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Research Article

# Effects of genistein and daidzein, in combination, on immunoexpression pattern of ER, PR and HER-2/neu during 7, 12-dimethylbenz (a) anthracene induced mammary carcinogenesis in rats

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**ABSTRACT**

The status of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2/neu) has been used as routine markers for diagnosis, prognosis and response to treatment of breast cancer. Aim of the present study was to investigate the effects of genistein and daidzein, in combination, on immunoexpression pattern of ER, PR and HER2/neu receptors during 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis in Sprague-Dawley rats. A single subcutaneous injection of DMBA (25 mg/rat) in the mammary gland developed mammary carcinoma in rats. Mammary tissues of control and experimental rats were routinely processed and paraffin embedded, 2-3µm sections were cut in a rotary microtome and mounted on clean glass slides. Immunohistochemical staining of mammary tissue was performed and each slide was microscopically analyzed and enumerated for the percentage of the positively stained cells semi-quantitatively and grading was then made. Immunohistochemical analysis showed over expression of ER, PR and HER2/neu in the mammary tissues of DMBA treated rats. Oral administration of genistein+daidzein (20mg+20mg/kg body weight) in combination, to DMBA treated rats significantly prevented the over expression of ER, PR and HER2/neu in the mammary tissues. Genistein and daidzein inhibited mammary epithelial cell proliferation probably by preventing the over expression of ER, PR and HER2/neu in the mammary tissues of Sprague-Dawley rats, during DMBA induced mammary carcinogenesis.

**Keywords:** Genistein; Daidzein; Estrogen receptor; Progesterone receptor; HER2/neu.

## INTRODUCTION

The term breast cancer refers to malignant breast neoplasm that has developed from the cells in the breast, most commonly from the inner lining of milk ducts or the lobules that supply the duct with milk. The incidence of breast cancer is rapidly increasing throughout the world, especially in developing countries including India. In 2009, around 192,370 newly diagnosed invasive and 62,280 non invasive breast cancer cases were reported in USA. Also, about 40,170 women were died due to breast cancer in USA in 2009 (Jemal, 2009). In India, 75,000 new cases of breast cancer are reported every year and increase in incidence has been reported in metropolitan cities (Murthy, et al., 2009).

Approximately 2/3 of breast cancers (both hereditary and sporadic) are estrogen sensitive. Lifetime exposure of estrogen has been implicated in the pathogenesis of initiation and promotion of breast cancer (Yager & Davidson, 2006). The most active estrogen, 17-β-estradiol (E2) regulates the growth, differentiation, and physiology of the reproductive process through the estrogen receptor. The estrogen receptors (ER) are nuclear receptors that modulate the transcription of target genes responsible for the proliferation of mammary cells. Increased ER status plays a major role in the progression and metastatic potential of breast cancer. ER isoforms have been characterized as estrogen receptor alpha (ER-α) and beta (ER-β), which are ligand dependent transcriptional activators of gene expression. Epidemiologic studies have found that significant correlation between ER+ breast cancer cells and life-style risk factors such as higher body mass index, earlier age at menarche, nulliparity, and diet (Colditz, et al., 2004). Breast epithelial cell proliferation is not only related to estrogen levels but also to progesterone, which acts through its own receptor, progesterone receptor (PR). The PR status was significantly elevated during mammary carcinogenesis (Shyamala, et al., 2002).

Human Epidermal Growth Factor Receptor-2 (HER-2), also known as c-erbB-2 or neu have substantial homology with the epidermal growth factor receptor (EGFR). HER family includes four structurally related members,

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HER-1 (erbB1, also known as EGFR), HER-2 (erbB2), HER-3 (erbB3) and HER-4 (erbB4). HER-2 plays an important role in cell growth, survival, and differentiation of normal cells. Over expression of HER-2 was reported in mammary carcinogenesis and its metastasis. The status of ER, PR and HER2/neu has been used as routine markers for diagnosis, prognosis and response to treatment of breast cancer (Engel & Kaklamani, 2007)

