

## RESEARCH ARTICLE

# Modulating Effect of Lupeol on the Expression Pattern of Apoptotic Markers in 7, 12-Dimethylbenz(a)anthracene Induced Oral Carcinogenesis

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### Abstract

Apoptosis, also known as cell suicide or programmed cell death, removes unwanted and genetically damaged cells from the body. Evasion of apoptosis is one of the major characteristic features of rapidly proliferating tumor cells. Chemopreventive agents inhibit or suppress tumor formation through apoptotic induction in target tissues. The aim of the present study was to investigate the pro-apoptotic potential of lupeol during 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Topical application of 0.5% DMBA three times a week for 14 weeks in the buccal pouches of golden Syrian hamsters resulted in oral squamous cell carcinoma. The expression pattern of apoptotic markers was analyzed using immunohistochemistry (p53, Bcl-2, Bax) and ELISA reader (caspase 3 and 9). In the present study, 100% tumor formation with defects in apoptotic marker expression pattern was noticed in hamsters treated with DMBA alone. Oral administration of lupeol at a dose of 50mg/kg bw completely prevented the formation oral tumors as well as decreased the expression p53 and Bcl-2, while increasing the expression of Bax and the activities of caspase 3 and 9. The present study thus indicated that lupeol might inhibit DMBA-induced oral tumor formation through its pro-apoptotic potential in golden Syrian hamsters.

**Keywords:** Lupeol - oral cancer - apoptosis - DMBA - Syrian hamster model

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### Introduction

Apoptosis, also known as programmed cell death, plays an important role in the tissue homeostasis. Apoptosis prevents abnormal cell proliferation and removes damaged cells. Any imbalance in the apoptotic pathway might lead to several disorders including cancer (Abel et al., 2005). Evasion of apoptosis is an important feature of cancer cells. Several genes have been identified as either inducers or repressors of apoptosis. p53 plays pivotal role in the stability of genome and normal cell growth. p53, a putative regulator of multiple cellular processes, accumulates in response to DNA damage and oncogene activation. It can initiate apoptosis if DNA damage proves to be irreparable. A large number of studies thus pointed out p53 as a clinical marker and novel therapeutic target of anticancer agents (Harrison, 2012; Utomo et al., 2012). Bcl-2, an anti-apoptotic protein, plays pivotal role in tumorigenesis and Bcl-2 inhibitors are currently under clinical trials for several malignancies (Chakravarti et al., 2012; Sun et al., 2012). Anti-apoptotic (Bcl-2) and pro-apoptotic proteins (Bax) determine the fate of damaged cells and imbalance in Bcl-2 to Bax ratio result in carcinogenesis (Gao et al., 2012). A family of cysteinyl aspartate-specific proteases, caspases, have a critical role in apoptosis. The

caspase cascade system is involved in the induction and amplification of intracellular apoptotic signals. Caspase 3 and 9 have pivotal role in the process of programmed cell death. A large number of studies pointed out that caspases are deactivated during carcinogenesis (Kim et al., 2012 Song et al., 2012).

Lupeol, a biologically active dietary triterpene, is found in many fruits and medicinal plants. Lupeol exhibited diverse pharmacological properties including anticancer, anti-inflammatory, antidiabetic and hepatoprotective activities (He et al., 2011; Siddique et al., 2011). Previous studies from our laboratory demonstrated the anti-clastogenic, antigenotoxic and chemopreventive potential of lupeol in experimental animal models (Palanimuthu et al., 2012a; 2012b; 2012c). The present study demonstrates the modulating effect of lupeol on the expression pattern of apoptotic markers (p53, Bcl-2, Bax caspase 3 and 9) during DMBA-induced hamster buccal pouch carcinogenesis.

### Materials and Methods

#### Animals

Male golden Syrian hamsters, aged 8-10 weeks, weighing 80-120 g, were purchased from the National Institute of Nutrition, Hyderabad, India and were

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maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided with a standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water ad libitum. The animals were maintained under controlled conditions of temperature ( $27\pm 2^{\circ}\text{C}$ ) and humidity ( $55\pm 5\%$ ) with a 12 h light/dark cycle.

