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RESEARCH

Lupeol, A Bioactive Triterpene, Prevents Tumor Formation During 7,12-Dimethylbenz(a)anthracene Induced Oral Carcinogenesis

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Abstract The oral cancer chemopreventive efficacy of lupeol, a bioactive triterpene, was assessed by monitoring the tumor incidence and using the status of phase I and II xenobiotic metabolizing enzymes, lipid peroxidation and antioxidants as biochemical end points during 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Oral tumors were developed in the buccal pouch of golden Syrian hamsters by painting with 0.5 % DMBA three times a week for 14 weeks. Well differentiated oral squamous cell carcinoma with marked abnormalities in the status of biochemical markers were noticed in hamsters treated with DMBA alone. Oral administration of lupeol at a dose of 50 mg/kg bw completely inhibited the formation of oral tumors and restored the status of biochemical markers during DMBA induced oral carcinogenesis. The present study thus demonstrates the chemopreventive potential of lupeol in DMBA induced oral carcinogenesis. The chemopreventive potential of lupeol is probably due to its antioxidant or free radical scavenging property and modulating effect on phase I and II xenobiotic metabolizing enzymes in favour of the excretion of carcinogenic metabolites during DMBA induced hamster buccal pouch carcinogenesis.

Keywords Antioxidants · DMBA · Lipid peroxidation · Lupeol · Oral cancer

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Introduction

Oral cancer, a preventable and avoidable malignancy, is the fifth most frequent cancer worldwide. Oral squamous cell carcinoma accounts for 90 % of all oral cancers. While oral cancer is one of the most common neoplasms worldwide, it is highly prevalent in South-East Asia. While oral cancer accounts form 3–4 % of all cancers in Western industrialized countries, this form of cancers accounts for 40–50 % of all cancers in developing countries including India, Pakistan, Bangladesh and Sri Lanka [1]. The use of tobacco and betel quid chewing, tobacco smoking and alcohol consumption are the major etiological or risk factors associated with the development of oral cancer. Tobacco and alcohol together enhanced the risk of oral carcinogenesis by exhibiting synergetic effect [2]. Despite extensive improvement in the diagnosis and advancement in the treatment strategy, oral cancer still contributes significantly to the morbidity and mortality as well as to low five-year survival rate of oral cancer patients [3].

7,12-dimethylbenz(a)anthracene (DMBA) mediates oral carcinogenesis in a step-wise manner, which proceeds from precancerous lesions (hyperplasia, hyperkeratosis, dysplasia) to malignant tumor. DMBA is metabolically activated to its ultimate carcinogenic metabolite, dihydrodiol epoxide, which subsequently binds to adenine and guanine residues of DNA and forms adducts, contributing to carcinogenesis. Golden Syrian hamsters are commonly used for in the induction of oral cancer and the cancer developed in the buccal pouch of these animals mimics the human oral tumor histopathologically, morphologically and at molecular level [4].

Liver has putative role in the metabolic activation and detoxification of carcinogens. Measurement of the activities of enzymes involved in these processes could thus help to

investigate the chemopreventive potential of natural products. Phase I enzymes (cytochrome P_{450}, b_5) are involved in the conversion of procarcinogen into its ultimate carcinogenic metabolites, which reacts and binds to DNA and initiating carcinogenesis. Phase II enzymes [Glutathione-S-transferase (GST), Glutathione reductase (GR) and Reduced glutathione (GSH) are involved in the detoxification and excretion of the carcinogenic metabolites, that are generated from phase I reaction, by conjugating the activated compounds to glutathione or glucuronic acid [5].

Reactive oxygen species (ROS) play pivotal role in phagocytosis and key role in several intracellular signal transduction pathways including apoptosis. Overproduction of ROS is involved in the initiation and promotion of carcinogenesis. Lipid peroxidation, a reactive oxygen species mediated chain reaction occurs in the biomembrane, has been implicated in the pathogenesis of several disorders including cancer [6]. Under normal circumstances, the deleterious effects of ROS are protected by non-enzymatic [Reduced glutathione (GSH) and vitamin E] and enzymatic [Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx)] antioxidants. Oxidative stress thus arises in the cell due to imbalance in the production of ROS and antioxidants. Altered lipid peroxidation by-products and antioxidants status has been reported in human and experimental oral cancer [7, 8].

