Chapter-VII

Quercetin inhibits chemically induced two-stage skin carcinogenesis in Swiss albino mice: Modulation of oxidative stress and inflammation
Introduction

Cancer is the second leading cause of death worldwide. Although, enormous progress made for the diagnosis and treatment of various types of cancer, still we do not have the single magic drug that can completely and selectively destroy cancerous cells.

Overall, present therapeutic approaches are painful and inadequate. Importance has been given to natural products to reduce the burden of this horrible disease. Different epidemiological and laboratory observation revealed that use of natural products seems to be a good promising approach to reduce cancer load (Surh, 2003). A Range of natural products derived from different plant sources have been reported to reduce the risk of various degenerative diseases due to their antioxidant and anti-inflammatory potential (Velasco et al., 2008; Thomasset et al., 2007; Wattenberg, 1993; Steimetz, 1991).

Quercetin (3, 3’, 4’, 5,6-pentahydroxyflavone) is a naturally occurring and widely distributed potent polyphenolic flavonoid found in many fruits, vegetables, nuts and red wine, possesses various biological activities including antioxidant, anti-inflammatory and ant-tumorigenic properties (Gibellini et al., 2011; Linsalata et al., 2010; Boots et al., 2008; Ruiz et al., 2007; Morand et al., 1998). Various previous findings showed that it has strong chemopreventive potential against different types of cancer and also capable enough to modulate different signaling pathways associated with cell inflammation, proliferation and survival (Xiao et al., 2011; Gibellini et al., 2011; Lee and Park, 2010; Lee et al., 2009; Choi et al., 2008).

Cancer is affected by alterations in various biological events, such as inflammation, differentiation and angiogenesis. The condition of chronic inflammation has been associated with most of the chronic human diseases, such as cancer, cardiovascular disease, diabetes, obesity, pulmonary and neurologic disorders. Chronic inflammation plays vital role in cellular transformation, promotion, survival, proliferation, invasion,
angiogenesis and metastasis involved in carcinogenesis (Mantovani, 2005). Epidemiological studies show that chronic inflammation predisposes individuals to different types of cancer and about 15% of all global cancers are initiated by chronic inflammation (Balkwill and Mantovani, 2001).

A range of cellular and molecular inflammatory mediators, such as macrophages, neutrophils, eosinophils, dendritic cells, mast cells and lymphocytes are known to play important roles in tumor development. Most of these inflammatory cells are capable enough to produce cytokines, cytotoxic mediators including reactive oxygen/nitrogen species, proteases, MMPs, membrane-perforating agents and soluble mediators of cell killing (Coussens and Werb, 2002). Improper regulations of redox sensitive signal transduction pathways induce by various inflammatory stimuli have been implicated in carcinogenesis. Array of research finding delineates about the critical role of cyclooxygenase-2, iNOS and the ubiquitous redox sensitive transcription factor NF-kB in inflammation associated cancer development (Allavena et al., 2008).

Topical application of phorbol ester induces inflammatory responses which are closely associated with promotion phase of carcinogenesis. In response to proinflammatory stimuli, activated inflammatory/immune cells generate ROS and RNS, over production of these free radicals lead to oxidative stress which plays important role in inflammation-driven carcinogenesis (Cerutti and Trump, 1991).

Promotion stage of carcinogenesis is a complex step that involves proliferation of the initiated cells and induction of genomic instability to these proliferating cells by progressive DNA damage. Inflammation and oxidative stress play important role in this stage. Double TPA treatment protocol is the most frequently used model to study the molecular, biochemical, cellular and histological changes associated with promotion stage of carcinogenesis and also to understand the role of inflammation, ROS and hyperplasia in cancer development (Riehl et al., 2010; Ha et al., 2006).
In light of the important roles of inflammatory mediators in carcinogenesis, present study was primarily designed to investigate the chemopreventive effects of quercetin on TPA induced oxidative stress, inflammation and tumor promotion in skin carcinogenesis.

Treatment regimen

The whole study is divided into two parts

(a) Short term

To observe the effect of quercetin against TPA induced cutaneous oxidative and inflammatory responses, animals were divided into four groups (I-IV) of six animal each (n=6). Dorsal skin of all the animals was shaved with the electric clipper two days prior to the start of the experiment. All the treatments were done topically onto the shaved area of dorsal skin.

