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Research Article

Hepatoprotective effects of lupeol and mango pulp extract of carcinogen induced alteration in Swiss albino mice

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Lupeol, a triterpene present in mango and other fruits, is known to exhibit a number of pharmacological properties including antioxidant, antilithiatic, and antidiabetic effects. In the present study, chemopreventive properties of lupeol and mango pulp extract (MPE) were evaluated against 7,12-dimethylbenz(a)anthracene (DMBA) induced alteration in liver of Swiss albino mice. Lupeol (25 mg/kg body weight, bw) or 1 mL of 20% w/v aqueous MPE/mouse were daily given once for 1 wk after a single dose of DMBA (50 mg/kg bw). Lupeol/MPE supplementation effectively influenced the DMBA induced oxidative stress, characterized by restored antioxidant enzyme activities and decrease in lipid peroxidation. A reduction of apoptotic cell population in the hypodiploid region was observed in lupeol and MPE supplemented animals. The inhibition of apoptosis was preceded by decrease in reactive oxygen species (ROS) level and restoration of mitochondrial transmembrane potential followed by decreased DNA fragmentation. In DMBA treated animals, downregulation of antiapoptotic Bcl-2 and upregulation of proapoptotic Bax and Caspase 3 in mouse liver was observed. These alterations were restored by lupeol/MPE, indicating inhibition of apoptosis. Thus, lupeol/MPE was found to be effective in combating oxidative stress induced cellular injury of mouse liver by modulating cell-growth regulators.

Keywords: Apoptosis / DMBA / Lupeol / MPE / Oxidative stress

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1 Introduction

Mango (*Mangifera indica* L.) is one of the most commonly eaten fruits in the tropical countries around the world. The beneficial health effects of mango pulp include antilithiatic and free radical scavenging properties by reducing lipid peroxidation and mango containing herbal formulation led to enhanced activities of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) against isoproterenol as reported in kidney and heart of rats [1]. Mango extract has further been shown to be antimutagenic in *Salmonella typhimurium* strain TA1538 [2]. In addition, mango pulp extract (MPE) has been shown to modulate the neoplastic transformation of cancer cells *in vitro* suggesting its chemopreventive properties [3]. However, *in vivo* data supporting these effects is rather scarce. Lupeol (Lup-20 (29)-en-3-β-ol) is a naturally occurring pentacyclic triterpene of mango (Fig. 1). Lupeol has antitumor promoting effects on the 7,12-dimethylbenz(a)anthracene (DMBA) induced mouse skin tumorigenesis model [4]. The triterpene has also been shown to exhibit strong anti-inflammatory, antiarthritic, antimutagenic, and antimalarial effects [5]. The pretreatment of lupeol changed the tissue redox system against cadmium chloride by scavenging the free radicals and by improving the antioxidant status of the liver [6]. We tested both lupeol and MPE in the present study in order to check their efficacy against oxidative stress.

Free radicals or reactive oxygen species (ROS) are generated by a variety of environmental pollutants such as cigar-

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Abbreviations: CAT, catalase; **CDNB,** 1-chloro-2, 4-dinitrobenzene; **DCFH-DA,** dichlorodihydrofluoroscein diacetate; **DMBA,** 7,12-dimethyl-benz(a)anthracene; **FCCP,** carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; **GR,** glutathione reductase; **gr.**, grounp; **GSH,** reduced glutathione; **GSSG,** oxidized glutathione; **GST,** glutathione S-transferase; **MFI**, mean fluorescence intensity; **MPE,** mango pulp extract; **NADH,** nicotinamide adenine dinucleotide reduced; **NADPH,** nicotinamide adenine dinucleotide phosphate reduced; **PI,** propidium iodide; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase

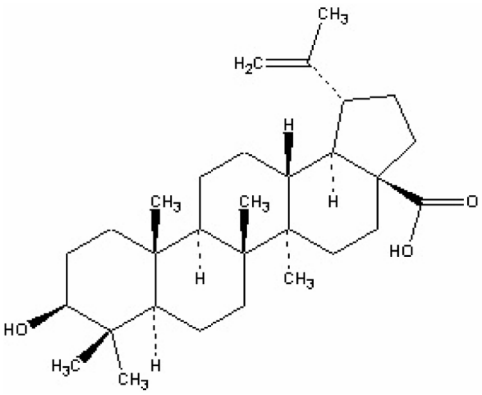

Figure 1. Chemical structure of lupeol.

