PROTECTIVE EFFECT OF QUERCETIN AGAINST 7, 12 DIMETHYLBENZ(A)-ANTHRACENE INDUCED BREAST CANCER IN RATS

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Abstract
The present study aimed to analyze the anticancer property of quercetin on inhibition of breast carcinoma induced by 7,12- dimethylbenz(a) anthracene (DMBA) in experimental animals. At the end of experimental period, 100% tumor formation was observed in DMBA induced animals. Quercetin at the dose of 100mg/kg body weight was given orally to DMBA induced animals for 30 days after a palpable tumor was observed in the breast. The serum and mammary tissues of experimental rats were analysed for serum tumor markers and levels of antioxidant enzymes. Quercetin effectively increased the levels of antioxidants in DMBA induced cancerous rats and also reduced the levels of tumor markers on quercetin supplementation. Our results demonstrate that quercetin may perhaps maintain the antioxidant levels and reduce the serum tumor markers thereby exerting chemopreventive potential.

Key words: Quercetin, Antioxidant, DMBA, Breast cancer.

Background
Breast cancer is the most common cancer among women. According to the American breast cancer society, it has been estimated that 1 in 8 females may develop breast cancer. Breast cancer death rates have been decreased by 34% from 1990 to 2010 (3.1% younger than 50 yrs and 1.9% above 50yrs) (1). Use of natural compounds in the treatment of cancer played an important role in the last half decade. There is an emerging need for the discovery of a natural drug, since the cancer cells are gaining resistance to the existing chemotherapeutic agents. Though the currently available drugs kill the cancer cells, there also exist the issue of toxicity. Hence, there is a need to identify more natural compounds for the treatment of cancer.

Phytocompounds contain flavonoids, polyphenolic compounds with protective effects against various diseases. Quercetin is one such plant-derived flavonoid, which is widely distributed in human diet like onion, apple,
Quercetin helps prevent cancer by blocking the flow of nutrients and oxygen to cancerous cells, effectively cutting off their food supply. Quercetin is also a phytoestrogen, or a plant hormone that mimics the effects of estrogen in the human body. Quercetin binds to estrogen-receptor in place of estrogen, so that breast cancers that need estrogen to flourish are no longer stimulated to grow.

Laboratory studies have shown that quercetin from citrus fruits can reduce the growth rate of breast cancer cells by as much as 50 percent. In addition, quercetin has been shown in animal and in vitro studies to inhibit the growth of colon, prostate, breast, lung cancer cells and as an anti-depressant (4,5).

Free radical induced oxidative stress in cancer is the major debate in the last two decades. The antioxidant enzymes can minimise free radical toxicity, out of which superoxide dismutase (SOD), catalase and glutathione peroxidise (GPx) play a major role in neutralising the free radicals. Therefore, the present study aims to investigate the effect of quercetin as an antioxidant and anticarcinogenic flavonoid in DMBA induced mammary carcinoma.

**Materials and methods**

**Source of Chemicals**

Quercetin and 7,12 – dimethylbenz(a)-anthracene (DMBA) were purchased from Sigma (St. Louis, USA) and all other chemicals used were of analytical grade.

**Experimental animals**

Healthy Wistar female rats of 20-30 days, weighing 80-120g were obtained from Tamil Nadu University of Veterinary and Animal Sciences (TANUVAS), Chennai. The animals were housed, three per polypropylene cage, in controlled environmental conditions of temperature (24 ± 4°C) and relative humidity (60 ± 5%) on alternate 12h light/dark cycles. After 1 week of acclimatization, rats were randomly divided into different groups of six animals each and allocated in plastic cages covered with a metal grid and bedded with sawdust. All animals were fed standard pellet diet (Amrut Laboratory Animal Feed, Bangalore, India; containing protein 22.06%, oil 4.28%, fibre 3.02%, ash 7.8, sand (silica) 1.37% w/w, together with a mixture of vitamins) and water ad libitum.

