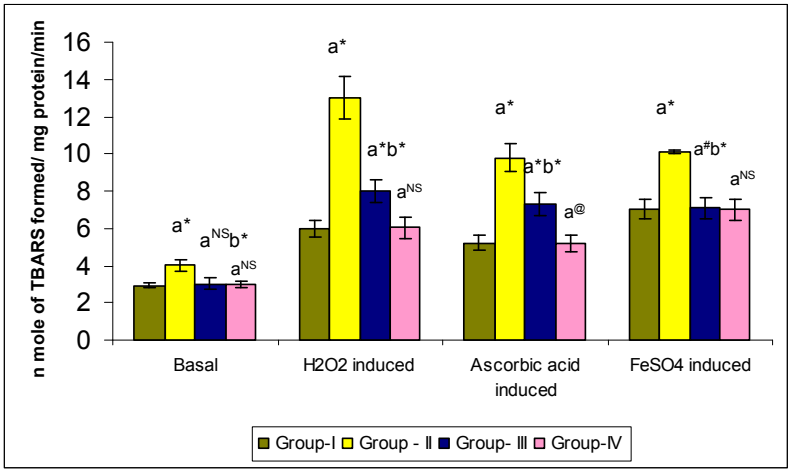
Unit are expressed as: SOD=units /mg protein: CAT=µmoles of $H_2O_2$ consumed /mg protein/min: GPx=µ g of GSH utilized/mg protein/min: GSH= µg/mg protein/min; VIT-E and VIT-C=mg/g of wet tissue.
Each value represents mean ± SD of six animals; a – Group II,III & IV compared with Group I; b – Group III compared with Group II. Statistical significance-$^{*}$p<0.001; $^{\#}$p<0.01; $^{@}$p<0.05; $^{NS}$ – No significant.

Activities of enzymic and non-enzymic antioxidants in liver and breast of control and experimental animals are presented in Table 1&2. Group II cancer-bearing animals showed a significant reduction in both enzymic and non-enzymic antioxidant levels when compared to control animals. Administration of D-Pinitol in Group III animals significantly increased the antioxidant levels when compared to Group II animals. No significant changes were observed in Group IV drug control animals when compared to Group I animals.

***(ii)Lipid peroxidation:***

**Graph: 1**

***The levels of lipid peroxidation in basal and in the presence of inducers in breast of control and experimental animals.***




Each value represents mean ± SD of six animals
a – Group II, III & IV compared with Group I
b – Group III compared with Group II
*p<0.001; #p<0.01; @p<0.05; NS – No significant

Graph 1 presents the activity of LPO in breast of control and experimental animals. Breast of Group II cancer-bearing animals showed a significant increase in LPO levels when compared to Group I control animals. However, the levels of LPO were decreased significantly in Group III animals when compared with Group II animals. No significant changes were found in drug control animals when compared to control animals.

***(iii)Biotransformation enzymes:***

**Table: 3**

***Activities of Phase I and Phase II biotransformation enzymes in liver microsomes of control and experimental animals.***

| Parameters | Group I (control) | Group II (DMBA) | Group III (DMBA+ *D-Pinitol*) | Group IV (*D-Pinitol*) |
|---|---|---|---|---|
| **Phase I enzymes:** Cytochrome $P_{450}$ | 0.91±0.04 | 0.44±0.038$^{a*}$ | 0.73±0.035 $^{a* b*}$ | 0.94±0.0041 $^{a NS}$ |
| Cytochrome $b_5$ | 0.74±0.015 | 0.31±0.019$^{a*}$ | 0.59±0.032$^{a* b*}$ | 0.71±0.017 $^{a NS}$ |
| NADPH Cytochrome 'C' reductase | 16.39±0.71 | 11.58±1.13 $^{a*}$ | 13.41±1.06 $^{a* b*}$ | 15.24±0.21 $^{a NS}$ |
| **Phase II enzymes:** Glutathione-S-Transferase | 1.68±0.047 | 3.10±0.15$^{a*}$ | 2.45±0.22 $^{a*b*}$ | 1.55±0.062 $^{a NS}$ |
| UDP-Glucronyl transferase | 47.89±1.79 | 61.24±4.79$^{a*}$ | 55.87±2.84 $^{a*b\#}$ | 47.14±1.06 $^{a NS}$ |