Phytoestrogens are biologically active phenolic compounds of plant origin that structurally mimic the mammalian estrogen, 17β-estradiol. Genistein and daidzein are such compounds that act as ligands for estrogen receptor and initiate estrogen-dependent transcription (Cos, et al., 2003). Manjanatha et al. (2006) reported that consumption of soy phytoestrogen mixture instead of single compounds was more protective against 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis. Previous study from our laboratory reported that genistein and daidzein in combination, inhibited mammary carcinoma in 80% of DMBA treated rats (Pugalendhi & Manoharan, 2010). The present study investigates the effects of genistein and daidzein in combination, on immunoexpression pattern of ER, PR and HER2/neu receptors during DMBA induced mammary carcinogenesis in Sprague-Dawley rats.

## MATERIALS AND METHODS

### Rats

Female Sprague-Dawley rats, six weeks old, 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±2°C) with relative humidity 55±5%, in an experimental room. In Annamalainagar, the LD (light: dark) cycle is almost 12:12 h. The rats were provided with standard pellet diet and water *ad libitum*. The experimental design (Proposal No. 578 dated. 25.07.2008) was approved by the Annamalai University animal ethical committee (Register number 160/1999/CPCSEA), Annamalainagar. 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.

### Chemicals

Genistein and daidzein were purchased from Shaanxi Sciphar Biotechnology Co. Ltd, China. DMBA and dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Other chemicals and solvents used were of analar grade.

## EXPERIMENTAL DESIGN

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 and 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 in 1ml of sunflower oil and physiological saline (Kolanjiappan & Manoharan, 2005). Group 2 rats received no other treatment. Group 3 rats were orally administered with genistein+daidzein (20mg+20mg/kg body weight, dissolved in 1 ml of 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 (20mg+20mg/kg body weight, each dissolved in 1 ml of 2% DMSO) alone throughout the experimental study. The experiment was terminated at $16^{th}$ week and all rats were sacrificed by cervical dislocation. The mammary tissues were harvested and preserved in 10% buffered formalin used for immunohistochemical studies.

### Immunohistochemical staining

Mammary tissues were routinely processed and paraffin embedded, 2-3µm sections were cut in a rotary microtome and mounted on clean poly lysine coated glass slides, dried at 37˚C. Paraffin embedded tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% $H_2O_2$ in methanol for 10 min. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid/L D. $H_2O$; 0.37 g EDTA/L D. $H_2O$; 0.2 g trypsin, pH 6.0) for 10 min, followed by washing step with tris-buffered saline (8 g NaCl; 0.605 g Tris, pH 7.6). The tissue section was then incubated with power Block$^{TM}$ reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent for 15 min at room temperature to block non-specific binding sites. The tissue sections were then incubated with the respective primary antibody (DAKO/ ER, PR and HER-2/neu) overnight at 4°C. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA) for 30 min at room temperature. After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3,3′-diaminobenzidine, the substrate of horseradish peroxidase. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium.

### Grading of immunostaining

Each slide was microscopically analyzed and enumerated for the percentage of the positively stained cells semi-quantitatively. Nuclear staining for ER, and PR were graded as 1+ (<10% of the cells are stained), 2+ (10–50% of the cells are stained), and 3+ (>50% of the cells are stained). Grades 2+ and 3+ were considered as positive, whereas absence of staining and 1+ staining were considered as negative (Anim, et al., 2005).

Membrane staining for HER2/neu was graded similarly in terms of intensity of staining and only grade 3+ (high intensity) was considered as positive, in accordance with the recommendation of Witton et al. (2003).

**RESULTS**

The immunoexpression pattern and intensity of positively stained cells of ER, PR and HER2/neu in control and experimental rats in each group are shown in figures 1 (a-d) – 3 (a-d) and table 1 respectively.

