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Research paper

Chemotherapeutic effect of tangeretin, a polymethoxylated flavone studied in 7, 12-dimethylbenz(a)anthracene induced mammary carcinoma in experimental rats

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A B S T R A C T

Globally, breast cancer is the second most prevalent cancer among women and its incidence is amplifying alarmingly. Since genetics is believed to account for only 10% of the reported cases, the environmental factors including diet are thought to play a significant role in predisposing breast cancer. Many bioactive compounds of plant origin have been reported for their anticancer potential. Tangeretin, a pentamethoxy flavone, is a naturally occurring phytoconstituent found to be present in significant amounts in the peel of citrus fruits. Tangeretin possess a wide array of pharmacological activities such as cytostatic, anti-proliferative and antioxidant properties. In the absence of systemic studies in the literature, the present study was aimed to evaluate the chemotherapeutic potential of tangeretin in 7, 12-dimethyl benzo(a)anthracene (DMBA) induced mammary carcinoma in rats. Oral treatment of tangeretin (50 mg/kg BW) to breast tumor bearing rats daily for four weeks was found to be effective against DMBA induced mammary gland carcinogenesis in female Wistar rats. The increase of AST, ALT, ALP, ACP, 5'-ND, γ-GT and LDH in serum of control and experimental breast cancer rats were significantly (p < 0.05) decreased to near normal levels. Further, the levels of lipid peroxide (TBARS), enzymatic antioxidants such as SOD, CAT, GPx and non-enzymatic antioxidants such as GSH, Vitamin C, Vitamin E and Phase I (cytochrome P450, cytochrome b5, EROD, MROD and PROD) and Phase II detoxification (glutathione S-transferase (GST), quinone reductase (QR)) were decreased significantly by administration of tangeretin. Immunohistochemical and western blotting studies for estrogen receptor, progesterone receptor and HER2/neu status exemplified the chemotherapeutic effect of tangeretin. Further, the histological and ultrastructural analysis of breast tissues evidenced the anti-tumorigenic nature of tangeretin. Thus, the results of the present study clearly indicate that tangeretin significantly suppresses DMBA induced breast cancer in rats.

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1. Introduction

Cancer is one of the major devastating diseases worldwide which is characterized by unregulated cell growth, decreased apoptosis and metastasis. Breast cancer is one of the most common types of cancer in women and accounts for the highest morbidity and mortality worldwide [1]. About 9,00,000 new cases of breast cancer have been diagnosed worldwide annually of which 4,00,000 women die of this disease [2]. In India, breast cancer is the second most common cancer next to cervical cancer with an estimated 1,15,251 new cases every year [3]. Despite the advances in diagnosis and treatment, the observed 5 year survival rate of breast cancer patients in India is less than 50% [4].

Breast cancer development and metastasis is a multistep process, a result of the dysfunction of several regulatory features that keep the cells in check [5]. Most of the chemotherapeutic drugs currently used for treating breast cancer cause deleterious side effects such as severe nausea, vomiting, fatigue and hair loss. Hence there is a need to develop plant based chemotherapeutic drugs with minimal side effects.

In addition to the general risk factors in the development of breast cancer, free radicals producing environmental contaminants such as polycyclic aromatic hydrocarbons (PAH) that are released from industries, domestic oil furnaces, gasoline and diesel engines are also known to cause breast cancer. PAH induces damage to cellular biomolecules through the formation of reactive oxygen species (ROS). Increased ROS generation leads to the formation of
DNA adduct which in turn leads to malignant transformation. The major source of ROS released is from the electron transport chain of the mitochondria [6].

The rat mammary carcinogenesis model is the best known animal system for studying the effects of chemotherapeutic agents. 7, 12-Dimethylbenz(a)anthracene (DMBA), a type of PAH initiates the production of reactive metabolic intermediates (peroxides and superoxide anion radicals) that induce oxidative stress leading to carcinogenesis in rats. This model mimics human breast cancer morphologically and histologically [7,8]. Oxidation of DMBA impacts carcinogenic and mutagenic activities via the transcriptional activation of cytochrome P450 that binds to the xenobiotic response element of the promoter region of cytochrome P450 1A1 and 1B1. The ultimate carcinogen, diol epoxides and other toxic metabolites of DMBA bind to the adenine residues of DNA resulting in mutagenesis and carcinogenesis [9]. The mammary tumors thus induced are hormone-dependent adenocarcinomas arising from terminal end buds (TEBS) on incompletely differentiated glands. Since, these tumors are similar to human breast cancer histologically and in their hormone response patterns, we have employed DMBA induced mammary carcinoma model to study the effect of tangeretin [10,11].

Many bioactive compounds of plant origin have the potential to subside the biochemical imbalances induced by the formation of free radicals. Hence, naturally occurring antioxidants such as flavonoids have been viewed as promising chemotherapeutic drugs for the management of cancer without causing damage to normal cells [12]. A series of flavonoids have been shown to possess anti-cancer property [13].

Tangeretin (5, 6, 7, 8, 4’-pentamethoxy flavone) is a polymethoxylated flavone found in significant amounts in the peel of citrus fruits (Fig. 1) [14]. Recent studies have shown that polymethoxylated flavones possess greater anti-tumor, anti-metastatic and anti-inflammatory properties compared to hydroxylated flavonoids [15]. Tangeretin acts as a natural resistance factor against pathogenic fungi in citrus fruits [16]. Tangeretin was found to possess several biological activities such as inhibition of cancer cell proliferation in lung [17], breast [18], as well as colorectal carcinoma cells [19], induce apoptosis in leukemia cells [20], enhance gap junctional intercellular communication to counteract tumor promoter induced inhibition of intercellular communication [21,22] and inhibit proliferation and migration of aortic smooth muscle cells [23]. It is found to be anti-allergic [24], hypolipidemic [25] and inhibit extracellular signal-regulated kinase (ERK) phosphorylation [26]. Tangeretin has also been shown to be a potent inhibitor of xenobioc-t induced genotoxicity in vitro [27] and hence decreases the levels of Phase I enzymes cytochrome P450 1A1 and 1A2 with a time course of induction of 4–8 days. Systemic effects of tangeretin for various diseases have been previously reported. Tangeretin prevents LPS induced bone loss in mice [28] and offers neuroprotection in rat model of Parkinson’s disease [29]. However, in the absence of systemic reports of tangeretin in rat mammary carcinoma, the present study was aimed to validate the anticancer effect of tangeretin in 7,12-DMBA induced mammary carcinoma in rats.

2. Materials and methods

2.1. Chemicals

7, 12-DMBA was purchased from Sigma chemical company (St. Louis MO, USA) and tangeretin from Indofine chemical company, USA. All other chemicals used were of analytical grade procured from local commercial sources.

2.2. Experimental animals

Virgin female Wistar rats, 7 weeks of age were purchased from Tamilnadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai and were used in the experiment. They were housed spacious in individual cages and maintained under standard experimental conditions: temperature 25 ± 1 °C, relative humidity 60 ± 5% and 12 ± 1 h (light/dark cycle) and fed with a balanced diet of commercially available pellet diet (Amruth laboratory Animal Feed, Bangalore, India; containing protein 22.06%, oil 4.28%, fiber 3.02%, ash 7.8%, sand (silica) 1.37% w/w) and water ad libitum. The animals were acclimatized for 2 days prior to the start of experiments. The experimental design was performed in accordance with the current ethical norms approved by the Ministry of Social Justice and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC No: 01/059/09).

2.3. Experimental design

Graded doses (25, 50, 100 and 200 mg/kg BW) of tangeretin dissolved in 1 ml of PBS containing 0.1% DMSO were administered to tumor bearing rats for 4 weeks to fix the optimum dosage of tangeretin. It was observed that oral tangeretin treatment at doses of 50, 100 and 200 mg/kg BW significantly altered the levels of tumor markers as well as activities of liver marker enzymes in serum to near normal levels in DMBA-induced rats (data not shown). However, there was no significant difference in the levels of tumor markers and liver marker enzymes activity between the groups of rats treated with 50, 100 and 200 mg of tangeretin. Hence, a minimum dose of 50 mg/kg was fixed as the optimum dose for the study.

The rats were divided into four groups of six rats each as follows:

- **Group 1:** normal control rats administered with 1 ml of PBS containing 0.1% DMSO.
- **Group 2:** control rats administered with tangeretin (50 mg/kg BW dissolved in 1 ml of PBS containing 0.1% DMSO/rat) for 4 weeks by oral gavage.
- **Group 3:** DMBA induced breast cancerous rats.
- **Group 4:** DMBA induced breast cancerous rats administered with tangeretin (50 mg/kg BW dissolved in 1 ml of PBS containing 0.1% DMSO/rat) for 4 weeks by oral gavage.

2.4. Carcinogen treatment

Mammary tumors were induced by DMBA using the “airpouch technique” as described by Arun et al. 1984 [30] with slight modifications. Briefly, the air pouch was produced using a 10 ml capacity glass syringe in the region of the second and third mammary glands. About 2—3 ml of air was drawn into the syringe, and a rubber cork was fixed to the needle tip in such a way that the plugging was airtight. The whole set was wrapped with aluminum foil and autoclaved at 15 psi for 20 min. The sterile air (1—2 ml) was carefully injected subcutaneously just beneath the mammary fat pad so as to produce a pouch containing sterile air. The air inside

![Fig. 1. Structure of tangeretin.](image-url)
was allowed to remain for a day to form a pouch. A single dose of 7, 12-DMBA (25 mg/kg BW/rat) in 0.5 ml of corn oil was vortexed to obtain a uniform emulsion and carefully injected into the air pouch.

2.5. Drug treatment

All rats were palpated every week to monitor the onset of tumorigenesis. Tumor yield and size were stabilized 90 days after the initiation with DMBA. Tangeretin (50 mg/kg BW dissolved in 1 ml of PBS containing 0.1% DMSO/rat) was given for 4 weeks by oral gavage (according to the optimum dosage fixation study).

After the experimental period, all animals were fasted overnight and sacrificed by sodium pentothal anesthesia followed by cervical decapitation. Blood was collected with and without anticogulant (EDTA) and the serum was centrifuged at 5000 rpm for 15 min to obtain a clear supernatant and stored at −80 °C until its use for further biochemical analysis. Mammary tissues from control and experimental groups of rats were immediately excised, washed in ice-cold PBS to remove the blood stains, blotted, weighed and homogenized in Tris-ice-cold PBS to remove the blood stains, blotted, weighed and homogenized in Tris–HCl buffer (0.1 M, pH 7.4) using a Teflon homogenizer to prepare 10% (w/v) tissue homogenate. This homogenate was centrifuged at 12,000 g for 30 min at 4 °C to obtain a clear supernatant. This supernatant was pooled and used for further analysis.

2.6. Body and tumor weight

The total body weight gain of the control and experimental animals was recorded periodically throughout the experimental period. The tumor volume was estimated according to the method of Schneider (1957) [32] and the DNA and RNA were quantified by the method of Burton (1956) [33] and Rawal et al. (1977) [34] respectively.