Animals of group I received topical application of only vehicle (0.2 ml acetone) and served as control. Group II animals were given topical application of TPA (10 nmol) in 0.2 ml acetone. Animals of the groups III were given topical pretreatment of the quercetin at the dose of 24 μmol in 0.2 ml acetone 30 min before TPA (10 nmol in 0.2 ml acetone) application. Group IV animals were given topical application of quercetin (24 μmol in 0.2 ml acetone) only. The treatments were carried out for 2 days at the interval of 24 hrs. Animals of the all the groups were sacrificed by cervical dislocation 1 h after the last TPA treatment and skin tissue was processed for the evaluation of different parameters.

(b) Long term

For skin tumor induction, classical DMBA-TPA model was used as described in chapter VI. Animals were divided into four groups (I-IV) of seven animal each (n=7). Dorsal skin of all the animals was shaved with the electric clipper two days prior to the start of the experiment. All the treatments were carried out topically on the dorsal shaved area of mouse skin.
Group I (Control) - Mice were given vehicle (0.2ml acetone) only.

Group II (DMBA+TPA) - Mice were treated with a single topical application of DMBA (50 μg) in 0.2 ml acetone. One week after the initiation, TPA (2.0 μg) in 0.2 ml acetone was applied twice a week for 16 consecutive weeks.

Group III - Mice were pre-treated with quercetin at a dose of 24 μmol /0.2 ml acetone/mice/day for 14 days. On day 15, DMBA (50 μg) was given to each animal and then after one week TPA (2.0 μg) was applied twice a week until the termination of the experiment at 16 weeks (Anti-initiation group).

Group IV - Mice were given single topical application of DMBA (50 μg). One week after, animals were pre-treated with quercetin (24 μmol) 30 minutes prior to the TPA (2.0 μg) application twice a week until the termination of the experiment at 16 weeks (Anti-promotion group).
Animals in all the groups were watched for any apparent signs of toxicity and mortality during the entire period (16 weeks) of study.

**Results**

**Short Term:**

**Effect of quercetin on TPA-induced myeloperoxidase (MPO) activity**

TPA application in the animals of group II showed significant elevation in MPO activity (p<0.001) when compared with the vehicle treated group I (control group). However, pre-treatment with quercetin in the group III significantly (p<0.05) inhibits MPO activity when compared with group II. There was no significant difference observed between group I and IV (Fig. 1a).

**Effect of quercetin on TPA-induced lipid peroxidation in mouse skin**

TPA application in the animals of group II showed significant elevation in the level of MDA when compared with vehicle treated group I (p<0.001). Pre-treatment of quercetin in group III before TPA application significantly (p<0.05) inhibits MDA formation as compared to group II. There was no significant change observed in the level of MDA between control and only quercetin treated animals (Fig. 1b).

**Effect of quercetin on TPA-induced cutaneous xanthine oxidase (XO) activity**

TPA application in the group II results in increased XO activity (p<0.001) in comparison with the acetone treated group I. However, pre-treatment with quercetin in the group III significantly (p<0.001) inhibits XO activity as compared to group II. There was no significant difference observed between group I and IV (Fig. 1c).

**Effect of quercetin and TPA on cutaneous glutathione status**

Pre-treatment of quercetin before TPA application was found effective in restoring the endogenous anti-oxidant GSH. There was significant depletion in the level of GSH in group II as compared to group I (p<0.001). Pretreatment with quercetin in group III shows significant increase in the level of GSH (p<0.05) as compared with group II.
There was no significant difference in the GSH content between group I and IV (Fig. 2a).

**Effect of quercetin and TPA on cutaneous antioxidant and phase II metabolizing enzymes**

The effect of quercetin pre-treatment on TPA induced alteration in the activity of antioxidant and phase II enzymes (SOD, CAT and GST) was assayed and the results were shown in figure 2 b, 2c and 2d respectively. We have observed that there was a significant (p<0.001) decrease in the activity of cytoprotective enzymes in TPA treated group II as compared to only vehicle treated group I. However pre-treatment of quercetin in group III before TPA application leads to the significant (p<0.01, p<0.001) restoration in the activity of cytoprotective enzymes as compared to the TPA treated group II. There was no significant difference observed between group I and IV as for as the activity of cytoprotective enzymes were concerned.

**Effect of quercetin on the TPA-induced cutaneous histological alterations**

Effect of topically applied quercetin was observed on cutaneous histological changes caused by TPA application as shown in figure 3. We found that TPA application causes pronounced increase in leukocyte infiltration and epidermal thickening in group II (Fig. 3 B) as compared to group I (Fig. 3 A). Pre-treatment of quercetin in the group III (Fig. 3 C) diminished TPA induced leukocyte infiltration as well as hyperplasia. There was no distinguished change observed between the group I (Fig. 3 A) and IV (Fig. 3 D) as far as leucocytes infiltration and hyperplasia is concern.