Chemoprevention has evolved as an useful and novel strategy to investigate the anticancer potential of natural products and synthetic agents. Cancer of the oral cavity is one among the few human cancers with the vast potential for prevention. Triterpenes are important structural components of plant membranes are natural components of human diets. Epidemiological data suggest that the phytosterols content of diet is associated with a reduction in common cancers including cancers of the colon, breast and prostate [9]. A number of triterpenoids have shown promise as antineoplastic agents and exhibit antiproliferative activity when tested against various cancer cell lines [10]. Lupeol (Fig. 1), a bioactive triterpene is found in vegetables such as white cabbage, pepper, cucumber, tomato, in fruits such as olive, fig, mango, strawberry, red grapes and in several medicinal plants etc. Lupeol has been shown to exhibit diverse pharmacological activities under in vitro and in vivo conditions [11]. It has potent protective effect against inflammation, cancer, arthritis, diabetes, heart diseases, renal toxicity and hepatic toxicity [12]. Lupeol and lupeol linolate treatment has been observed to decrease the lipid peroxidation levels and to increase enzymatic and non-enzymatic antioxidants. Lupeol inhibited the chemically induced DNA damage under in vitro conditions. Topical application of lupeol (200 μg/mouse) prevented DMBA-induced DNA damage (DNA strant breaks) in murine skin [13]. Topical application of lupeol (40 mg/kg/three times a week) for 28 weeks has been shown to significantly decrease tumor burden, tumor multiplicity and increase tumor latency period in the Mouse models [14]. Of late, lupeol has attracted interest in content to chemoprevention attributable in large part to its antioxidant, apoptosis inducing, antiproliferative, antimutagenic and anti-inflammatory properties as well as its efficacy in inhibition of in vitro and in vivo cancer growth [15, 16]. Though the anticancer effect of lupeol (lupane triterpene) has been demonstrated in various cancer cell lines [16], there were no reports on anticarcinogenic effect in DMBA-induced experimental hamster buccal pouch carcinogenesis. The present study was therefore designed to provide scientific validity for the anticancer potential of lupeol in experimental oral carcinogenesis.

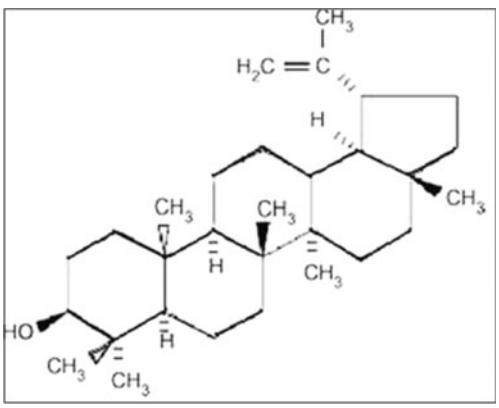

Fig. 1 Chemical structure of lupeol

Materials and Methods

Chemicals

The carcinogen, DMBA and lupeol were obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade, purchased from Hi-media Laboratories, Mumbai, India.

Experimental Protocol

The local 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 animals were orally given lupeol at a dose of 50 mg/kg body weight/day, starting 1 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 alone throughout the experimental period. The experiment was terminated at the end of 16th week and all hamsters were sacrificed by cervical dislocation. Biochemical studies were conducted on plasma, liver and buccal mucosa of control and experimental hamsters in each group. For histopathological studies, buccal mucosa tissues were fixed in 10 % formalin and routinely processed and embedded with paraffin, 2–3 μm sections were cut using a rotary microtome and stained with haematoxylin and eosin.

Biochemical Analysis

Estimation of Lipid Peroxidation by-products

Thiobarbituric acid reactive substances (TBARS) in plasma and buccal mucosa were determined by the methods of Yagi [17] and Ohkawa et al. [18], respectively. Briefly, plasma and buccal mucosa samples was reacted with thiobarbituric acid, then the pink color formed were measured at 530 and 532 nm, respectively. The absorbance was directly proportional to TBARS level.

Determination of Enzymatic and Non Enzymatic Antioxidants

Superoxide dismutase activity in plasma and buccal mucosa was assayed by the method of Kakkar et al. [19]. The enzyme assay was based on the 50 % inhibition of formation of Nicotinamide adenine dinucleotide—phenazine methosulphate nitroblue tetrazolium formation and the color developed was read at 520 nm. One unit of enzyme activity was taken as the amount of enzyme required to give 50 % inhibition of nitroblue tetrazolium reduction. The activity of catalase in plasma and buccal mucosa was assayed by the method of Sinha [20]. The enzyme assay was based on the utilization of H_2O_2 by the enzyme and the color developed was read at 620 nm. One unit of the enzyme was expressed as micromoles of H_2O_2 utilized per minute. The activity of Glutathione peroxidase in plasma and buccal mucosa was determined using the method of Rotruck et al. [21]. The reduced glutathione level in plasma and buccal mucosa was determined by the method of Beutler and Kelly [22]. The yellow derivative obtained by the reaction of erythrocytes, liver and skin tissues with 5,5-dithiobis-2-nitrobenzoic acid was measured at 412 nm. The absorbance was directly proportional to the reduced glutathione level. The oxidised glutathione level in the buccal mucosa was determined by the method of Tietze [23]. The oxidised glutathione content in the buccal mucosa was measured enzymically using glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate. The vitamin E level in the plasma was determined colorimetrically using the method described by Desai [24]. Vitamin E presents in the lipid residue forms a pink colored complex with bathophenanthroline–phosphoric acid reagent, which was measured at 536 nm. Buccal mucosa vitamin E was measured using the fluorimetric method described by Palan et al. [25]. The lipid extracts were dried under nitrogen and the residues were suspended in 66 % ethanol, followed by the addition of 4 ml of hexane and 0.6 ml of 60 % sulphuric acid. The tubes were vortexed and centrifuged. The upper hexane phase was removed and its fluorescence intensity was measured at an excitation of 295 nm and emission of 320 nm, with α-tocopherol used to determine the standard curve.