2.7. Biochemical analysis

Nucleic acids from breast tissues were extracted by the method of Schneider (1957) [32] and the DNA and RNA were quantified by the method of Burton (1956) [33] and Rawal et al. (1977) [34] respectively.

2.7.1. Estimation of liver marker enzymes

The activity of cytosolic marker enzymes such as AST and ALT were assayed by the method of Bergmeyer et al. (1978) [35]. Alkaline phosphatase and Acid phosphatase activities were estimated by the method of King (1965a) [36] as described by Bala- subramanian et al. (1983) [37] using disodium phenyl phosphate as substrate. The 5'-nucleotidase was assayed by the method of Luly et al. (1972) [38] using 5'-adenosine monophosphate as substrate and the activity of γ-glutamyl transpeptidase was assayed by the method of Rosalki and Rau, (1972) [39] using L-γ-glutamyl-p-nitroanilide as substrate. Lactate dehydrogenase (LDH) was assayed by the method of King (1965b) [40] using lithium lactate as substrate.

2.7.2. Estimation of Phase I and Phase II enzymes

The breast tissues of control as well as experimental rats were removed aseptically, washed with ice-cold sterile 1.15% KCl and homogenized with a Potter-Elvehjem homogenizer using a Teflon pestle. The homogenates were centrifuged at 4000 × g for 20 min and the resulting supernatant was further centrifuged at 105,000 × g for 1 h at 4 °C to obtain the microsomalS9 fraction which was used for further analysis. Phase I detoxification en- zymes such as cytochrome P450 and cytochrome b5 were assessed by the method of Omura and Sato (1964) [41]. The ethoxyresorufin O-decarboxylase (EROD), methoxyresorufin O-decarboxylase (MROD), pentoxyresorufin O-decarboxylase (PROD) activities were determined by measuring the formation of resorufin spectrofluorimetrically at excitation and emission wavelength of 536 and 585 nm respectively [42]. Phase II detoxification enzymes such as glutathione S-transferase (GST) [43] and quinone reductase (QR) [44] were also measured.

2.7.3. Oxidative stress markers in plasma and breast tissues

TBARS in plasma and breast tissue was estimated by measuring the concentration of malondialdehyde (MDA) according to the method of Ohkawa et al. 1979 [45] and calculated according to the molar absorption coefficient of MDA, e = 1.56 × 105 M−1 cm−1 at 535 nm. Conjugated dienes from breast tissues were measured by the method of Rao and Recknagel (1968) [46]. Briefly, lipids from the homogenate were extracted in 20 ml of chloroform: methanol (2:1). Lipids in the chloroform phase were recovered by evaporating the chloroform under oxygen-free N2. The final lipid extract was dissolved in cyclohexane (1 mg/ml) and then scanned from 220 to 300 nm against a cyclohexane blank in a spectrophotometer. Total lipid content was measured and detected for diene conjugates.

2.7.4. Assay for breast and serum enzymatic and non-enzymatic antioxidants

The enzymatic antioxidant, superoxide dismutase (SOD) activity was measured spectrophotometrically as the degree of inhibition of autoxidation of pyrogallol in an alkaline pH at an absorbance of 420 nm [47]. In the catalase (CAT) activity assay, dichromate in acetic acid was reduced to chronic acetate when heated in the presence of hydrogen peroxide (H2O2) with the formation of perchloric acid as unstable intermediate and chronic acetate thus formed was measured spectrophotometrically at 570 nm. The results were expressed in terms of μmol H2O2 liberated/min/mg protein [48]. For the glutathione peroxidase (GPx) activity assay, the reaction mixture containing 0.2 ml of EDTA (0.8 mM pH 7.0), 0.4 ml of phosphate buffer (10 mM) and 0.2 ml of enzyme source were incubated with 0.1 M of H2O2 and 0.2 ml of glutathione for 10 min. Oxidation of gluta- thione by the enzyme was measured spectrophotometrically at 420 nm. The activity of GPx, was expressed as μmol glutathione oxidized/mg protein [49].

The non-enzymatic antioxidants Vitamin C, Vitamin E and reduced glutathione (GSH) were also estimated. In the reduced glutathione (GSH) assay, 1 ml of the sample was precipitated with 1 ml of TCA and centrifuged at 1200 × g for 20 min. To 0.5 ml of supernatant, 2 ml of 5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB) was added and the color developed was read immediately at 412 nm using a spectrophotometer [50]. Vitamin E (α-tocopherol) assay estimated the levels of ferric ions which were reduced to ferrous ions in the presence of tocopherol and bathophenanthro- line to form a pink colored substance, which was read at 520 nm using a spectrophotometer [51]. In the Vitamin C (ascorbic acid) assay, 1 ml of ethanol was added to 1 ml of sample and then mixed thoroughly after which 3 ml of petroleum ether was added and the reaction mixture was centrifuged. The supernatant was evaporated to dryness and 0.2 ml each of bathophenanthroline, ferric chloride and β-phosphoric acid were added to reach a total volume of 3 ml with ethanol. The color developed was measured at 530 nm [52].
2.7.5. Assay for serum tumor markers
Tumor markers AFP, CEA and CA 15-3 (specific for breast cancer) were quantified based on solid phase enzyme linked immunosorbent assay method using UBI MAGIWELL (USA) enzyme immunoassay kit [53].

2.8. Histopathology

The breast tissue was immediately fixed in 10% neutral buffered formalin, embedded in paraffin, 5 μm section was cut using a microtome and then rehydrated with xylene and graded series of ethanol. The specimens were then stained with Hematoxylin and Eosin. The H & E stained breast specimens were examined by a pathologist to histopathologically classify the tumors as described by Russo et al. (1990) [8].

2.9. Transmission electron microscopy

The tissues were transferred into a primary fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4) to better preserve the ultrastructures before further cutting. They were cut into 1 mm thick slices in planes perpendicular to the plain of the first cut, finally, into ~1 mm blocks, transferred into a fresh portion of the fixative in which they were cut and incubated for 4–8 h at 4 °C. The fixed tissue blocks were washed with 0.1 M sodium cacodylate–HCl buffer pH 7.4 (3 × 15 min) and post fixed in 1% osmium tetroxide (OsO4) in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 h at 4 °C. The excess fixative was washed with the same buffer thrice for 10 min each time as before. The specimen blocks were then treated with graded series of acetone 30%, 50%, 70%, 80%, 90% with 10 min for each concentration and with 100% acetone twice for 10 min each time followed by propylene oxide treatment twice with 10 min each. The blocks were then moulded, trimmed and sectioned using ultramicrotome fitted with a diamond knife. The ultrathin sections were collected on copper grids and stained with uranyl acetate followed by lead citrate. The sections were screened in JEOL JEM 1400 Transmission Electron Microscope at 80 kV. The micrographs were taken using Olympus Keenview CCD camera attached to the microscope.

2.10. Immunohistochemical expression

Immunohistochemical analysis was performed on 5 μm paraffin embedded breast tissue section on poly-l-lysine coated glass slides. The tissue slides were deparaffinized by placing the slides in an oven at 60 °C for 10 min and then rinsed twice in xylene for 5 min each. The slides were then hydrated in graded ethanol series for 10 min each and then finally washed in double distilled water for 5 min. The sections were incubated with 1% H2O2 in double distilled water for 15 min at 22 °C, to quench the endogenous peroxidase activity. Then the sections were rinsed with Tris–HCl containing 150 mM NaCl (pH 7.4) and blocked with blocking buffer (1 × TBS, 0.05% Tween 20, 5% NFDM) for 1 h at 22 °C. After washing with 1 × TBS containing 0.05% Tween 20, the sections were incubated with primary antibodies anti-estrogen receptor α, anti-progesterone receptor and anti-HER2/neu overnight at 4 °C, followed by incubation with the respective secondary antibodies IgG-HRP conjugates for 1 h at 4 °C. After washing with 1 × TBS containing 0.05% Tween 20, the immunoreactivity was developed with 0.01% DAB and H2O2 for 1–3 min and the sections were observed (20 × and 100 ×) for brown color formation under bright field in a microscope.

2.11. Western blotting analysis of ER, PR and Her2/neu

The tissues were removed from animals, resuspended in Tris/sucrose buffer with protease inhibitors and homogenized immediately. The protein was quantified by Bradford method and 20 μg of protein was separated by electrophoresis on 10% SDS-PAGE gels. It was then transferred to nitrocellulose membrane and blocked with 5% BSA in 0.1% TBST (Tris-buffered saline with 0.1% Tween-20) for 3 h. The membranes were incubated overnight with primary antibodies: anti-ER, anti-PR, anti-Her2/neu and anti-β-actin (ER, PR and β-actin antibodies were procured from Santa Cruz, CA and Her2/neu was procured from Abcam). The membranes were washed with 0.1% TBST for 5 min each and probed with secondary antibody (antimouse horse radish peroxidase conjugated secondary antibody) for 1 h at room temperature. The bands in the membranes were detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences, UK).

2.12. Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago) statistical package. The results were expressed as mean ± Standard Error Mean (SEM). One-way analysis of variance (ANOVA) followed by post hoc test least significant difference (LSD) was used to correlate the difference between the variables. Values were considered statistically significant if p < 0.05.

3. Results

3.1. Effect of tangeretin on body and tumor weight

Table 1 shows the change in body weight of the control and experimental group of animals. There was a significant decrease in the body weight of tumor induced rats (p < 0.05) compared to control rats. On the contrary, treatment with tangeretin prevented the decrease in the body weight of tumor bearing animals (p < 0.05). However, there was no significant difference in body weight in the animals administered with tangeretin alone when compared to control animals.

There was a significant increase in breast tumor weight in tumor induced rats. Whereas the tumor weight was found to be significantly reduced in tumor bearing animals treated with tangeretin (p < 0.05). However, there was no change observed in breast of both tangeretin alone treated animals and control animals.

3.2. Effect of tangeretin on DNA and RNA levels

The total DNA and RNA content increases with the degree of malignancy indicating greater proliferation in tumor condition. Tumor induced rats showed a significant increase in nucleic acid content (p < 0.05) in the breast tissue compared to control animals (Table 2). However, on the administration of tangeretin to tumor

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Tumor weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>220.86 ± 4.22</td>
<td>-</td>
</tr>
<tr>
<td>Control + tangeretin</td>
<td>214.84 ± 7.35</td>
<td>7.13 ± 0.31*</td>
</tr>
<tr>
<td>DMBA</td>
<td>155.83 ± 4.74*</td>
<td>3.84 ± 0.19#</td>
</tr>
<tr>
<td>DMBA + tangeretin</td>
<td>177.61 ± 3.07*</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follow: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.
bearing animals, the levels of nucleic acids in breast tissue was found to be significantly decreased (p < 0.05). The nucleic acid levels remained the same in control and tangeretin alone treated animals.

3.3. Effect of tangeretin on liver marker enzymes in serum

Marker enzymes play a significant role as indicators of malignant conditions. The levels of liver marker enzymes in the serum, such as transaminases, alkaline phosphatases, 5'-nucleotidase, transpeptidase and LDH in the control and experimental group of animals are shown in Table 3. A significant increase in the levels of serum marker enzymes was observed in tumor induced rats (p < 0.05) compared to control animals. In contrast, these levels decreased to near normal in tumor bearing animals treated with tangeretin (p < 0.05). However, there was no significant change in marker enzyme activities observed in tangeretin alone treated animals compared to control animals.

