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## Chemopreventive Potential of Genistein and Daidzein in Combination during 7,12-dimethylbenz(a)anthracene (DMBA) Induced Mammary Carcinogenesis in Sprague-Dawley Rats

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**Abstract:** The chemopreventive potential of two major soy isoflavones, genistein and daidzein, in mammary carcinogenesis remains enigmatic. The aim of the present study was to investigate the chemopreventive potential of orally administered genistein, daidzein and genistein+daidzein in 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis in Sprague-Dawley rats. The chemopreventive potential was assessed by monitoring the tumor incidence and tumor volume as well as by analyzing the status of biochemical markers (17  $\beta$ -estradiol (E2), enzymatic and non-enzymatic antioxidants and phase I and phase II detoxification enzymes) during DMBA-induced mammary carcinogenesis. A single subcutaneous injection of DMBA (25 mg rat<sup>-1</sup>) in the mammary gland developed mammary carcinoma in female Sprague-Dawley rats. Oral administration of genistein (20 mg kg<sup>-1</sup> b.wt.), daidzein (20 mg kg<sup>-1</sup> b.wt.) and genistein+daidzein (20mg+20 mg kg<sup>-1</sup> b.wt.) to DMBA treated rats significantly prevented the tumor incidence and tumor volume as well as brought back the status of above said biochemical variables. Genistein and daidzein in combination have shown pronounced chemopreventive potential than either as genistein or daidzein alone. The present study revealed the chemopreventive potential of genistein+daidzein in combination during DMBA induced mammary carcinogenesis. The chemopreventive potential of genistein+daidzein is probably due to their antilipid peroxidative efficacy and modulatory effect on phase I and phase II detoxification cascade during DMBA induced mammary carcinogenesis.

**Key words:** Mammary cancer, chemoprevention, antioxidants genistein, daidzein, DMBA

### INTRODUCTION

Breast cancer accounts for the second largest cause of death in women in Western countries (Lester, 2007). The incidence of breast cancer in Asian populations is indeed 6 to 7-fold lower as compared to Western populations (Duffy *et al.*, 2007). Globally more than 700,000 women are diagnosed with breast cancer every year. In USA, approximately 182,460 new cases of invasive and 67,770 new cases of non-invasive (*in situ*) breast cancers were diagnosed in women. In India, 70,000 new cases of breast cancer and 35,000 deaths due to this cancer are reported every year (Newman *et al.*, 2006). Though genetic factor accounts for 10-15% of all breast cancer case, life style and environmental factors play a significant role in predisposing women to this form of cancer.

DMBA-induced mammary carcinogenesis is a suitable model to investigate the chemopreventive

potential of natural and synthetic entities (Manjanatha *et al.*, 2006; Goswami and Das, 2009). In mammary epithelial cells, DMBA undergo metabolic activation to form its active metabolite, dihydrodiolepoxides, which can damage DNA and form DMBA-DNA adduct, contributing to carcinogenesis. Over production of reactive oxygen species occur during metabolic activation of DMBA to diolepoxide, can also cause oxidative damage to structure and functions of DNA, proteins and lipids, contributing to neoplastic transformation (Ray and Husain, 2002). In recent years, women of Western societies consume soy phytoestrogen rich diet for their apparent benefits against breast cancer. Profound studies suggest that the anticancer potential of soy isoflavone rely on estrogenic/antiestrogenic activity, anti-cell proliferative activity, induction of cell-cycle arrest and apoptosis, induction of detoxification enzymes, regulation of the host immune system and free radical scavenging properties (Constantinou *et al.*, 2001;

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Ruffer and Kulling, 2006; Mishra *et al.*, 2009). Despite numerous investigations, the exact mechanisms of phytoestrogens in breast cancer have yet to be elucidated. Messina *et al.* (2006) and Mense *et al.* (2008) reviewed the possible mechanism of action of phytoestrogen against breast cancer prevention and concluded that the mechanism of phytoestrogens action in breast cancer have yet to be elucidated due to the dual (chemopreventive and adverse effect) role of the phytoestrogens. Constantinou *et al.* (2001) reported the chemopreventive effects of soy protein and purified soy isoflavones in DMBA-induced mammary tumors in female Sprague-Dawley rats. Kijkuokool *et al.* (2006) reported that genistein increased tumor cross sectional area, increased tumor multiplicity, elevated the percentage of proliferative cells in tumors and increased the weight of estrogen dependent mammary adenocarcinomas in rat models of mammary cancer. Yuan *et al.* (1995) have detected no association between phytoestrogen intake and breast cancer risk. Manjanatha *et al.* (2006) have shown that dietary administration of genistein in combination with daidzein commencing two weeks prior to carcinogen treatment reduced DMBA mediated carcinogenicity than its individual treatment.

