Chapter-IV

Geraniol attenuates phorbol ester-induced inflammatory responses in mouse skin: possible role of p38 MAP Kinase and NF-kB
Introduction

Dietary natural products have shown protection against various degenerative diseases including cancer and in the present era, therapeutic or medicinal importance has been given to them because of the virtue of their anti-oxidant and anti-inflammatory potential. Epidemiological findings also implicate that substantial intake of fruits and vegetables in the diet have protective effects against different types of cancer (Singleton, 2000). Isoprenoids, originate from the mevalonate pathway, are one of the important groups of natural product present in diverse plant based products including fruits, vegetables and essential oils, have been shown to possess promising chemopreventive potential (Mo and Elsonm, 2004; He et al., 1997). Geraniol (GOH) is an important isoprenoid and one of the main constituents of the essential oils of various rose species. GOH has been shown to possess antioxidant (Tiwari and Kakkar, 2009), anti-inflammatory, anti-apoptotic and anti-carcinogenic potential (Shoff et al., 1991). Previous findings reflect that GOH has potent anti-tumour activity against different types of malignancies in animal models (Cardozo et al., 2011; Yu et al., 1995).

Elevated level of ROS and pro-inflammatory cytokines play important roles in the promotion stage of cancer development. ROS are the important tumor promotional trigger in the multistage cancer development as they interfere with the signalling components of a number of pathways and the expression of genes (Henricks and Nijkamp, 2001).

Inflammatory responses play important role in multistage carcinogenesis, including initiation, promotion, malignant conversion, invasion and metastasis (Balkwill and Mantovani, 2002; Coussens and Werb, 2002). During inflammation, leucocytes and mast cell are recruited first to produce ROS and various other mediators like chemokines and cytokines which further recruit inflammatory cells to produce ROS (Henricks and Nijkamp, 2001; Lin and Karin, 2007). Uncontrolled production of ROS or change in
intracellular antioxidants level/activity causes damage or modification of cellular macromolecules which ultimately result in the up or down expression of regulators of normal cellular physiology (Droge, 2002; Waris and Ahsan, 2006).

Aberrant production of inflammatory mediators like proinflammatory cytokines TNF-α, IL-6 and IL-1β and nitric oxide have critical impact on multistage cancer development, including initiation, promotion, malignant conversion, invasion, and metastasis (Hussain and Harris, 2007; Barker et al., 1991; Hong et al., 2000).

Most of the tumour promoting agents and other stimuli like oxidative stress, TNF-α, and lipopolysaccharides (LPS) can induce activation of NF-kB (Bowie and O’Neill, 2000). NF-kB, one of the most important ubiquitous redox sensitive transcription factor known to regulate the expression of genes involved in inflammation, cell proliferation and survival (Karin and Delhase, 2000). Although, various signalling cascade components have been implicated in promotional stage of cancer development but those that congregate with NF-kB has prominent link with tumour promotion (Balkwill and Mantovani, 2002). One of the downstream targets of NF-kB is COX-2, a key regulatory enzyme in the synthesis of prostaglandins. Uncontrolled expression of COX-2 has been observed in different premalignant as well as in malignant stage (Williams et al., 1999).

TPA is one of the most widely used distinguished promoting agents to better understand the cellular and molecular alterations associated with skin carcinogenesis (Nakadate, 1989). Previous studies revealed that it is a well-known model to understand the role of ROS production, inflammation and hyperplasia in the promotional stage of carcinogenesis (Ha et al., 2006).

Considering all these above findings, we postulated that GOH, a natural compound having antioxidant and anti-inflammatory effects might be an effective candidate in the prevention of damage caused by topically applied TPA-induced early tumor promotional events in the mouse skin. To test this hypothesis, we have studied the effects of GOH on the TPA-induced cutaneous oxidative stress and various biomarkers of inflammation.
**Treatment schedule**

To observe the effect of GOH on TPA induced cutaneous oxidative and inflammatory responses, animals were divided into four groups (I-IV) of six animal (n=6) each. 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.

**Group I**- Animals of this group were given topical application of vehicle (0.2 ml acetone) only and served as control group.