$Cyt.P_{450}$, $Cyt.b_5$ – n mole/mg microsomal protein/min.
NADPH Cyt. 'C' reductase – n mole of Cytochrome'c' reduced/mg protein/min.
GST – n moles of CDNB utilized/mg protein/min; UDPGT – units/mg microsomal protein/min.
Each value represents mean ± SD of six animals; a – Group II, III & IV compared with Group I
b – Group III compared with Group II; $^{*}p<0.001$; $^{\#}p<0.01$; $^{@}p<0.05$; $^{NS}$ – No significant

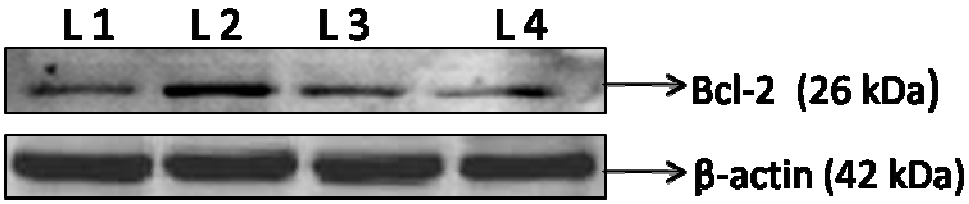
The activities of Phase I and Phase II biotransformation enzymes in liver microsomes of control and experimental animals were performed and the observed results were presented in Table 3. In Group II cancer-bearing animals, the levels of phase I biotransformation enzymes such as Cytochrome $P_{450}$, Cytochrome $b_5$, NADPH Cytochrome 'C' reductase, were found to be decreased significantly when compared to the control animals. On the other hand, phase II biotransformation enzymes such as UDP-Glucronyl transferase and Glutathione–S-transferase were significantly increased in group II cancer bearing animals when compared to the control. On contrary a significant increase in phase I enzymes and a concomitant decrease in phase II enzymes were observed in D-Pinitol treated group III animals in comparison to Group II cancer bearing animals. However, there were no significant changes in Group IV, D-Pinitol alone treated animals when compared to group I control animals.

***(iv) Western blotting:***

**Fig 1**

***Western blot analysis of Bcl-2 protein expression in breast of control and experimental animals.***

**[A]**




L1-Group I (Control); L2- Group II (DMBA);
L3- Group III (DMBA+D-Pinitol); L4- Group IV (D-Pinitol)

**[B]**

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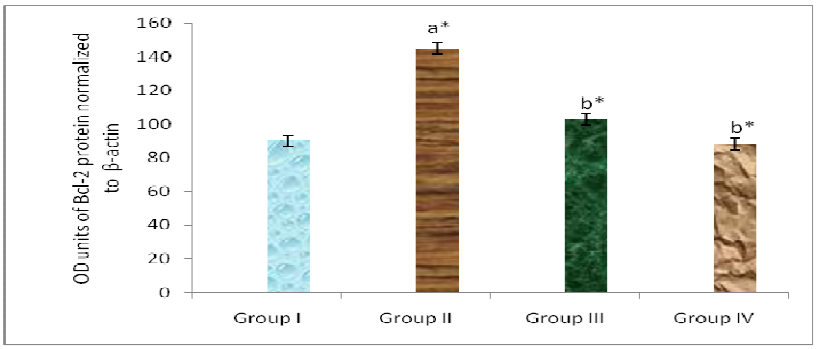


Band intensities were analyzed by quantity one software using β–actin as an internal control. Values are expressed as Mean ± SD.
a- Group II compared with Group I
b- Group III compared with Group II
*p<0.05

Fig.1 shows the Bcl-2 protein expression on DMBA induced mammary carcinoma in breast of control and experimental animals. The expression of Bcl-2 protein was found to be significantly increased in Group II cancer bearing animals when compared to Group I control animals. On the other hand, in Group III D-Pinitol treated animals, the expression of Bcl-2 protein was significantly decreased when compared to group-II cancer bearing animals. However, there was no significant change in the expression of Bcl-2 protein observed in group IV D-Pinitol alone treated animals when compared to group I control animals.