3.4. Effect of tangeretin on biotransformation enzyme activities in breast microsomal fraction of control and experimental animals

The levels of Phase I and Phase II biotransformation enzymes in the breast tissue of control and experimental group of animals is shown in Table 4. The levels of Phase I enzymes such as cytochrome P450, cytochrome b5, EROD, MROD and PROD were found to be increased significantly in the breast tissue of tumor induced rats (p < 0.05) when compared to control animals. However, the Phase II enzymes such as glutathione S-transferase (GST) and quinone reductase (QR) decreased significantly in the breast tissue of tumor induced rats (p < 0.05). On the contrary, the administration of tangeretin to tumor bearing animals significantly increased the levels of Phase II enzymes and increased the levels of Phase I enzymes when compared to control animals. This proves the chemotherapeutic effect of tangeretin.

3.5. Effect of tangeretin on the levels of TBARS and conjugated dienes in control and experimental animals

The activities of TBARS in plasma and mammary tissue and the activity of Conjugated Dienes (CD) in mammary tissue are represented in Figs. 2 and 3 respectively. The lipid peroxidation marker enzymes TBARS and CD levels were significantly increased in tumor induced rats compared to control animals (p < 0.05). The administration of tangeretin to tumor bearing animals significantly decreased the TBARS and CD levels to near normalcy. However the administration of tangeretin alone to animals did not cause any significant change in TBARS and CD levels compared to control animals.

3.6. Effect of tangeretin on the levels of enzymatic antioxidants in serum and mammary tissue of control and experimental animals

The effect of tangeretin treatment on the activities of enzymatic antioxidants in serum and mammary tissue is represented in Figs. 4 and 5 respectively. The levels of SOD, CAT and GPx were significantly lowered in tumor induced rats compared to control animals (p < 0.05). The administration of tangeretin to tumor bearing animals increased the enzymatic antioxidant levels to near normalcy. However the administration of tangeretin alone to animals did not cause any significant change in the antioxidant levels compared to control animals.

3.7. Effect of tangeretin on the levels of non-enzymatic antioxidants in serum and mammary tissue of control and experimental animals

The effect of tangeretin treatment on the activities of non-enzymatic antioxidants in serum and mammary tissue is represented in Table 5. The levels of Vitamin C, Vitamin E and GSH were significantly decreased in tumor induced rats compared to control animals (p < 0.05). The administration of tangeretin to tumor bearing animals significantly increased the non-enzymatic antioxidant levels to near normalcy (p < 0.05). However the administration of tangeretin to control animals did not cause any significant change in the antioxidant levels compared to control animals.

3.8. Effect of tangeretin on the levels of serum tumor markers of control and experimental animals

Fig. 6 depicts the effect of tangeretin on the levels of serum tumor markers alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and breast cancer specific marker (CA 15-3) in control and experimental group of animals. Tumor induced rats showed a significant increase in the levels of these markers when compared to control animals (p < 0.05). However, oral tangeretin treatment to tumor bearing animals reduced these levels significantly (p < 0.05). This proves the chemotherapeutic effect of tangeretin.

### Table 2

Effect of tangeretin on the levels of nucleic acids in mammary tissue of control and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>DNA (mg/g wet tissue)</th>
<th>RNA (mg/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.95 ± 0.14</td>
<td>2.14 ± 0.11</td>
</tr>
<tr>
<td>Control + tangeretin</td>
<td>2.91 ± 0.22</td>
<td>2.32 ± 0.12</td>
</tr>
<tr>
<td>DMBA</td>
<td>4.94 ± 0.10*</td>
<td>3.54 ± 0.18*</td>
</tr>
<tr>
<td>DMBA + tangeretin</td>
<td>3.84 ± 0.13*</td>
<td>2.79 ± 0.15*</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.

### Table 3

Effect of tangeretin on the levels of nucleic acids in breast tissue of control and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>DNA (mg/g wet tissue)</th>
<th>RNA (mg/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38.00 ± 2.45</td>
<td>22.27 ± 2.05</td>
</tr>
<tr>
<td>Control + tangeretin</td>
<td>35.12 ± 2.27</td>
<td>23.41 ± 1.23</td>
</tr>
<tr>
<td>DMBA</td>
<td>65.35 ± 3.28*</td>
<td>39.99 ± 2.09*</td>
</tr>
<tr>
<td>DMBA + tangeretin</td>
<td>43.17 ± 3.61*</td>
<td>27.36 ± 1.62*</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.
epithelial architecture. Tumor bearing animals induced with DMBA showed altered ductal epithelial lining indicating invasive ductal carcinoma. Tumor bearing animals treated with tangeretin showed improved ductal architecture proving the chemotherapeutic effect of tangeretin.

3.10. Ultrastructural changes in breast tissue of control and experimental groups of animals

Fig. 8 shows the ultrastructural changes in the mammary tissue of control and experimental group of rats. Control and tangeretin alone treated rats showed normal ultrastructure of cells with intact nucleus and normal mitochondria. DMBA induced rats showed cells with dislodged nuclear membrane, fragmented nucleolar contents and swollen mitochondria that support the tumorigenic events. Tumor bearing animals treated with tangeretin showed chromatin condensation, disintegration of nuclear membrane, swollen endoplasmic reticulum and reduced number of swollen mitochondria which signifies the initiation of apoptotic events suggesting the chemotherapeutic effect of tangeretin.

3.11. Immunohistochemical expression of ER, PR and HER2/neu in breast tissue of control and experimental groups of animals

Figs. 9–11 represents the immunohistochemical analysis of hormones and receptor status of estrogen receptor (ER), progesterone receptor (PR) and Her2/neu respectively in control and experimental animals. Control and tangeretin alone treated rats showed normal expression of ER, PR and Her2/neu, whereas DMBA

### Table 4

Effect of tangeretin on the levels of Phase I and Phase II enzymes in mammary tissue of control and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cyt P450</th>
<th>Cyt b5</th>
<th>EROD</th>
<th>MROD</th>
<th>PROD</th>
<th>GST</th>
<th>QR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.35 ± 0.028</td>
<td>0.61 ± 0.037</td>
<td>54.21 ± 2.24</td>
<td>41.99 ± 2.81</td>
<td>29.10 ± 1.91</td>
<td>3.56 ± 0.22</td>
<td>0.24 ± 0.018</td>
</tr>
<tr>
<td>Control + tangeretin</td>
<td>0.42 ± 0.036</td>
<td>0.57 ± 0.045</td>
<td>51.42 ± 2.60</td>
<td>43.74 ± 2.61</td>
<td>28.30 ± 1.73</td>
<td>3.79 ± 0.26</td>
<td>0.22 ± 0.029</td>
</tr>
<tr>
<td>DMBA</td>
<td>1.55 ± 0.064*</td>
<td>1.68 ± 0.082*</td>
<td>83.49 ± 3.27*</td>
<td>65.34 ± 3.24*</td>
<td>55.60 ± 2.90*</td>
<td>3.49 ± 0.31*</td>
<td>0.18 ± 0.021*</td>
</tr>
<tr>
<td>DMBA + tangeretin</td>
<td>1.02 ± 0.056*</td>
<td>1.16 ± 0.070*</td>
<td>61.44 ± 3.72*</td>
<td>50.32 ± 2.43*</td>
<td>43.04 ± 1.98*</td>
<td>6.34 ± 0.30*</td>
<td>0.37 ± 0.033*</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.

Cyt P450 and cyt b5 expressed in nmol/mg protein, EROD, MROD and PROD expressed in μmol of resorufin formed/min/mg protein, GST expressed in μmol of CDNB conjugated with GSH/min/mg protein, QR expressed in μmol of DCPIP reduced per h/mg protein.

**Fig. 2.** Effect of tangeretin on the levels of TBARS in control and experimental animals. Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.

**Fig. 3.** Effect of tangeretin on the levels of CD in mammary tissues of control and experimental animals. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.

**Fig. 4.** Effect of tangeretin on the levels of enzymatic antioxidants in serum of control and experimental animals. Units: SOD is expressed as the amount of enzyme required to give 50% inhibition of NBT reduction, Catalase is expressed in μmol of H2O2 utilized per minute and GPx is expressed in μmol of GSH utilized per min. Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.


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induced rats showed higher expression of the hormones and their receptors. However, tangeretin treatment to tumor bearing animals significantly decreased the expression of the hormones and receptors showing chemotherapeutic effect of tangeretin. The representative graph shows the percentage of cells stained positively for estrogen receptor (a), progesterone receptor (b) and Her2/neu receptor (c) status in control and experimental group of rats. Positive staining was assessed and measured objectively by two independent observers and gave similar results. For quantitation, data expressing the respective stain was quantified by counting the positively stained cells in ten fields/section by three independent observers in blinded fashion, and the average was used to denote the total no. of positively stained cells, that was presented as “fold changes” as compared with control and experimental group of rats. Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: ∗control rats; ∗∗DMBA induced rats. Values are statistically significant at p < 0.05.

3.12. Western blotting of ER, PR and HER2/neu in breast tissue of control and experimental groups of animals

Fig. 13 shows the levels of expression of ER, PR and HER2/neu in mammary tissues of control and experimental group of rats. There was a significant increase (p < 0.05) in the levels of ER, PR and HER2/neu in DMBA induced animals. However, the levels of these receptors were reduced significantly (p < 0.05) in induced rats treated with tangeretin. Control and tangeretin alone treated animals showed very low expression of these receptors.

4. Discussion

Among the multiple experimental animal models employed for analyzing the various aspects of mammary carcinogenesis, the induction of mammary tumors in rats by chemical carcinogens is one of the models most utilized.

DMBA is a polycyclic aromatic hydrocarbon, widely used as a carcinogen to induce mammary tumors in rodents [54] and it is employed to assess the efficacy of chemotherapeutic agents in vivo for the treatment of this disease [55]. Like many other PAHs, DMBA is metabolized to highly reactive PAH diol-epoxides by cytochrome P450 monooxygenases and epoxide hydrolase followed by further epoxidation by CYP isoforms CYP1A1 and CYP1B1 to form the ultimate carcinogen DMBA-3,4-dihydro-diol-1,2-epoxide. This compound binds covalently to DNA to form PAH–DNA adducts, which play a crucial role in PAH-induced carcinogenesis [56, 57]. Further, the oxidation of DMBA catalyzed by cytochrome P450 increases the free radical generation and induces single- and double-strand DNA breaks [58, 59]. Thus, the compound that scavenges free radical and detoxifies the carcinogens may serve as potent anticancer agent in PAH-induced carcinogenesis [60].

Various mechanisms contribute to weight loss of the host in cancerous condition [61]. Though the food and water intake of the control and experimental animals remained unaltered, there was significant decrease in body weight of tumor induced rats. This goes in accordance with the findings of Cheng et al., 2003 [62].