**Group II**- Animals of this group were given topical application of TPA (10 nmol) in 0.2 ml acetone.

**Group III**- Animals of the groups III were given topical pretreatment of GOH at the dose of 250 µg in 0.2 ml acetone 30 minutes before TPA [(10 nmol) in 0.2ml acetone] application.

**Group IV**- Animals were given topical application of GOH (250 µg) in 0.2 ml acetone.

The treatments were carried out for 2 days at the interval of 24 h. Animals of the entire group were sacrificed by cervical dislocation 1 h after the last TPA treatment and skin tissue was processed for the evaluation of different parameters.

**Results**

**Effect of GOH on skin edema formation**

Application of TPA causes massive cutaneous inflammation which was assessed by edema formation. TPA application in the group II results in increased edema formation (p<0.001) in comparison with the only vehicle treated group I. However, pre-treatment with GOH in the group III significantly (p<0.01) inhibits edema formation when compared with group II. There was no significant difference observed between group I and IV (Figure 1a).
Effect of GOH on the cutaneous NO production

Topical application of TPA resulted in the elevated cutaneous NO production in group II as compared with the only vehicle treated group I (p<0.001). We observed that pre-treatment with GOH was significantly effective in reducing NO production in group III when compared with the group II (p<0.01). There was no significant difference observed between group I and IV as far as NO production is concern (Figure 1b).

Effect of GOH on TPA-induced skin lipid peroxidation

Double application of TPA resulted in significant elevation in the level of MDA in group II when compared with group I (p<0.001). GOH pre-treatment significantly (p<0.001) decreased the level of MDA in group III when compared with group II. There was no significant change observed in the level of MDA between only vehicle treated group I and group IV (Figure 2a).

Effect of GOH on the cutaneous GSH content

Pretreatment of GOH before the TPA application was found effective in restoring the level of GSH. There was significant depletion in the level of GSH content in group II when compared with group I (p<0.001). Pretreatment with GOH in group III showed significant increase in the level of GSH (p<0.01) as compared with group II. There was no significant difference in the GSH level between group I and IV (Figure 2b).

Effect of GOH on the skin anti-oxidant and phase II enzymes

The effect of GOH pre-treatment on TPA induced alteration in the activity of different cytoprotective enzymes was examined and the results were shown in table 1. We have observed that there was a significant (p<0.001) depletion in the activity of different cytoprotective enzymes in TPA treated group II as compared to only vehicle treated group I. However pretreatment with GOH in the group III before TPA application significantly (p<0.05, p<0.01) restored the activity of cytoprotective enzymes when compared with the TPA treated group II. There was no significant difference observed between group I and IV.
Effect of GOH on cutaneous proinflammatory cytokines

We have assessed the effect GOH on TPA induced cutaneous TNF-α, IL-6 and IL-1β level, as shown in figure 3. We found that there was a significant increase in the level of proinflammatory cytokines in TPA treated group II as compared to only vehicle treated group I (p<0.001). Pre-treatment with GOH significantly inhibit their production in the group III when compared with the group II (p<0.01, p<0.001). There was no significant difference found between group I and IV.

Effect of GOH on the TPA-induced cutaneous immunohistochemical expression of p38-MAPK, NF-kB (p65), COX2 and PCNA

Cutaneous expression of the above mention proteins are shown in the figures 4, 5, 6 and 7 respectively. Brown colour clearly indicates the more number of cells having phospho-p38 MAPK, NF-kB, COX-2 and PCNA expression in the group II as compare to that of group I. Pretreatment with GOH in the group III reduced the number of cells showing expression of phospho-p38 MAPK, NF-kB, COX-2 and PCNA. However there was no significant difference observed in the expression of these proteins in group IV as compared to group I. For immunohistochemical analysis, brown colour indicates specific immunostaining of phospho-p38 MAPK, NF-kB, COX-2 and PCNA and light blue colour indicates haematoxylin staining. Original magnification: x10 and x40.

