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ORIGINAL ARTICLE

D-Carvone Attenuates Biochemical and Molecular Expression via Oncogenic Signaling in Aryl Hydrocarbon-Induced Hamster Mucosal Carcinogenesis

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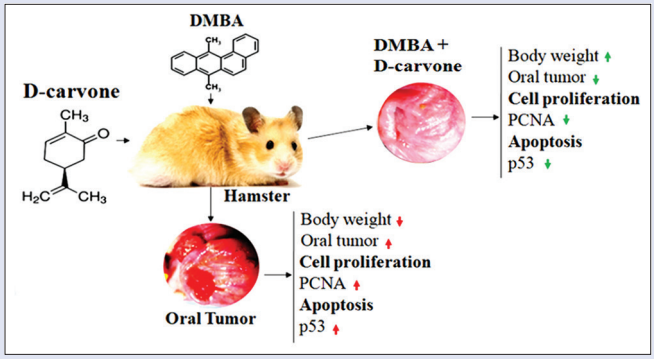
ABSTRACT

Background: Chemo prevention through nutritional constituents has appeared as an innovative methodology to control the oral cancer incidence. The main target of this research exists to explore the chemopreventive effect of D-carvone by way of biochemical and molecular prototype during 7,12-dimethylbenz[a] anthracene (DMBA)-stimulated hamsters mucosal carcinogenesis. **Materials and Methods:** Topical application of 0.5% DMBA in liquid paraffin, thrice a week, for 10 weeks well developed oral squamous cell carcinoma in hamsters cheek pouch (HCP). All the same 100% tumor formation was perceived in hamsters induced with DMBA alone, but intragastric administration of D-carvone, at a dose of 10 mg/kg bw, to DMBA-treated hamster totally prevented the formation of oral tumor. **Results:** D-carvone significantly lessens lipid peroxidation (LPO) by-products and enhanced the status of enzymatic, non-enzymatic antioxidants, and varied the status of Phase I and II xenobiotic enzymes, favoring the secretion of cancer metabolites through downregulation of proliferating cell nuclear antigen and p53 expression during oral tumor hamsters. **Conclusion:** The nearby study suggests that D-carvone relies on its anti-LPO, antioxidant, xenobiotic metabolic enzymes as well as anti-cell proliferation and induced apoptosis during DMBA-induced hamster oral mucosal carcinogenesis.

Key words: 7,12-dimethylbenz[a]anthracene, apoptosis, cell proliferation, D-carvone, hamsters, P53, proliferating cell nuclear antigen

SUMMARY

- D-Carvone has multiple pharmacological actions such as antioxidant, antimicrobial, antihypertensive, and antihyperlipidemia effect
- D-Carvone successful defense toward 7,12-dimethylbenz[a]anthracene treated HBPC by amending the levels of antioxidant, lipid peroxidation, xenobiotic-metabolizing enzymes further inhibits cell proliferation, and develops tumor cell apoptosis.



Abbreviations used: HOC: Human oral cancer; DMBA: 7, 12-Dimethylbenz[a]anthracene; HMC: Hamsters mucosal carcinogenesis; OSCC: Oral squamous cell carcinoma; HCP: Hamsters cheek pouch; LPO: Lipid peroxidation.

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INTRODUCTION

Cancer, common most fatal diseases, engages morphological, cellular transformation, uncontrolled cellular proliferation, deregulation of cell death, invasion, blood vessel formation, and metastasis with substantial economic, physiological, and pathological impacts.[1] OC mainly represents the tongue, cheek, and palate. Squamous cell carcinoma (SCC), at a preliminary stage, reduces the survival rate as well as the lifestyle and quality of patients.[2] The multistep procedure of oncogenesis branded by a large number of cells and apoptosis on the cellular and molecular status that finally undertake uncontrolled cell proliferation, is suspected in tumor progression.[3] 7,12-dimethylbenz[a]anthracene (DMBA), an aryl hydrocarbon, a potent procarcinogen, mediated carcinogenesis. During metabolism, aryl hydro carbon generates a active metabolites of dihydrodiol epoxide, by xenobiotic metabolising enzymes later binds to adenine and quinine residues.[4]