ette smoke, automobile exhaust fumes, radiation, and exposure to xenobiotic compounds. In biological systems, ROS are continuously generated by the body's normal use of oxygen [7, 8]. Due to their high chemical reactivity, free radicals are able to induce cellular damage in a number of ways [9]. The most deleterious effects of free radicals include DNA damage [10], which can lead to a number of pathological conditions including cancer. However, ROS can act as damaging entities, but at the same time also carry out some important beneficial biological effects [11, 12]. ROS has been shown to be mediators, triggers or executioners of essential protective mechanisms such as apoptosis, phagocytosis, and detoxification reactions. Increase of ROS concentration by depletion of antioxidants, enhances apoptosis and thereby inhibits neoplastic growth. Excessive antioxidants decrease ROS level, inhibit apoptosis and suppress the elimination of cancer cells [11, 12].

Apoptosis is a highly organized cell death process, characterized by loss of plasma membrane phospholipid asymmetry, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the cells into membrane-bound apoptotic bodies [13]. Genetic changes resulting in loss of apoptosis or derangement of apoptosis-signaling pathways in the transformed cells are likely to be critical components of carcinogenesis [14, 15]. Additionally, induction of apoptosis of cancer cells is recognized as a valuable tool for management of cancer [16]. The execution process of cells by apoptosis is mediated by Caspase-3, one of a family of cysteine proteases [17]. It is thought to be regulated by Bax and Bcl-2. Proapoptotic Bax forms pores in the outer mitochondrial membrane, releasing cytochrome *c* while antiapoptotic Bcl-2 prevents the opening of mitochondrial transition pore by binding with Bax [18, 19]. ROS have been suggested to act as an upstream signal for Caspase-3 activation [20, 21].

The present study was designed to evaluate the effect of lupeol/MPE on DMBA induced hepatic alterations on ROS generation, lipid peroxidation, status of the antioxidant enzymes, mitochondrial membrane potential and events involved in apoptosis such as modulation of Bcl-2, Bax and Caspase-3, and DNA fragmentation in Swiss albino mice.

2 Materials and methods

2.1 Chemicals

DMBA, lupeol, dichlorodihydrofluorescein diacetate dye (DCFH-DA), Rhodamine 123, propidium iodide (PI), carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), phenazine methosulfate (PMS), 1-chloro-2,4-dinitrobenzene (CDNB), 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP), nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide phosphate reduced (NADPH), nicotinamide adenine dinucleotide reduced (NADH), reduced glutathione (GSH) oxidized glutathione (GSSG), and β-actin (clone AC-74) were purchased from Sigma (St. Louis, USA). The Bcl-2 (polyclonal antirabbit IgG), Bax (polyclonal antirabbit IgG), and Caspase-3 (antimouse IgG) antibody were procured from Oncogene Research Products (Cambridge, USA). The antimouse and antirabbit horseradish-peroxidase conjugate secondary antibodies were obtained from Banglore Genei (India). The nitrocellulose membrane was obtained from Millipore, (Bedford, Massachusetts, USA). The rest of the chemicals were of analytical grade of purity and procured locally.

2.2 Preparation of MPE

MPE was prepared by using modified protocol of Iagher *et al.* [22]. Briefly, fresh mango pulp (20 g) from ripe fruit (*M. indica* cv. Dashehari) was mixed with 100 mL of 0.1 M (pH 7.0) phosphate buffer (PBS). The resulting homogenate was filtered through four-layered muslin cloth and then centrifuged at $6000 \times g$ for 20 min at room temperature. Supernatant was used as MPE [22].

2.3 Animals and treatment

Adult, Swiss albino mice (25 ± 2 g body weight, bw) were taken from ITRC animal colony and acclimatized for 1 wk under standard conditions (25 ± 2°C, relative humidity 57 ± 2% and 12/12 h light/dark phase). The animals were randomly divided into six groups (gr.), each comprising of six animals. During the course of experiment animals were kept under same standard conditions and were fed with synthetic pellet diet (M/S Ashirwad, Chandigharh, India). Mice in gr. I were fed with corn oil, which was used as vehicle for DMBA, by gavage, whereas the animals of gr. III and V were given lupeol (25 mg/kg bw daily once for 1 wk) by gavage. The dose and duration of lupeol supplementation was selected on the basis of our pilot experiments and it found to be effective at this dose which is 50% of the dose where lupeol has been shown to protect from hepatotoxicity [23]. In gr. IV and gr. VI, animals were given 1.0 mL MPE

daily for 1 wk. Single dose of DMBA (50 mg/kg bw dissolved in 0.2 mL corn oil) was given by gavage to the gr. II (positive controls), III, and IV 24 h before lupeol/MPE administration. The feeding regimen of lupeol/MPE was followed for 7 day continuously. Animals were examined every day for distinct morphological changes during the study period. On 8th day, all the animals were killed humanely by cervical dislocation. Liver from each animal was excised and immediately washed with ice-cold saline and stored at -80°C till further analysis.