**Effect of quercetin on TPA-induced cutaneous DNA fragmentation**

We analyzed the effect quercetin pretreatment on skin DNA fragmentation induced by TPA by gel electrophoresis (Fig. 4). We found that there was more DNA damage in TPA treated group II (Fig. 4, Lane B) as compared to vehicle treated group I (Fig. 4, Lane A). Pre-treatment of quercetin in the group III showed less fragmentation (Fig. 4, Lane C) in
comparison to TPA-treated group II (Fig.4, Lane B). There was no distinguished change observed between the group I (Fig.4, Lane A) and IV (Fig.4, Lane D) as far as DNA fragmentation is concern.

**Effect of quercetin on TPA-induced cutaneous immunohistochemical expression of NF-kB (p56), COX2 and iNOS**

Cutaneous expression of the above mention proteins were shown in the figures 5, 6 and 7 respectively. Brown color clearly indicates the more number of cells having NF-kB, COX-2 and iNOS expression in the group II as compare to that of group I. Pretreatment with quercetin in the group III reduced the number of cells showing expression of NF-kB, COX-2 and iNOS. However there was no significant difference observed in the expression of these proteins in group IV in comparison to group I. For immunohistochemical analysis, brown color indicates specific immunostaining of NF-kB, COX-2, and iNOS and light blue color indicates haematoxylin staining. Original magnification: x40; x100; scale bar-50μm.

**Long Term:**

**Effect of quercetin on DMBA initiated and TPA promoted skin tumor incidence**

Topical application of quercetin resulted in the strong protection in both, tumor initiation and tumor promotion, experimental animals (Fig.8, Table 1). In the group II, 100 % tumor incidence was found as shown in table 1. Quercetin pre-treatment in both anti-initiation (group III) and anti-tumor promotion (group IV) groups effectively suppressed the tumor incidence as compared to group II.

In case of anti-initiation group III, application of quercetin for 14 days before DMBA application effectively suppressed the tumorigenesis. At the termination of the experiment at 16 weeks, compared to 100 % animals with skin tumors in the group II, only 57 % of the animals in the quercetin pre-treated group III, exhibited skin tumors accounting for 43 % inhibition in tumor incidence (Table 1).
In case of anti-tumor promotion experimental group IV, application of quercetin prior to each TPA application resulted in strong prevention against TPA-induced tumor promotion in DMBA-initiated mouse skin with reference to tumor incidence (Table 1).

On the termination of the experiment at 16 weeks, compared to 100% animals with skin tumors in the group II, only 71% of the animals in the quercetin pre-treated group IV, exhibited skin tumors accounting for 29% inhibition in tumor incidence (Table 1).

**Discussion**

The key finding of the present study is that quercetin, an antioxidant and inflammatory natural product, showed strong anti-tumorigenic potential against two-stage skin tumorigenesis in mouse suggesting that quercetin could be a useful cancer chemopreventive agent.

Carcinogenesis consists of three distinct stages initiation, promotion and progression. Data from biochemical and histological observations revealed that quercetin effectively attenuates TPA induced oxidative stress and inflammation. Double application of TPA to mouse skin causes massive ROS production that result in oxidative stress. Each application of TPA induces two discrete biochemical events, namely priming, the first application lead to recruitment of cellular (leucocytes) inflammatory mediators and activation, the second application is characterized by ROS production from accumulated leukocytes and further recruitment of leucocytes (Slaga, 1983; DiGiovanni, 1992; Murakami et al., 2000). Hence, generation of inflammatory responses is an integral part of mouse skin to TPA (Nakamura et al., 1998). In accordance with this, we have also observed that double application of TPA on mouse skin resulted in massive induction of neutrophils infiltration.