Table 5

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vitamin C (µg/mg protein)</th>
<th>Vitamin E (µg/mg protein)</th>
<th>GSH (mg/100 g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Mammary tissue</td>
<td>Serum</td>
</tr>
<tr>
<td>Control</td>
<td>1.39 ± 0.08</td>
<td>2.47 ± 0.13</td>
<td>1.49 ± 0.08</td>
</tr>
<tr>
<td>Control + tangeretin</td>
<td>1.35 ± 0.06</td>
<td>2.16 ± 0.13</td>
<td>1.44 ± 0.06</td>
</tr>
<tr>
<td>DMBA</td>
<td>0.53 ± 0.04*</td>
<td>1.30 ± 0.09*</td>
<td>0.61 ± 0.03*</td>
</tr>
<tr>
<td>DMBA + tangeretin</td>
<td>1.07 ± 0.06*</td>
<td>1.88 ± 0.16*</td>
<td>1.09 ± 0.06*</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: ∗control rats; ∗∗DMBA induced rats. Values are statistically significant at p < 0.05.
Fig. 7. Photomicrographs of histopathological changes in the mammary tissues of control and experimental animals (hematoxylin and eosin staining showed at 20×). Histology on mammary tissues of control (A) and tangeretin control (B) rats showing normal ductal epithelial architecture of the terminal epithelial buds; mammary tissues of DMBA induced rat (C) showing invasive ductal carcinoma with abnormal cellular proliferation and infiltrated ducts; mammary tissues of tumor bearing rats treated with tangeretin (D) showing improved ductal architecture.

Fig. 8. Ultrastructure of mammary cells of control and experimental group of animals. Electron microscopic examination of mammary cells of control (A) and tangeretin treated control (B) rats showing normal nucleus, mitochondria and endoplasmic reticulum; Electron microscopic examination of mammary cells of DMBA induced rat (C) showing membrane damage, dislodged nuclear membrane, swollen mitochondria and high electron density in nucleus that support tumorigenesis; electron microscopic examination of mammary cells of DMBA induced rats treated with tangeretin (D) showing chromatin condensation, disintegration of nuclear membrane, swollen endoplasmic reticulum and reduced number of swollen mitochondria that signifies the initiation of apoptotic events.
Conversely, upon oral tangeretin treatment in tumor bearing animals, these levels were altered to near normalcy.

Nucleic acids play an important role during neoplastic transformation which is an index of proliferative activity in tumor conditions. The increased level of DNA synthesis in tumors of cancer induced rats may be due to the increased expression of enzymes necessary for differentiated cell functions. Generally the synthesis of DNA is least in normal cells which have a very slow rate of cell division. It is demonstrated that PAHs interrupt in uracil nucleotide dependent biosynthesis of nucleic acid and this results in DNA damage leading to uncontrolled DNA replication. The RNA level in breast tissues of cancer induced rats was increased. This may be due to the increased DNA content which might have led to an increased transcription and thereby elevated RNA content in cancerous condition. On the contrary, DNA and RNA levels were normalized in tangeretin treated tumor bearing animals. Thus, the administration of tangeretin controlled the nucleic acid biosynthesis and exhibits anti-proliferative effect during treatment.

Biochemical marker enzymes are used to screen cancer conditions particularly for differential diagnosis, prognosis, monitoring the progress and for assessing the response to therapy. The structural integrity of the cells has been reported to be damaged in toxicity induced animals and this results in cytoplasmic leakage of enzymes into the blood stream. Aspartate transaminase (AST) and alanine transaminase (ALT) are the enzymes associated with the conversion of amino acids to ketoacids. They are pathophysiological marker enzymes used to assess tissue damage. The increase in the activities of ALT, AST and ALP may be primarily due to leakage of these enzymes from liver cytosol into the blood stream as a result of tissue damage. Tissue damage is the vulnerable feature in cancerous environment. Hence, elevation of the liver specific enzymes such as AST and ALT were observed in breast cancer condition and is a possible indicator of progression of tumor growth. The elevated serum ALP levels observed in the study could be due to the toxic effect of DMBA in the liver. Dao et al. (1959) have reported that the increased activity of 5'-nucleotidase seems to have

Fig. 9. Immunohistochemical analysis of estrogen receptor (ER) status in the mammary tissues of control and experimental animals. ER status of mammary tissue of control (A) and tangeretin treated control (B) animals showing normal levels of estrogen receptor expression; ER status of mammary tissue of DMBA induced rat (C at 20\(\times\) and C1 at 100\(\times\)) showing increased expression of estrogen receptor; ER status of mammary tissue of tumor bearing rats treated with tangeretin (D at 20\(\times\) and D1 at 100\(\times\)) showing comparatively decreased expression of estrogen receptor.
originated from the proliferating breast cells and Walia et al. (1995) [64] reported higher activities of 5'-nucleotidase in breast cancer patients. Gamma glutamyl transferase (γ-GT), a key enzyme in glutathione metabolism which provides high intracellular levels of GSH required for conjugation by glutathione S-transferase, is involved in protecting cells against toxins and carcinogens. It has been reported that there is an increased risk of breast cancer in individuals with elevated levels of serum γ-GT [65]. Lactate dehydrogenase (LDH) enzyme is a tetrameric isoenzyme recognized as a marker with potential use in assessing the progression of the proliferating malignant cells. Numerous reports revealed the elevated levels of LDH in various types of cancers. The rise in LDH may also be due to the high glycolysis rate in the cancerous condition, which is the only energy-producing pathway for the uncontrolled proliferating malignant cells [66,67]. The increase in the activities of LDH in tumor induced rats could be attributed to over production of enzymes by proliferated cells and further release of their isoenzymes from destructed cells thus making it a sensitive marker for solid neoplasm. In the present investigation the increased levels of AST, ALT, 5'-nucleotidase, gamma glutamyl transferase and LDH observed in tumor induced rats were normalized upon treatment with tangeretin indicating the non toxic nature of tangeretin.

Human body deals with carcinogens with the help of carcinogen-metabolizing enzymes namely, the Phase I enzymes and Phase II enzymes. These two enzyme groups work together to metabolize any foreign substances, or xenobiotics, that enter or make contact with the body. The Phase I enzymes, including the cytochrome P450, cytochrome b5 and epoxide hydrolases work by adding a polar functional group to the original molecule, producing reactive compounds. These reactive intermediates can then bind to DNA and cause a mutation, or they can become a substrate for the second class of detoxification enzymes. The Phase II enzymes, including glutathione S-transferase and quinone reductase,

Fig. 10. Immunohistochemical analysis of progesterone receptor (PR) status in the mammary tissue of control and experimental animals. PR status of mammary tissue of control (A) and tangeretin treated control (B) animals showing normal levels of progesterone receptor expression; PR status of mammary tissue of DMBA induced rat (C at 20× and C1 at 100×) showing increased expression of progesterone receptor; PR status of mammary tissue of tumor bearing rats treated with tangeretin (D at 20× and D1 at 100×) showing comparatively decreased expression of progesterone receptor.
conjugate these reactive intermediates, adding a hydrophilic functional group and making them water-soluble. These secondary products are then more easily excreted from the body [68]. Breast tumor induced rats showed elevated levels of Phase I enzymes and reduced levels of Phase II enzymes. However, tangeretin treatment to tumor bearing animals significantly altered these enzyme levels. Increased level of TBARS in plasma and tumor tissues and of conjugated dienes in tumor tissues have been reported earlier [69]. Overproduction of oxygen free radicals in mammary cancer tissues have been demonstrated [70]. The status of plasma TBARS serve as an index to assess the extent of tissue damage. Elevated levels of plasma TBARS in mammary cancer induced rats could therefore be related to the excessive production and diffusion from the mammary tissues and other damaged tissues. The levels of TBARS and conjugated dienes were also significantly increased in breast tissue of tumor induced rats. The tumor cells in breast tissue showed a distinctly low level of peroxidation creating a favorable atmosphere for the proliferation of cancer cells [71]. Tangeretin supplementation significantly altered the lipid peroxidation status indicating its anti-lipoperoxidative property.

Overproduction of free radicals may result in a variety of pathological processes including cancer, inflammation and other diseases. Free radicals play an imperative role in tumor promotion by direct chemical reaction or alteration of cellular metabolic processes [72] and their scavengers (antioxidants) represent inhibitors at different stages of carcinogenesis [73]. Superoxide dismutase, being the primary antioxidant enzyme plays a greater role in the protection of cells against the oxidative damage induced by DMBA. Catalase catalyzes the formation of water and oxygen from hydrogen peroxide and has a rapid turnover rate. Catalase is shown to decrease the chromosomal aberrations and delay the onset of neoplastic transformation in mouse fibroblasts epidermal keratinocytes [74]. Glutathione is required for the stability of erythrocytes. Oxidation of glutathione leads to hemolysis. Non-enzymic
antioxidants, such as GSH, Vitamins C and E, also play an important role in protecting the cells from oxidative stress. GSH and its oxidized counterpart, glutathione disulphides, are the major non-enzymic antioxidants present in the cell. The level of this enzyme is found to be decreased in tumor induced rats, which may be due to increased LPO, thereby resulting in increased utilization. Vitamin E is a powerful antioxidant, which reduces hydroperoxides generated during the process of peroxidation, thereby protecting cell structures against damage [75]. Vitamin C or ascorbic acid is a hydrophilic antioxidant in plasma and disappear faster than other antioxidants upon exposure to reactive oxygen species [76]. The decreased level of ascorbic acid may be due to either increased utilization as an antioxidant defense against increased reactive oxygen species or due to a decrease in glutathione level, since glutathione is required for the recycling of ascorbic acid [77]. Tangeretin treatment rejuvenated the levels of the antioxidant

![Graph](image1)

**Fig. 12.** a–c: The representative graph shows the percentage of cells stained positively for estrogen receptor (a), progesterone receptor (b) and Her2/neu receptor (c) status in control and experimental group of rats. Positive staining was assessed and measured objectively by two independent observers and gave similar results. For quantitation, data expressing the respective stain was quantified by counting the positively stained cells in ten fields/section by three independent observers in blinded fashion, and the average was used to denote the total no. of positively stained cells, that was presented as “fold changes” as compared with control and experimental group of rats. Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.

![Graph](image2)

**Fig. 13.** Effect of tangeretin on the protein expression of ER, PR and Her2/neu in mammary tissue of rats. Beta-actin was used as an internal standard. Lane 1: mammary tissue of control animals (group I); lane II: mammary tissue of tangeretin control rats (group II); lane III: mammary tissue of DMBA induced rats (group III); lane IV: mammary tissue of DMBA induced rats treated with tangeretin. Values are given as mean ± S.E.M. for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: *control rats; #DMBA induced rats. Values are statistically significant at p < 0.05.
enzymes in breast tissue as well as in serum of tumor bearing animals indicating its antioxidant potential. Serum tumor markers are proteins liberated into the blood stream by cancer cells. A number of serum tumor markers like CEA, c-myc, p53 and c-erbB2 have been proposed for breast cancer detection of which CEA, AFP and CA 15-3 are most widely used [78]. Carcinoembryonic antigen (CEA), an oncofetal glycoprotein, is expressed in normal mucosal cells and overexpressed in adenocarcinoma of the breast [79]. Alpha-fetoprotein (AFP) is the major protein of fetal serum formed by the yolk sac and the liver during fetal development, but falls to an undetectable level after birth. It is also used as a tumor marker for primary malignancies like hepatocellular carcinoma, germ cell tumors and metastatic cancers [80]. CA 15-3 (Cancer Antigen 15-3) is a tumor marker used to monitor certain cancers, especially metastatic breast cancer [81]. Elevated CA 15-3, in conjunction with alkaline phosphatase (ALP), was found to be associated with an increased chance of early recurrence in breast cancer. All the above serum markers are elevated in the tumor induced rats wherein tangeretin treatment brought the markers to near normal levels hence showing the chemotherapeutic potential of tangeretin.