All animals received human care and the study was conducted in compliance with the ethical norms approved by the Ministry of Social Justice and Empowerment, Government of India and Animal Ethics Committee Guidelines, University of Madras, Chennai, India (IAEC No.01/038/08).
Experimental design and induction of mammary carcinoma

Previous research suggests that 30mg/kg to 500mg/kg of body weight of quercetin for different time periods, given orally to DMBA induced rats gave significant tumor reduction. However, a pilot study was done to fix the optimum dose of quercetin. Quercetin was dissolved in warm water as it is sparingly soluble in water. Four different doses (50, 100, 150 and 200mg/kg body weight) of quercetin were selected for the study, out of which 100mg/kg body weight of quercetin given orally did not show toxicity in liver (Data not shown). Hence, 100mg/kg body weight dose was fixed for this study. Animals were weighed every day and observed every morning and evening for mortality, if any. The maximum gain in weight compared to the control was recorded. The induction processes were terminated after 30 days and all the animals were sacrificed by cervical dislocation after overnight fasting. Blood was collected in tubes containing anticoagulant, from which plasma was separated. The experimental animals were divided into four groups, with six animals in each group. Group 1 animals served as normal control. Group 2 animals were treated with quercetin alone (100mg/kg of body weight / rat) for 30 days to study the cytotoxicity (if any) induced by quercetin. Group 3 animals were induced for mammary carcinogenesis, by a single ‘air-pouch’ administration of DMBA (25mg/ kg body weight), dissolved in 0.5ml corn oil. Group 4 animals were post-treated with a daily oral dose of quercetin (100mg/Kg body weight) for 30 days, after a palpable tumor was observed. Blood was collected from tail vein of the control and experimental groups of rats, the day before the euthanasia. All the groups of animals were fasted overnight at the end of the experimental period and euthanized using sodium pentothal anesthesia followed by cervical decapitation. The mammary tissues were excised and washed with ice-cold PBS, small part of tissues were fixed with 10% neutral buffered formalin for histological evaluation and the remaining tissues were snap frozen in liquid nitrogen and immediately stored at -80°C for further analysis.

Assay of tumor markers: The tumor markers Alpha-feto Protein (AFP), Carcinoembryonic antigen (CEA) and breast cancer specific marker (CA 15-3) were quantified based on solid phase enzyme linked immunosorbant assay method using UBI MAGIWELL (USA) enzyme immunoassay kit. The levels of all the markers were expressed in ng/ml in serum.

Enzymic antioxidants assay

The activities of antioxidant enzymes like superoxide Dismutase (SOD) (6), catalase (CAT) (7), glutathione peroxidase (GPx) (8) were assayed in serum and mammary tissue homogenates. The levels of non-enzymic antioxidants GSH, vitamin C and vitamin E were assayed in serum.
Histological examination

A portion of tissues was fixed in 10% neutral buffered formalin. The specimens were dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Tissue blocks were sectioned into 5µm thickness using a microtome. Then the sections were stained with Harris hematoxyline and eosin. The stained sections were examined under light microscope and photographs were captured.

Body weight observation

The changes in the body weight of experimental animals were monitored during the experimental period. Tumor volume was calculated using the equation \( V = \frac{4}{3} \pi r^3 \), where \( r \) is tumor radius.

Preparation of tissue homogenate

100mg of the mammary tissue was accurately weighed and homogenised in tissue homogenising buffer containing 25mM HEPES pH 7.4, 150mM NaCl, 1% NP-40, 10% glycerol, 25mM NaF, 10mM MgCl₂, 1mM EDTA and protease inhibitor cocktail using Teflon homogenizer at 4°C. The homogenate was centrifuged at 12000xg for 30 min at 4°C and the supernatant was pooled and used for estimations.

Statistical analysis

The values are expressed as mean values of six rats in each group ± standard deviation. Data analysis was done with SPSS 7 student software. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by post hoc testing performed with least significance difference test. The value of \( p < 0.05 \) was considered to indicate statistical significance.