#### Chemicals

The carcinogen, 7, 2-dimethylbenz(a)anthracene and lupeol was obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. p53, Bcl-2 and Bax, primary antibodies were purchased from Dako, Carpinteria, CA, USA. Power Block™ reagent and secondary antibody conjugated with horseradish peroxidase were purchased from BioGenex, San Ramon, CA, USA.

The caspase 3 and -9 colorimetric assay kits were purchased from Biovision, Mountain View, CA, USA.

#### Experimental design

The institutional animal ethics committee (Register number 160/1999/CPCSEA), Annamalai University, Annamalainagar, India, approved the experimental design. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with Indian National Law on animal care and use. A total number of 40 hamsters were randomized into four groups of ten hamsters in each. Group I hamsters served as control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Groups II and III hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group III hamsters were orally given lupeol at a dose of 50 mg/kg bw/day, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the end of the experiment. Group IV hamsters received oral administration of lupeol 50 mg/kg bw/day alone throughout the experimental period. The experiment was terminated at the end of 16th week and all hamsters were sacrificed by cervical dislocation.

#### Immunohistochemical staining

Paraffin embedded tissue sections were dewaxed and rehydrated through grade ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3%  $\text{H}_2\text{O}_2$  in methanol for 10 minutes. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid/L D. $\text{H}_2\text{O}$ ; 0.37 g EDTA/L D. $\text{H}_2\text{O}$ ; 0.2 g Trypsin) (pH 6.0) for 10 minutes, followed by washing step with Tris-buffered saline (8g NaCl; 0.605g Tris) (pH 7.6). The tissue section was then incubated with power Block™ reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent, for 15 minutes at room temperature to block non-specific binding sites. The tissue sections were then incubated with the respective primary antibody (p53, Bcl-2 and Bax - Dako, Carpinteria, CA, USA) overnight at  $4^{\circ}\text{C}$ . The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish

peroxidase (BioGenex, San Ramon, CA, USA) for 30 minutes at room temperature. After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3,3'-diaminobenzidine, the substrate of horseradish peroxidase. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium. Each slide was microscopically analyzed and enumerated the percentage of the positively stained cells semi-quantitatively. The percentage of positive cells was scored according to the method of Nakagawa et al. (1994) as follows: 3+=strong staining, more than 50% of cells were stained; 2+=moderate staining, between 20 and 50% of cells were stained; 1+=weak staining, between 1 and 20% of cells were stained; 0=negative, less than 1% of cell staining.

#### Estimation of caspase 3 and 9 activities by enzyme linked immunosorbent assay (ELISA)

The activities of caspase-3 and 9 were assayed in the buccal mucosa using ELISA kits for caspase-3 and 9 according to the manufacturer's instructions. The caspase-3 and 9 assays are based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA and LEHD-pNA respectively at 405nm in a microtiter plate reader.

#### Statistical analysis

Values are expressed as mean±standard deviation (SD). Statistical comparisons were performed by one-way analysis of variance followed by Duncan's Multiple Range Test. The results were considered statistically significant if the p values were less than 0.05.

## Results

The tumor incidence, tumor volume, tumor burden and histopathological changes of control and experimental hamsters are shown in Table 1. We observed 100% tumor formation with mean tumor volume ( $258.62\text{ mm}^3$ ) and tumor burden ( $672.41\text{ mm}^3$ ) in hamsters treated with DMBA alone. The tumor was histopathologically confirmed as well differentiated squamous cell carcinoma. Oral administration of lupeol at a dose of 50 mg/kg bw completely prevented the tumor incidence, tumor volume and burden in hamsters treated with DMBA. Also, lupeol administration significantly reduced the severity of precancerous lesions in hamsters treated with DMBA. We noticed only mild hyperplasia, hyperkeratosis and dysplasia in hamsters treated with DMBA+lupeol. Tumors were not formed in control hamsters painted with liquid paraffin alone as well as hamsters administered with lupeol alone.