Hormone receptors are distributed in the human body mainly in the female reproductive organs but also in non-reproductive organs as well. The ER and PR levels vary in different tissues. Their levels are also influenced by factors such as age, obesity, and existence of cancer. Breast cancers that are ER, PR receptor-negative are associated with the worst outcomes and require aggressive treatment right from the time of diagnosis. Targeted therapies are available for cancers that are ER/PR+ve or HER2+ve before they metastasize. ER, PR, and HER2 receptor determination is vital for standard and state of art breast cancer management. The optimal management of breast cancer cannot be accomplished without the determination of ER, PR, and HER2 status. These receptor determinants have diagnostic, therapeutic and prognostic implications. Breast cancer patients with tumors that are receptor positive have a lower risk of mortality after their diagnosis compared to women with negative hormone receptors. In the present study, the IHC and Western blotting results showed that DMBA induced cancerous rats expressed high levels of the hormone receptors wherein tangeretin treatment brought the expression of the hormone receptor levels to near normalcy.

5. Conclusion

In conclusion, the results of the present study clearly establish the anticancer efficacy of tangeretin against DMBA induced mammary carcinoma in rats. The attenuation of oxidative stress and improved antioxidant status upon tangeretin treatment exemplifies the antioxidant potential of tangeretin. Also, the alteration in the levels of tumor markers indicates the antitumor activity of tangeretin. Our results underlie the potency of tangeretin as an effective chemotherapeutic agent in the treatment of breast cancer. However, further studies are warranted to elucidate the exact molecular mechanism underlying the action of tangeretin in ameliorating the toxic effects of DMBA in experimental mammary carcinoma.

Conflict of interest

The authors declare no conflict of interest.

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Tangeretin ameliorates oxidative stress in the renal tissues of rats with experimental breast cancer induced by 7,12-dimethylbenz[a]anthracene

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HIGHLIGHTS
- Tangeretin attenuated DMBA induced oxidative stress by upregulating Nrf2.
- Tangeretin ameliorated DMBA induced renal inflammatory cytokines.
- Tangeretin protected the ultrastructure of DMBA induced kidney.
- Tangeretin reduced the DMBA induced kidney DNA damage.

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ABSTRACT
Tangeretin, a citrus polymethoxyflavone, is an antioxidant modulator which has been shown to exhibit a surfeit of pharmacological properties. The present study was hypothesized to explore the therapeutic activity of tangeretin against 7,12-dimethylbenz[a]anthracene (DMBA) induced kidney injury in mammary tumor bearing rats. Recently, we have reported the chemotherapeutic effect of tangeretin in the breast tissue of DMBA induced rats. Breast cancer was induced by “air pouch technique” with a single dose of 25 mg/kg of DMBA. Tangeretin (50 mg/kg/day) was administered orally for four weeks. The renoprotective nature of tangeretin was assessed by analyzing the markers of oxidative stress, proinflammatory cytokines and antioxidant competence in DMBA induced rats. Tangeretin treatment revealed a significant decline in the levels of lipid peroxides, inflammatory cytokines and markers of DNA damage, and a significant improvement in the levels of enzymatic and non-enzymatic antioxidants in the kidney tissue. Similarly, mRNA, protein and immunohistochemical analysis substantiated that tangeretin treatment notably normalizes the renal expression of Nrf2/Keap1, its downstream regulatory proteins and the inflammatory cytokines in the DMBA induced rats. Histological and ultrastructural observations also evidenced that the treatment with tangeretin effectively protects the kidney from DMBA-mediated oxidative damage, hence, proving its nephroprotective nature.

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1. Introduction
Breast cancer is a major global public health problem in both the developed and developing countries, and accounts for the highest morbidity and mortality worldwide (Siegel et al., 2014). Australia, Northwest Europe and North America report the highest rates of breast cancer, whereas the Middle-East, Africa and Asia report the lowest rates (Jemal et al., 2011). India contributes to 7% of global burden of breast cancer, making it the second most common malignancy after cervical cancer among women (Datta et al., 2012). The geographic variation in the incidence suggests that hereditary factors play only a minor role, whereas environmental and dietary factors play a major role. Chemotherapeutic drugs used for treating breast cancer cause deleterious side effects, for which plant based bioactive compounds with minimal side effects is desired.

Ubiquitous chemical compounds such as polycyclic aromatic hydrocarbons (PAHs), are established cancer initiators that bio-accumulate and persist in the environment, the major sources being automobile exhaust, cigarette smoke, oil furnaces and charbroiled food (Gelboin, 1980). These PAHs are metabolized and transformed...
into DNA attacking electrophiles in the body, producing PAH–DNA adducts which are found in human breast tumors (Leung et al., 2009). This leads to the formation of highly reactive and toxic free radicals in vivo. The synthetic PAH, 7,12-DMA initiates the production of free radicals that induce carcinogenesis in rodents which mimic human cancers morphologically and histologically (Costa et al., 2002). Therefore, DMBa induced mammary carcinogenesis is an ideal model to study the therapeutic effect of natural and synthetic agents in experimental animals.

The transcription factor, nuclear factor erythroid 2-related factor-2 (Nrf2), activates the antioxidant response element which induces the phase II and antioxidant enzymes (Kaspar et al., 2009; Flocyk et al., 2010). Generally, under physiological conditions, Kelch-like echilcholorhydrin-associated protein 1 (Keap1), the cytosolic inhibitor of Nrf2 induces its ubiquitin-dependent proteasomal degradation. However, upon activation by oxidative stimuli, Nrf2 translocates to the nucleus, wherein it binds to the antioxidant response element (ARE). This leads to the activation of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases (GPX) (Li et al., 2012) and phase II detoxification enzymes such as NQO1 and GST (Baird and Dinkova-Kostova, 2011; Ma and He, 2012) which help to eliminate toxic reactive intermediates generated via xenobiotic metabolism. Other important downstream targets of Nrf2 are γ-glutamyl cysteine synthase (γGCS) and heme oxygenase-1 (HO-1). Recent studies have established the key role of Nrf2 signaling in impeding DMBa-induced oxidative stress and tissue injury (Becks et al., 2010; Kavitha et al., 2013).

Kidney cells, being exposed to high concentrations of chemicals during their excretion and being endowed with xenobiotic-metabolizing activity, are the main targets of several carcinogens (Robbiano et al., 1999) not only of ultimate carcinogens, but also of procarcinogens bio-transformed into reactive species in the kidney, inducing injury to the cells. DMBa is a well known cytoxic, carcinogenic, mutagenic and immunosuppressive agent known to induce free radical mediated injury in various tissues (Miyata et al., 2001; Buters et al., 2003). It has been reported that DMBa induces substantive nephrotoxicity that is characterized by renal tubular necrosis, increased number of lysosomes, dilated mitochondria and chromat condensation in the nucleus (Ozturk et al., 2006; Yehia et al., 2007).

Recently, there is a growing interest in the role and usage of natural antioxidants such as flavonoids as a strategy to prevent and protect oxidative damage in various health disorders (Pandey and Rizvi, 2009). Citrus fruit juices are a rich source of flavonoids and have been known to possess a number of biological activities such as antioxidant, anti-inflammatory, anti-allergic, vasodilatory and anticarcinogenic properties (Kawai et al., 1999). Recent studies have shown that polymethoxyl flavones possess greater anti-tumor, anti-metastatic and anti-invasive properties compared to hydroxylated flavonoids (Walle, 2007). Tangeretin, a pentamethoxyflavone, found in citrus peel has polymethylation of the hydroxylated groups, resulting in lipophility, increased metabolic stability and membrane transport in the intestine and liver and hence improved bioavailability (Manthey et al., 2011). Tangeretin was found to possess several biological activities such as inhibition of cancer cell proliferation in lung (Chen et al., 2007) and colorectal carcinoma cells (Pan et al., 2002), induce apoptosis in leukemia cells (Hirano et al., 1995) and inhibit proliferation and migration of aortic smooth muscle cells (See et al., 2011). It was found to be neuroprotective (Datla et al., 2001), hypolipidemic (Kuwowska and Manthey, 2004) and a potent inhibitor of xenobiotic-induced genotoxicity in vitro (Lake et al., 1999). More recently, we have reported the chemotherapeutic effect of tangeretin in the breast tissue of DMBa induced rat mammary carcinoma (Lakshmi and Subramanian, 2014). However, there are no studies demonstrating the protective effect of tangeretin in DMBa induced kidney injury.

Hence, in continuation with our previous report, we have studied the nephroprotective effect of tangeretin in renal tissues of DMBa induced mammary tumor bearing rats.

The present study aims to observe the alterations in antioxidative defense enzymes, inflammatory cytokines and renal morphology after DMBa administration in rats and to investigate the possible therapeutic effects of tangeretin in DMBa-induced kidney injury.

2. Materials and methods

2.1. Chemicals

DMBa was purchased from Sigma Chemical Company (St. Louis, MO, USA) and tangeretin from Indofine Chemical Company, USA. All other chemicals used were of analytical grade procured from local commercial sources.

2.2. Experimental animals

Virgin female Wistar rats, seven weeks of age were purchased from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai and were used in the present study. Rats were housed sparsely in individual cages and maintained under standard experimental conditions: temperature 25 ± 1°C, relative humidity 60 ± 5% and 12 ± 1h (light/dark cycle) and fed with commercially available balanced pellet diet (Amrut laboratory Animal Feed, Bangalore, India) and water ad libitum. The animals were acclimatized for one week prior to the initiation of experiments. The experimental design was performed in accordance with the current ethical norms approved by the Ministry of Social Justice and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC No.: 01/059/09).

2.3. Induction of breast cancer

Mammary tumors were induced by DMBa using the “air pouch technique” as described by Arun et al. (1984) with slight modifications. Sterile air (1–2 ml) was carefully injected subcutaneously just beneath the mammary fat pad so as to produce a pouch containing sterile air. The air inside was allowed to remain for a day to form a pouch. A single dose of DMBa (25 mg/kg/rat) in 0.5 ml of corn oil was carefully injected into the air pouch. All rats were palpated every week to monitor the onset of tumorigenesis. Tumor mass and size was stabilized 90 days after the injection with DMBa.

2.4. Experimental design

The rats were divided into four groups each comprising of six rats as detailed below:

- **Group I:** rats received oral administration of 1 ml of PBS containing 0.1% DMSO and served as control.
- **Group II:** rats received oral administration of tangeretin alone for the entire experimental period and served as drug control.
- **Group III:** rats received subcutaneous injection of DMBa in mammary tissues and served as breast cancer model.
- **Group IV:** rats received DMBa as in Group III and treated with tangeretin for four weeks, served as tangeretin treated model.