The analysis showed a positive staining for ER, PR and HER2/neu in tumor tissues, which is more pronounced as compared to normal tissues. It was observed 70%, 65% and 50% positive staining for ER, PR and HER2/neu respectively in tumor tissues (group 2). We observed nuclear expression for ER and PR, and membrane staining for HER2/neu in the mammary tissues of DMBA alone treated rats.

It was found that ER, PR and HER2/neu less than 10% of the cells are stained in the mammary tissues of control rats. In normal mammary epithelium, lower expression of ER, PR and HER2/neu were observed. Oral administration of genistein+daidzein (group 3) to DMBA treated rats significantly down regulated the expression ER, PR and HER2/neu receptors. Rats treated with genistein+daidzein alone showed no significant difference in the expression pattern of ER, PR and HER2/neu receptors as compared to control rats (group 1).

**DISCUSSION**

The ER/PR pathways play a critical role in the pathophysiology of human breast cancer. The mutation or over expression of HER2 could directly lead to tumorigenesis as well as metastasis. DMBA, a potent organ and site specific polycyclic aromatic hydrocarbon, is mostly used to study the hormone-dependent mammary carcinogenesis in experimental models (Manjanatha, et al., 2006) since the estrogen metabolite 16α-hydroxyestrogen levels were significantly elevated in DMBA administered rats (Bradlow, et al., 1984). Estrogens activate expression of genes through the estrogen receptors, which in turn regulate cell differentiation and growth (Bourdeau, et al., 2008). ERs are extremely important components of a complex signal transduction pathway that specifically regulates the growth and development of target tissues and tumors. Breast cancer is often susceptible to treatment with inhibitors that block the interaction between estrogen and the estrogen receptor. Aberrant roles for ER have been demonstrated in breast cancer.