Determination of Phase I and Phase II Xenobiotic Metabolising Enzymes

The levels of cytochrome P_{450} and b_5 in liver and buccal mucosa were determined according to the method of Omura and Sato [26]. Cytochrome P_{450} was measured by the formation of pigment on reaction between reduced cytochrome P_{450} and carbon monoxide. The pigment was read with an absorbance maximum at 450 nm. The difference spectrum between reduced and oxidized cytochrome was used as an index to measure the level of cytochrome b5. The activity of glutathione-S-transferase in liver and buccal mucosa was assayed by the method of Habig et al. [27]. The enzyme assay was based on the formation of 1-chloro-2,4 dinitrobenzene–reduced glutathione conjugate, which was measured at 540 nm. Glutathione reductase activity in liver and buccal mucosa was assayed by the method of Carlberg and Mannervik [28]. The enzyme activity was assayed at 340 nm by measuring the formation of reduced glutathione, when the oxidized glutathione was reduced by reduced nicotinamide adenine dinucleotide phosphate.

Protein Determination

The protein content was determined by the method of Lowry et al. [29]. The peptide bonds (–CONH–) in polypeptide chain react with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of proteins cause reduction of the phosphomolybdate and phosphotungstate components of the Folin–Ciocalteau reagent to give bluish products read at 640 nm, which contribute towards enhancing the sensitivity of this method.

Statistical Analysis

The data are expressed as mean ± standard deviation (SD) Statistical comparisons for biochemical parameters 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

Table 1 and Fig. 2 shows the tumor incidence and histopathological changes in control and experimental hamsters in each group. We observed 100 % tumor formation with mean tumor volume (292.43 mm^3) and tumor burden (827.2 mm^3) in hamsters treated with DMBA alone and the tumors were histopathologically confirmed as well differentiated squamous cell carcinoma. Also, we noticed severe keratoses, hyperplasia and dysplasia in hamsters treated with DMBA alone (Fig. 3b). Oral administration of lupeol at a dose of 50 mg/kg bw completely prevented the tumor incidence and also reduced the severity of histopathological changes in hamsters treated with DMBA (Fig. 3c). A well defined intact epithelium was noticed in hamsters treated with lupeol alone (Fig. 3d) and control hamsters (Fig. 3a).

Figure 4 shows the status of plasma TBARS and antioxidants (SOD, CAT, GPx, GSH and vitamin E) in control and experimental hamsters in each group. The concentration of TBARS was increased whereas antioxidants activities were decreased in DMBA treated hamsters as compared to control hamsters. Oral administration of lupeol at a dose of 50 mg/kg bw restored the concentration of TBARS and antioxidants to near normal range in DMBA treated hamsters. Hamsters treated with lupeol alone showed no significant difference in TBARS and antioxidants status as compared to control hamsters.

Figure 5 shows the status of buccal mucosa TBARS and antioxidants (SOD, CAT, GPx, GSH and vitamin E) in control and experimental hamsters in each group. The concentration of TBARS and activities of SOD and CAT were decreased whereas the status of GPx, GSH and vitamin E were increased in DMBA treated hamsters as compared to control hamsters. Oral administration of lupeol at a dose of 50 mg/kg bw, restored the concentration of TBARS and antioxidants in DMBA treated hamsters to near normal range. Hamsters treated with lupeol alone showed no significant difference in TBARS and antioxidants status as compared to control hamsters.

Figure 6 shows the status of phase I (cytochrome P_{450} and b_5) and phase II (GR, GST and GSH) detoxification agents in the liver of control and experimental hamsters in each group. The status of phase I detoxification agents was significantly increased whereas phase II agents were decreased in the liver of DMBA treated hamsters as compared to control hamsters. Oral administration of lupeol to DMBA treated hamsters brought back the status of phase I and phase II detoxification agents to near normal range in the liver. Oral administration of lupeol alone showed no significant difference in the status of phase I and II detoxification agents as compared to control hamsters.