2.4 Biochemical determinations

The liver tissue was homogenized individually in ice-cold phosphate buffer (pH 7.4) containing 0.15 M KCl. The homogenate was centrifuged at $9000 \times g$ for 10 min and the supernatant was taken as enzyme source. Activity of the enzyme SOD was analyzed according to Kakkar *et al.* [24]. Briefly, the assay mixture in a final volume of 3 mL contained 0.052 M sodium pyrophosphate buffer (pH 8.3), 186 μM PMS, 300 μM NBT, 780 μM NADH, enzyme source, and Milli-Q water. The reaction was initiated by addition of NADH followed by incubation at 37°C for 90 s. The reaction was stopped by the addition of 1.0 mL of glacial acetic acid and the contents were shaken vigorously with 4.0 mL of *n*-butanol, allowed to stand for 10 min before they were centrifuged and butanol layer was separated. The color intensity of chromogen in butanol was measured against butanol using a spectrophotometer (Thermo Spectronic, Genesys, USA). A reaction mixture without of enzyme served as control. A single unit of enzyme activity is defined as the quantity of SOD required for 50% inhibition of reaction. The activity of enzyme CAT was analyzed according to the method of Sinha [25] using H_2O_2 as substrate. In brief, the reaction in a final volume of 3 mL consisted of phosphate buffer (pH 7.0), 0.2 M H_2O_2, and enzyme protein. The enzyme activity measured following the disappearance of H_2O_2 at 570 nm and was expressed as micromoles of H_2O_2 consumed/min/mg protein. Glutathione reductase (GR) activity was determined by the procedure of Carlberg and Mannervic [26]. Briefly, the assay mixtures in a final volume of 3.0 mL contained 0.067 M phosphate buffer (pH 6.6), NADPH, 7.5×10^{-3} GSSG (pH 6.6) enzyme, and water. The reaction was initiated with enzyme preparation. The difference in OD *per* 30 s was measured for 3 min at 340 nm against a reference cuvette without GSSG and NADPH. The activity was expressed in nanomoles/min/mg protein. Glutathione S-transferase (GST) was analyzed by the method of Habig *et al.* [27]. The assay mixture in a final volume of 3.0 mL contained 0.2 M phosphate buffer (pH 6.5), GSH, CDNB, enzyme, and water. The reaction was initiated by addition of CDNB. The difference in OD *per* 30 s was measured at 340 nm for 3 min against a reference cuvette without enzyme. The activity was expressed in nanomoles CDNB – GSH conjugate/min/mg protein. Lipid peroxidation was analyzed by the method of Ohkawa *et al.* [28]. The reaction mixture in a final volume of 3.0 mL contained enzyme, 100 μL of 10% sodium dodesyl sulfate (SDS), 600 μL of 20% glacial acetic acid, 600 μL of 0.8% TBA, and water. The mixture was placed in boiling water bath 1 h and immediately shifted to crushed ice bath for 10 min. The mixture was centrifuged at $2500 \times g$ for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed was assayed by measuring OD of supernatant at 535 nm against a blank without enzyme. The activity was expressed as *n* moles of TBARS/mg of tissue protein using TMP as standard. The protein content of the tissue was determined by the method of Lowry *et al.* [29] using BSA as standard.

2.5 Flow cytometric analysis of apoptosis

Single cell suspension of liver tissue was prepared by using Madimachine (Becton-Dickinson, San Jose, CA, USA) and centrifuged at 2000 rpm for 10 min at 4°C. The cell pellet resuspended in 50 μL cold PBS and fixed in 2 mL of 70% ice-cold ethanol. Cells were centrifuged from the fixative and treated with 0.1% Triton X-100 for 5 min. After incubation, cells were again centrifuged and resuspended in 1 mL of PBS, ribonuclease (100 μg/mL) was added, and the cells were incubated at 37°C for 30 min. After further centrifugation, cells were resuspended in 1 mL of PBS and 50 μg/mL PI and incubated for 18 h at 4°C. The 10 000 cells were acquired and analyzed on a flow cytometer using "Cell Quest 2.0" software [30].