Results

Toxicological observations

The effect of DMBA and quercetin on body weight gain is shown in table 1. The changes in body weight in quercetin treated rats (group 2) were monitored and compared with control rats during the treatment period to determine the toxic effect of quercetin, if any. The initial whole body weight of each rat was measured which was almost similar in all groups of animals. The recorded body weight of experimental rats after administration of quercetin showed no evidence of decrease in body weight. Quercetin treated rats had increased body weight over 4 weeks of time and gained body weight to near normal. No detectable changes were observed between group 1 and group 2 rats showing the non-toxic nature of quercetin at the selected dose.
Table-1 Percentage increase in body weight of experimental group of animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body weight</th>
<th>Final body weight</th>
<th>Body weight gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86.44±5.83</td>
<td>186.521±6.12</td>
<td>53.76±0.66</td>
</tr>
<tr>
<td>Quercetin alone</td>
<td>83.67±2.46</td>
<td>190.51±5.77</td>
<td>55.78±4.76</td>
</tr>
<tr>
<td>DMBA-Induced</td>
<td>97.92±5.45</td>
<td>148.18±6.113</td>
<td>33.78±4.65(^a)</td>
</tr>
<tr>
<td>DMBA-Induced + quercetin</td>
<td>98.165±6.28</td>
<td>170.52±9.57</td>
<td>42.35±5.85(^b)</td>
</tr>
</tbody>
</table>

Changes in body weight observed during the experimental period. Each value is expressed as mean±SD for six animals in each group. Statistical significance at \(p <0.05\). Comparison is made as \(^a\) DMBA-Induced Vs Control and \(^b\) DMBA-Induced Vs quercetin treated.

Effect of quercetin on breast tumor mass

The effect of quercetin on tumor volume and size are shown in table 2. Group 3 experimental rats were treated with DMBA and followed for mammary tumor production. Within 8-12 weeks of DMBA treatment, the animals formed a palpable tumor in the breast. The tumor incidence in DMBA treated rats was 100\% whereas normal and quercetin alone treated rats showed 0\% incidence. The tumor mass was measured and scaled for each animal, before and after quercetin treatment. A significant increase in the tumor volume (1.31 cm\(^3\)) was observed in DMBA induced group 3 rats as compared to normal rats. Quercetin treatment significantly reduced the tumor volume (0.3 cm\(^3\)) to 50\% (Figure 1).

Table-2: Anticancer effect of quercetin in experimental group of animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean tumor volume (cm(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Number of animals</td>
<td>6</td>
</tr>
<tr>
<td>Tumor incidence</td>
<td>0</td>
</tr>
<tr>
<td>Tumor regression</td>
<td>0</td>
</tr>
<tr>
<td>Tumor volume (cm(^3))</td>
<td>0</td>
</tr>
</tbody>
</table>

Reduction of tumor volume observed after treatment with quercetin in DMBA induced group of animals. Statistical significance at \(p <0.05\). Comparison is made as \(^a\) DMBA-Induced Vs Control and \(^b\) DMBA-Induced Vs quercetin treated.
Histological examination

Figure 1 represents the histological examination of mammary tissue sections of experimental groups of rats. Group 1 and group 2 rats showed normal ducts and lobules architecture which are the signs of normal growing cells. DMBA induced cancerous rats showed abnormal mitosis, cellular hyperplasia with high levels of infiltrating cells and loss of extracellular matrix which were the signs of malignancy. However, the cancerous rats treated with quercetin showed reduced cellular infiltration, as normal architecture when compared to the control rats. This result proves the chemopreventive potential of quercetin.

Effect of quercetin on the activities of enzymic antioxidants

Figure 2 shows the activity of enzymic antioxidants such as SOD, catalase and GPx in mammary tissues of control and experimental animals. A significant decrease in the activity of enzymic antioxidants was observed in DMBA induced rats when compared to the control rats. Treatment of quercetin to DMBA induced rats significantly increased the activity of enzymic antioxidants.

The effect of quercetin on enzymic antioxidant levels in experimental animals.
Effect of quercetin on serum tumor markers

The levels of serum tumor markers like Alpha-feto protein (AFP), carcinoembryonic antigen (CEA) and breast cancer specific marker (CA-15-3) were analysed in the serum of control and experimental groups of animals. The DMBA induced rats showed a significant increase in levels of tumor markers when compared to control rats. However, the cancerous rats treated with quercetin significantly reduced the levels of tumor markers which proves the effect of quercetin on reduction of tumor cells (Figure 3).

Levels of tumor markers in serum of experimental animals.

![Graph showing levels of tumor markers](image)

**Fig 3:** Levels of alpha-feto protein (AFP), breast cancer specific marker CA 15-3 and Carcinoembryonic antigen (CEA) in the serum of control and experimental group of animals. Each value is expressed as mean±SD for six animals in each group. Statistical significance is \( p < 0.005 \). Comparison is made as group 1 Vs group 3\(^a\) and group 3 Vs group 4\(^b\). CA 15-3 is expressed as units/ml and AFP and CEA are expressed as ng/ml.