Figure 7 shows the status of phase I and phase II detoxification agents in the buccal mucosa of control and experimental hamsters in each group. The status of phase I (cytochrome P_{450} and b_5) and phase II detoxification agents (GST and GSH) were significantly increased whereas GSSG content was decreased in tumor-bearing hamsters as compared to control hamsters. Oral administration of lupeol to DMBA treated hamsters significantly brought back the status of GSH, GSSG and GST to near normal range. Hamsters treated with lupeol alone showed no significant difference in the status of GSH, GSSG and GST as compared to control hamsters.

Table 1 Incidence of oral neoplasm and histopathological changes in control and experimental hamsters in each group (n=10)

Parameter	Group I Control	Group II DMBA alone	Group II (DMBA + Lupeol)	Group V (Lupeol alone)
Tumor incidence (oral squamous cell carcinoma)	0	100 %	0	0
Total number of tumour/animals	0	28/10	0	0
Tumour volume (mm^3)/animals	0	292.43±27.78	0	0
Tumour burden (mm3)/animals	0	827.2±77.78	0	0
Hyperplasia	–	Severe	Mild	–
Hyperkeratosis	–	Severe	Mild	–
Dysplasia	–	Severe	Mild	–

Tumor 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) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal

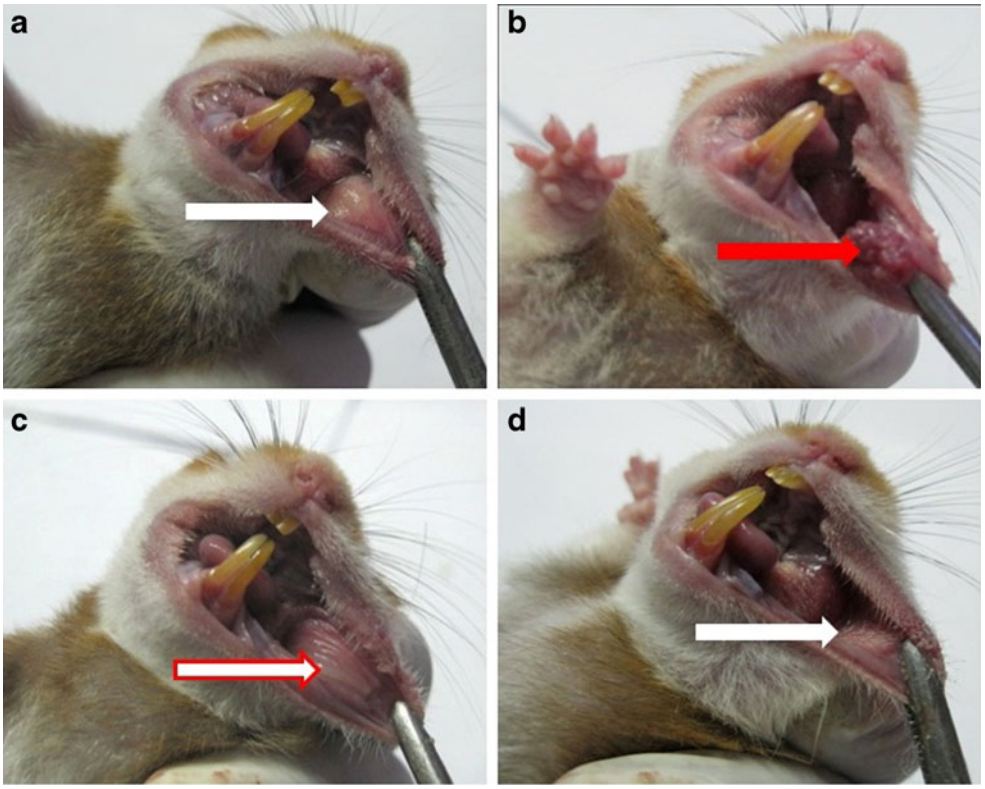

Fig. 2 Gross appearance of buccal mucosa in control and experimental animals in each group. **a & d** [⇨] Normal buccal pouch. **b** [➡] well differentiated squamous cell carcinoma. **c** [⇨] Precancerous oral epithelial layers

Discussion

DMBA-induced hamster buccal pouch carcinogenesis is the best and well-characterized experimental model for the development of oral cancer as well as to study the chemopreventive potential of natural and synthetic agents. Extensive studies pointed out that DMBA induces several types of cancer including oral cancer through chronic inflammation, oxidative stress and impairing the activities of phase I and II xenobiotic metabolizing enzymes [30, 31]. Natural products or synthetic