2.6 Measurement of ROS generation

ROS production was monitored by flow cytometry (Becton-Dickinson, San Jose, CA, USA) using DCFH-DA dye as described by Esposti and McLennan [31]. Single cell suspension was prepared as described above in PBS supplemented with 50 mM glucose and incubated with 10 μM DCFH-DA at 37°C for 1 h. The fluorescence increase due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH) by some nonspecific cellular esterases and its subsequent oxidation by peroxides was measured. Values were given in terms of mean fluorescence intensity (MFI) using software Cell Quest 2.0.

2.7 Mitochondrial membrane potential (ψm) analysis

Mitochondrial membrane potential was assessed by using Rhodamine 123. Rhodamine 123 accumulates in normal mitochondria and decline of ψm will lead to leakage of Rhodamine 123 from mitochondria, while its fluorescent intensity is reduced. The single cell suspensions were incubated with rhodamine 123 (5 μg/mL) for 60 min in dark at 37°C, harvested and suspended in PBS. Prior to Rhodamine

123, a mitochondrial uncoupler FCCP added for 10 min in one sample of control. The mitochondrial membrane potential was measured using flow cytometry (Becton – Dickinson, San Jose, CA, USA) by the fluorescence intensity (FL-1) of 10 000 cells [32].

2.8 DNA fragmentation

The DNA fragmentation pattern was carried out by agarose gel electrophoresis [33]. Untreated and treated cells of liver pelleted by centrifugation at 2000 rpm for 10 min and the pellet lysed with 0.5 mL lysis buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% Triton X-100) on ice for 30 min. The DNA in lysed solution was extracted with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5.2) and chilled ethanol. The purity of DNA was determined by measuring OD at 260 and 280 nm and the ratio obtained between 1.7 and 1.9. DNA (2 μg) was then loaded on 0.8% agarose gel and electrophoresis was carried out. The bands were visualized by ethidium bromide staining under UV light and recorded using a gel documentation system (Herolab, Germany).

2.9 Western blotting

Western blotting was carried out as described earlier [34] in the liver tissue homogenate. Protein contents were estimated [29]. Proteins (30 μg) were resolved on 10% gel followed by electro-transfer onto an immobile PVDF membrane using semidry transfer (BioRad Laboratories, Hercules, CA, USA). The membranes were blocked overnight with 5% nonfat dry milk and probed with primary antibody of Bcl-2/Bax/Caspase-3 at dilutions recommended by the suppliers. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. Finally, the intensity of the bands was quantitated using Easy Win 32 software on a gel documentation system (Herolab). Densitometry measurements of the scanned bands were performed using digitalized scientific software program UNSCAN. Data were expressed as mean values ± SE of three separate sets of experiments.

2.10 Statistical analysis

Significance difference of variance in antioxidant enzymes activity data between DMBA treated positive control (gr. II) and experimental groups (gr. III – VI) was analyzed using student's *t*-test and $p < 0.05$ and $p < 0.01$ were considered to be significant.

3 Results

3.1 Activities of antioxidant enzymes

Lupeol and MPE administration resulted in a significant ($p < 0.05$) protection against DMBA induced alteration in antioxidant enzyme activities. A significant decrease of the enzyme SOD, CAT, GR and GST in liver was recorded in the animals of gr. II, following DMBA administration. The activities of SOD, CAT, GR and GST decreased upto an extent of 39, 45, 44, and 48% respectively in comparison to the untreated control (gr. I). The protection by lupeol was upto an extent of 31, 26, 40, and 39% respectively in comparison to animals of gr. II. The MPE administration along with DMBA (gr. IV) also resulted in protection against antioxidant enzymes upto an extent of 28, 38, 36, and 33% in SOD, CAT, GR, and GST activity respectively (Table 1). These results clearly demonstrated that lupeol/MPE can effectively combat oxidative stress induced by DMBA at the major site of metabolism, *i. e.*, liver.