Effect of quercetin on expression of heat shock proteins

Figure 4 represents the expression of heat shock protein 70 (HSP70) in control and experimental groups of animals. The DMBA induced rats showed a significant increase in the expression of HSP70 when compared to control, whereas the cancerous rats treated with quercetin significantly reduced the expression of HSP70 when compared to the induced group of animals.
Western blot analysis showing the expression of HSP70 protein in control, induced and treated mammary tissues.

Lane 1. Control, Lane 2. DMBA-induced animal, Lane 3. Quercetin treated animal after tumor induction

Fig 4: Immunoblot analysis of HSP70 (~70 KDa) in control, DMBA induced and quercetin treated animals. Bar graph shows normalized densitometry readings (using Image J software) indicating the protein density compared to that of β-actin and are expressed as Mean ± SD. The data shown were combined from three independent experiments with statistical significance at \( p<0.05 \).

Discussion

In the present study, we investigated the activities of enzymic antioxidants and non-enzymic antioxidants to elucidate the role of quercetin in mammary carcinoma. Antioxidants play a vital role in cellular defence mechanism. SOD converts \( O_2^- \) into oxygen and \( H_2O_2 \), whereas GPx and catalase convert \( H_2O_2 \) into water. In this way the antioxidants help to convert the harmful substances in the body to harmless products (9, 10). Biochemical analysis of SOD, catalase and glutathione peroxidase is a routine step in determining the oxidative stress experienced by a cell (11) and to determine the antioxidative properties of plant extracts (12). In the present study, DMBA induced rats showed decreased levels of SOD, catalase and GPx activity when compared to the control rats. However, quercetin treated
rats significantly increased the levels of antioxidant enzyme activity to near normal when compared to the DMBA induced rats.

The histological examination also revealed that DMBA induced cancerous rats showed signs of malignancy like high infiltrating cellular morphology, abnormal mitotic cells and loss of extracellular matrix whereas the quercetin treated cancerous rats showed normal cellular architecture. This result clearly revealed the chemopreventive potential of quercetin against breast carcinoma.

The tumor markers are substances found in the body usually in blood or urine when cancer is present. The detection of serum markers in breast cancer helps in early diagnosis, determining prognosis, response to specific therapies and surveillance after surgery (13). A large number of tumor markers have been proposed in breast cancer out of which AFP, CEA and CA 15-3 are most widely used in breast cancer (14). All the three tumor markers are found to be elevated in the DMBA induced rats when compared to the control rats whereas the quercetin treated rats showed suppressed levels of tumor markers when compared to the induced group of rats. This result clearly revealed that quercetin acts as an anticancer agent by suppressing the tumor markers, thereby preventing the initiation and progression of breast cancer.

Heat Shock Proteins (HSP’s) are induced by heat shock or by any other stress and they function as intra-cellular chaperons for other proteins. Their important role in protein-protein interaction is to maintain proper folding of the protein and maintain protein conformation and avoid unwanted protein aggregation. In cancer, HSP70 helps cancerous cells to escape from apoptosis by inhibiting caspase activation. Activation of caspase-3 results in DNA fragmentation, formation of apoptotic bodies and finally uptake by phagocytic cells (15). Hence HSP’s are highly involved in cancer progression. The expression of HSP70 was significantly increased in DMBA induced rats when compared to the control rats. This may be due to the fact that heat shock proteins promote cancer cell survival, growth and metastasis by allowing continuous translation and cellular proliferation (16). However, quercetin treated rats showed a suppressed expression of HSP70. This result predicts that quercetin could inhibit the breast cancer growth by controlling the expression of heat shock protein 70 which possibly allows activation of caspase-3 thereby allowing the cells to undergo apoptosis (17).

Conclusion: In conclusion, quercetin, due to its antioxidant and anticancer property acts as a chemopreventive agent against mammary carcinoma. The supplement of quercetin maintains the antioxidant levels thereby preventing and regressing the development of mammary carcinoma in DMBA induced cancerous wistar rats.
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Conflict of interest: The authors declare no conflict of interest.

References


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