In addition, pretreatment with quercetin prior to TPA application effectively diminishes neutrophils infiltration.
Inflammation play important role in the ROS production as the various inflammatory mediators are the critical source of ROS production. It is well established that over production of ROS can alter the normal cellular physiology and play important role in carcinogenesis. Phorbol ester induced ROS generation play vital role in the process of lipid peroxidation and deregulation of different signalling cascade (Nakamura et al., 2003). Lipid peroxidation is a crucial marker of oxidative stress and critically associated with cancer development (Valko et al., 2006; Saintot et al., 1996; Gerber et al., 1997). In the present study level of MDA was found elevated many fold after TPA application. Pre-treatment with quercetin attenuated MDA formation, which suggests the strong antioxidant potential of quercetin. Apart from increased MDA formation level and activity of different endogenous antioxidant and phase II metabolizing enzymes was also altered which is in agreement with the previous observations (Sharma and Sultana, 2004). Pre-treatment with quercetin normalizes these antioxidants possibly by boosting endogenous antioxidant defence machinery via triggering their transcriptional up-regulation (Granado-Serrano et al., 2012; Arredondo et al., 2010; Tanigawa et al., 2007).

Inflammatory responses play important roles in skin tumorigenesis. Array of cellular and molecular inflammatory mediators are involve in the inflammation (Stanley et al.1991; DiGiovanni, 1992). We have found that pre-treatment of quercetin diminish various inflammatory markers, such as MPO activity, leukocyte infiltration, inhibition of NF-kB, COX2 and iNOS expression.

COX-2 and iNOS the two important enzymes involved in inflammation, are the well known biomarkers of inflammation and tumor promotion. They act as important mediators of inflammatory process (Smith et al.,1996; Herschman,1994). Various study findings show that both COX-2 and iNOS play important role in the skin inflammation, cell proliferation and skin cancer development suggesting that their inhibition will not only ease inflammation but also prevent carcinogenesis (Sarfaraz et al.,2008; Kim et al.,
Multiple lines of evidence indicate that over expression of COX-2 and iNOS is found in malignancy of different organs including skin. Use of selective pharmacological inhibitors of COX-2 and iNOS or their functional inactivation provides substantial evidence about their role in tumorigenesis (Kundu and Surh, 2008; Chun et al 2004). Findings of the present study reveal that quercetin inhibits TPA-induced cutaneous expression of COX-2 and iNOS which is in agreement with previous findings (Xiao et al., 2011; Dias et al., 2005).

NF-kB has been recognised as a key molecular bridge between inflammation and cancer and also as a prime target for intervention strategies of cancer prevention (Pikarsky et al., 2004). Aberrant and constitutive activation of NF-kB play central role in the development of almost all human diseases including malignancies due to its ubiquitous presence and multiple functions. Induction of various inflammatory mediators, for example proinflammatory cytokines, chemokines, iNOS, COX-2, MMPs, and a number of adhesion factors are generally mediated by DNA binding of NF-kB (Tak and Firestein, 2001). So, the unregulated NF-kB activation may lead to the over expression of the above mentioned proteins thereby creating a tumorigenic environment (Karin, 2006). Considerable body of compelling evidence suggest that deregulated activation of NF-kB plays important role in the development of various skin pathological conditions including initiation, promotion and progression of skin cancer development (Bell et al., 2003). Our study demonstrates that quercetin strongly suppressed the activation of NF-kB that might be mediated via interfering with a pathway that leads to the phosphorylation or degradation of IkB as previous findings implicate that natural products inhibit NF-kB activation by blocking the modification of IkB (Surh and Na, 2008; Surh et al., 2005; Surh et al., 2001). These inhibitory effects of quercetin against TPA mediated responses in the mouse skin suggest that the primary effect of quercetin may be against inflammatory responses, which may then result in the inhibition of tumor induction.
Findings of the long term study revealed the strong chemopreventive potential of quercetin against chemically induced skin tumorigenesis as it strongly suppressed the tumor incidence. The underlying mechanism may involve inhibition of inflammatory responses, hyper proliferation and oxidative stress. Induction of antioxidant and phase II detoxifying enzymes also provides substantial evidence for the anti-tumorigenic potential of quercetin. In conclusion, findings of the present study show that quercetin strongly inhibited TPA induced cutaneous tumor promotional changes via boosting of endogenous defense system, suppression of phase I enzymes, inhibition of oxidative stress and inflammation. Theses finding suggest the strong anti-tumorigenic potential of quercetin and hence, may serve as one of the important strategies for the prevention of cancer.
Fig. 1. Effect of quercetin pre-treatment on TPA-induced cutaneous (a) MPO activity (b) Lipid peroxidation (LPO) and (c) Xanthine oxidase (XO) activity.

Values are expressed as mean ± SEM. (n = 6). **p<0.001 shows significant difference in TPA treated Group II [TPA (10 nmol)] as compared to only vehicle treated Group I. #p<0.05, ###p<0.001 shows significant difference in the quercetin pre-treated Group III [Q (24 µmol) + TPA (10 nmol)] as compared to TPA treated Group II.