Due to controversial reports on chemopreventive potential of genistein and daidzein, the present study investigated the same effect individually as well as in combination by monitoring the tumor incidence and tumor volume as well as by analyzing the status of biochemical markers (E2, enzymatic and non-enzymatic antioxidants and phase I and phase II detoxification enzymes) during DMBA-induced mammary carcinogenesis.

## MATERIALS AND METHODS

**Chemicals:** Genistein and daidzein were purchased from Shaanxi Sciphar Biotechnology Co. Ltd, China. DMBA, reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), 1, 1', 3, 3'-tetramethoxypropane, were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Other chemicals and solvents used were of analar grade.

**Rats:** Ninety female Sprague-Dawley rats (Fifty rats for preliminary study and Forty rats for subsequent chemoprevention study), 6 weeks old, weighing  $120.0 \pm 9.0$  g, were obtained from National Institute of Nutrition, Hyderabad and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The rats were housed in polypropylene cages at room temperature ( $27 \pm 2^\circ\text{C}$ ) with relative humidity  $55 \pm 5\%$ , in an experimental room. In Annamalainagar, the LD (light: dark) cycle is almost

12: 12 h. The rats were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian National Law on animal care and use. The rats were provided with standard pellet diet (Amrut Laboratory Animal Feed, Mysore Feeds Limited, Bangalore, India) and water *ad libitum*.

**Induction of mammary carcinogenesis:** Mammary carcinogenesis was induced in Sprague-Dawley rats using a single subcutaneous injection of 25 mg of DMBA in 1 mL emulsion of sunflower oil (0.75 mL) and physiological saline (0.25 mL) to each rat (Kolanjiappan and Manoharan, 2005).

## Experimental design

**Preliminary design:** We carried out this study from June 2008 to September 2009, in the department of Biochemistry and Biotechnology, Annamalai University.

Fifty rats were divided into five groups and each group contained 10 rats. Group 1 rats received the excipient (single dose of 1 mL of emulsion of sunflower oil and physiological saline, s.c.) and 1 mL of 2% DMSO (p.o.) throughout the experimental period and served as vehicle treated control. Rats in groups 2-5 were treated with single subcutaneous injection of DMBA (25 mg). Group 2 rats received no other treatment. Group 3-5 rats received oral administration of genistein ( $20 \text{ mg kg}^{-1}$  b.wt.), daidzein ( $20 \text{ mg kg}^{-1}$  b.wt.) and genistein+daidzein ( $20 \text{ mg} + 20 \text{ mg kg}^{-1}$  b.wt.) starting one week before the exposure of the carcinogen and continued till the end of experimental period. The experiment was terminated at 16th week to evaluate the tumor incidence and tumor volume in control and experimental rats in each group.

**Chemoprevention study:** From our preliminary experimental design, we found that the combined dose of genistein and daidzein significantly prevented the tumor formation in experimental rats. Therefore, the same combinational dose was used for further studies.

Forty rats were divided into four groups and each group contained ten rats. Group 1 rats received the excipient (single dose of 1 mL of emulsion of sunflower oil and physiological saline, s.c.) and 1 mL of 2% DMSO (p.o.) throughout the experimental period served as vehicle treated control. Rats in groups 2 and 3 were induced mammary carcinogenesis by providing single subcutaneous injection of 25 mg of DMBA. Group 2 rats received no other treatment. Group 3 rats were orally administered with genistein+daidzein ( $20 \text{ mg} + 20 \text{ mg kg}^{-1}$  b.wt., dissolved in 2% DMSO) starting one week before the exposure of the carcinogen and

continued till the experimental period. Group 4 rats were orally administered with genistein+daidzein (dissolved in 2% DMSO) alone throughout the study. The experiment was terminated at 16th week to evaluate the chemopreventive effect of genistein+daidzein during DMBA-induced mammary carcinogenesis. All rats were sacrificed by cervical dislocation at the end of experimental period. For histopathological studies, tumor tissues and normal mammary gland tissues were fixed in 10% formalin and were routinely processed and paraffin embedded, 2-3 µm sections were cut in a rotary microtome and were stained with hematoxylin and eosin.

