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Research Article

# Modifying role of apigenin in angiogenesis and anti-oxidant status in experimentally induced breast cancer in rats

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## ABSTRACT

**Background:** Angiogenesis and agents which inhibit it are currently an alternative approach to anti-cancer therapy. Tumor metastasis and resistance to chemotherapy are factors to be considered in the management of breast cancer. Apigenin, a flavone is documented to possess anti-inflammatory, anti-oxidant, and anti-proliferative effects *in vitro* studies, but activity *in vivo* is still hypothetical.
**Methods:** Apigenin at doses of 50,100, 200 mg/kg body weight and tamoxifen at 50 mg/kg was administered to female albino Wistar rats and 7,12, dimethylbenzanthracene was used to induce mammary carcinogenesis. The anti-oxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase were estimated in breast tissue and erythrocyte lysate with thiobarbituric acid reactive substances as an indicator of lipid peroxidation. Immunohistochemical staining for vascular endothelial growth factor (VEGF) protein expression was done to study its role in angiogenesis. The statistical significance of the data was determined using one-way analysis of variance and Dunnett's multiple range test.
**Results:** Apigenin at doses of 100 mg/kg and 200 mg/kg (<0.05) was most effective in modifying the anti-oxidant status in breast tissue and in inhibiting VEGF expression in the immunohistochemical analysis in comparison with tamoxifen.
**Conclusion:** The results of our study implicate that apigenin, an innocuous agent could help alleviate the oxidative stress in breast cancer tissues, minimize toxicity of anti-cancer drugs and also slow down the process of angiogenesis in breast cancer.

**Keywords:** Angiogenesis, Thiobarbituric acid reactive substances, Apigenin, Anti-oxidant enzymes, 7,12, dimethylbenzanthracene, Tamoxifen, Vascular endothelial growth factor

## INTRODUCTION

Cancer is a major public health burden worldwide, both in developed and developing countries with a fatality of more than 7 million every year. By definition, cancer is the uncontrolled growth and spread of malignant cells that may affect any tissue, presenting as different forms in each area of the body. On a global scale, breast cancer has been the focus of intensive study as it is the 2nd most common type of cancer in women. In spite of readily accessible methods of diagnosis, it continues to exact a heavy toll in Indian women next to cervical cancer.[1] Molecular genetics involving specific mutational changes alters the functional control of growth, promoting proteins (i.e.,) gene amplification that increases protein expression, which accounts for resistance to chemotherapy. Metastasis of tumors by blood or lymphatics also accounts for poor prognosis.[2]

Angiogenesis inhibition and anti-oxidant potential of substances have recently become the focus of study for cancer therapy and prevention. Such drugs inhibit growth of new blood vessels that provide oxygen and nutrients, essential for tumor growth. Vascular endothelial growth factor (VEGF) expression is a marker implicating tumor growth, invasion, and metastasis. VEGF-A receptors are expressed in breast cancers signifying tumor growth and metastasis. Inhibition of VEGF expression now serves as a target in therapy.[3]

Several chemopreventive agents are in vogue to diminish the morbidity and mortality of cancer by delaying the progress of carcinogenesis. Treatment modalities involving chemotherapeutic agents are expensive, highly toxic to normal tissues and produce variable responses, which serves as a major limiting factor. Newer targeted strategies include chemoprevention, which aims at prevention or reversal of

initiation phase of carcinogenesis or arrests progression of growth by the administration of pharmacological drugs or natural agents.[4]

Flavonoids[5] are the most common widely distributed polyphenolic compounds, ubiquitously present in a variety of fruits and vegetables. Apigenin, a naturally occurring plant flavone (4',5,7,-trihydroxyflavone) present in parsley,[6] onions, oranges, chamomile and wheat sprouts, has been shown to possess anti-inflammatory, anti-oxidant and anti-proliferative effects in various malignant cell lines. Activity *in vivo* is yet to be substantiated. Therefore, the objective of our present study with the flavone apigenin is to evaluate its anti-oxidant effect and inhibition of angiogenesis in comparison with tamoxifen, a selective estrogen receptor (ER) modulator (SERM) used in the treatment of breast cancer.[7]