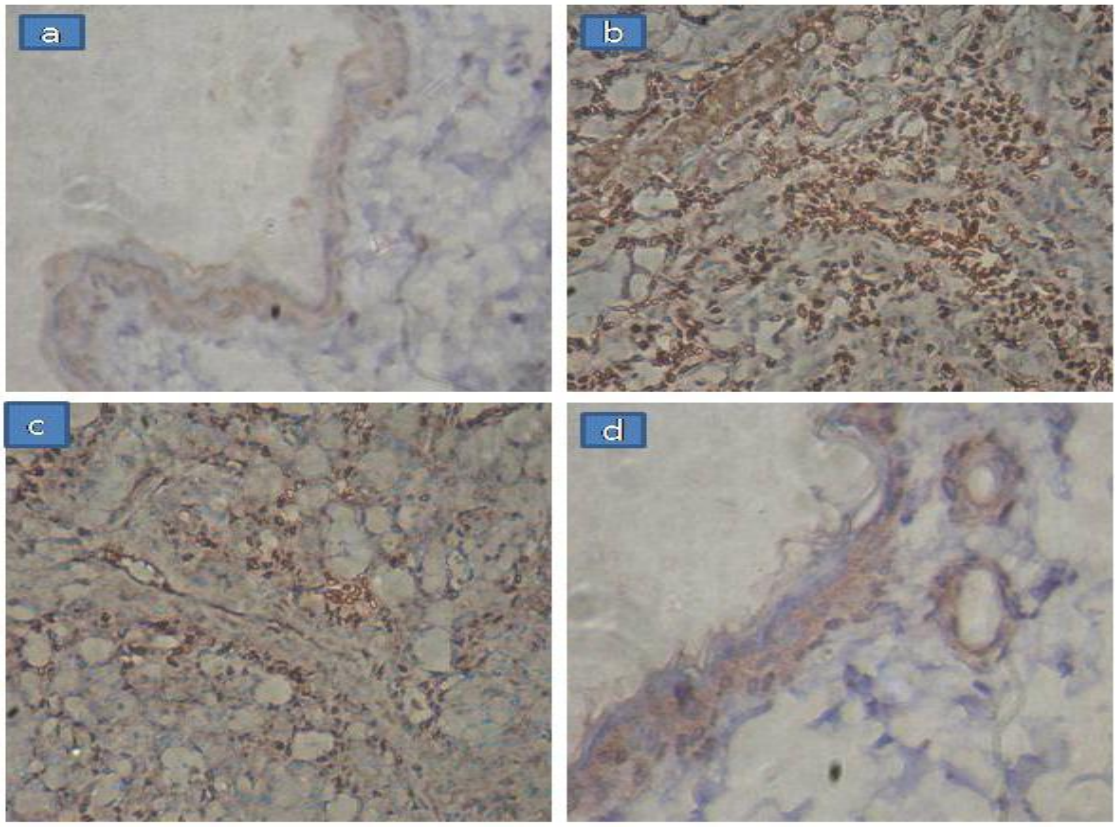


**Figure 1: Depicts immunoexpression of ER in (a) control rats (mild expression), (b) DMBA alone treated rats (over expression), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (mild expression)**

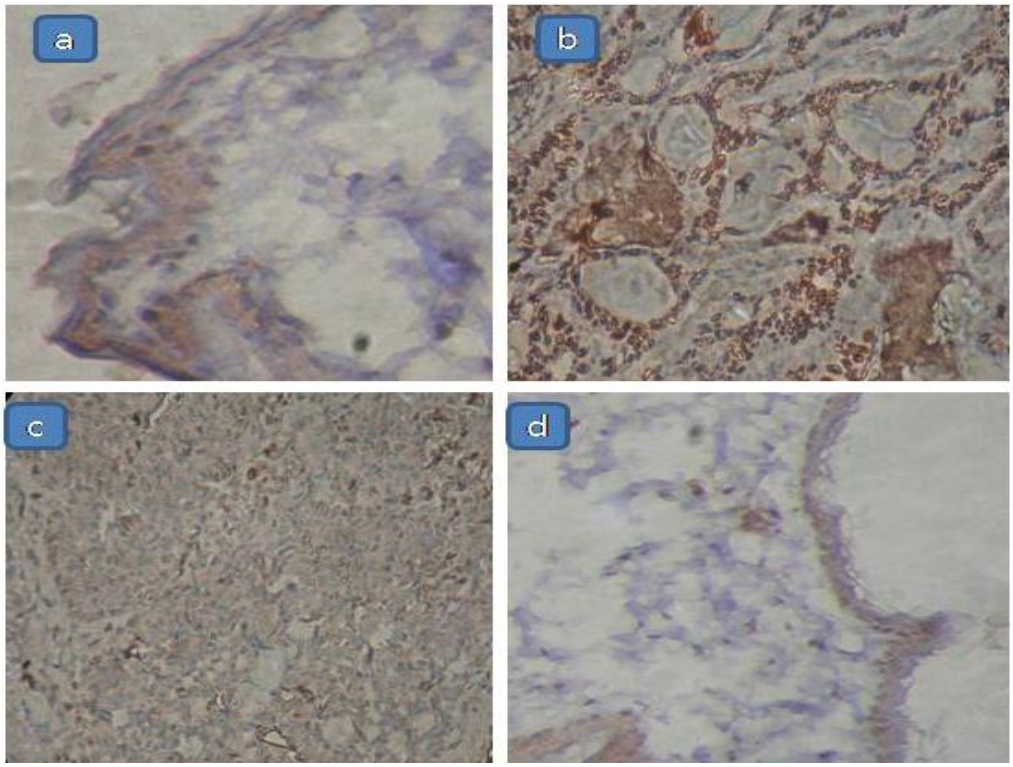


**Figure 2: Depicts immunoexpression of PR in (a) control rats (mild expression), (b) DMBA alone treated rats (over expression), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (mild expression)**