Effect of GOH on the cutaneous histological alterations

Effect of topically applied GOH was seen on cutaneous histological changes caused by TPA application with reference to neutrophils infiltration and epidermal hyperplasia, figure 8. We found that TPA application causes pronounced increase in leukocyte infiltration and epidermal thickening in group II (Figure 8B) as compared with the group I (Figure 8A). Pre-treatment with GOH in the group III (Figure 8C) diminished TPA induced leukocyte infiltration as well as hyperplasia. There was no distinguished change observed between the group I (Figure 8A) and IV (Figure 8D) as far as leucocytes infiltration and hyperplasia is concern.
Discussion

Cancer development is a multistep process viz., initiation, promotion and progression. Since initiation is an irreversible stage the most effective intervention would be at the promotion stage. Topical applications of different tumor promoters have been shown to induce oxidative and inflammatory responses which have close association with the development of skin tumor. Therefore, TPA induced oxidative stress, inflammation and their mediators are recognised as an important regulator of tumor promotion.

Findings of this study implicates that GOH, a monoterpene, exhibits strong preventive potential against TPA-induced oxidative and inflammatory events that are mainly associated with tumor promotion. Biochemical, molecular and histological observations delineates that protective effects of GOH is attributed to its antioxidative and antinflammatory potential.

Findings of the present study revealed that status of antioxidants were altered after TPA application. Generation of superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (·OH) by TPA application play important 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 the promotion stage of cancer development (Saintot et al., 1996; Gerber et al., 1997). In the present study level of MDA was found elevated many fold after TPA application. Pretreatment with GOH attenuated MDA formation, which suggests the strong anti-tumor promoting potential of GOH. Apart from increased MDA formation level and activity of different cytoprotective (GSH, GR, GPx, SOD, QR, and Catalase) elements was also altered after TPA application which was in agreement with the previous observations (Sharma and Sultana, 2004; Tiwari and Kakkar, 2009). Glutathione and its dependent enzymes are the major chelators/detoxifying agents. Antioxidants play pivotal role in the endogenous defence system by scavenging toxic moieties so alteration in their function
results in the over production of highly reactive species which are often associated with tumour promotion (Pillai, 2002). Pre-treatment with GOH normalizes the level and activity of these cytoprotective elements. Present study data suggest the critical protective role of antioxidants and detoxifying enzymes against phorbol ester-induced early tumor promotional events. Pre-treatment with GOH suppress the oxidative damages possibly by boosting cytoprotective endogenous defence machinery via triggering their transcriptional up-regulation.

TPA induces inflammatory responses (leucocytes infiltration, increase production of ROS, edema formation, and hyperplasia), proinflammatory cytokines production and over expression of COX-2 and iNOS which are closely associated with skin tumor promotion (DiGiovanni, 1992; Murakami et al., 2000; Slaga, 1984). Therefore, interception of these inflammatory markers could be an important approach for chemoprevention of skin carcinogenesis. In the present study, we examined the potential role of GOH against TPA induced inflammatory responses (cutaneous edema, hyperplasia and neutrophil infiltration) and the expression of NF-kB and COX-2. Proinflammatory molecules like cytokines IL-6, TNF-α, IL-1β and NO act as cell-to-cell signalling messenger and play a key role in the promotion stage of cancer development. In spite of meditating inflammatory responses they also acts as major signalling component for the production of adhesion molecules, growth factors, eicosanoids, nitric oxide, angiogenic factor like VEGF, and activation of the NF-kB (Kundu and Surh, 2008).

TNF-α, IL-1β and IL-6, induction has been associated with the promotion phase of skin tumor growth as confirmed by the previous findings which show that mouse deficient in TNF-α, IL-6 and IL-1β are resistant to skin tumor formation and chronic inflammation, neoplasia and tumor metastasis respectively (Moore et al., 1999; Tricot, 2000; Vidal-Vanaclocha et al., 2000). These cytokines are also well known for their roles in tumor
growth as studies of humans and mice shows that elevated level of these cytokines play important role in the development of microenvironment in tumor progression (Smith, 1998). Our data suggest that GOH effectively suppressed the production of these cytokines which implicates its chemopreventive potential.