## METHODS

### *Chemicals and carcinogen*

Apigenin was purchased from Ruland Chemicals, Hong Kong, the purity of apigenin was estimated to be 98.12% and the botanical source *Apium graveolens* var. dulce. Dimethyl sulfoxide (DMSO) from Sun Pharma, India. 7,12, dimethyl benzanthracene (DMBA) was purchased from Sigma Chemical Company (St. Louis, M.O, USA). Tamoxifen from Cipla Pharma, India. All other chemicals and reagents used were of analytical grade.

### *Preparation of the drug*

Apigenin powder was used as a suspension in 1% DMSO, and each rat received a daily dose of 50, 100 and 200 mg/kg body weight in 2 ml of suspension by oral gavage. DMBA 25 mg/kg,[8] dissolved in an emulsion of sunflower oil (0.75 ml) and physiological saline given subcutaneous in the 4th and 5th week, was prepared just prior to use. Tamoxifen was dissolved in distilled water and administered by oral gavage.

### *Animals and diet*

About 36 female albino Wistar rats 6-8 weeks old weighing 150-180 g were purchased from the Central Animal House, Rajah Muthiah Medical College, Annamalai University, Chidambaram, Tamil Nadu, South India. The approval of Institutional Animal Ethical Committee was obtained (proposal no: 748) prior to research. The animals were randomized into 6 groups of 6 rats in each group. The rats were housed at room temperature. (25±1°C) and humidity (55±5%) with 12 light/dark cycle. Animals were maintained under standard laboratory conditions as per the principles and guidelines of the Ethical Committee for Animal Care of Annamalai University in accordance with the Indian National Law on Animal Care and Use (Reg. No. 160/1999/CPCSEA). The animals were fed with pellet feed and Bengal gram and water *ad-libitum*. The study was carried out for a period of 16-week.

### *Experimental protocol*

Breast cancer was induced by DMBA, a site-specific pro-carcinogen with selectivity for inducing breast cancer in experimental rats.[8] The dose selected was 25 mg/kg subcutaneous, once a week in the 4th and 5th week. The carcinogenic activity of this drug requires metabolic activation by myeloperoxidase in rat liver microsomes. The dihydrodiol peroxide binds with adenine residues and DNA resulting in mutagenesis and carcinogenesis.

The mammary tumors appeared by 7-9 weeks of the experimental period. While Groups IV-VI received apigenin at doses of 50 mg, 100, 200 mg/kg/day. Group III was administered tamoxifen 40 mg/kg/d as a standard anti-cancer drug. The weight of the animals and signs of morbidity/mortality was observed at weekly intervals until the end of the study period. Rats in all groups received normal pellet feed and water *ad-libitum*.

| | | |
|---|---|---|
| Group I | Control | Pellet feed and water DMSO orally for 16 weeks |
| Group II | 7,12 DMBA | 7,12 DMBA (25 mg/kg subcutaneous) once a week in the 4th and 5th week |
| Group III | Tamoxifen | Tamoxifen 40 mg/kg orally by gavage for 16 weeks+7,12 DMBA |
| Group IV | Apigenin 50 mg/kg | Apigenin 50 mg/kg orally by gavage for 16 weeks+7,12 DMBA |
| Group V | Apigenin 100 mg/kg | Apigenin 100 mg/kg orally for 16 weeks+7,12 DMBA |
| Group VI | Apigenin 200 mg/kg | Apigenin 200 mg/kg for 16 weeks+7,12 DMBA |

DMSO: Dimethyl sulfoxide, DMBA: 7,12, dimethylbenzanthracene

The experiment was terminated by the 16th week and all the animals sacrificed. Blood samples for biochemical analysis and breast tissue samples were excised and sent for anti-oxidant enzymatic estimation and immunohistochemical evaluation of VEGF.