The immunoeexpression pattern of apoptotic (p53, Bcl-2 and Bax) markers and the score of positively stained cells in control and experimental hamsters in each group are depicted in Figure 1 and Table 2 respectively. Over expression of p53 and Bcl-2 and decreased expression of Bax was noticed in hamsters treated with DMBA alone. Oral administration of lupeol at a dose of 50 mg/kg bw

to hamsters treated with DMBA significantly restored the expression of above markers. Hamsters treated with lupeol alone revealed expression similar to that of control hamsters.

**Table 1. Incidence of Oral Neoplasm and Histopathological Changes in the Control and Experimental Hamsters in Each Group (n=10)**

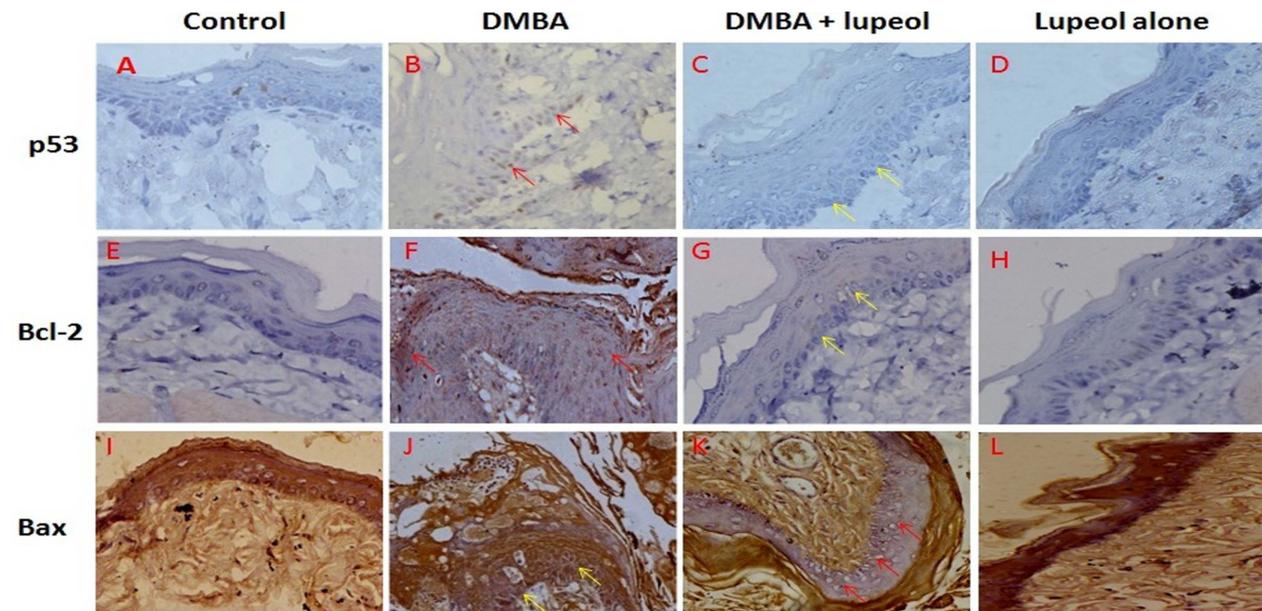
Parameter	Group			
	I	II	III	V
Tumor incidence (oral squamous cell carcinoma)	0	100%	0	0
Total number of tumour /animals	0	26/10	0	0
Tumour volume (mm <sup>3</sup> )/animals	0	258.62±21.47	0	0
Tumour burden (mm <sup>2</sup> )/animals	0	672.41±55.82	0	0
Hyperplasia	0	+++	+	0
Hyperkeratosis	0	+++	+	0
Dysplasia	0	+++	+	0
Well differentiated squamous cell carcinoma	0	+++	+	0

\*Group I Control, Group II DMBA alone, Group III (DMBA+andrographolide) and Group V (Andrographolide alone). Tumour volume was measured using the formula,  $v=(4/3)\pi [D_1/2][D_2/2][D_3/2]$  where  $D_1$ ,  $D_2$  and  $D_3$  are the three diameters (mm<sup>3</sup>) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal. 0=Absent; +++=Severe; ++=Moderate; +=Mild