3.2 Lipid peroxidation levels

The rate of lipid peroxidation is a reliable marker for xenobiotic induced oxidative stress. DMBA administration resulted in a significant ($p < 0.05$) increase in the TBARS

Table 1. Activities of antioxidant enzymes and lipid peroxidation induced by lupeol/MPE against DMBA in liver of Swiss albino mice

Treatment	Superoxide dismutase (U/mg protein)	Catalase (μmol/min/mg protein)	Glutathione reductase (nmol/min/mg protein)	Glutathione S-transferase (nmol/min/mg protein)	Lipid peroxidation (nmol TBARS/mg protein)
Untreated	7.1 ± 0.6	290.4 ± 26.6	74.9 ± 8.8	25.4 ± 3.4	1.40 ± 0.5
DMBA	4.3 ± 0.5 (39.4%)[a)]	161.2 ± 20.6 (44.5%)[a)]	41.9 ± 4.0 (44.1%)[a)]	13.1 ± 2.3 (48.3%)[a)]	2.62 ± 0.2 (87.1%)[c)]
DMBA + Lupeol	5.6 ± 0.6 (30.5%)[b)]	202.5 ± 25.5 (25.6%)[b)]	58.7 ± 6.1 (40%)[b)]	18.1 ± 2.4(38.5%)[b)]	1.77 ± 0.2 (32.2%)[d)]
DMBA + MPE	5.5 ± 0.5 (28.4%)[b)]	222.3 ± 18.6 (37.8%)[b)]	57.1 ± 6.2 (36.1%)[b)]	17.5 ± 2.2 (33.2%)[b)]	1.93 ± 0.2 (26.3%)[d)]
Lupeol	7.2 ± 0.8	296.4 ± 25.5	76.8 ± 7.5	26.5 ± 2.4	1.40 ± 0.1
MPE	7.2 ± 0.7	312.4 ± 28.2	78.6 ± 8.0	26.2 ± 2.3	1.38 ± 0.2

Values are expressed as mean ± SE of six animals. Data are significant as $p < 0.05$.DMBA – 50 mg/kg bw.
a) Represent significant decrease over untreated control group.
b) Represent significant increase over DMBA treated group.
c) Represent significant increase over untreated control group.
d) Represent significant decrease over DMBA treated group.

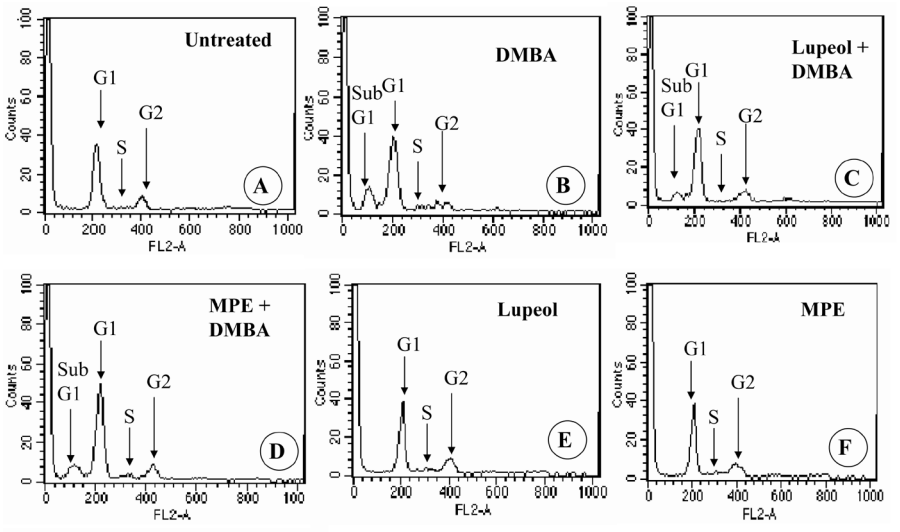

Figure 2. Effect of lupeol/MPE on apoptosis. Mouse liver of untreated (A) as well as lupeol/MPE (E, F) showing no sub-G1 peak while DMBA treated showing significantly increased peak (B) indicating cell death. The increased cell death (sub-G1 peak) was reduced by lupeol (C) as well MPE (D). PI fluorescence was measured using a flow cytometer with FL-2 filter and were expressed as histogram.