**Biochemical estimations:** Blood samples were collected into heparinized tubes. Plasma was separated by centrifugation at 1000x g for 15 min. Tissue samples from rats were washed with ice cold saline and dried between folds of filter paper, weighed and homogenized using appropriate buffer in an All-glass homogenizer with teflon pestle and used for biochemical estimations

Lipid peroxidation was estimated as evidenced by the formation of thiobarbituric acid reactive substances (TBARS). TBARS in plasma were assayed by the method of Yagi (1987). Tissue lipid peroxidation was done by the method of Ohkawa *et al.* (1979). The activities of superoxide dismutase (SOD), catalase (CAT) and Glutathione Peroxidase (GPx) were assayed by the method of Kakkar *et al.* (1984), Sinha (1972) and Rotruck *et al.* (1973) respectively. The GSH level in plasma, liver and mammary tissues was determined by the method of Beutler and Kelley (1963).

The activity of Glutathione-S-Transferase (GST) and Glutathione Reductase (GR) in liver and mammary tissue homogenate was assayed by the method of Habig *et al.* (1974) and Carlberg and Mannervik (1985), respectively. The activity of DT-Diaphorase (DTD) was determined by the method of Ernster (1967). Cytochrome P450 and cytochrome b5 were assayed by the method of Omura and Sato (1964). Chemiluminescent immunoassay (CLIA) was used for the estimation of serum 17 β-estradiol (E2) (Buscarlet *et al.*, 2001).

**Statistical analysis:** The values are expressed as Mean±SD. The statistical comparisons were performed by one way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT), using SPSS

version 12.0 for windows (SPSS Inc. Chicago; <http://www.spss.com>). The values are considered statistically significant if the p-value was less than 0.05.

## RESULTS

Table 1 shows the incidence of mammary tumors in DMBA, DMBA+genistein, DMBA+daidzein and DMBA+genistein+daidzein treated rats. The present study observed 100% tumor incidence in rats treated with DMBA alone. The tumors were histopathologically confirmed as moderately and poorly differentiated adenocarcinoma. Oral administration of genistein, daidzein and genistein+daidzein to DMBA-treated rats reduced the tumor incidence (60, 50 and 80%, respectively) and tumors in this group (40, 50 and 20%) were histopathologically confirmed as well-differentiated adenocarcinoma. The size of the tumor and tumor volume observed in DMBA+genistein+daidzein treated rats (2 rats) were very small as compared to rats treated with DMBA alone. Figure 1a, b and 2a-f show the gross appearance of mammary tumors and histological features in DMBA, DMBA+genistein, DMBA+daidzein and DMBA+genistein+daidzein treated rats.

In control rats and rats treated with genistein+daidzein alone, the epithelium frequently contained multiple layers of cells. Rats treated with DMBA alone exhibited higher level of epithelial cell proliferation as evidenced by multi-layered epithelium with very high density and cellular atypia. Oral administration of genistein+daidzein to DMBA treated rats significantly prevented the mammary epithelial cell proliferation.

The level of plasma E2 was significantly increased in DMBA treated rats as compared to control rats (Table 2). Oral administration of genistein+daidzein to DMBA-treated rats as well as control rats significantly (p<0.05) decreased the level of E2.

The levels of TBARS were significantly increased whereas GSH content and activities of SOD, CAT and Gpx in plasma were decreased in DMBA treated rats as compared to control rats (Table 3). Oral administration of genistein+daidzein to DMBA-treated rats significantly (p<0.05) decreased the levels of TBARS and improved the levels of GSH and activities of SOD, CAT and GPx. Rats treated with

Table 1: Incidence of mammary tumor in control and experimental rats in each group

Groups	Control (vehicle)	DMBA alone	DMBA+Genistein	DMBA+Daidzein	DMBA+Genistein+Daidzein
Tumor incidence (mammary tumor)	0	100%	40%	50%	20%
Total number of tumors	0	(10)/10	(4)/10	(5)/10	(2)/10
Tumor volume (cm <sup>3</sup> ) rat <sup>-1</sup>	0	15.42±1.4 <sup>a</sup>	1.23±0.10 <sup>b</sup>	1.46±0.15 <sup>b</sup>	0.76±0.05 <sup>c</sup>

Tumor volume was measured using the formula:  $V = (4/3)\pi(D_1/2)(D_2/2)(D_3/2)$  where, D<sub>1</sub>, D<sub>2</sub> and D<sub>3</sub> are the three diameters (in cm) of the tumor. ( ) indicates total number of rats bearing tumors. Values that are not sharing a common superscript letter in the same row differ significantly at p<0.05 (DMRT)