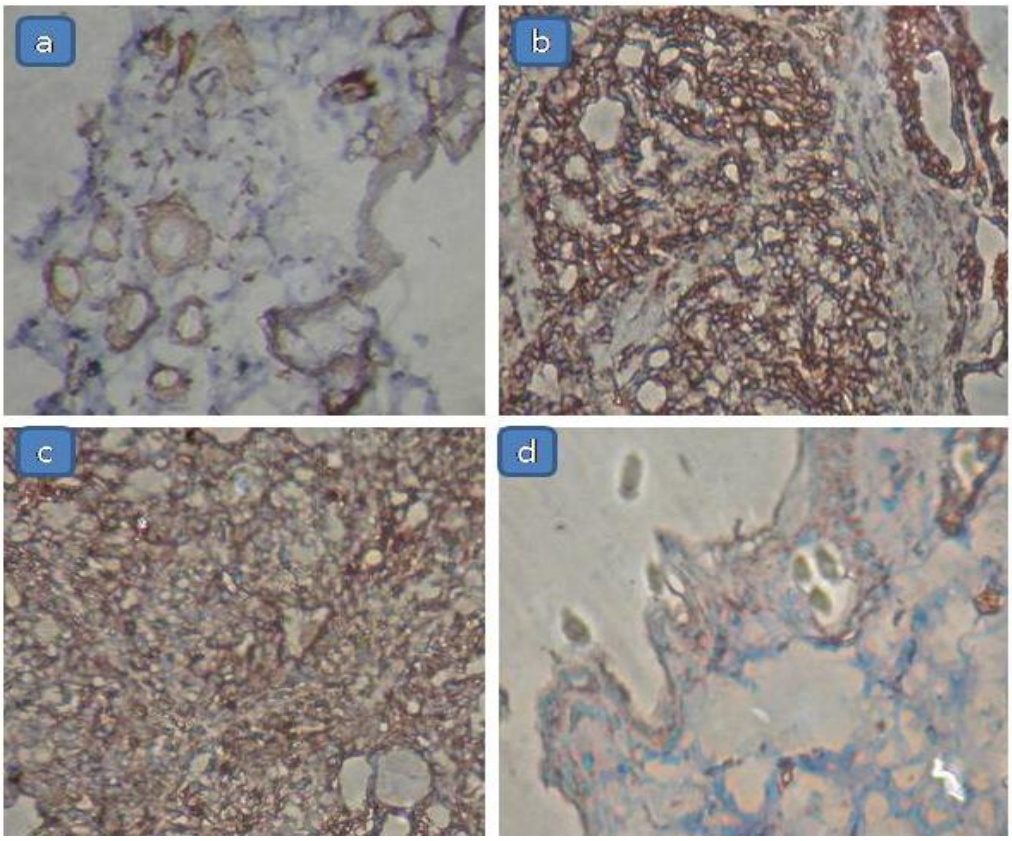


**Figure 3: depicts immunoexpression of HER2/neu in (a) control rats (mild expression), (b) DMBA alone treated rats (over-expression), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (mild expression)**

**Table 1: Shows the grading of positively stained cells of ER, PR and HER2/neu in control and experimental rats in each group**

| Groups/ Markers | ER | | | PR | | | HER2/neu | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1+ | 2+ | 3+ | 1+ | 2+ | 3+ | 1+ | 2+ | 3+ |
| Control (Vehicle) | 10 | 0 | 0 | 10 | 0 | 0 | 10 | 0 | 0 |
| DMBA | 0 | 3 | 7 | 1 | 4 | 5 | 0 | 9 | 1 |
| DMBA+ Genistein+ Daidzein | 6 | 3 | 1 | 7 | 3 | 0 | 7 | 3 | 0 |
| Genistein+ Daidzein alone | 10 | 0 | 0 | 10 | 0 | 0 | 10 | 0 | 0 |

Values are given as number of rats (n = 10).

The percentage positive cells were graded as: **3+** = >50% of the cells are stained; **2+** = 10–50% of the cells are stained; **1+** = <10% of the cells are stained; **0** = Absence of staining: Grade 2+ and 3+ were considered as positive for ER and PR. and 1+ staining were considered as negative. For HER2/neu, score 3+ (high intensity) was considered as positive.