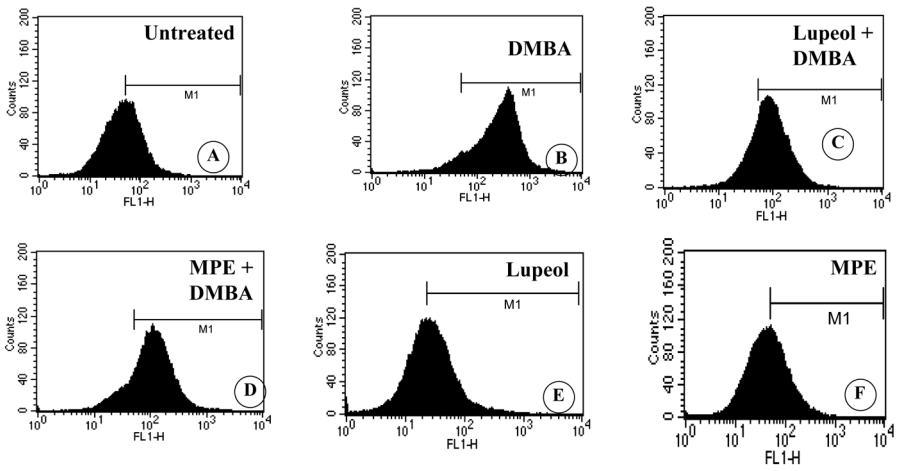

Figure 3. A flow cytometric analysis ROS level. DMBA treatment resulted significant production of ROS (B) in comparison to untreated control (A), which was quenched by lupeol/MPE in mouse liver (C, D). No significant increase in ROS level was found by lupeol/MPE alone treatment (E, F).

upto an extent of 87% over control. However, lupeol and MPE administration resulted in significant difference in the level of TBARS upto an extent of 32 and 26% respectively (Table 1).

3.3 Inhibition of cell death

In mouse liver, ROS produced by DMBA metabolism in gr. II, increased the apoptotic cell death, this was observed by the appearance of sub-G1 peak, whereas no such peak was seen in untreated control gr. I and lupeol/MPE alone treated gr. V and VI (Fig. 2A – F). Considerable reduction in cell death was recorded after lupeol and MPE supplementation in gr. III and IV.

3.4 Levels of ROS

The intracellular ROS levels were determined by mean fluorescence intensity (MFI) of 2,7′- dichlorofluorescein using flow cytometry. DMBA treatment significantly increased the ROS level (MFI 65.90) in comparison to untreated gr. I (MFI 28.34) (Figs. 3A and B). These induced levels of ROS were reduced significantly by both lupeol (MFI 44.73) (gr. III) and MPE (MFI 54.12) (gr. IV) administration (Figs. 3C and D). However, there was no significant change observed in lupeol/MPE alone treated animals of gr. V (MFI 28.67) and VI (MFI 29.05) (Figs. 3E and F).

3.5 Restoration of mitochondrial membrane potential (ψm)

During apoptosis, dying cells undergo an ordered series of events resulting in the activation of proteases, accumulation of intracellular peroxidase, protein release, and, most importantly, alteration in plasma membrane. We examined changes in mitochondrial membrane potential (ψm) during DMBA treatment by flow cytometry. FCCP dissipates the mitochondrial electrochemical gradient. As predicted, addi-

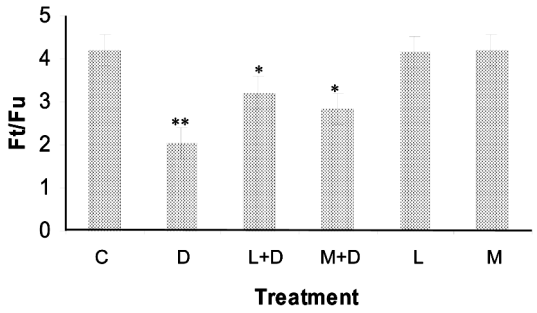

Figure 4. Effect of lupeol/MPE on mitochondrial membrane potential. Diagram showing significant ($p < 0.01$) loss of mitochondrial membrane potential in DMBA treated mouse liver, represented by ratio between total fluorescence and fluorescence after mitochondrial uncoupling (Ft/Fu), which was significantly ($p < 0.01$) restored by lupeo/MPE. No significant ($p < 0.01$) change in mitochondrial membrane potential was found by lupeol/MPE alone treatment [untreated control (C), DMBA alone (D), Lupeol + DMBA (L + D), MPE + DMBA (M + D), lupeol alone (L), MPE alone (M)]. Rhodamine 123 fluorescence was measured using a flow cytometer with FL-1 H filter. Values are expressed as mean + SE of six animals. * Significant ($p < 0.01$) over untreated group; ** significant ($p < 0.01$) over DMBA treated group.