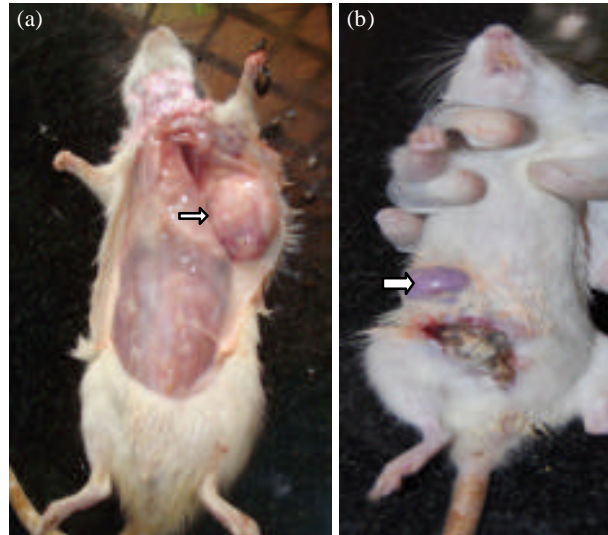


Fig. 1: The gross appearance of the mammary adenocarcinoma in DMBA and DMBA+ genistein+ daidzein treated female Sprague-Dawley rats. (a) the gross appearance of the mammary adenocarcinoma in DMBA treated female Sprague-Dawley rats and (b) the gross appearance of the mammary adenocarcinoma in DMBA+ genistein+ daidzein treated female Sprague-Dawley rats

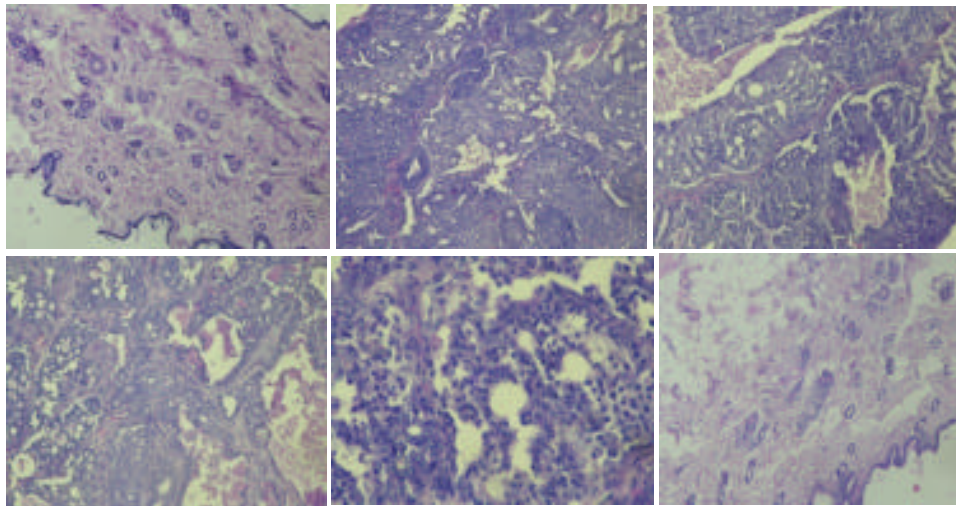


Fig. 2: The histopathological changes observed in control and experimental rats in each group. (a) Microphotograph showing normal glandular structure in control rat. (b) Microphotograph showing adenocarcinoma in DMBA treated rats (100% of rat). (c) Microphotograph showing ductal hyperplasia in DMBA + genistein treated rat (40% of rats). (d) Microphotograph showing ductal hyperplasia in DMBA + daidzein treated rats (60% of rats). (e) Microphotograph showing moderate to severe ductal dysplasia in DMBA + genistein + daidzein treated rats (20% of rats). (f) Microphotograph showing normal glandular structure in genistein + daidzein alone treated rats

genistein+daidzein alone showed no significant difference in the status of TBARS, GSH and activities of SOD, CAT and GPx as compared to control rats.

The levels of TBARS, GSH and activity of GPx were increased whereas the activities of SOD and CAT were

decreased in the tumor tissues as compared to normal tissues of control rats (Table 4). Oral administration of genistein+daidzein to DMBA-treated rats significantly ( $p<0.05$ ) restored the status to near normal. Rats administered with genistein+daidzein alone orally showed no significant difference in the status of

TBARS and activities of antioxidants as compared to control rats.