Chemoprevention is a strategy to completely stop the progress of cancer development by prevailing in the method of carcinogenesis.[5] Carvone is cyclic unsaturated monoterpenoid ketones which takes out from a lot of herbal plants such as caraway and dill and spearmint utilized on the widespread food products. Among the carvones, D-carvone has multiple pharmacological actions, for instance, antioxidant, antimicrobial, antihypertensive, as well as antihyperlipidemias capacity.[6] D-carvone has been reported as the best anticancer agent for MCF-7 and HT-29 cells, to slow down p38 MAPK signaling, and invasion on malignant cells.[7] In addition, D-carvone exhibits 1,2-dimethylhydrazine brought colon cancer by avoiding the abnormal activation of biotransforming enzymes and preneoplastic lesions oxidative stress.[8]

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P53, a tumor-suppressing protein, guards a genome alongside infection during the regulation of the cell cycle, apoptosis, and repairing of DNA. Ahead of DNA break and stress, mutant p53 obtained spreading toward cells altering usual metabolisms. Wild type p53 were suppressed, which is inhibit the cell proliferation and induces apoptosis in normal cells, it contrast in malignancy.[9] Proliferating cell nuclear antigen (PCNA), cell proliferative markers, non-histamine nuclear protein in DNA polymerase delta, which is majorly employed to successful tumor proliferation, is being used like a biomarker for cancer diagnosis and prognosis. Cell proliferation is recognized as a necessary step in cancer development and has become a fascinating goal for anticancer drug development.[10] Cell proliferative marker of PCNA and apoptotic marker of P53 were over expressed and down regulated form in human cancer around 90%. Our exhibition of PCNA and p53 abnormal expression at some stage in DMBA-has provoked hamster buccal pouch carcinogenesis (HBPC) that was in accordance with an earlier description.[11] In addition, we investigate things to see an oral treatment of D-carvone radically lower PCNA expression while increasing a p53 on DMBA-alone tumor hamsters.

Oxidative stress arises due to the unevenness in antioxidant status and is related to the deterioration of normal cellular processes that has a role in cell differentiation and proliferation. lipid peroxidation (LPO) is a process that occurs due to the oxidative deterioration of lipids. LPO has been occupied in the pathogenesis of carcinogenesis.[12] Multicellular organism has, however, been inbuilt with enzymatic as well as non-enzymatic antioxidant protective system to counteract the oxidative stress. Antioxidants protect the oxidative tissue damage by scavenging terribly accreted reactive oxygen species (ROS). Both antioxidants take a pivotal responsibility toward the ROS-intervened oxidative damage.[13] The liver has toxins eliminating surge for actively metabolizing as well eliminating toxic metabolites of xenobiotic agents. While Phase I enzymes contribute their role during the metabolic commencement of the pro-cancerous, Phase II enzymes participate on the secretion of toxic metabolites on conjoining to diminished glutathione. Any deviation on the equilibrium among those detoxification cascades could result in neoplastic transformation.[12,14]

D-carvone has possessed multifunctional properties; however, there is no paucity of information on the promising effectual of D-carvone to DMBA-challenged squamous cell malignancy. We have analyzed the tumor incidence, histological modification, LPO statuses, antioxidant, and xenobiotic enzymes on experimental hamsters. Further, appearance of cell proliferation (PCNA) and apoptosis (p53) was analyzed using immunohistochemistry (IHC).

MATERIALS AND METHODS

Chemicals and reagents

D-carvone (≥96% Purity) and DMBA (≥95% Purity) were acquired at Sigma-Aldrich Chemicals Pvt. Ltd (Bengaluru, Karnataka, India). Antibodies against PCNA, p53, and horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

Eight-to-ten-week-old male golden Syrian hamsters (n = 24) were purchased and housed in polypropylene cages. A total number of twenty-four hamsters were randomly divided into four groups; six hamsters in each group and maintained in a controlled room temperature (27°C ± 2°C) and moisture (55% ±5%) with a light/dark cycle at 12/12 h.

Preparation of D-carvone

D-carvone was dissolved in 1% DMSO previous action and administered orally with the effective dose, 10 mg/kg bw, of thrice a week rotation to DMBA application, opening a week by painting the carcinogen till 14 weeks on alternative days of drug treatment.