2.5. Drug treatment

After the onset of tumor, tangeretin (50 mg/kg dissolved in 1 ml of PBS containing 0.1% DMSO/rat) was administered orally for four weeks. At the end of the experimental period, the tumor volume
was estimated according to the method of Geran et al. (1972). Briefly, the resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axes. The two short axes were measured with vernier calipers. The tumor volume was calculated using the formula:

\[ \text{Tumor volume} = \frac{\text{length (cm)} \times \text{width}^2 (\text{cm})}{2} \]

All animals were fasted overnight and sacrificed by sodium pentothal anesthesia followed by cervical decapitation. Blood was collected with and without anticoagulant and the serum was centrifuged at 5000 rpm for 15 min to obtain a clear supernatant and stored at –80°C until its use for further biochemical analysis. Kidney tissues from control and experimental groups of rats were immediately excised, washed in ice-cold PBS to remove the blood stains, blotted, weighed and homogenized in Tris–HCl buffer (0.1 M, pH 7.4) using a Teflon homogenizer to prepare 10% (w/v) tissue homogenate. This homogenate was centrifuged at 12,000 \( \times g \) for 30 min at 4°C to obtain a clear supernatant. This supernatant was pooled and used for further analysis.

2.6. Assessment of renal dysfunction

The plasma levels of blood urea nitrogen (BUN) and the levels of plasma and urinary creatinine were determined using diagnostic kits from Span Diagnostics Ltd., Surat, India. The activities of pathophysiological enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were also assayed in the kidney tissue homogenate of control and experimental groups of rats (King, 1965a,b).

2.7. Assay of xenobiotic enzymes

Cytochrome P450 and cytochrome b\(_5\) content was assayed in the kidney tissue homogenate by the method of Omura and Sato (1964).

2.8. Assay of renal superoxide radical, hydroxyl radical and total nitric oxide (NO)

The levels of free radicals generated in the kidney tissue homogenate of control and experimental groups of rats were assayed as follows. The levels of superoxide radical was measured spectrophotometrically at 510 nm using the cytochrome c method and the superoxide radicals generated were expressed as absorbance at 550 nm/15 min (Fridovich, 1986). The levels of hydroxyl radical was measured spectrophotometrically at 510 nm and was expressed as absorbance at 510 nm/30 min (Halliwell and Gutteridge, 1986) and the levels of NO was indirectly measured by determining the nitrite level using a colorimetric method based on the Griess reaction (Green et al., 1982). Further, the levels of lipid peroxides (Ohkawa et al., 1979), hydroperoxides (Jiang et al., 1992) and protein carbonyls (Uchida and Stadtman, 1993) were determined in the kidney tissue homogenate of control and experimental groups of rats.

2.9. Assay of renal antioxidant status

The activities of enzymatic antioxidants such as superoxide dismutase (SOD) (Misra and Fridovich, 1972), catalase (Takahara et al., 1960), glutathione peroxidase (GPx) (Rotruck et al., 1973), glutathione-S-transferase (GST) (Habig et al., 1974) and glutathione reductase (GR) (Carlberg and Mannervik, 1985) and the levels of non-enzymatic antioxidants such as vitamin C (Omaye et al., 1979), vitamin E (Desai, 1984) and GSH (Sedlak and Lindsay, 1968) were estimated in the kidney tissue homogenate of control and experimental groups of rats.

2.10. Assay of TNF-α, IL-1β, IL-6 and NF-κB/p65 subunit

The levels of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in kidney tissue homogenate of control and experimental groups of rats were determined by ELISA kits from Biosource, Camarillo, CA. The nuclear level of free p65 was quantified using ActivELISA kit from Imgenex, San Diego, CA.

2.11. Histopathology

The kidney tissue was immediately fixed in 10% neutral buffered formalin, embedded in paraffin, 5 μm section was cut using a microtome and then rehydrated with xylene and graded series of ethanol. The specimens were then stained with haematoxylin and eosin and examined by a pathologist.

2.12. Pathological studies

5 μm sections of the paraffin embedded tissues were cut using a microtome and then rehydrated with xylene and graded series of ethanol. The specimens were then stained accordingly as mentioned below. Glycoprotein in the kidney tissues was stained with periodic acid–Schiff (PAS) according to the method of Yamabayashi (1987). Collagen deposition was analyzed by staining with Masson’s trichrome. Lipid accumulation in the kidney tissue was studied by Oil Red-O staining. Histochemical analysis of mast cells by toluidine blue staining was carried out by the method of Migliaccio et al. (2003).

2.13. Transmission electron microscopy (TEM)

A portion of the kidney tissue from control and experimental groups of rats was fixed in 3% gluteraldehyde in sodium phosphate buffer and post-fixed in 1% osmium tetroxide. Then, the samples were dehydrated with graded series of ethanol and embedded in Araldite. Thin sections were cut with ultramicrotome using a diamond knife, mounted on a copper grid and stained with 2% uranyl acetate and Reynolds lead citrate. The sections were screened in JEOL JEM 1400 transmission electron microscope at 80 kV. The micrographs were taken using Olympus Keenview CCD camera attached to the microscope.

2.14. Semi-quantitative and quantitative PCR analysis

Total RNA was extracted from the renal tissues from control and experimental groups of rats by Trizol reagent (Invitrogen, Carlsbad, CA) and purified by Qiagen RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Purified total RNA was reverse transcribed into single strand cDNAs, which were successively analyzed semi-quantitatively using the GoTaq PCR master mix and quantitatively using the SYBR GREEN PCR master mix (Applied Biosystems, Foster City, CA). Gene expression was evaluated using Nrf2, Keap1, NQO1, HO-1, NF-κB and TNF-α primer pairs based on published rat sequences (Table 1). GAPDH was used as the internal control.

2.15. Protein extraction and Western blotting

Total protein, cytoplasmic and nuclear extracts of the kidney tissues were used for Western blotting. For extracting the total protein, 50 mg of each tissue was homogenized in 0.1 M Tris–HCL buffer (pH 7.4) containing protease inhibitor. The nuclear and cytoplasmic extracts were prepared as described by Legrand-Poels et al. (2000). SDS-PAGE was performed using equivalent protein extracts (50 μg) from each sample. The resolved proteins were electrophoretically transferred to nitrocellulose membranes. The blots were incubated in 0.1% TBS containing 5% non-fat dry milk
for 1 h to block nonspecific binding sites. The blot was incubated with 1:1000 dilution of primary antibodies for Nrf2, Keap1, γ-GCS, μ-GST, NQO1, HO-1 and 8-OHdG and inflammatory markers NF-κB/p65, TNF-α, iNOS, COX-2 and GAPDH (internal control) overnight at 4 °C. All antibodies were procured from Santa Cruz Biotech, USA. After washing, the blots were incubated with 1:2500 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After extensive washes, the bands in the membranes were detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences, UK) and quantified with Image J software.

### 2.16. Immunohistochemical analysis

Paraffin embedded tissue sections were processed as described earlier and were then immunostained with the primary antibodies for Nrf2, Keap1, NQO1, HO-1, NF-κB/p65, iNOS, COX-2 and 8-hydroxydeoxyguanosine (8-OHdG) from Santa Cruz Biotech, USA at a concentration of 1 μg/ml with 3% BSA in TBS and incubated overnight at 4 °C. After washing the slides thrice with TBS, the sections were then incubated with the respective HRP conjugated secondary antibodies (Bangalore Genei, India), diluted 1:200 with 3% BSA in TBS and incubated for 2 h at room temperature. Sections were then washed with TBS and incubated for 5–10 min in a solution of 0.02% diaminobenzidine (DAB) containing 0.01% hydrogen peroxide. Counter staining was performed using hematoxylin, and the slides were visualized under a light microscope (Nikon XDS-18).

### 2.17. Comet assay and DNA fragmentation analysis

Comet assay was performed under alkaline conditions essentially according to the procedure of Olive and Banath (2006). The parameter taken to assess cellular DNA damage was tail length (migration of DNA from the nucleus). Slides were scored using an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope. For the DNA fragmentation assay, isolation of DNA was performed according to the method described by Sambrook et al. (1989). Analysis of DNA fragments was performed with a 1.2% agarose gel in 1x TBE buffer.

### 2.18. Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago) statistical package. The results were expressed as mean ± SEM. One-way ANOVA followed by post hoc test LSD was used to correlate the difference between the variables. Values were considered statistically significant if *p* < 0.05.

### 3. Results

#### 3.1. Tangeretin improves renal function in DMBA induced rats

Table 2 shows the breast tumor weight in DMBA induced and tangeretin treated rats. There was significant increase in breast tumor weight in DMBA induced rats. Whereas the tumor weight was found to be significantly reduced in tangeretin treated rats (*p* < 0.05). However, there was no change observed in breast of both drug control and control rats.

#### 3.2. Effect of tangeretin on kidney weight, creatinine clearance, BUN and the levels of activities of renal AST, ALT and ALP in the kidney of control and experimental animals

Table 3 shows the effect of tangeretin on the kidney weight, creatinine clearance, BUN and the activities of renal AST, ALT and ALP in the control and experimental groups of rats. There was a significant increase (*p* < 0.05) in the organ weight, BUN and the activities of renal AST, ALT and ALP in breast cancer rats with

---

**Table 1**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Sense strand</th>
<th>Antisense strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>5'-TTGGCAGAGACATCCCAT-3'</td>
<td>5'-GCTGCCAGTCACACTGGG-3'</td>
</tr>
<tr>
<td>Keap1</td>
<td>5'-ATCACAACAGCAGGCTT-3'</td>
<td>5'-GATCTCCTGTCCTGCCTC-3'</td>
</tr>
<tr>
<td>NQO1</td>
<td>5'-ATGGTACGCGGCACTACA-3'</td>
<td>5'-GGCCCATTGTACATTAGG-3'</td>
</tr>
<tr>
<td>HO-1</td>
<td>5'-ACAACAGACAGACACCTC-3'</td>
<td>5'-AGGTAACGGGATTACGCT-3'</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5'-ACCTGAGTCTCCGACGCCG-3'</td>
<td>5'-CCAGGCTTCATCGTCAACTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ACACACGACTCCGATCAC-3'</td>
<td>5'-TCCACCACCTTGTTGCTA-3'</td>
</tr>
</tbody>
</table>

---

**Table 2**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Tang control</th>
<th>DMBA</th>
<th>DMBA + tang</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor weight (g)</strong></td>
<td>-</td>
<td>-</td>
<td>7.13 ± 0.31*</td>
<td>3.84 ± 0.19**</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM for groups of six rats in each. One-way ANOVA followed by post hoc test LSD.

Values are statistically significant at *p* < 0.05.

* Statistical significance was compared within the group vs control rats.