The level of liver tissue GSH and activities of phase II detoxification enzymes were significantly decreased whereas the activities of phase I enzymes were increased in DMBA treated rats as compared to control rats (Table 5). Oral administration of genistein+daidzein to DMBA-treated rats significantly ( $p < 0.05$ ) restored the

status of above said biochemical parameters to near normal. Rats administered with genistein+daidzein alone orally showed no significant difference in the status of GSH and activities of phase I and phase II detoxification enzymes as compared to control rats.

The activities of mammary tissue phase I enzymes were significantly increased and phase II detoxification enzymes were decreased in DMBA treated rats as compared to control rats (Table 6). Oral administration of genistein+daidzein to DMBA-treated rats significantly ( $p < 0.05$ ) decreased the activities of phase I and increased the activities of phase II detoxification enzymes. Rats administered with genistein+daidzein alone orally showed no significant difference in the activities of phase I and phase II detoxification enzymes as compared to control rats.

Table 2: Status of plasma 17- $\beta$  estradiol (E2) in control and experimental rats in each group

Groups	17- $\beta$ estradiol (pg mL <sup>-1</sup> )
Control	46.3 $\pm$ 4.1 <sup>a</sup>
DMBA alone	58.2 $\pm$ 5.5 <sup>b</sup>
DMBA+Genistein+Daidzein	50.1 $\pm$ 4.6 <sup>c</sup>
Genistein+Daidzein alone	44.0 $\pm$ 4.2 <sup>a</sup>

Values are expressed as Mean $\pm$ SD (n = 10). Values that are not sharing a common superscript letter in the same column differ significantly at  $p < 0.05$  (DMRT)

Table 3: Status of plasma TBARS and antioxidants in control and experimental rats in each group

Groups	TBARS (nmoles mL <sup>-1</sup> )	SOD (U <sup>a</sup> mL <sup>-1</sup> )	CAT (U <sup>b</sup> mL <sup>-1</sup> )	GPx (U <sup>c</sup> L <sup>-1</sup> )	GSH (mg dL <sup>-1</sup> )
Control (Vehicle)	1.86 $\pm$ 0.16 <sup>a</sup>	2.98 $\pm$ 0.25 <sup>a</sup>	1.56 $\pm$ 0.14 <sup>a</sup>	154 $\pm$ 13.0 <sup>a</sup>	31.0 $\pm$ 2.72 <sup>a</sup>
DMBA alone	3.24 $\pm$ 0.30 <sup>b</sup>	1.82 $\pm$ 0.16 <sup>b</sup>	1.10 $\pm$ 0.09 <sup>b</sup>	110 $\pm$ 10.8 <sup>b</sup>	18.0 $\pm$ 1.64 <sup>b</sup>
DMBA+Genistein+Daidzein	2.08 $\pm$ 0.21 <sup>c</sup>	2.69 $\pm$ 0.25 <sup>c</sup>	1.37 $\pm$ 0.14 <sup>c</sup>	137 $\pm$ 12.7 <sup>c</sup>	28.0 $\pm$ 2.60 <sup>c</sup>
Genistein+Daidzein alone	1.85 $\pm$ 0.14 <sup>a</sup>	3.02 $\pm$ 0.32 <sup>a</sup>	1.57 $\pm$ 0.16 <sup>a</sup>	156 $\pm$ 13.6 <sup>a</sup>	32.0 $\pm$ 3.30 <sup>a</sup>

Values are expressed as Mean $\pm$ SD (n = 10). Values that are not sharing a common superscript letter in the same column differ significantly at  $p < 0.05$  (DMRT).  
<sup>a</sup>: The amount of enzyme required to inhibit 50% nitroblue-tetrazolium (NBT) reduction. <sup>b</sup>: Micromoles of H<sub>2</sub>O<sub>2</sub> utilized sec<sup>-1</sup>. <sup>c</sup>: Micromoles of glutathione utilized min<sup>-1</sup>

Table 4: Status of mammary tissues TBARS and antioxidants in control and experimental rats in each group