Preparation of 7,12-dimethylbenz[a]anthracene

The 0.5% DMBA (mixed with liquid paraffin) was painted on the left buccal pouches using a no. 4 painting brush thrice a week for 10 weeks.[15]

Tumor assessment

To measure the DMBA-induced hamsters oral tumor by the side of the end of scarification of the 14 week study, hamsters cheek pouch (HCP) tumor was removed and flushed with saline. The incidence of whole tumor number in every HCP was carefully counted during a look at microscopically and every tumor's width was calculated by utilizing the vernier scale. Tumor volume was measured applying the formula $V = \frac{4}{3}\pi\left(\frac{D_1}{2}\right)\left(\frac{D_2}{2}\right)\left(\frac{D_3}{2}\right)$ where D_1, D_2, and D_3 were considered as diameters (mm^3) of tumors. The hamster oral tumor load was measured via the tumor volume multiplying with number of tumors per hamster.

Grouping

Twenty-four hamsters were divided into four groups consisting of six hamsters in every group; afterward, those are guaranteed to 2 weeks of natural as well found managing the adaptation prior to starting a trial. Group 1 hamsters were given a modified diet for 14 weeks and served as normal rats; Group 2–3 hamsters in 0.5% DMBA-were provoked thrice in a week up to 10 weeks; and Group 3–4 hamsters obtained D-carvone (10 mg/kg bw) orally on alternative days for 14 weeks.

The test was finished at the closing stages of 14 weeks, and then necrotized via the displacement of cervical bone. Hamster bodyweight was measured through deducting a starting and final weight. Oral buccal tissues were fixed in 10% foramlin, and fixed in paraffin, and then stained with hematoxylin and eosin for histopathological analysis. Biochemical analysis was conducted on the oral mucosa and plasma of all hamsters.

Biochemical investigations

Sample collection

Different biochemical parameters were analyzed in blood as well oral cheek pouch of experimental hamsters. Blood samples were analyzed, and plasma was removed through spinning at 1000 g for 15 min. Normal and human oral cancer (HOC) tissues were rinsed through chilled saline and homogenized by means of a suitable buffer at the homogenizer and they were utilized for biochemical examinations.

Estimation of lipid peroxidation and antioxidant

LPO was estimated spectrophotometrically by assessing the absorption of thiobarbituric acid reactive species (TBARS), lipid hydroperoxides (LOOH), and conjugated dienes (CD) through a method suggested by Ohkawa *et al.*,[16] Jiang *et al.*,[17] and Rao and Recknagel,[18] respectively. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) functions were assessed by the method proposed by Kakkar *et al.*,[19] Rotruck *et al.*,[20] and Sinha,[21] respectively. Reduced glutathione (GSH) on plasma and buccal mucosa, a Vitamin E status on plasma and buccal mucosa, was tested through the procedures of Beutler and Kelly,[22] Desai,[23] and Pala *et al.*[24]

Analysis of xenobiotic enzymes

The levels of the detoxification metabolizing agents such as cytochrome-p450 (Cyt-p450), cytochrome-b5, DT-diaphorase (DTD), glutathione-S-transferase (GST), glutathione reductase (GR), GSH, oxidized glutathione (GSSG) liver and oral mucosal cells were analyzed through the procedures suggested by Omura and Sato,[25] Lind *et al.*,[26] Habig *et al.*,[27] Carlberg and Mannervik[28] Anderson,[29] Tietze,[30] and Ernster,[31] respectively.

Estimation of protein

The proteinaceous substances were quantified through the procedure proposed by Lowry *et al.*[32]

Immunohistochemistry investigation

Oral mucosa tissues from experimental hamster in each group were fixed in paraformaldehyde solution. Specimens were dehydrated with graded ethanol and D H_2O, cleaned on xylene as well surrounded with paraffin. Tissue block sections were cut to 3–5 µM and were further used for IHC staining. Tissue sections were incubated in a blocking reagent and the sections were stained with the individual primary antibodies (PCNA and p53) overnight followed by a secondary antibody. HRP and 3,3'-diaminobenzidine substrate were used for detection. The slides were washed with counterstained hematoxylin staining for 30 s, covered, and mounted. IHC staining analysis of hamster buccal pouch sections was examined under a light microscope and the percentage of protein expression in positive cells was determined using the technique of Lyzogubov *et al.* (2005).[33]

Statistical tests

Statistical tests were conducted through the means of one-way ANOVA, pursued through Duncan's Multiple Range Test (DMRT) using Statistical package for the social sciences (SPSS) (SPSS Software for Windows release 17.0 SPSS Inc., Chicago, IL, USA). Results are presented as mean ± standard deviation and all the $P \leq 0.05$ values were considered statistically significant.

