CHAPTER - 4

EXPRESSION PATTERN OF INFLAMMATORY MARKER DURING DMBA-INDUCED CARCINOGENESIS

Introduction

Cancer is a multi-step process, which involves a series of epigenetic and genetic alterations that begin with genomic instability and end with the development of cancer (Smith et al., 2010). The activation of NF-κB, a marker of inflammation occurs due to oncogenes, carcinogens, tumor promoters and inflammatory stimuli. NF-κB activation regulates or controls the expression of genes that mediate transformation, proliferation, invasion, angiogenesis, apoptosis and metastasis. Over expression of NF-κB was shown in oral carcinogenesis (Karin, 2006 and Chang and Van, 2005). At the molecular level, this process involves activation of oncogenes, loss of function of tumor suppressor genes, cell cycle, apoptotic genes. Investigation of expression pattern of inflammatory marker with natural products in the target tissues could help to validate the anti-inflammatory potential of the natural products. Chemo prevention is an innovative area of cancer research that focuses on the prevention of cancer through pharmacologic, biologic, and nutritional interventions. As originally described, this involves the primary prevention of initiation and the secondary prevention, delay, or reversal of promotion and progression (Sporn, 1976 and Wattenberg, 1985).

Presently in this study the modulating effect of lupeol, kemferol, gallic acid on expression pattern of inflammatory (NFκB) marker during DMBA-induced carcinogenesis was investigated.
MATERIALS AND METHODS

Chemicals

Purified compounds (Lupeol, Kaempferol and Gallic acid) from S.grandiflora leaves. Reagent was purchased from BioGenex, San Ramon, CA, USA. Trizol reagent was purchased from Invitrogen, CA, USA. cDNA reverse transcriptase kit and SYBR green fluorophore assay reagents were purchased from Applied Biosystems, CA, USA. Oligo nucleotide primers were purchased from Genei, INDIA.

Animals

Hamsters, ten weeks old, weighing 80 grams were purchased from National Institute of Nutrition, Hyderabad and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages at room temperature. The animals were provided with standard pellet diet (Laboratory Animal Feed Limited, Bangaluru, India) and water. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University.

Tumor induction

Oral tumors were developed in the buccal pouch of hamsters by painting with 0.5% DMBA in liquid paraffin three times a week for 14 weeks. The total number of tumors in the hamsters were counted. The diameter of the each tumor was measured by using vernier scale. The tumor volume was calculated using the formula $(V= \frac{4}{3} \pi r^3)$ where $(r = D/2)$ and $D_1$, $D_2$, and $D_3$ are the three diameters (mm) of the tumor.
Experimental Design

The forty hamsters were divided into four groups of ten hamsters in each.

Group I: Control was painted with 0.5% DMBA in liquid paraffin three times a week for 15 weeks on their buccal pouches.

Groups II: Given Lupeol 50 mg bw/day alternate to DMBA painting until the end of the experiment (15 weeks).

Group III: Given Kemferol 50 mg bw/day alternate to DMBA painting until the end of the experiment (15 weeks).

Group IV: Given Gallic acid 50mg bw/day alternate to DMBA painting until the end of the experiment. The experiment was terminated at the end of 15th week and all animals were sacrificed by cervical dislocation.

Expression of inflammatory marker (NFκB) using real time PCR

RNA extraction: Total RNA from the hamster tissues were extracted with Trizol reagent which was based on the guanidine method. The RNA concentration was determined by agarose gel analysis. In brief, 50 mg tissue was homogenized using Trizol reagent. The homogenate was treated with 0.2 mL of chloroform and shook vigorously. The mixture was then centrifuged at 12,000rpm for 15 min. at 4°C. To the aqueous phase, 0.5 mL of isopropanol was added, and centrifuged at 12,000rpm for 10 min. at 4°C. The supernatant was discarded gently and the precipitated RNA was rinsed with ethanol. The RNA was resuspended in 100 µl of nuclease free water.
**cDNA Synthesis:** Isolated total RNA (1µg) was reverse-transcribed to cDNA using reverse transcriptase enzyme containing 2 µL of 10x reaction buffer, 0.8 µL of dNTP mixture and 2 µL of reverse transcriptase. The reaction mixture was incubated at 25°C for 10 min. and the reaction was terminated by heating at 85°C for 5 min. The resultant cDNA was further used.

**RT-PCR reaction:** RT-PCR was performed using a thermal cycler with SYBR green dye. Reactions were run in a total volume of 10 µL, including 5 µL of SYBR Green Super mix, 0.5 µL of each primer at 10 µM concentrations, 2 µL of the previously reverse transcribed cDNA template and 2 µL of sterile water.

**Table 13 - Primers Used for Real-time PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>NFκB</td>
<td>forward</td>
<td>5′-TTGGACGATCTGTGTCCCCC-3′</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-GGGTTACTCGGCAGATCCT-3′</td>
</tr>
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Thermo cycling conditions were 50°C for 2 min. and 95°C for 11 min. and extension at 60°C for 1 min. for 40 cycles. A melting curve analysis was made after each run to ensure a single amplified product for every reaction. The amount of target gene relative to a control was determined by the CT method.

**Results:** NFκB plot (Run Vs Cycle):
The NFκB mRNA expression and fold increase pattern of control and experimental animals in each group is given in Fig. The expression of NFκB was significantly higher in hamsters treated with DMBA alone as compared to compound treated animals. Oral administration of Gallic acid, Lupeol and Kempferol to animals treated with DMBA suppressed the expression of NFκB.
Anticancer activity of \textit{S. grandiflora} purified compound \textit{(in vivo)} – Animal study

Normal Animal (Control)

Buccal Pouch
CANCEROUS

C

Lupeol treated

D
Kempferol treated

![Image of Kempferol treated]

E

Gallic acid treated

![Image of Gallic acid treated]

F

Fig. 51 (A-F) - Anticancer activity of purified compounds from S.grandiflora
Discussion

The present study dealt with anticancer property of Lupeol, Kemferol and Gallic using Hamster chicks as animal model. All of them were found to be potential anticancer compounds. The results showed the order of potential was Lupeol, Gallic acid, Kemferol.

In the present investigation NFκB was used as a marker. The activation of NF-κB, a marker of inflammation, occurs due to activation of oncogenes and inflammatory stimuli. NFκB activation regulates or controls the expression of genes that mediate cell transformation, proliferation, invasion, angiogenesis, apoptosis and metastasis. NFκB, over expression negatively regulated the function of p53, contributing to tumorigenesis. It has been reported that NFκB inhibited p53 function by up regulating anti-apoptotic proteins, which can antagonize the pro-apoptotic functions of p53. Deregulation of NFκB has been implicated in the pathogenesis of several disorders including oral carcinoma.

In the present investigation NFκB marker is used to evaluate the efficiency of three isolated compounds viz., Lupeol, kemferol and Gallic acid in suppression of cancer development. Gallic acid reduced the inflammatory response by down regulating the expression of NFκB in lipopolysaccharide induced inflammatory response.

Lupeol, kemferol, gallic acid reduced the inflammatory response by down regulating the expression of NFκB. Oral administration of lupeol, kemferol, gallic acid at a dose of 50 µg/kg bw down regulated the expression of NFκB in hamsters treated with DMBA. The results thus suggested that lupeol, kemferol, gallic acid modulated the expression of NFκB molecular marker in favour of preventing neoplastic transformation during DMBA-induced hamster carcinogenesis.
The present study thus concludes the chemopreventive potential of lupeol, kemferol, gallic acid due to their anti-inflammatory potential during DMBA-induced hamster buccal pouch carcinogenesis.