Groups	TBARS (nmoles/100 mg tissue)	SOD (U <sup>a</sup> mg <sup>-1</sup> of protein)	CAT x (U <sup>a</sup> mg <sup>-1</sup> of protein)	GPx (U <sup>c</sup> mg <sup>-1</sup> of protein)	GSH (mg/100 mg tissue)
Control (Vehicle)	0.72 $\pm$ 0.06 <sup>a</sup>	12.54 $\pm$ 1.14 <sup>a</sup>	43.65 $\pm$ 4.5 <sup>a</sup>	18.06 $\pm$ 1.78 <sup>a</sup>	8.98 $\pm$ 0.64 <sup>a</sup>
DMBA alone	1.14 $\pm$ 0.09 <sup>b</sup>	8.90 $\pm$ 0.92 <sup>b</sup>	27.46 $\pm$ 2.62 <sup>b</sup>	27.44 $\pm$ 2.94 <sup>b</sup>	13.42 $\pm$ 1.04 <sup>b</sup>
DMBA+Genistein+Daidzein	0.64 $\pm$ 0.07 <sup>c</sup>	11.20 $\pm$ 0.92 <sup>c</sup>	38.42 $\pm$ 3.60 <sup>c</sup>	20.18 $\pm$ 1.88 <sup>c</sup>	10.37 $\pm$ 1.0 <sup>c</sup>
Genistein+Daidzein alone	0.70 $\pm$ 0.04 <sup>a</sup>	12.55 $\pm$ 1.0 <sup>a</sup>	44.43 $\pm$ 4.70 <sup>a</sup>	18.56 $\pm$ 1.42 <sup>a</sup>	8.98 $\pm$ 0.70 <sup>a</sup>

Values are expressed as Mean $\pm$ SD (n = 10). Values that are not sharing a common superscript letter in the same column differ significantly at  $p < 0.05$  (DMRT).  
<sup>a</sup>: The amount of enzyme required to inhibit 50% nitroblue-tetrazolium (NBT) reduction; <sup>b</sup>: Micromoles of H<sub>2</sub>O<sub>2</sub> utilized sec<sup>-1</sup>; <sup>c</sup>: Micromoles of glutathione utilized min<sup>-1</sup>

Table 5: Status of phase I and phase II detoxification agents in the liver of control and experimental rats in each group

Groups	Cytochrome P450 (U* g <sup>-1</sup> protein)	Cytochrome b <sub>5</sub> (U* g <sup>-1</sup> protein)	GSH (mg g <sup>-1</sup> tissue)	GST (U <sup>a</sup> mg <sup>-1</sup> protein)	GR (U <sup>b</sup> mg <sup>-1</sup> protein)	DT-diaphorase (U <sup>c</sup> mg <sup>-1</sup> protein)
Control (Vehicle)	0.81 $\pm$ 0.07 <sup>a</sup>	0.47 $\pm$ 0.03 <sup>a</sup>	1.78 $\pm$ 0.18 <sup>a</sup>	128.5 $\pm$ 12.7 <sup>a</sup>	22.14 $\pm$ 2.02 <sup>a</sup>	0.48 $\pm$ 0.05 <sup>a</sup>
DMBA alone	1.25 $\pm$ 0.10 <sup>b</sup>	0.74 $\pm$ 0.08 <sup>b</sup>	1.14 $\pm$ 0.10 <sup>b</sup>	90.44 $\pm$ 8.60 <sup>b</sup>	12.65 $\pm$ 1.20 <sup>b</sup>	0.32 $\pm$ 0.03 <sup>b</sup>
DMBA+Genistein+Daidzein	0.72 $\pm$ 0.07 <sup>c</sup>	0.53 $\pm$ 0.06 <sup>c</sup>	1.58 $\pm$ 0.13 <sup>c</sup>	113.2 $\pm$ 10.0 <sup>c</sup>	19.64 $\pm$ 1.90 <sup>c</sup>	0.42 $\pm$ 0.05 <sup>c</sup>
Genistein+Daidzein alone	0.80 $\pm$ 0.08 <sup>a</sup>	0.46 $\pm$ 0.05 <sup>a</sup>	1.79 $\pm$ 0.18 <sup>a</sup>	129.0 $\pm$ 12.2 <sup>a</sup>	22.65 $\pm$ 2.10 <sup>a</sup>	0.49 $\pm$ 0.05 <sup>a</sup>

Values are expressed as Mean $\pm$ SD (n = 10). Values that are not sharing a common superscript letter in the same column differ significantly at  $p < 0.05$  (DMRT).  
 \*: Micromoles of cytochrome per gram of tissue. <sup>a</sup>: Micromoles of CDNB-GSH conjugate formed h<sup>-1</sup>. <sup>b</sup>: Micromoles of NADPH oxidised h<sup>-1</sup>. <sup>c</sup>: Micromoles of 2,6-dichlorophenol indophenol reduced min<sup>-1</sup>