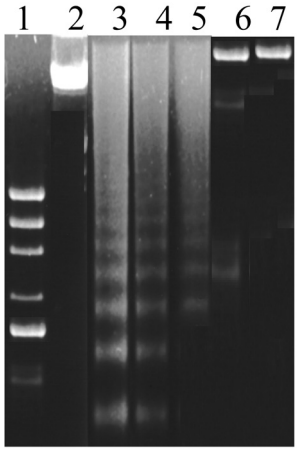

Figure 5. Effect of lupeol/MPE on DNA fragmentation by (0.8%) agarose gel electrophoresis. Untreated, lupeol, and MPE treatment (lanes 2, 6, and 7) showing single band or slight fragmentation of genomic DNA while DMBA treatment resulted DNA fragmentation indicated by clear ladder (lane 3), which was reduced by lupeol/MPE treatment (lanes 4 and 5). Lane 1 showing marker DNA.

tion of FCCP to the untreated control liver cells produced a rapid and marked decrease in fluorescence, indicating loss of potential. The loss of mitochondrial membrane potential was determined by the ratio between total fluorescence (Ft) and fluorescence after mitochondrial uncoupling (Fu). A significant loss of mitochondrial membrane potential (Fig. 4) was observed by administration of DMBA (Ft/Fu 2.05) ($p < 0.05$) in comparison to untreated control (Ft/Fu 4.2), which was restored by pretreatment of both lupeol (Ft/Fu 3.22) ($p < 0.05$) and MPE (Ft/Fu 2.85) ($p < 0.05$).

3.6 Detection of fragmented DNA by agarose gel electrophoresis

DNA fragmentation resulting in a ladder formation is a characteristic feature of apoptosis. A clear DNA fragmentation was observed in the cells of mice exposed to DMBA. DNA fragments (DNA ladder) showing varying sizes between 200 and 2000 bp were clearly visible by agarose gel electrophoresis (Fig. 5). Lupeol/MPE prevented DNA fragmentation efficiently, but not totally. No specific DNA fragments were detected in gr. I, V, and VI.

3.7 Lupeol/MPE modified the expression level of Bcl-2, Bax, and Caspaes-3

The levels of Bcl-2, Bax, and Caspase-3 have been reported to play a crucial role in apoptotic response mediated by many chemopreventive agents. In the present study, Western blot analysis revealed that DMBA (gr. II) induced downregulation of Bcl-2 (~1.9-fold compared to untreated control) (Fig. 6A) and upregulation of Bax (Fig. 6B), and Caspase-3 protein (~1.5 and ~1.6-fold compared to control) (Fig. 6C) supporting evidence of apoptotic cell death. Densitometry of the immunoblot shows that lupeol/MPE supplementation significantly increased the Bcl-2 and decreased the Bax in gr. III and IV. Lupeol/MPE administration resulted in further downregulation of Caspase-3 levels. However, lupeol/MPE alone (gr. V and VI) had no effect on the levels of Bcl-2, Bax, and Caspase-3 protein (Fig. 6A–C) in liver.

4 Discussion

The metabolism of DMBA has been shown to lead to free radicals, which play a critical role in pathogenesis [35]. DMBA is metabolized by cytochrome P4501A1 and cytochrome P4501B1 in liver microsomes to form diol epoxides and other toxic ROS such as peroxides, hydroxyl, and superoxide anion radicals. Here we showed that DMBA exposure increased the ROS level during its *in vivo* metabolism. These results extend the earlier studies performed on liver and other tissues in other laboratories [36]. The metabolites of DMBA including diol epoxides are capable of binding to adenine residues of DNA causing chromosomal damage [37].

Because of the pivotal role of ROS in triggering apoptosis, antioxidants can inhibit this protective mechanism by depleting ROS [38, 39]. Our data demonstrate that, indeed, apoptosis induced by DMBA is accompanied by an increase in ROS generation. The inhibition of apoptosis in DMBA