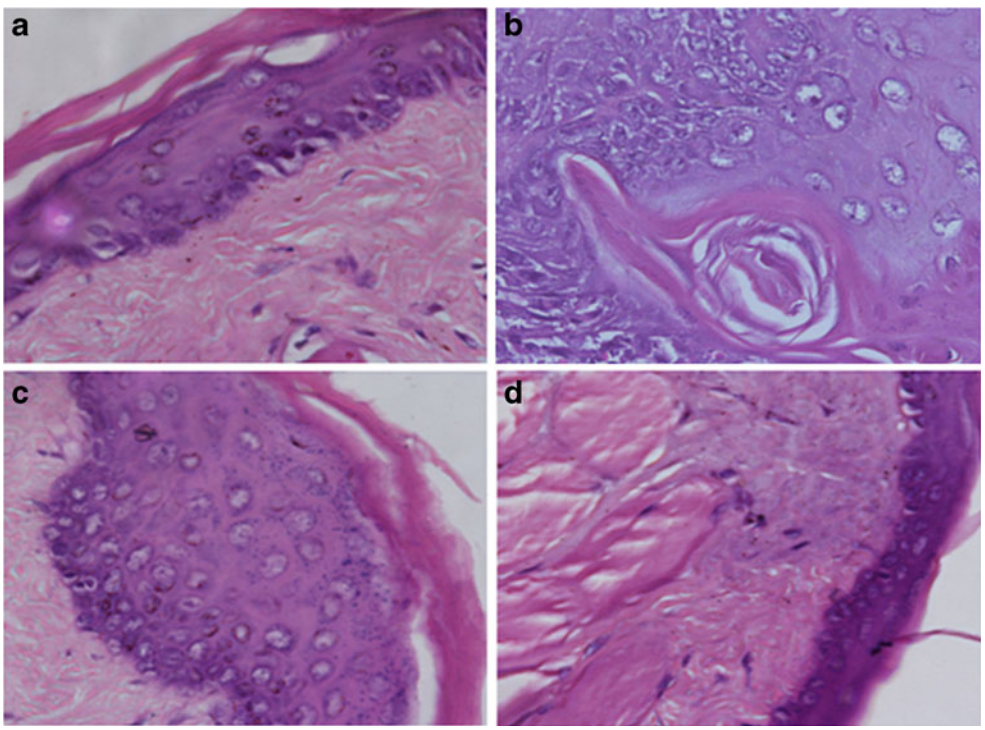

Fig. 3 Histopathological changes in the buccal mucosa of control and experimental hamsters in each group. **a & d** Photomicrographs showing well-defined buccal pouch epithelium from control and lupeol alone treated hamsters respectively (H & E, 40X). **b** Photomicrograph showing well-differentiated squamous cell carcinoma with keratin pearls in hamsters treated with DMBA alone (H & E, 40X). **c** Photomicrograph showing hyperplastic and mild dysplastic epithelium in hamsters treated with DMBA+ lupeol (H & E, 40X)

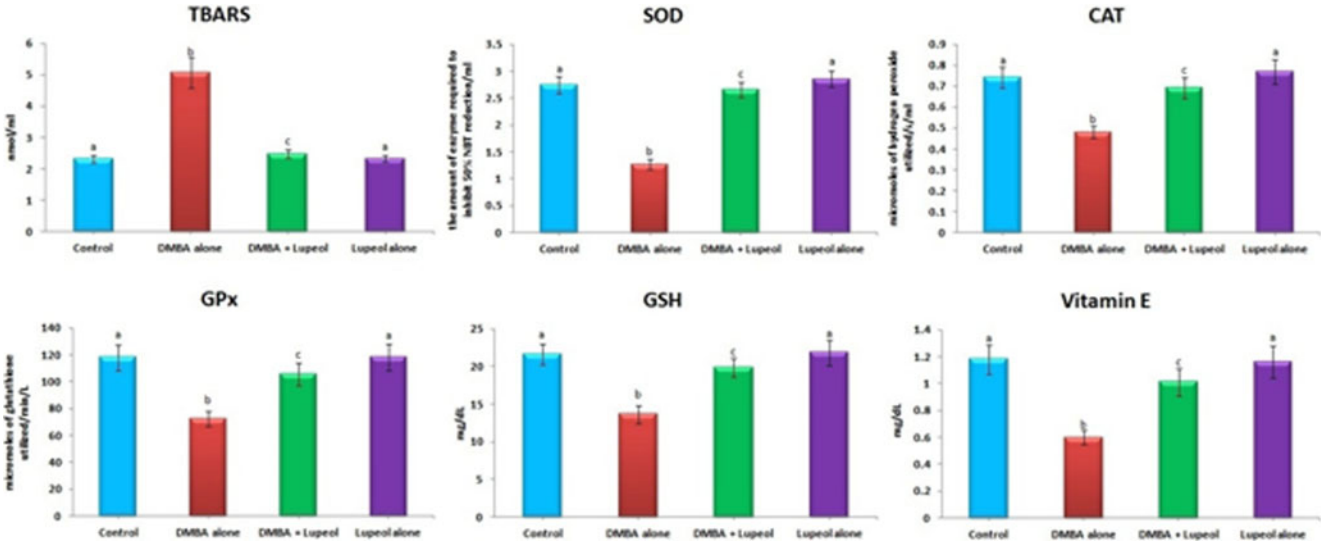

Fig. 4 Status of TBARS and antioxidants in the plasma of control and experimental hamsters in each group. Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript between groups differ significantly at $p<0.05$. (DMRT)

agents that possess anti-inflammatory and antioxidant properties as well as modulating effect on phase I and II xenobiotic metabolizing enzymes in favour of the removal of carcinogenic metabolites are thus considered to have potent chemopreventive efficacy against carcinogenesis [32].