**Table 2. The Score of Positively Stained Cells of p53, Bcl-2 and Bax in Control and Experimental Hamsters in Each Group**

Groups/Markers	P53				Bcl-2				Bax			
	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+
Control	10	0	0	0	10	0	0	0	3	4	2	1
DMBA	0	1	3	6	0	1	2	7	9	1	0	0
DMBA + lupeol	6	2	1	1	7	2	1	0	1	3	2	4
Lupeol alone	10	0	0	0	9	1	0	0	3	3	2	2

\*Values are given as number of hamsters (n=10). The percentage positive cells were scored as: 3+=strong staining, more than 50% of cells were stained, 2+=moderate staining, between 20 and 50% of cells were stained 1+=weak staining, between 1 and 20% of cells were stained, 0=negative, less than 1% of cell staining

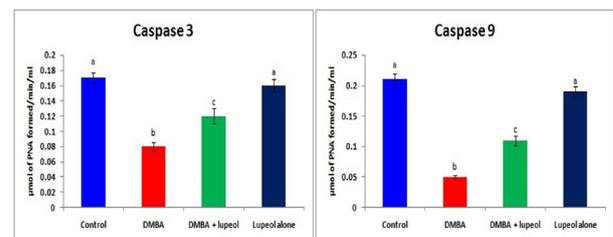


**Figure 1. Immunexpression Pattern of p53, Bcl-2 and Bax Proteins Observed in the Buccal Mucosa of Control and Experimental Hamsters in Each Group (40X).** P53: A and D) Control and lupeol alone (expression not detectable), B) DMBA alone (over expressed [←]), C) DMBA + lupeol (down regulated [←]). Bcl-2: E and H) Control and lupeol alone (expression not detectable); F) DMBA alone (over expression [←]); G) DMBA + lupeol (down regulated [←]). Bax: I and L) Control and lupeol alone (nuclear expression positive); J) DMBA alone (nuclear expression negative [←]); K) DMBA+lupeol (nuclear and cytoplasmic expression positive [←])

The status of caspase 3 and 9 in the buccal mucosa of control and experimental hamsters in each group is shown in Figure 2. The activities of caspase 3 and 9 were decreased in hamsters treated with DMBA alone. Oral administration of lupeol to hamsters treated with DMBA brought back the status of above markers to near normal range. No significant difference was noticed in the status of above markers in control hamsters and hamsters administered with lupeol alone.

## Discussion

Apoptosis is essential for the maintenance of tissue homeostasis and elimination of unwanted or damaged cells from multi-cellular organisms. Aim of the present study was to explore the pro-apoptotic potential of lupeol during DMBA-induced hamster buccal pouch carcinogenesis. In the present study, we noticed deregulation in the apoptotic markers (p53, Bcl-2, Bax, Caspase 3 and 9) during DMBA-induced oral carcinogenesis. Genomic aberrations in the apoptotic pathway are central to carcinogenesis. Deregulation in either pro-apoptotic or anti-apoptotic



**Figure 2. Caspase 3 and 9 Activities in Control and Experimental Hamsters in Each Group.** Values are expressed as mean±SD for 10 hamsters in each group. Values that do not share a common superscript letter between groups differ significantly at  $p < 0.05$  (Analysis of variance followed by DMRT)

pathways extends the survival of genetically altered cells and make them more prone to neoplastic transformation (Lee et al., 2007). p53, a molecular policeman, protects cells from wide array of genotoxic insult including chemical insults, radiation, mitotic stress and oncogene activation. Chemical mutagen or carcinogen induced DNA damage turn on p53 function to induce cell cycle arrest or apoptosis depending on the severity of DNA damage. If p53 function is impaired, the damaged cell may undergo neoplastic transformation. Aberrations of p53 gene are the most common genetic alterations in oral cancer (Nigam et al., 2010). It has been reported that lack of p53 activity decreases the transcription of bax, which in turn results in reduced apoptotic activity (Goloudina et al., 2012). Loss of p53 function or p53 mutation provides selective advantage for clonal expansion of preneoplastic and neoplastic cells (Yoon et al., 2012). Our results are in line with these findings.