Q-Quercetin
Fig. 2. Effect of quercetin and TPA on cutaneous anti-oxidant and phase II enzymes
(a) Glutathione (GSH) (b) Superoxide dismutase (SOD) (c) Catalase (d) Glutathione-s-transferase (GST)

Values are expressed as mean ± SEM. (n = 6). ***p<0.001 shows significant difference in TPA treated Group II [TPA (10 nmol) in 200 µl acetone] as compared to only vehicle treated Group I. #p<0.05, ##p<0.01, ###p<0.001 shows significant difference in the quercetin pre-treated Group III [Q (24 µmol) + TPA (10 nmol)] as compared to TPA treated Group II.

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Fig. 3. Effect of quercetin pre-treatment on TPA-induced cutaneous histological alterations

Representative photomicrographs (magnification ×10), (A) only vehicle (Group I), (B) TPA only (Group II), (C) Q + TPA (Group III) and (D) only Q (Group IV).

There was marked infiltration of neutrophils and epidermal thickness (arrow) in TPA treated animals (Group II) as compared with only vehicle treated animals (Group I). Quercetin pretreatment (Group III) attenuates TPA induced alterations significantly as compared with only TPA treated animals (Group II). However, there is no significant difference between group I and group IV. Q-Quercetin

Fig. 4. Effect of quercetin pre-treatment on TPA-induced cutaneous DNA fragmentation

Effect of quercetin pretreatment on TPA-induced cutaneous DNA fragmentation. Lane A- control (Group I), Lane B- TPA (Group II), Lane C- Q + TPA (Group III) and Lane D- Q only (Group IV). Q- Quercetin
Fig. 5. Effect of quercetin pre-treatment on TPA-induced cutaneous expression of NF-kB.

Representative photomicrographs (magnification ×40), (A) only vehicle (Group I), (B) TPA only (Group II), (C) Q + TPA (Group III) and (D) Q only (Group IV).

Brown color indicates NF-kB specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more NF-kB immunopositive staining (arrow) as compared with vehicle treated group (Group I). Quercetin pretreatment (Group III) reduces NF-kB expression as compared to Group II. However there was no significant difference in the NF-kB immunostaining in Group IV as compared to Group I. Q-Quercetin
Fig. 6. Effect of quercetin pre-treatment on TPA-induced cutaneous expression of COX-2

Representative photomicrographs (magnification ×100), (A) only vehicle (Group I), (B) TPA only (Group II), (C) Q + TPA (Group III) and (D) Q only (Group IV).

Brown color indicates COX-2 specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more COX-2 immunopositive staining (arrow) as compared with vehicle treated group (Group I). Quercetin pretreatment (Group III) reduces COX-2 expression as compared to Group II. However there was no significant difference in the COX-2 immunostaining in Group IV as compared to Group I. Q-Quercetin
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Fig. 7. Effect of quercetin pre-treatment against TPA-induced cutaneous expression of iNOS

Representative photomicrographs (magnification×40), (A) only vehicle (Group I), (B) TPA only (Group II), (C) Q + TPA (Group III) and (D) Q only (Group IV).

Brown color indicates iNOS specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more iNOS immunopositive staining (arrow) as compared with vehicle treated group (Group I). Quercetin pretreatment (Group III) reduces iNOS expression as compared to Group II. However there was no significant difference in the iNOS immunostaining in Group IV as compared to Group I. Q-Quercetin
Fig. 8. Effect of quercetin pre-treatment on chemically induced skin tumor appearance

Representative photographs of skin tumor morphology, (A) control (Group I), (B) DMBA + TPA (Group II), (C) Anti-initiation (Group III) and (D) Anti-promotion (Group IV).

Table 1. Effect of quercetin on chemically induced skin tumor incidence

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of mice</th>
<th>No. of Tumor-bearing mice</th>
<th>Tumor incidence (%)</th>
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<tbody>
<tr>
<td>I-Control</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II-DMBA + TPA</td>
<td>7</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>III-Anti-initiation</td>
<td>7</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>IV-Anti-promotion</td>
<td>7</td>
<td>5</td>
<td>71</td>
</tr>
</tbody>
</table>

Tumor incidence is expressed as the percentage of animals with one or more confirmed tumors.
Schematic representation of the chemopreventive action of quercetin against two-stage skin carcinogenesis.