In the present study topical application of DMBA three times for 14 weeks in the buccal pouch of golden Syrian hamsters resulted in well-differentiated squamous cell carcinoma, confirmed histopathologically by the oral pathologist. The incidence of tumor formation was 100 % in hamsters treated with DMBA alone. Also, severe hyperplasia, hyperkeratosis (4 weeks) and dysplasia (8 weeks) preceded well differentiated squamous cell carcinoma (10–14 weeks) in hamsters treated with DMBA alone. Oral administration of lupeol at a dose of 50 mg/kg bw to hamsters treated with DMBA not only prevented the tumor formation but also reduced drastically the severity of precancerous lesions such as hyperplasia, hyperkeratosis and dysplasia. The results of the present study thus suggest that lupeol exhibited significant chemopreventive potential by inhibiting or suppressing abnormal cell proliferation occurring in DMBA-induced hamster buccal pouch carcinogenesis. It has been reported that the groups at C-3 (hydroxyl) and C-28 (methyl) in lupeol showed apoptotic effects against the tested tumor cell lines [33]. The cytotoxic effect of lupeol is also due to its preventive role on binary complex formation between topoisomerase and DNA. Prasad et al. [34] reported that lupeol induced G2/M cell cycle arrest in

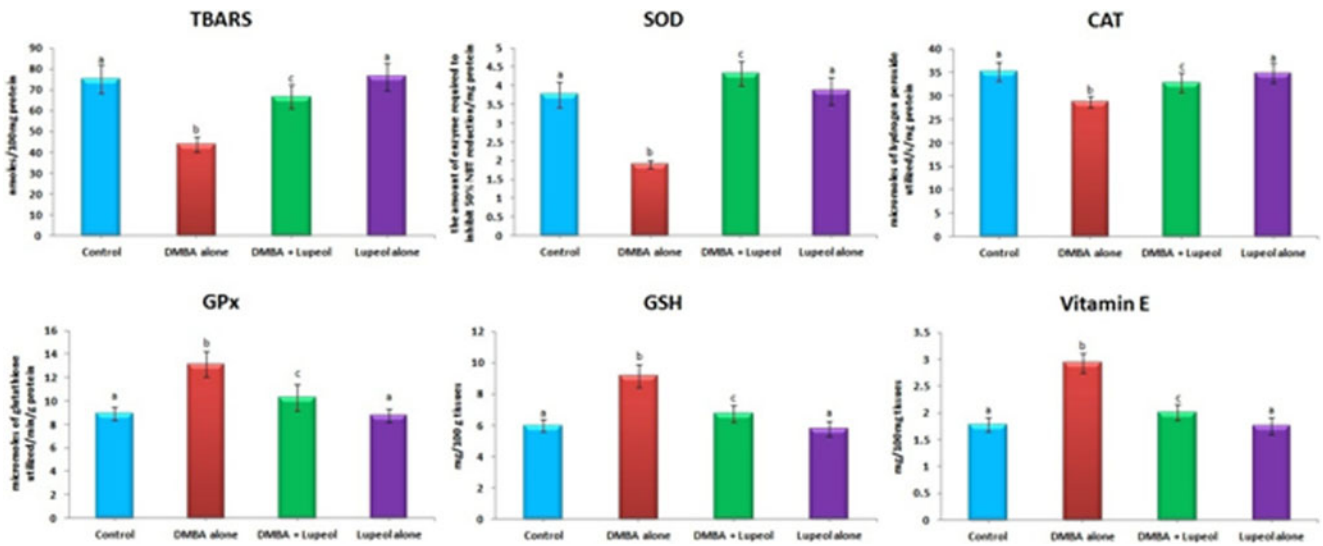

Fig. 5 Status of TBARS and antioxidants in the buccal mucosa of control and experimental hamsters in each group. Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript between groups differ significantly at $p<0.05$. (DMRT)