ER was expressed in 70–95% of invasive lobular carcinomas, and 70–80% of invasive ductal carcinomas, whereas PR was expressed in 60–70% of invasive breast carcinomas (Sastre-Garau, et al., 1996; Zafrani, et al., 2000). In the present study, DMBA-induced mammary tumor cells express significantly higher levels of ER and PR. Oral administration of genistein and daidzein in combination, to rats treated with DMBA, significantly down regulated the ER and PR expression.

Phytoestrogens may exert estrogenic, anti-estrogenic, or partial agonistic activity depending upon the cell and tissues type, concentration and other factors, such as age, endogenous E2 levels etc (Hwang, et al., 2006). Anti-estrogenic effects of isoflavone might reduce cell proliferation in their target organs (Xu, et al., 2000). It has also been shown that serum concentrations of E2 are 40% lower in Asian women as compared to their Caucasian counterparts (Peeters, et al., 2003). Soy isoflavones alter endogenous sex hormones levels and stimulate the production of sex hormone binding globulin (SHBG) in liver cells and thereby reduced the levels of circulating E2 (Pino, 2000). Genistein and daidzein possess estrogenic action because of similarity to E2 in their molecular structure and binding affinities to estrogen receptors (Setchell, 2001). Genistein and daidzein may thus inhibit mammary carcinogenesis by blocking the binding of potent estrogen with ER. Kuiper et al. (1998) reported that the binding affinity of genistein for ER-α was 4%, and for ER-β was 87%, as compared to estradiol.

HER2/neu over expression increases the cell migration rate, upregulates the activities of the matrix metalloproteinases MMP-2 and MMP-9, and increases the invasiveness of the cells (Hung & Lau, 1999; Tan, et al., 1997). The HER2/neu oncoprotein promotes cell multiplication, thereby triggering a cascade of protein kinases (Rajkumar, et al., 1994). HER2 receptor becomes an important therapeutic target for cancer therapy since up regulated HER2 level could cause tumorigenesis (Niehans, et al., 1993). Inhibition of HER2 dimerization prevents the activation of several intracellular signaling cascades including mitogen activated protein kinases (MAPK) pathways which can cause carcinogenesis (Agus, et al., 2005).

The expression of steroid hormone receptors (ER/PR) is inversely correlated with HER2/neu in the advanced stages of breast cancer (Gago, et al., 2006). The rat neu gene (rodent HER2 gene) was first recognized as a potent oncogenic mutant in neuroglioblastomas in rats (Schechter, et al., 1984). Although mutation of the neu gene is required for tumorigenesis in rodents, human HER-2 appears to hold tumorigenic potential through over expression of the wild-type HER-2 gene. Over expression of its protein product has been demonstrated in several malignant neoplasms including breast cancer (Slamon, et al., 1989: Ross & Fletcher, 1999). The protective effect of soy compounds in different tumors is associated with modulation of neu gene or cell cycle/apoptosis (Dalu, 1998). In the present study, DMBA-induced mammary tumor cells express significantly increased level of HER2/neu. Oral administration of genistein and daidzein in combination, to rats treated with DMBA, significantly down regulated the HER2/neu expression. Genistein is a known inhibitor of protein tyrosine kinase (PTK), which may attenuate the growth of cancer cells by inhibiting PTK-mediated signaling mechanisms (Akiyama, et al., 1987). Marked inhibition of tyrosine kinases was observed in high concentration of daidzein (370 µM) in murine mammary carcinoma cells (Scholar & Toews, 1994). Sakla, et al. (2007) recently reported that genistein inhibits the proto-oncogene HER-2 protein tyrosine phosphorylation in breast cancer cells as well as delayed tumor onset in transgenic mice that overexpress the HER2 gene. These data support its potent anti-cancer role in breast cancer.

## CONCLUSION

The present study thus concludes that genistein and daidzein inhibited mammary epithelial cell proliferation probably by preventing the over expression of ER, PR and HER2/neu in the mammary tissues of Sprague-Dawley rats, during DMBA induced mammary carcinogenesis.

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