## DISCUSSION

Carcinoma of the breast is the second most common cancer worldwide, which accounts for the highest morbidity and mortality. The prevention strategies could reduce the risk of developing breast cancer and potentially reduce the number of breast cancer related deaths. However, current treatments used for breast cancer such as radiation, anti-hormonal

therapy and chemotherapy produce various side effects. Considering these effects, it is of interest that innovative new strategies will be required to treat breast cancer. Therefore searching for effective chemotherapeutic agents is important to improve the survival rate of patients with advanced or recurrent breast cancer. Hence, the development of novel drugs of natural origin or plant derived compounds is desired. The natural chemotherapeutic agents have low side effects, toxicity and are involved in the carcinogenic potential by modulating carcinogen detoxification, inhibiting lipid peroxidation, or by improving *in vivo* antioxidants defense mechanism.

In the present study, D-Pinitol was selected as a compound from natural origin to evaluate anticancer potency in DMBA induced breast cancer animals. Lipid peroxidation (LPO) has been implicated in several pathologic conditions including aging, hepatotoxicity, hemolysis and cancer. It is regarded as one of the basic mechanism of cellular damage caused by free radicals. Increased lipid peroxidation alters membrane fluidity and membrane potential and there by leading to loss of cellular function and cell death[21]. Malondialdehyde is the major end product of LPO and readily reacts with DNA to form DNA-MDA adduct. The increased levels of LPO in Group-II cancer bearing animals of the present investigation may be due to the free radicals induced by DMBA. However the administration of D-Pinitol decreased the LPO levels in drug treated animals indicating that it is a good free radical scavenger. Naturally there is a dynamic balance between the amount of free radicals generated in the body and antioxidant defense system that scavenge them and protect the body against their deleterious effects[22]. In our study, the antioxidants such as SOD, CAT ,GPx ,VIT-E and VIT-C activities were significantly lowered in Group-II cancer bearing animals which could be due to altered antioxidant status caused under oxidative stress condition and increased concentration of Reactive oxygen species(ROS) by DMBA. On contrary, upon administration of D-Pinitol the levels were reverted to near normal when compared to the control animals, thus signifying its role in scavenging the free radicals, which in turn may readily account for its antioxidant nature. Xenobiotics may exert their pathological effects through generation of ROS which is related to the etiology of cancer. In general carcinogens are converted into DNA reactive metabolites by phase I and phase II xenobiotic metabolizing enzymes that are involved in the activation and detoxification of xenobiotics[23].

In our study the decreased activity of phase I enzymes were observed in Group-II cancer bearing animals this may be due to utilization of these enzymes to excrete the DMBA metabolites. UDP-GT and GST are known to be important proneoplastic and neoplastic markers to evaluate the extent of free radical damage caused by exposure to various carcinogens. D-pinitol administration to DMBA treated rats restored the activities of phase I and phase II enzymes to near those of the control rats, by elevating the phase I enzyme activities and lowering the phase II enzyme activities. The probable mechanism behind this could be that due to the efficacy of D-Pinitol to arrest the formation of free radicals and thereby oxidative threat to the animals generated by DMBA. Bcl-2 family proteins reside in the mitochondrial outer membrane and have been implicated in the regulation of mitochondrial permeability transition (MPT) pore opening and release the of apoptogenic proteins from mitochondria in to the cytosol which leads to apoptosis. The increased levels of Bcl-2 expression in cancer condition conferred cell survival by inhibiting apoptosis[24]. In our study, the expression of Bcl-2 protein was decreased on treatment with D-Pinitol. This shows the apoptotic property of D-Pinitol. On the other hand, the expression of Bcl-2 didn't differ significantly in alone treated animals when

compared with control suggest that it doesn't have deleterious effects in normal breast tissue.

## CONCLUSION

The data of this experiment suggest that, D-Pinitol positively modulated the antioxidant activity and reduce the lipid peroxidation by quenching and detoxifying the free radicals induced by DMBA against mammary carcinoma.

## ACKNOWLEDGEMENT

Financial assistance from Department of Science and Technology (DST), New Delhi in the form of DST-PURSE-JRF is gratefully acknowledged.

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