Table 6: Status of phase I and phase II detoxification agents in the mammary tissues of control and experimental rats in each group

Groups	Cytochrome P450 (U* g <sup>-1</sup> protein)	Cytochrome b <sub>5</sub> (U* g <sup>-1</sup> protein)	GST (U <sup>a</sup> mg <sup>-1</sup> protein)	GR (U <sup>b</sup> mg <sup>-1</sup> protein)	DT-diaphorase (U <sup>c</sup> mg <sup>-1</sup> protein)
Control (Vehicle)	0.95 $\pm$ 0.07 <sup>a</sup>	0.68 $\pm$ 0.06 <sup>a</sup>	2.15 $\pm$ 0.22 <sup>a</sup>	3.55 $\pm$ 0.29 <sup>a</sup>	0.28 $\pm$ 0.03 <sup>a</sup>
DMBA alone	1.45 $\pm$ 0.14 <sup>b</sup>	1.16 $\pm$ 0.11 <sup>b</sup>	1.34 $\pm$ 0.10 <sup>b</sup>	2.16 $\pm$ 0.20 <sup>b</sup>	0.16 $\pm$ 0.01 <sup>b</sup>
DMBA+Genistein+Daidzein	1.06 $\pm$ 0.11 <sup>c</sup>	0.77 $\pm$ 0.08 <sup>c</sup>	1.90 $\pm$ 0.18 <sup>c</sup>	3.21 $\pm$ 0.30 <sup>c</sup>	0.25 $\pm$ 0.02 <sup>c</sup>
Genistein+Daidzein alone	0.93 $\pm$ 0.08 <sup>a</sup>	0.68 $\pm$ 0.08 <sup>a</sup>	2.16 $\pm$ 0.20 <sup>a</sup>	3.56 $\pm$ 0.26 <sup>a</sup>	0.29 $\pm$ 0.03 <sup>a</sup>

Values are expressed as Mean $\pm$ SD (n = 10). Values that are not sharing a common superscript letter in the same column differ significantly at  $p < 0.05$  (DMRT).  
 \*: Micromoles of cytochrome per gram of protein. <sup>a</sup>: Micromoles of CDNB-GSH conjugate formed h<sup>-1</sup>. <sup>b</sup>: Micromoles of NADPH oxidised h<sup>-1</sup>. <sup>c</sup>: Micromoles of 2,6-dichlorophenol indophenol reduced min<sup>-1</sup>

## DISCUSSION

The present study demonstrated the chemopreventive potential of genistein and daidzein (1:1 ratio) in combination during DMBA-induced mammary carcinogenesis. The mechanistic pathway for the chemopreventive potential was assessed by analyzing the status of biochemical markers that are related to carcinogenic process. Estrogens have been implicated in the initiation and promotion stages of breast cancer and lifetime estrogen exposure is a major risk factor for breast cancer development (Yager and Davidson, 2006). Mense *et al.* (2008) reviewed lower serum estrogen levels and a lower incidence of DMBA-induced mammary tumors in rats fed soy-containing diet as compared to rats fed soy-free diet. It has also been shown that serum concentrations of E2 are 40% lower in Asian women compared with their Caucasian counterparts (Peeters *et al.*, 2003). Estrogen receptor (ER- $\alpha$  to ER- $\beta$ ), a group of receptor that are activated by the E2 function as DNA binding transcription that regulates gene expression. Immuno expression pattern of E2 receptors has been well documented in breast carcinogenesis. Gallo *et al.* (2001) reported that dietary supplementation of soy significantly down regulated the expression of E2 receptors in mammary tumor bearing rats. These reviews support our results. Oral administration of genistein+daidzein to DMBA treated rats significantly decreased serum E2 levels, which indicate the antiestrogenic activity of these isoflavones during DMBA-induced mammary carcinogenesis.

Strategies for protecting cells from tumor initiation events include decreasing the activities of metabolic enzymes responsible for generating excess reactive oxygen species (phase I enzymes) and increasing the activities of phase II enzymes that deactivates excessively generated free radicals and electrophiles. Epoxides that are produced during metabolic activation of DMBA in the mammary gland could damage DNA molecule and contributing to carcinogenesis. Cytochrome P450 and cytochrome b5 are the key enzymes responsible for the metabolic activation of DMBA. DT-diaphorase protects the cell from oxidative damage by preventing oxyradical formation. GSTs are a family of enzymes that catalyze the conjugation of reactive oxygen species with GSH and thereby facilitating the excretion of reactive metabolites (Yoshimasa *et al.*, 2000). Glutathione Reductase (GR) maintains the levels of reduced glutathione in the cells by catalyzing the NADPH-dependent reduction of glutathione disulfide to glutathione (Yeh *et al.*, 2005). Increase in phase I and decrease in phase II detoxification enzymes in the liver and mammary tissues were reported