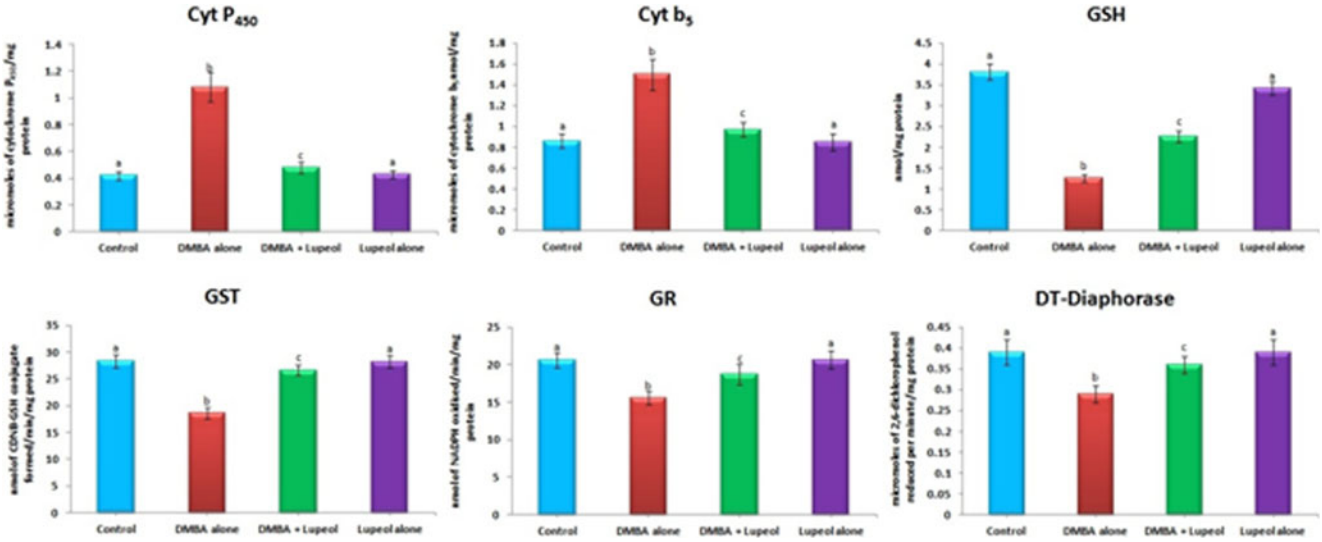

Fig. 6 Status of phase I and phase II enzymes in the liver of control and experimental hamsters in each group. Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript between groups differ significantly at $p<0.05$. (DMRT)

cancer cells by inhibiting the cyclin regulated signaling pathway inducing apoptosis.

Chemopreventive agents that inhibit phase I enzymes and induce phase II enzymes are considered to have significant anti-tumor initiating potential. Any disturbances in the homeostatic balance between the activities of these enzymes could result in malignant transformation. Increase in liver phase I and decrease in phase II enzymes in hamsters treated with DMBA implies that the liver detoxification cascade is significantly impaired during DMBA induced oral carcinogenesis. Repeated and long-term exposure to DMBA could account for increased phase I and II enzymes in the buccal mucosa of hamsters treated with DMBA alone. A large number of studies on DMBA induced oral carcinogenesis reported altered activities of phase I and II enzymes in liver and buccal mucosa [35, 36]. Our results are in line with these findings. Oral administration of lupeol at a dose of 50 mg/kg/bw restored the status of phase I and II enzymes in the liver and buccal mucosa of hamsters treated with DMBA. The results of the present study thus suggests that lupeol modulated the activities of phase I and II enzymes in favour of the excretion of carcinogenic metabolite during DMBA induced hamster buccal pouch carcinogenesis.

ROS play dual role in biological organisms. Excessive generation of ROS can cause extensive damage to vital biomolecules including lipids, protein and DNA, contributing to neoplastic transformation [37]. Antioxidants play a pivotal role in protecting the cells from the oxidative damage caused by reactive oxygen species. Over productive