** Table 3**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Tang control</th>
<th>DMBA</th>
<th>DMBA + tang</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney weight (g)</strong></td>
<td>0.92 ± 0.03</td>
<td>0.94 ± 0.04</td>
<td>1.54 ± 0.048*</td>
<td>1.17 ± 0.035**</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>2.27 ± 0.35</td>
<td>2.31 ± 0.32</td>
<td>1.39 ± 0.068*</td>
<td>2.14 ± 0.015**</td>
</tr>
<tr>
<td>BUN</td>
<td>14</td>
<td>15.2 ± 0.41</td>
<td>29.30 ± 2.72*</td>
<td>21.10 ± 1.91**</td>
</tr>
<tr>
<td>AST</td>
<td>58.08 ± 2.20</td>
<td>56.29 ± 2.32</td>
<td>104.70 ± 5.64*</td>
<td>76.30 ± 2.19**</td>
</tr>
<tr>
<td>ALT</td>
<td>148.67 ± 9.36</td>
<td>142.50 ± 4.31</td>
<td>283.17 ± 9.71*</td>
<td>176.87 ± 5.55**</td>
</tr>
<tr>
<td>ALP</td>
<td>0.52 ± 0.03</td>
<td>0.48 ± 0.03</td>
<td>1.03 ± 0.008*</td>
<td>0.72 ± 0.006**</td>
</tr>
</tbody>
</table>

Units: creatinine clearance is expressed in ml/min/100 g body weight; BUN is expressed in mg/dl protein; AST and ALT are expressed in μmoles of pyruvate liberated/mg protein/min; ALP is expressed in μmoles of p-nitrophenol liberated/mg protein/min.

Values are given as mean ± SEM for control and experimental. One-way ANOVA followed by post hoc test LSD.

Values are statistically significant at *p* < 0.05.

* Statistical significance was compared within the group vs control rats.

** Statistical significance was compared within the group vs DMBA induced rats.
notable ($p < 0.05$) decline in their creatinine clearance rate. Tangeretin treated rats showed significantly ($p < 0.05$) improved levels of renal functional markers when compared with DMBA induced rats. However, drug control rats did not reveal any statistical significant differences on these indices when compared to that of control rats.

3.2. Tangeretin decreases the levels of xenobiotic enzymes in renal tissues

The activities of xenobiotic enzymes in kidney tissue of control and experimental animals are presented in Fig. 1. In DMBA induced rats, the levels of xenobiotic enzymes such as cytochrome P$_{450}$ and cytochrome b$_5$ were found to be increased significantly ($p < 0.05$) when compared to control rats. On contrary, a significant decrease ($p < 0.05$) in these enzymes was observed in tangeretin treated rats. However, there were no significant changes in drug control rats when compared with control rats ($p < 0.05$).

3.3. Tangeretin scavenges free radicals and decreases the formation of oxidative stress markers in DMBA induced kidney

Fig. 2 and Table 4 illustrate the effect of tangeretin on the levels of superoxide anion, hydroxyl radical and NO and the levels of lipid peroxides, hydroperoxides and protein carbonyls respectively in the renal tissues of control and experimental groups of rats. The levels of free radicals and oxidative stress markers in DMBA induced rats were significantly ($p < 0.05$) escalated when compared with control rats. Nevertheless, tangeretin treated rats demonstrated a marked ($p < 0.05$) decrement in these levels. No considerable statistical variation was experienced in the drug control rats.

3.4. Tangeretin improves the activities of enzymatic and non-enzymatic antioxidants in renal tissues of DMBA induced rats

Fig. 3 and Table 5 indicate the effect of tangeretin on the activities of renal enzymatic antioxidants such as SOD, catalase, GR, GPx and GST and non-enzymatic antioxidants such as vitamin C, vitamin E and GSH respectively in the control and experimental groups of rats. The activities were significantly ($p < 0.05$) diminished in the renal tissues of DMBA induced rats. Tangeretin treated rats showed significantly ($p < 0.05$) altered activities of these antioxidants to near normalcy in renal tissues. However, no significant changes were observed in drug control rats in comparison to that of control rats.

3.5. Tangeretin ameliorates proinflammatory cytokines in DMBA induced kidney

The effect of oral treatment of tangeretin on the levels of TNF-$\alpha$, IL-1$\beta$, IL-6 and NF-$\kappa$B/p65 subunit in renal tissues of control and
Table 4
Effect of tangeretin on the levels of lipid peroxides, hydroperoxides and protein carbonyls in the kidney tissues of control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Tang control</th>
<th>DMBA</th>
<th>DMBA + tang</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxides</td>
<td>1.36 ± 0.12</td>
<td>1.22 ± 0.07</td>
<td>3.64 ± 0.29</td>
<td>1.98 ± 0.08</td>
</tr>
<tr>
<td>Hydroperoxides</td>
<td>53.12 ± 2.13</td>
<td>53.22 ± 0.07</td>
<td>87.02 ± 2.81</td>
<td>61.89 ± 2.17</td>
</tr>
<tr>
<td>Protein carbonyls</td>
<td>3.95 ± 0.18</td>
<td>3.41 ± 0.2</td>
<td>12.12 ± 0.71</td>
<td>7.73 ± 0.32</td>
</tr>
</tbody>
</table>

Units: lipid peroxides expressed in mmoles of TBARS/100 g tissues; hydroperoxides expressed in mmoles/100 g tissues; protein carbonyls expressed in mmoles/mg protein. Values are given as mean ± SEM for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Values are statistically significant at p < 0.05.

* Statistical significance was compared within the group vs control rats.

b Statistical significance was compared within the group vs DMBA induced rats.

Fig. 3. Effect of tangeretin on the levels of enzymatic antioxidants in renal tissue of control and experimental animals. Activity is expressed as: 50% of inhibition of epinephrine autoxidation/min for SOD; μ moles of hydrogen peroxide decomposed/min/mg protein for catalase; μ moles of glutathione oxidised/min/mg protein for GR; units/min/mg protein for GST; μ moles of DTNB–GSH conjugate formed/min/mg protein for GPx. Values are given as mean ± SEM for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: * vs Control rats; # vs DMBA induced rats. Values are statistically significant at p < 0.05.

3.6. Tangeretin protects the morphology and ultrastructure of the kidney against DMBA induced oxidative stress

Fig. 4 represents the histological observations of haematoxylin–eosin staining of kidney tissue of control and experimental groups of rats. Fig. 4A and B shows the section of kidney tissues of control and drug control rats exhibiting normal kidney morphology and architecture with normal appearance of glomerulus; DMBA induced rats showed glomerular degeneration (Fig. 5C) and tangeretin treated rats showed almost normal morphology of the glomerulus (Fig. 5D). Section of kidney tissue of control and drug control rats shows normal appearance of medulla region (Fig. 5E and F) with tubules; DMBA induced rats showed medullar and tubular degeneration (Fig. 5G) and tangeretin treated rats showed almost normal morphology of the medullar and tubular cells of the kidney (Fig. 5H).

Fig. 6 shows the electron microscopic examination of the kidney tissues of control and experimental groups of rats. Ultrastructural examination of the renal cortex of the control and drug control rats revealed normal aspects of kidney cells with euchromatic nuclei (Fig. 6A and B); DMBA induced rats revealed multiple alterations of the kidney cells showing distorted nuclei with chromatin condensation (Fig. 6C) and tangeretin treated rats showed a marked improvement of the ultrastructural aspects with

Table 5
Effect of tangeretin on the levels of non-enzymatic antioxidants in kidney tissue of control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Tang control</th>
<th>DMBA</th>
<th>DMBA + tang</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C (μg/mg protein)</td>
<td>1.04 ± 0.02</td>
<td>1.21 ± 0.05</td>
<td>0.61 ± 0.14</td>
<td>0.98 ± 0.12</td>
</tr>
<tr>
<td>Vitamin E (μg/mg protein)</td>
<td>3.04 ± 0.12</td>
<td>3.22 ± 0.15</td>
<td>2.02 ± 0.21</td>
<td>2.82 ± 0.16</td>
</tr>
<tr>
<td>GSH (mg/100 g tissue)</td>
<td>19.05 ± 1.11</td>
<td>20.41 ± 1.2</td>
<td>11.16 ± 1.7</td>
<td>18.71 ± 2.02</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Values are statistically significant at p < 0.05.

* Statistical significance was compared within the group vs control rats.

b Statistical significance was compared within the group vs DMBA induced rats.
intact nucleus (Fig. 6D). Also, the control and drug control rats revealed normal basement membrane with mitochondria (Fig. 6E and F); DMBA induced rats showed distorted basement membrane with lysosomes (Fig. 6G) and tangeretin treated rats showed marked improvement in the basement membrane (Fig. 6H). The control and drug control rats revealed normal mitochondria with cristae (Fig. 6I and J); DMBA induced rats showed swollen mitochondria with disappearance of cristae (Fig. 6K) and

Fig. 5. Photomicrographs of hematoxylin–eosin staining of renal tissues of control and experimental animals. Histological photograph of control (A, E) and drug control (B, F) sections of kidney tissues exhibiting normal morphology and architecture with normal appearance of glomerulus and medulla region. DMBA induced animals showed glomerular (C) and medullar degeneration (G) along with tubular degeneration and glomerular atrophy. Tangeretin treated rats showed almost normal morphology of the glomerular (D), medullar (H) and tubular cells of the kidney (20× magnification).
tangeretin treated rats showed normal mitochondria with cristae (Fig. 6L). Additionally, the proximal and distal tubules of DMBA induced rats were also affected with increase in invagination of tubules and disappearance of mitochondria (Fig. 6M) which were reverted in tangeretin treated rats (Fig. 6N). Presence of electron dense lipid globules (Fig. 6O) was also observed in DMBA induced rats which was reduced significantly in tangeretin treated rats (Fig. 6P), which was comparable to that of the control rats.

3.7. Tangeretin reduces the accumulation of mast cells, collagen, polysaccharide and lipids in DMBA induced rat kidney

Fig. 7A–D, E–H, I–L and M–P shows the effect of tangeretin on the histochemical analysis of toluidine blue staining for mast cells, Masson’s trichrome staining for collagen, PAS staining for polysaccharides and Oil Red-O staining for lipids, respectively. In the kidney sections of control and experimental animals, Toluidine blue staining (Fig. 7A–D) revealed that DMBA induced rats showed significantly increased mast cell population; Masson’s trichrome staining for collagen deposition (Fig. 7E–H) showed significantly increased collagen content in the DMBA induced group; PAS staining for glycoprotein content in the kidney tissue (Fig. 7I–L) showed significantly increased polysaccharide content in the DMBA induced kidney and Oil Red-O staining demonstrated large number of intracellular lipid droplet accumulation (Fig. 7M–P) in the kidney of DMBA induced rats, which were altered in tangeretin treated rats. Control and drug control rats exhibited very low accumulation of mast cells, collagen, polysaccharides and intracellular lipids.