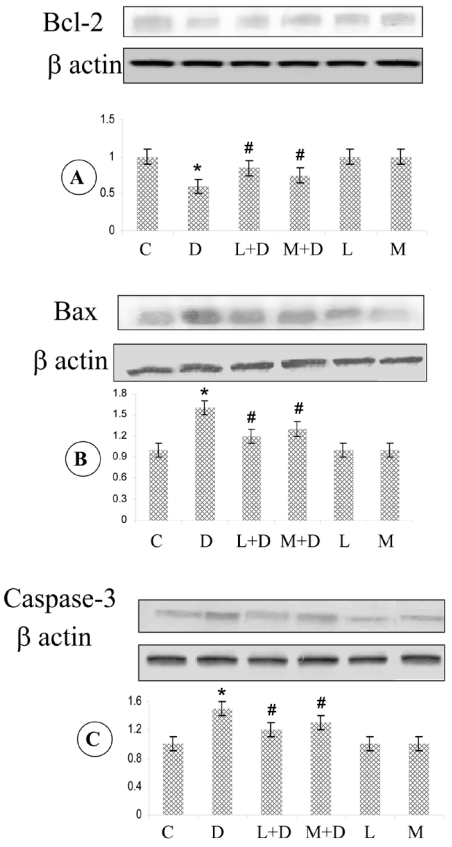

Figure 6. Western blot and its densitometric analysis of (A) Bcl-2, (B) Bax, and (C) Caspase-3 expression in mouse liver tissue in different groups [untreated control (C), DMBA alone (D), lupeol + DMBA (L + D), MPE + DMBA (M + D), lupeol alone (L), MPE alone (M)]. Equal loading was confirmed by reprobing the membrane with β-actin. The bands shown here are from a representative experiment repeated three times with similar results.*,Values are significantly different over untreated group, $p < 0.01$.#,Values are significantly different over DMBA treated group, $p < 0.05$.

induced mouse hepatocytes is consistent with the view that lupeol/MPE deactivates preexisting apoptosis machinery. Treatment with lupeol/MPE in our study caused an induction of Bcl-2 and suppression of Bax and Caspase-3, associated with the decrease in fragmentation of nuclear DNA, which inhibit the DMBA induced onset of apoptosis. Mitochondrial transmembrane potential is often employed as an indicator of cellular viability and its disruption has been implicated in a variety of apoptosis phenomena [40]. Mitochondria are a source of ROS during apoptosis and reduced mitochondrial membrane potential leads to increased generation of ROS and apoptosis [19]. In the present study, lupeol/MPE restored the DMBA induced reduced mitochondria membrane potential and prevented normal cell death.

It has been shown that the Bcl-2 family plays an important regulatory role in apoptosis, either as activator (Bax) or as inhibitor (Bcl-2) [41 – 44]. Since Bcl-x_L can bind to Bax and prevent Bax insertion into the outer membrane of mitochondria, the decrease in total and mitochondrial Bcl-x_L promotes the changes of $\Delta\psi m$. It has also been demonstrated that the gene products of Bcl-2 and Bax play important roles in apoptotic cell death [45, 46]. In the present study, inhibition of DMBA induced cell death by lupeol/MPE in mouse liver was associated with decreased level of Bcl-2 and increased level of Bax and Caspase-3. Analysis of data indicates that lupeol/MPE may disturb the Bcl-2 and Bax level and, therefore, lead to decrease in apoptosis.

Oxidative stress due to increased ROS levels coupled with deficiency of host antioxidant defense mechanism might be a determining factor contributing in the development of cancer. To protect cells from oxidative stress, radical, and nonradical reactive species including peroxides and superoxides need to be inactivated enzymatically by CAT, SOD, and GPx [47], antioxidant enzymes that provide the cellular defense against the intermediates of dioxygen reduction (superoxide radical, hydrogen peroxide, and hydroxyl radicals). SOD converts superoxide radicals into hydrogen peroxide, which in turn has to be removed by CAT and GPx. Thus, CAT and GPx protect SOD against inactivation by hydrogen peroxides. Reciprocally, SOD protects CAT and GPx from inhibition by superoxide radicals [48]. There was a significant decline in the activities of SOD and CAT after DMBA administration, indicative of oxidative stress. These decreased antioxidant enzyme activities in liver were restored by the supplementation of lupeol/MPE. The protection provided by lupeol/MPE is possible by scavenging of superoxide and peroxyl radicals. GST catalyzes the detoxification of electrophilic species including metabolites of DMBA to protect the cells against peroxidative damage [49]. The reduced activity of GST observed by DMBA administration may be partly due to the lack of its substrate (GSH) [50], which occurs also due to reduced activity of GR. Our results nicely support the data from another report in which pretreatment of lupeol for a week caused reversion of aflatoxin induced peroxidative hepatic damage [51].

Lipid peroxidation is one of the main manifestations of oxidative damage initiated by ROS. It has been linked to the altered membrane structure and enzyme activation. It is initiated by the abstraction of a hydrogen atom from the side chain of PUFAs in the membrane [52]. The present data reveal that DMBA exposure causes a marked oxidative impact. The increase in lipid peroxides might result from increased production of free radicals and a decrease in antioxidant status. Lupeol/MPE significantly lowered lipid peroxidation and the values were comparable with that of the control animals. Recently, the mitigating role of lupeol has been shown on hepatic lipemic oxidative injury in rats *via* the same mechanism [23].

In summary, this study has demonstrated that lupeol is more effective than MPE and has antioxidant and antiapop-

totic effects. The data also imply that antioxidant enzymes and mitochondrial cell death pathway can be used as targets for studies on chemoprevention and that lupeol/MPE merits further investigations in future.

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