### *Blood collection and biochemical analysis*

After sacrifice blood had been collected in vacutainer and centrifuge at 5000 rpm for 10 mins then serum was separated and analyzed for:

*Estimation of erythrocyte thiobarbituric reactive acid substitutes (TBARS)*[9]

Serves as an indicator of lipid peroxidation TBARS in erythrocytes was estimated by the method of Donnan. Red blood cell (RBC) lysate was prepared by lysing a known volume of erythrocytes by addition of two volumes of

distilled water to packed erythrocytes and centrifuged at 3000 rpm for 10 mins at 4°C to separate the erythrocyte lysate. The reaction mixture in a total volume of 1.7 ml containing 0.2 ml F erythrocytes/erythrocyte membrane and 1.5 ml of 10% trichloroacetic acid was filtered through Whatmann filter paper. Thiobarbituric acid was added to the portion of filtrate (0.6-0.8 ml) and heated in a boiling water bath for 15 mins, cooled to room temperature, and the color developed measured at 620 nm.

*Estimation of tissue TBARS*[9]

Lipid peroxidation in tissue (TBARS) was estimated by the method of Ohhawa et al. To 0.2 ml of tissue homogenate, 0.2 ml of 811% sodium dodecyl sulfate and 1.5 ml of glacial acetic acid 20% were added. pH was adjusted to 3.5 with NaOH, and then 1.5 ml of 0.8% TBA was added to the mixture and volume made up to 4 ml. Reaction mixture was heated in oil bathat 95°C for 60 mins. 1 ml distilled water added after cooling with 5 ml N-butanol pyridine and shaken vigorously. After centrifugation at 4000 rpm for 10 mins, the supernatant was removed and absorbance was read at 620 nm.

*Estimation of anti-oxidant enzymes*

Estimation of superoxide dismutase (SOD) (Kakkar et al.,).[10] Catalase (CAT) was assayed by the method of Sinha[11] and glutathione peroxide by the method of Rotruck et al.,[12] The results were compared with Groups I and III and tabulated.

*Immunohistochemical analysis-VEGF*[13]

Two to three sections of 3 µm thickness were prepared and taken in precoated slides. The blocking of endogenous peroxides by 3% hydrogen peroxide for 10 mins followed by protein block to avoid cross-reaction. After a buffer wash, sections were covered with optimally diluted VEGF monoclonal mouse antibody (Dako Denmark) for 1 hr, then washed with buffer and treated with poly horseradish peroxidase enzyme [Dako Real cn Vision] for 30 mins. AB) Immunostaining for 5 mins with freshly prepared 3, 3′-diaminobenzidine (DAB) for 5 mins and visualization was carried out using (DAB) (DakoCytomation, Germany) as a chromogen. Counter staining with Mayer's hematoxylin for 1 min. Sections were dehydrated in graded alcohol, air dried and mounted with DPX. Sections were analyzed using a Nicon Eclipse 80i microscope.

***Statistical analysis***

The statistical significance of the data was determined using one-way analysis of variance, and the significant difference between the treatment groups was evaluated by Dunnett's multiple range test. Fischer's exact test was used to signify the VEGF expression. The results were considered as statistically significant at $p<0.05$.

## RESULTS

Mortality >80% with weight loss was observed in the DMBA treated groups when compared with other drug treated groups. No statistically significant difference was observed between drug treated groups in regard to weight gain/loss.

***Effect on lipid peroxidation***

TBARS in plasma and breast tissue were significantly elevated (<0.05) in the DMBA treated group (Tables 1 and 2) indicating an increase in tissue lipid peroxidation. Apigenin at doses of 100 and 200 mg/kg (Graphs 5 and 6) showed a significant reduction in these levels comparable to normal controls. A similar reduction was also observed with tamoxifen (Group III).