3.8. Tangeretin normalizes the mRNA expression of Nrf2–Keap1 pathway genes and inflammatory cytokines in DMBA induced kidney

The effect of tangeretin on the mRNA expression of oxidative stress markers Nrf2, Keap1, NQO1, HO-1 and inflammatory markers NF-κB/p65 and TNF-α in the kidney tissues of control and experimental groups of rats by semi-quantitative and quantitative PCR analysis is depicted in Fig. 8. The mRNA expression of Nrf2 and its
Fig. 7. Representative images showing the effect of tangeretin on the histochemical analysis of toluidine blue staining (A–D), Masson’s trichrome staining (E–H), PAS staining (I–L) and Oil Red-O staining (M–P) respectively in renal tissue of control and experimental rats. The control (A, E, I and M) and drug control rats (B, F, J and N) showed normal kidney staining; the DMBA induced rats (C, G, K and O) showed increased mast cell accumulation, collagen deposition, polysaccharides and lipid accumulation when compared with control rats. However, tangeretin treated rats showed decreased mast cell accumulation, collagen deposition, polysaccharides and intracellular lipids (D, H, L and P) in the kidney tissues (20× magnification).

downregulatory genes such as NQO1 and HO-1 were significantly (p < 0.05) declined with simultaneous elevation of Keap1, NF-κB/p65 and TNF-α genes in the renal tissues of DMBA induced rats. However, these altered mRNA expressions were significantly (p < 0.05) normalized in the tangeretin treated rats. On the contrary, drug control rats did not demonstrate any statistical difference in comparison to control rats.

3.9. Tangeretin normalizes the protein expression of Nrf2–Keap1 pathway genes and inflammatory cytokines in DMBA induced kidney

Fig. 9 depicts the effect of tangeretin on the protein expression of oxidative stress markers Nrf2, Keap1, γ-GCS, μ-GST, NQO1, HO-1 and 8-OHdG and inflammatory markers NF-κB/p65, TNF-α, iNOS and COX-2 in the kidney tissues of control and experimental groups of rats. The expression of Nrf2 and its downregulatory proteins such as γ-GCS, μ-GST, NQO1 and HO-1 was significantly (p < 0.05) decreased with concomitant elevation in Keap1, NF-κB/p65, TNF-α, iNOS, COX-2 and 8-OHdG genes in the renal tissues of DMBA induced rats. However, these altered protein expressions were significantly (p < 0.05) normalized in the tangeretin treated rats. On the contrary, drug control rats did not demonstrate any statistical difference in comparison to control rats.

3.10. Tangeretin normalizes the expression of Nrf2–Keap1 pathway genes and inflammatory cytokines in the kidney of DMBA induced rats

Fig. 10 shows the immunohistochemical expression pattern of Nrf2, Keap1, NQO1 and HO-1 respectively in control and experimental groups of rats. There was significant (p < 0.05) decrease in the levels of Nrf2, NQO1 and HO-1 genes and increase in the level of Keap1 gene in DMBA induced rats. However, the levels of these markers was altered significantly (p < 0.05) in tangeretin treated rats. The control and drug control rats showed normal expression of these markers.

Fig. 11 shows the immunohistochemical expression pattern of NF-κB/p65, iNOS, COX-2 and 8-OHdG respectively in control and experimental groups of rats. There was significant (p < 0.05) increase in the levels of all these markers in DMBA induced rats which was altered significantly (p < 0.05) in tangeretin treated rats. The control and drug control rats showed very low expression of these markers.
3.11. Tangeretin reduces the DNA damage in the kidney of DMBA induced rats

Fig. 12 shows the effect of tangeretin and DMBA treatments on kidney DNA damage. Comet assay (Fig. 12A) shows that DMBA induction significantly ($p < 0.05$) augmented the DNA damage in the kidney cells showing a longer tail length in comparison with the results from control and drug control animals. However, tangeretin treated rats showed reduced DNA damage. DMBA induced rats also exhibited marked DNA fragmentation (Fig. 12B) showing a ladder-like pattern suggesting that DMBA might induce DNA fragmentation via an apoptosis-like mechanism. The tangeretin treated rats showed a lesser extent of nuclear DNA damage and apoptosis in the kidney cells. On the contrary, control and drug control animals showed no shearing of DNA and apoptosis.

4. Discussion

The present work demonstrates the nephroprotective potential of tangeretin against DMBA induced oxidative stress in mammary tumor bearing rats. It is well known that PAHs are widely distributed in environment and they induce physiological, biochemical and histological disorders. The main target sites for the potent carcinogenicity of DMBA, a synthetic PAH, in rodents include skin and mammary gland and to some extent the liver and kidney (Izzotti et al., 1999). The tumors developed in the present work were mammary adenocarcinomas which are similar to human mammary carcinomas morphologically and histologically. Based on our previous study, the present work demonstrates that rats intoxicated with DMBA display a pronounced impairment in kidney function which is evidenced from the enhancement of plasma creatinine, blood urea nitrogen and histopathological and ultrastructural alterations (Lakshmi and Subramanian, 2014). Previous reports have also shown that DMBA induces oxidative stress that contributes to kidney injury, which is characterized by nuclear and mitochondrial degeneration, renal tubular necrosis and chromatin condensation in the nucleus (Ozturk et al., 2006; Yehia et al., 2007).

The increased kidney weight, elevated activities of renal AST, ALT, ALP and BUN and diminished creatinine clearance in the
DMBA induced group of rats indicate the development of renal hypertrophy, glomerular injury and renal dysfunction. However, oral treatment with tangeretin to DMBA induced group of rats normalized these altered levels to near normalcy, suggesting that tangeretin treatment effectively protects the kidney from DMBA induced oxidative stress. DMBA induces the production of various free radicals such as superoxide anion, hydroxyl radical and NO that damage macromolecules, triggering renal cell injury (Frenkel et al., 1995). Similarly, markers of oxidative stress such as LPO, hydroperoxides and protein carbonyls are also produced during lipid peroxidation. The present study shows that DMBA induction increases the levels of these free radicals and tangeretin treatment significantly scavenges them. This normalization may be accomplished by the antioxidant and free-radical quenching nature of tangeretin. The enzymatic and non-enzymatic antioxidants play a major role in scavenging these reactive free radicals in the DMBA induced kidney. The antioxidants SOD, CAT, and GPx function as the first-line of defense against oxidative stress by virtue of their ability to catalyze the disproportionation reactions of their substrate free radicals that are spontaneously generated via in vivo oxidative phosphorylation, cytochrome P450 metabolism, and inflammatory processes (Uddin and Ahmad, 1995). DMBA induction notably decreases the levels of intracellular antioxidants and elevates the formation of pro-oxidants such as reactive free radicals and electrophilic substances that eventually results in renal dysfunction and deterioration which was normalized upon oral tangeretin treatment. Thus, the increase in the expression of antioxidants observed in the present study on administration of tangeretin implies that the activation of Nrf2 signaling stimulates the expression of these antioxidant enzymes thereby offering protection against DMBA induced oxidative stress (Fig. 13). This data is in accordance with a previous report where the nephroprotective effect of melatonin against DMBA-induced renal damage by the elevation of antioxidant status was reported (Ozturk et al., 2006).

Proinflammatory cytokines such as TNF-α, IL-1β and IL-6 produced in the renal tissues have been suggested to play a significant role in the development and progression of kidney injury. NF-κB regulates the genes of inflammatory mediators such as TNF-α, IL-6, iNOS and COX-2, which suggested that NF-κB might be one of the key regulators of inflammation after kidney damage (Tak and Firestein, 2001). The nuclear and mitochondrial DNA is a common target of oxidative damage, and 8-OHdG is used as a measure to monitor DNA oxidation. In the kidney, COX-2 is constitutively expressed for the maintenance of renal blood flow and is highly regulated in response to alterations in intravascular volume resulting from oxidative stress. The present study indicates that the oral administration of tangeretin to DMBA induced group of rats significantly attenuates the elevated levels of renal inflammatory and DNA damage markers to near normalcy, thereby moderating the consequence of inflammation and DNA damage mediated oxidative tissue damage.

Nrf2 is the key transcription factor that regulates antioxidant response element–mediated expression of detoxifying antioxidant enzymes (Florczyk et al., 2010). Several naturally occurring antioxidants and phytochemicals have been demonstrated to activate Nrf2.
Fig. 10. Immunohistochemical analysis of expression pattern of Nrf2, Keap1, NQO1 and HO-1 in kidney tissue of control and experimental rats. Values are given as mean ± SEM for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: * – vs control rats; # – vs DMBA induced rats. Values are statistically significant at p < 0.05.
Fig. 11. Immunohistochemical analysis of expression pattern of NF-κB/p65, iNOS, COX-2 and 8-OHdG in kidney tissue of control and experimental rats. Values are given as mean ± SEM for groups of six rats in each. One-way ANOVA followed by post hoc test LSD. Statistical significance was compared within the group as follows: * – vs control rats; # – vs DMBA induced rats. Values are statistically significant at p < 0.05.
signaling by disrupting the interactions between Nrf2 and Keap1 (Su et al., 2013). Increase in nuclear Nrf2 expression observed in the present study on oral tangeretin treatment to DMBA induced animals indicate that tangeretin protects the renal tissue against the deleterious effects of DMBA by activating Nrf2/Keap1 signaling.

The nephroprotective nature of tangeretin in control and experimental groups of rats was ascertained by histological as well as ultrastructural studies, respectively. The oxidative stress induced by DMBA is accounts for major morphological abnormalities such as tubular cell swelling, cellular vacuolization, medullary congestion, severe lesions and necrosis in proximal tubular cells and glomerulus hypertrophy. Transmission electron microscopic studies also potentiate the histological observations showing degeneration of nuclei with chromatin condensation and loss of mitochondria between the folds of proximal convoluted tubules. Presence of lipid globules and swollen mitochondria showing the disappearance of cristae was also observed. However, administration of tangeretin to DMBA induced rats ameliorated all of those histological and ultrastructural alterations in the kidney tissues establishing its protective nature during oxidative stress. This is the first study showing the protective nature of tangeretin on the kidney tissue in DMBA induced oxidative stress.

Presence of inflammatory mast cells creates a microenvironment that reflects a persistent inflammatory state, thereby fostering tissue damage. Lipid accumulation is associated with increased oxidative stress and DMBA has been reported to induce lipid accumulation in several tissues such as breast, liver and kidney. Several reports have suggested that renal lipid accumulation, lipotoxicity, is associated with the development of renal injury. Oxidative stress prevents the production or assembly of the type IV collagen network, which is an important structural component of basement membranes in the kidney, eventually leading to kidney failure. PAS staining is used to highlight basement membranes of glomerular capillary loops and tubular epithelium. Any abnormality in the distribution of basement membrane results in improper filtration of waste products from the blood and denotes renal injury. In the present study, excess accumulation of mast cells, collagen, lipids and derangement in basement membrane structure were observed in DMBA induced group of rats. However, oral tangeretin treatment to DMBA induced animals showed that tangeretin protects the kidney from DMBA induced oxidative stress.

The present study reports for the first time to our knowledge that tangeretin ameliorates DMBA mediated oxidative stress in the
kidney tissues which was evidenced by the decline in oxidant and proinflammatory cytokine production. Moreover, tangeretin treatment to DMBA induced rats attenuated the oxidative stress, thereby improving the ultrastructure of the kidney cells.

**Conflict of interest**

The authors declare no conflict of interest.

**Transparency document**

The Transparency document associated with this article can be found in the online version.

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