COX-2, a key enzyme play important role in inflammatory signalling, has been sorted out as one of the prime target for chemoprevention. A number of stimuli like pro-oxidant /proinflammatory cytokines, anomalous regulation of signalling regulated by kinases and transcription factors results in abnormal expression of COX-2 (Chun et al., 2004). Approaches for cancer prevention demonstrated the importance of COX-2 in tumour promotion (Philip et al., 2004). Since the aberrant expression of COX-2 is recognised as a connecting link between inflammation and tumour promotion, the findings of the current study reflects the anti-inflammatory and anti-tumor property of GOH which inhibits TPA induced expression of COX-2 in mouse skin. TPA induced expression of COX-2 in mouse skin regulated by array of redox sensitive transcription factors, including NF-kB (Kim and Fischer, 1998) as the promoter region of COX-2 gene contain consensus sequence for binding with NF-kB. In resting cells NF-kB resides along with its cytosolic repressor inhibitory protein IkB. Exposure with different stimuli like oxidative stress/phorbol esters causes extensive modification of IkB which results in the nuclear translocation of NF-kB. Our findings demonstrate that GOH strongly suppresses the activation of NF-kB in TPA treated mouse skin. Various findings implicate that natural antioxidants inhibit NF-kB activation by blocking the modification of IkB (Surh et al., 2001).

Application of TPA results in the recruitment of inflammatory cells, massive production of proinflammatory cytokines, ROS and RNS thereby causing damage to cellular macromolecules (Bowden et al., 1995). This further stimulates redox sensitive transcription factors by activating mitogen-activated protein kinase (MAPK) signalling pathways (Goetz and Luch, 2008; Hou et al., 2004; Schulze-Osthoff et al., 1997).
p38MAP kinase, one of the most extensively studied cell signalling cascades, play pivotal role in the inflammation as it regulates the induction of COX-2 and activation of NF-kB in response to various external stimuli (Kyriakis and Avruch, 2001). Production of proinflammatory cytokines, known for their critical roles in tumor promotion, is regulated by p38 MAPK pathway through transcriptional activation of transcription factors like NF-kB (Kumar et al., 2003). Our results demonstrate that GOH significantly suppressed the activation of p38 MAPK which further supports the anti-inflammatory nature of GOH.

In conclusion the biochemical, molecular and histological findings of present study revealed the antioxidant and anti-inflammatory properties of GOH against TPA induced tumor promotional events in mouse skin. Inhibition of COX-2 expression, proinflammatory cytokine production, activation of NF-kB and p38 MAPK by GOH provide the molecular basis of the anti-inflammatory potential of GOH. The findings of the present study suggest that GOH could be one of the potential chemopreventive agents against inflammation associated chronic human diseases including cancer.
Figure 1. Effect of GOH on TPA-induced cutaneous, (a) Edema formation (b) Nitric oxide production

Values are expressed as mean ± SEM. (n = 6). ***p<0.001 shows significant difference in Group II [TPA (10 nmol) in 200 µl acetone] when compared with Group I (only acetone). ##p<0.01 shows significant difference in the Group III [GOH (250 µg) + TPA (10 nmol)] when compared with Group II.

Figure 2. Effect of GOH on TPA-induced cutaneous (a) Lipid peroxidation and (b) GSH content

Values are expressed as mean ± SEM. (n = 6). ***p<0.001 shows significant difference in Group II [TPA (10 nmol) in 200 µl acetone] as compared to Group I (only acetone). ##p<0.01, ###p<0.001 shows significant difference in the group III [GOH (250 µg) + TPA (10 nmol)] as compared to TPA treated Group II.
Table 1. Effects of GOH and TPA on the activities of cytoprotective (antioxidant and phase II) enzymes