***Effect on anti-oxidant enzymes***

The levels of RBC lysate and breast anti-oxidant enzymes, SOD and glutathione peroxidase (Gpx) were significantly decreased in DMBA treated rats (Tables 1 and 2). Similar reduction was evident in CAT levels in Groups II and III, suggestive of oxidative damage to breast tissues. Apigenin at 100 mg/kg and 200 mg/kg replenished these deficiencies

**Table 1: Effect of apigenin and DMBA on tissue TBARS and anti-oxidant enzyme levels.**

| Groups | Tissue TBARS (nmoles/mg tissue) | Tissue SOD (U/mg protein) | Tissue GPx (U/mg protein) | Tissue CAT (U/mg protein) |
|---|---|---|---|---|
| Group I | 13.33±0.68 | 10.17±0.24 | 89.01±4.55[b] | 86.54±1.28 |
| Group II | 53.55±0.50 | 1.20±0.25 | 50.09±5.58 | 9.46±0.89[a] |
| Group III | 18.17±0.73 | 6.24±0.43 | 71.95±3.52 | 49.35±4.84 |
| Group IV | 22.01±1.99 | 6.88±0.30 | 60.73±0.44[c] | 58.98±4.75 |
| Group V | 19.09±0.86 | 6.44±0.23 | 67.85±1.47 | 65.04±1.14 |
| Group VI | 15.82±1.06[d] | 8.54±0.51 | 80.87±2.50 | 78.08±1.54 |

All the values are expressed as mean±SD of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other ($p<0.05$) DMRT. DMBA: Dimethylbenzanthracene, TBARS: Thiobarbituric acid reactive substances, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase, SD: Standard deviation, DMRT: Dunnett's multiple range test

**Table 2: Effect of Apigenin and DMBA on plasma TBARS and anti-oxidant enzyme levels.**

| Groups | Plasma TBARS (nmoles/mg tissue) | Plasma SOD (U/mg protein) | Plasma GPx (U/mg protein) | Plasma CAT (U/mg protein) |
|---|---|---|---|---|
| Group I | 3.35±1.07 | 20.68±0.57 | 76.55±0.41[a] | 6.15±0.23 |
| Group II | 22.56±0.53 | 1.77±0.29 | 16.26±0.54 | 0.05±0.36[b] |
| Group III | 8.63±0.40 | 10.84±0.45 | 60.43±0.77[b] | 3.27±0.35 |
| Group IV | 6.70±0.28 | 6.94±0.81 | 55.83±0.72 | 2.78±0.26 |
| Group V | 5.80±0.46 | 8.53±0.24 | 37.74±0.39[c] | 3.12±0.33 |
| Group VI | 4.30±0.50[d] | 13.00±0.82 | 66.02±0.95 | 4.02±0.21 |

All the values are expressed as mean±SD of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other ($p<0.05$) DMRT. DMBA: Dimethyl benzanthracene, TBARS: Thiobarbituric acid reactive substances, SOD: Superoxide dismutase, DPx: Glutathione peroxidase, CAT: Catalase, DMRT: Dunnett's multiple range test, SD: Standard deviation

**Table 3: VEGF expression in DMBA treated, experimental and control groups.**

| Groups | VEGF expression | | Percentage |
|---|---|---|---|
| | Positive | Negative | |
| Group I | – | ++++++ | 100 (negative) |
| Group II | ++++++ | – | 100 (positive) |
| Group III | ++ | – | 30 (negative)* |
| Group IV | +++ | – | 40 (negative)* |
| Group V | +/– | +++++ | 80 (negative)** |
| Group VI | – | +++++ | 95 (negative)** |

(+++) When >50% of cells staining positive, (++) when 25-50% of cells staining positive, (+) when 5-25% of cells staining positive, (-) when <5% of cells staining positive were observed. The statistical significance for two variables was done with Fischer's exact test. The results are expressed as percentages of control and represented as mean SE; n=6; *$p<0.05$; **$p<0.01$, VEGF: Vascular endothelial growth factor, DMBA: Dimethylbenzanthracene

and restored enzymes to normalcy (Tables 1 and 2).