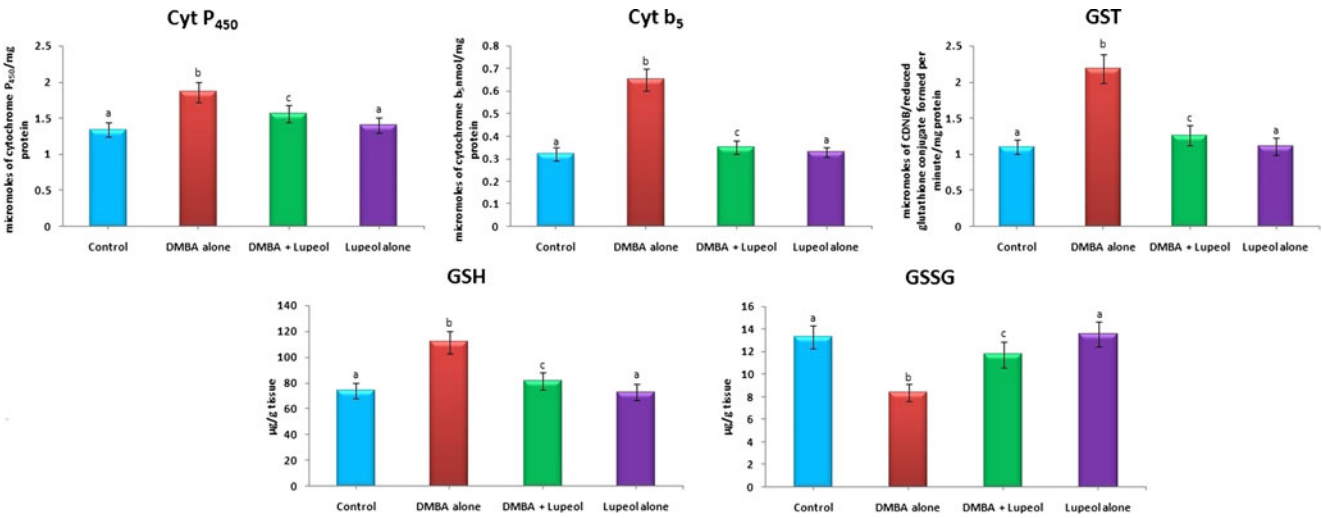

Fig. 7 Status of phase I and phase II enzymes in the buccal mucosa of control and experimental hamsters in each group. Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript between groups differ significantly at $p<0.05$. (DMRT)

ROS and decrease in antioxidants in circulation has been reported in several pathological conditions including oral carcinogenesis [8]. Our results corroborate these observations. A poor antioxidant defense mechanism as evidenced by low levels of non-enzymatic antioxidants and decreased activities of enzymatic antioxidants could account for increased levels of plasma TBARS.

A fine balance should exist between the rate of cell proliferation and cell death to maintain normal physiological processes. An inverse association between cell proliferation and lipid peroxidation has been reported in oral tumor tissues [38]. Oral tumors with abnormal and rapid cell proliferation have low levels of TBARS and PUFA, the substrate of lipid peroxidation [4]. Our results corroborate these observations.

SOD, CAT and GPx (enzymatic antioxidants) and vitamin E and GSH (non-enzymatic antioxidants) form the first line of defense against oxidative stress and ROS mediated oxidative damage. Altered glutathione metabolism may lead to several pathological conditions due to its diverse biochemical functions in the cell. GSH, a biologically important non-enzymatic antioxidant, scavenges a large number of reactive oxygen species and conjugates readily with an electrophilic centre of carcinogenic substances to aid their excretion. Vitamin E, the major lipophilic antioxidant, has pivotal role in the prevention of cancer and in scavenging ROS [39]. Tumor cells sequester vitamin E and glutathione from circulation to meet their nutrients demand as well as to protect themselves from oxidative damage. Glutathione and GPx have putative role in the regulation of cell proliferation. Extensive studies reported tumors with high glutathione content have lowered TBARS levels [32, 40]. Increase in vitamin E, GSH and GPx activity in tumor tissues of hamsters treated with DMBA alone could account for lowered lipid peroxidation. Increase in superoxide radicals and hydrogen peroxide were reported in oral tumor tissues [35]. Lowered activities of SOD and CAT in the tumor tissues are probably due to exhaustion of these enzymes to scavenge excessively generated superoxide and hydrogen peroxide at the site of tumors. Oral administration of lupeol at a dose of 50 mg/kg bw restored the status of TBARS and antioxidants in plasma and buccal mucosa of hamsters treated with DMBA. Yamashita et al. [41] reported that lupeol significantly scavenged arachidonic acid induced superoxide radicals in human neutrophils. Lupeol improved the antioxidant status in liver by scavenging the free radicals during cadmium induced hepatotoxicity [15]. The results of the present study thus suggest that lupeol might have exerted potent free radical scavenging property during DMBA-induced hamster buccal pouch carcinogenesis.

The present study thus demonstrates the chemopreventive potential of lupeol during DMBA-induced hamster buccal pouch carcinogenesis. The chemopreventive potential of lupeol relies on C-3 hydroxyl group and C-28 methyl group present on its molecular structure. The chemopreventive potential of lupeol is also due to its antioxidant potential and modulating effect on phase I and II enzymes during DMBA-induced hamster buccal pouch carcinogenesis.

Conclusion

The present study explores lupeol as a potent chemopreventive agent against oral carcinogenesis. The antioxidant potential and modulating effect on detoxification cascade, the important general mechanisms of chemoprevention, could partly account for the anti-tumor initiating potential of lupeol during DMBA induced oral carcinogenesis. In conclusion, lupeol can be used as a promising chemopreventive candidature in primary chemoprevention programme directed or targeted at patients with oral premalignant lesions. Further studies are under investigation to focus the effect of lupeol on the expression pattern of molecular markers that are related to oral carcinogenesis.

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