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAT</th>
<th>GR</th>
<th>GPx</th>
<th>G6PD</th>
<th>SOD</th>
<th>QR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
<td>93.87 ± 2.86</td>
<td>125.83 ± 2.18</td>
<td>307.09 ± 10.34</td>
<td>132.51 ± 9.79</td>
<td>22.12 ± 2.21</td>
<td>123.91 ± 6.9</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td>52.74 ± 5.8***</td>
<td>81.5 ± 1.94***</td>
<td>154.48 ± 6.95***</td>
<td>73.59 ± 4.85***</td>
<td>8.22 ± 0.32***</td>
<td>52.67 ± 5.7***</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td>75.2 ± 3.14##</td>
<td>97.33 ± 4.10##</td>
<td>207.22 ± 5.25##</td>
<td>106.54 ± 4.31##</td>
<td>16.86 ± 1.28##</td>
<td>92.89 ± 4.0##</td>
</tr>
<tr>
<td><strong>Group IV</strong></td>
<td>94.4 ± 2.31</td>
<td>128.66 ± 1.62</td>
<td>308.34 ± 10.34</td>
<td>133.32 ± 10.03</td>
<td>21.82 ± 2.43</td>
<td>124.85 ± 7.6</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. of six animals. ***p<0.001 shows significant difference in Group II [TPA (10 nmol) in 200 µl acetone] when compared with Group I (only acetone).

#p<0.05, ##p<0.01 shows significant difference in the group III [GOH (250 µg) + TPA (10 nmol)] when compared with only TPA treated Group II.

Catalase (CAT) - as nmol H$_2$O$_2$ consumed/min/mg protein,
Glutathione reductase (GR) - as nmol NADPH oxidized/min/mg protein,
Glutathione peroxidase (GPx) - as nmol NADPH oxidized/min/mg protein,
Glucose-6-phosphate dehydrogenase (G6PD) - as µmol NADP reduced/min/mg protein,
Superoxide dismutase (SOD) - as units/mg protein
Quinone reductase (QR) - as µmol of DCPIP reduced/min/mg protein.
Figure 3. Effect of GOH pre-treatment on TPA-induced cutaneous proinflammatory cytokines, (a) TNF-α (b) IL-6 (c) IL-1β.

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] when compared with only vehicle treated Group I (only acetone). ##p<0.01, ###p<0.001 shows significant difference in the GOH pre-treated Group III [GOH (250 μg) + TPA (10 nmol)] when compared with TPA treated Group II.
Figure 4. Effect of GOH on TPA-induced cutaneous p38 MAPK expression

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

Brown color indicates phosphor-p38 specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more phosphor-p38 immunopositive staining (arrows) as compared with vehicle treated group (Group I). GOH pretreatment (Group III) reduces phosphor-p38 expression as compared to Group II. However there was no significant difference in the phospho-p38 immunostaining in Group IV as compared to Group I.
Figure 5. Effect of GOH on TPA-induced cutaneous NF-kB expression

Representative photomicrographs (magnification x40), (A) only acetone (Group I), (B) TPA only (Group II), (C) GOH+TPA (Group III) and (D) only GOH (Group IV).

Brown color indicates NF-kB (p65) specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more NF-kB immunopositive staining (arrows) as compared with vehicle treated group (Group I). GOH 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.
Figure 6. Effect of GOH on TPA-induced cutaneous COX-2 expression

Representative photomicrographs (magnification x40), (A) only acetone (Group I), (B) TPA only (Group II), (C) GOH+TPA (Group III) and (D) only GOH (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 (arrows) as compared with vehicle treated group (Group I). GOH 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.
Figure 7. Effect of GOH on TPA-induced cutaneous expression of PCNA

Representative photomicrographs (magnification x40), (A) only acetone (Group I), (B) TPA only (Group II), (C) GOH+TPA (Group III) and (D) only GOH (Group IV).

Brown color indicates PCNA specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more PCNA immunopositive staining (arrows) as compared with vehicle treated group (Group I). GOH pretreatment (Group III) reduces PCNA expression as compared to Group II. However there was no significant difference in the PCNA immunostaining in Group IV as compared to Group I.
Figure 8. Effect of GOH on TPA-induced cutaneous histopathology

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

There was marked infiltration of neutrophils (arrow head) and epidermal thickness (arrow) in TPA treated animals (Group II) as compared with only vehicle treated animals (Group I). GOH pretreatment (Group III) attenuates TPA induced alterations effectively as compared with TPA treated animals (Group II). However there was no significant difference in the cutaneous histology in the animals only treated with GOH (Group IV) as compared to only vehicle treated animals (Group I).
Graphical representation for putative mechanisms of GOH action against TPA–induced oxidative stress and inflammation.