### *VEGF expression*

Fischer's exact test was applied to analyze the contingency tables when the sample size is small because the significance of deviation for a null hypothesis can be calculated exactly than relying on an approximation (Figure 1 and Table 3).

The protein expression was scored as follows:

1. (+++) When >50% of cells staining positive
2. (++) When 25-50% of cells staining positive
3. (+) When 5-25% of cells staining positive
4. (-) when <5% of cells staining positive were observed.

## DISCUSSION

Angiogenesis a prerequisite for tumor growth and metastasis has been recognized as a critical element in primary tumor growth and has become a target for the development of new anticancer agents.[14] VEGF-A is a vascular endothelial mitogen and a stimulator of angiogenesis. As a critical

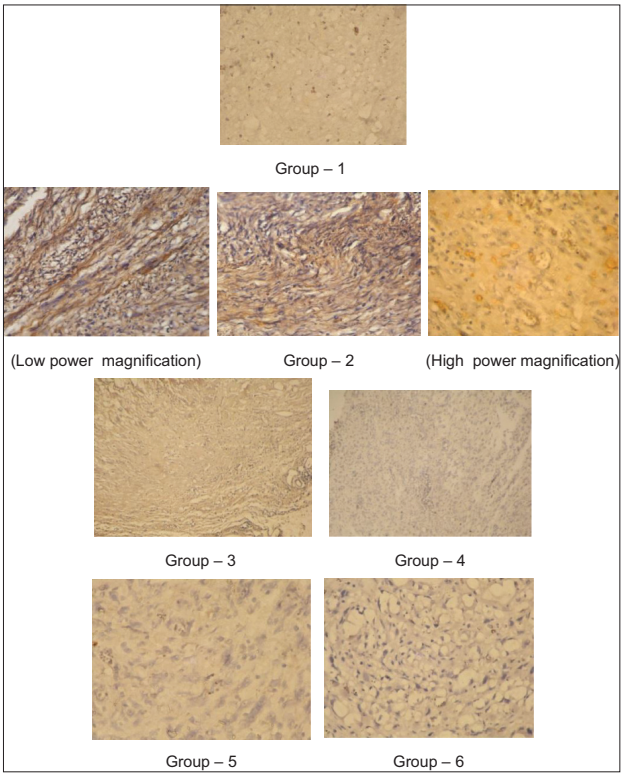


**Figure 1: Vascular endothelial growth factor expression in dimethylbenzanthracene treated, experimental and control groups.**

regulator of tumor vascularization it stimulates endothelial cell survival, mitogenesis,migration, differentiation and self-assembly of vascular elements. VEGF expression, both in tumor and circulation correlates with poor patient prognosis in cancer. Cancer cells expressing VEGF proteins[15] constituently provide a paracrine mechanism to induce angiogenesis.

Our present study which includes immunohistochemical staining of rat breast tissue showed maximal VEGF protein expression in DMBA treated animals. This was greatly reduced in the apigenin-treated groups at doses of 100 mg/kg and 200 mg/kg. Of the several mechanisms postulated, apigenin possibly exerted its anti-angiogenic action by inhibition of ERK½ and PI3K/Akt pathways.[16]

Chen et al., demonstrated the proteosome chymotrypsin -like inhibitory effect of apigenin in cultured breast cancer MDA-MB-231 cells,[17] which further substantiates it is apoptotic and tumor inhibitory effects.