in DMBA treated rats (Mathivadhani *et al.*, 2007). Present results are in line with these findings. Oral administration of genistein+daidzein to DMBA treated rats reversed the status of phase I and phase II detoxification agents in the liver and mammary tissues, which suggest that genistein in association with daidzein might have either inhibited the metabolic activation of DMBA or stimulated the activities of detoxification agents to excrete the active metabolite of DMBA, dihydrodiolepoxides. Choi and Kim (2008) reported that daidzein modulates the expression of hepatic CYP1A1, 1B1 and AhR by DMBA treated mice. Steiner *et al.* (2007) reported that genistein protects human epithelial cells from benzo(a)pyrene by modulating the glutathione/glutathione-s-transferase system. Our results corroborate these findings.

Oxidative stress has been implicated in the pathogenesis of several cancers including mammary carcinoma. Increased level of TBARS in plasma and tumors tissues has been well documented in both human and experimental carcinogenesis (Kolanjiappan and Manoharan, 2005). Over production of oxygen free radicals in mammary cancer tissues have been demonstrated (Mishra *et al.*, 2008). The status of plasma TBARS serve as an index to assess the extent of tissue damage. Elevated levels of TBARS in plasma of mammary cancer rat could therefore be related to overproduction and diffusion from the mammary tumor tissues and damaged host tissues. Isoflavones and their metabolites are able to efficiently scavenge peroxy radicals in a hydrophilic environment and act as inhibitors of lipid peroxidation through different mechanisms in a lipophilic environment (Ruffer and Kulling, 2006). Oral administration of genistein+daidzein to DMBA treated rats brought back the status of TBARS to near normal range. Inhibition of lipid peroxidation has shown to be a possible mechanism in the antioxidant function of genistein, daidzein and its metabolite equol (Ferretti *et al.*, 2004).

Enzymatic and non-enzymatic antioxidants form the first line of defense mechanism to scavenge excessively generated ROS in the system. Abnormalities in the status of antioxidants lead to several disorders including cancer (Khataibeh *et al.*, 2007; Suresh *et al.*, 2006; Renju *et al.*, 2007). Lowered activities of plasma SOD, CAT and GPx were demonstrated well in mammary cancer (Kolanjiappan and Manoharan, 2005). Lowered content of plasma glutathione is probably due to utilization by mammary tumor tissues to reduce reactive oxygen species during carcinogenic process. Present results are in line with these findings

Profound studies have shown that the activities of SOD and CAT were decreased in mammary tumor tissues

(Kolanjiappan and Manoharan, 2005). Lowered activities of SOD and CAT are probably due to exhaustion of these enzymes to scavenge excessively generated reactive oxygen species in mammary tumor tissues. High content of GSH and enhanced activity of GPx were well documented in several cancers including mammary cancer (Kolanjiappan and Manoharan, 2005; Mishra *et al.*, 2008). The results of the present study are in line with these findings. Antioxidant potential of genistein and daidzein has been documented well in several diseased conditions (Arora *et al.*, 1998; Russo, 2007). Mishra *et al.* (2009) reported that daidzein prevented mammary carcinogenesis by enhancing the activities of antioxidant enzymes. Genistein and daidzein stimulated the activities of antioxidant CAT, SOD, GPx and glutathione reductase (Adlercreutz, 2002; Mishra *et al.*, 2009). Present results corroborate these findings. Oral administration of genistein+daidzein brought back the status of these enzymes to near normal level, which indicates their free radical scavenging properties.

Histopathological studies also showed that the tumor is very necrotic in the isoflavones treated rats which might be due to induction of apoptotic and necrotic cell death by these isoflavones. Li *et al.* (1999) reported that genistein mediates apoptosis in breast cancer cells by up-regulation of the pro-apoptotic (bax) and down-regulation of anti-apoptotic (bcl-2 and p<sup>53</sup>) markers.

The present study thus demonstrated the chemopreventive potential of genistein and daidzein in combination during DMBA induced mammary carcinogenesis. Further studies warranted to elucidate the exact mechanistic pathway for the chemopreventive potential of combined administration of genistein and daidzein by analyzing the status of molecular markers of mammary carcinogenesis.

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