The Bcl-2 family, which includes 17 or more members in mammalian cells, function as a 'life/death switch' that integrates diverse inter and intracellular cues to determine whether or not the stress apoptosis pathway should be activated (Wang et al., 2012). Bcl-2, an anti-apoptotic protein, prolongs the survival of genetically damaged cell by repressing apoptosis. Over expression of Bcl-2 and down regulation of Bax has been reported in several types of tumor tissues including oral tumors (Manikandan et al., 2008; Manoharan et al., 2011). Over expression of Bcl-2 in tumors is also associated with resistance to chemotherapeutic treatment (Letchoumy et al., 2007). Upregulation of Bcl-2 in endothelial cells in oral squamous cell carcinoma is shown to enhance tumor angiogenesis and accelerated tumor growth. Expression of Bcl-2 in tumor cells around the vasculature of tumors was found to be an indicator of poor prognosis (Subapriya et al., 2006). Administration of anti-Bcl-2 ribozymes into oral cancer cells resulted in apoptotic induction (Gibson et al., 2000). Extensive studies have shown over expression of Bcl-2 proteins in the early phase of carcinogenesis, which indicates impairment in apoptotic process (Liu et al., 2011; Plati et al., 2011). Bcl-2 over expression was shown in oral premalignancies and carcinomas (Balakrishnan et al., 2010). Our results corroborate these findings.

Bax expression was noticed in the cytoplasm uniformly in all cell layers of the normal squamous epithelium. Decreased expression of Bax in the cancerous tissues may reduce apoptotic cell death as well as accelerate their growth (Zhang et al., 2006). A positive association between loss of Bax immune positivity and shorter survival of patients with metastatic breast adenocarcinoma has been shown (Bukholm et al., 2002). An inverse correlation between p53 and Bax immunostaining has been reported in oral carcinoma (Manoharan et al., 2011). Our results support these observations.

Caspase 3 and 9 have been identified as being a key mediator of apoptosis in mammalian cells. Extensive studies reported that chemotherapeutic drugs induce apoptosis through caspase activation (Harish et al., 2010; Ye et al., 2012). Decreased levels of caspase 3 and 9 were reported in experimental carcinogenesis (Manoharan et al., 2011). While the pro-apoptotic protein promotes the

activation of caspase cascade, the anti-apoptotic protein inhibits caspase cascade. Decreased levels of caspase 3 and 9 noticed in hamsters treated with DMBA alone could thus be due to imbalance in Bcl-2/Bax ratio.

In the present study, oral administration of lupeol at a dose of 50 mg/kg bw restored the expression pattern of p53, Bcl-2 and Bax and the activities of caspase 3 and 9 in the buccal mucosa of hamsters treated with DMBA. Saleem et al. (2005) reported that lupeol specifically activated the fas receptor-mediated apoptotic pathway in androgen-sensitive prostate cancer cells. Nigam et al. (2009) reported that lupeol exhibited antiproliferative and apoptotic potential by inducing p53 and cyclin-B mediated G2/M cell cycle arrest as well as through up regulating Bax and caspase 3 and down regulating Bcl-2 in DMBA induced alterations in mouse skin. Saleem et al. (2008) pointed out that lupeol inhibited the growth of human metastatic melanoma cells in vitro and in vivo by up regulating Bax and caspase 3 and down regulating Bcl-2. Prasad et al. (2009) reported that lupeol induced apoptosis in prostate cancer cells by up regulating Bax, caspase 3 and 9 and down regulating Bcl-2 genes. The present study are in line with other reported findings on apoptotic potential of lupeol. The pro-apoptotic potential of lupeol noticed in the present study might have inhibited the oral tumor formation during DMBA-induced hamster buccal pouch carcinogenesis. Our results thus conclude that lupeol could be used as an adjuvant in the prevention of early stages of oral carcinogenesis.

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