Carcinogenesis is a multistage process involving reactive oxygen species generated during the metabolism of drugs. Cytotoxic metabolites so formed tilts, the normal cell redox balance, leading to oxidative stress,[18] triggering healthy cell damage and predisposing to cancer. Lipid peroxidation is a consequence of oxygen species such as hydroxyl radicals, superoxide anion and singlet oxygen targeting the polyunsaturated fatty acids in the cell membranes. Lipid peroxidation is also strongly associated with aging and carcinogenesis.[19] In the presence of oxidative stress, more lipid peroxidation products are formed and released into the serum due to cell damage. However, living systems are protected from active oxygen species and oxidative stress by cellular anti-oxidant enzymes such as SOD, Gpx and CAT.

Anti-oxidant enzymes SOD, Gpx and CAT play a pivotal role in the cellular defense mechanisms,[20] in the setting of oxidative stress by scavenging the free radicals. SOD and CAT are the two major enzymes that are directly involved in the elimination of ROS. Present in cytosol and mitochondria, it converts superoxide anion to hydrogen peroxide and water. SOD protects tissues against oxygen free radicals by catalyzing the removal of superoxide that damages the membrane and biological structure. SOD and CAT levels showed a statistically significant reduction in the DMBA treated group but were elevated to near normal values in the apigenin-treated groups, as observed in our present study.

Gpx is a cytoplasmic enzyme that catalyzes the detoxification of hydrogen peroxide to $H_2O_2$ using the reducing equivalents of glutathione. Gpx is a seleno-enzyme[21] two-third of which (in liver) is present in the cytosol and one third in the mitochondria. It catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulfide and the reduction product of the hydroperoxide. Yet another role is the stability of erythrocytes by glutathione, preventing hemolysis. Apigenin in doses of 100 mg/kg and more effectively at 200 mg/kg supplemented the reduced glutathione stores in the drug treated groups.

Mammary quiescence was induced with the SERM, tamoxifen,[22] an agent known to suppress proliferation of mammary epithelium in ER-positive tumor cells. In our study, this expected anti-proliferative effects of tamoxifen on mammary epithelium was observed. Acquired resistance of breast cancer cells to the antiestrogens tamoxifen and fulvestrant[23] is accompanied by the dysregulation of ERα-dependent signaling molecules, such as co-activators, as well as altered receptor-independent growth pathways, such as protein kinases. To effectively block proliferation of antiestrogen-resistant breast cancer cells, it may be necessary to identify a drug(s) that can target both of these important pathways. At high concentrations, apigenin down regulates protein levels of ERα and AIB1 and inhibits protein kinases p38, MAPK, PKA, and AKT/PI3K, leading to growth inhibition.[24] An exciting aspect of this study is that apigenin has the potential to inhibit both ERα-dependent pathway and protein kinase-mediated growth factor signaling pathways. As both pathways are commonly altered in antiestrogen-resistant breast cancer, these broad effects of apigenin[25] may be synergistic in combination with antiestrogens in growth inhibition of antiestrogen-resistant breast cancer cells.

Chemoprevention aims at prevention or reversal of the initiation phase of carcinogenesis or arrest at the progression of carcinogenesis through the administration of naturally occurring constituents or pharmacological agents. Considerable attention has been devoted to identifying plant-derived dietary agents which could be developed as promising agents in amelioration of cancer toxicity and improving the host defense systems.[26] Apigenin thus fits into a recently proposed novel paradigm for the treatment of drug-resistant breast cancer by signal transduction inhibitors in combination with antiestrogen therapy. Based on its ability to target both ERα-dependent and-independent pathways, apigenin warrants further investigation as a therapeutic agent for both antiestrogen-sensitive and-resistant breast cancer.

## CONCLUSION

This article highlights the role of apigenin in decreasing VEGF expression in rat breast tissue *in vivo* and in increasing the anti-oxidant enzymes which help to supplement/scavenge reactive oxygen species in plasma and tissues. Taken together, apigenin an innocuous natural substance appears to be a promising novel anti-cancer agent which when used as a chemosensitizer and/chemopreventive agent, which may help mitigate toxicity and boost the effectiveness of current chemotherapeutics.

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