RESULTS

The effect of D-carvone in hamsters bodyweight

Figure 1 shows the effects of D-carvone administration in the last of the 14th week on the initial and final body weights in control as well as tested hamsters. At the end of the 14th week, DMBA-alone painted hamsters showed significantly ($P < 0.05$) diminished body weight as compared to controls hamsters (Group 1 and IV). However, this significant body weight loss was prevented on oral supplementation by way of an effective dose of D-carvone (10 mg/kg bw) to DMBA hamsters.

Effect of D-carvone in oral tumor incidence

Table 1 shows the hamsters oral tumor development of normal and tested hamsters. Findings of this test exhibited 100% of hamster oral tumor development through average tumor volume (193.29 mm^3) and tumor burden (2845.22 mm^3) in DMBA-induced oral SCC (OSCC) hamsters. However, supplementation of D-carvone significantly reduced the tumor development during DMBA-painted hamsters as compared to control and D-carvone-only hamsters.

Hematoxylin and eosin staining of hamsters oral tumor

The gross appearance of the oral tumor observed on hamster challenged through DMBA-induced SCC is shown in Figure 2. Histopathological changes on untreated as well as tested hamsters of all groups are shown in Figure 3 and Table 2. Hamsters exposed to DMBA alone had changed in the histopathological stern, hyperkeratosis, hyperplasia, dysplasia along with prominently distinguished SCC of epithelial. HCP of DMBA + D-carvone hamsters showed moderate mild keratosis and mild hyperplastic epithelium. Control and D-carvone-alone hamsters showed normal intact epithelial layers.

Effect on D-carvone on lipid peroxidation

Table 3 shows the effects of D-carbon absorption of LPO byproducts on plasma as well as oral mucosal tissue of normal and tested hamsters. DMBA-only OSCCs hamsters concentration of LPO by-products were significantly ($P < 0.05$) higher on plasma and lower on oral mucosa cells (Group 2) that are in contrast or control. Orally administered D-carvone with DMBA-treated hamsters significantly ($P < 0.05$) restored the levels of LPO as compared with control hamsters. No significant changes were observed in control and D-carvone hamsters.

Effects of D-carvone on enzymatic antioxidants

Table 4 shows the present status of enzymatic antioxidants on the plasma and oral tissues of normal and tested hamsters. Our results point out that the activities of enzymatic antioxidant status on the plasma and oral buccal pouch were signed ($P < 0.05$) lower, except GPx (augmented) in the HCP treated with DMBA only. D-carvone administered with DMBA-painted hamsters showed that the elevated levels of enzymatic antioxidant, brought back status near to normal level, which were similarly ($P < 0.05$) evaluated in control and D-carvone alone treated hamsters.

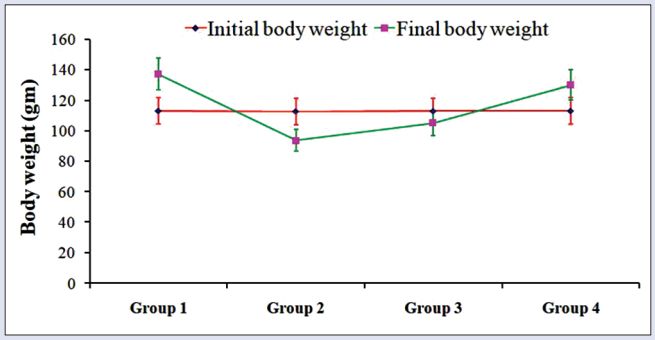

Figure 1: Body weight of the initial and final body of the control and experimental hamsters. Values are expressed as mean ± standard deviation for six hamsters in each group. Values not sharing a common superscript letter differ significantly at $P < 0.05$

Table 1: Tumor incidence, number, volume, and burden of experimental hamsters

Groups/treatment	Group 1	Group 2	Group 3	Group 4
Tumor incidence	0	100%	0	0
Total number of tumor/hamsters	0	8±0.61	0	0
Total volume (mm^3)/hamsters	0	193.29±14.72	0	0
Tumor burden (mm^3)/hamsters	0	2845.22±216.69	0	-

Values are expressed as mean±SD for six hamsters in each group. Values not sharing a common superscript letter differ significantly at $P<0.05$. SD: Standard deviation

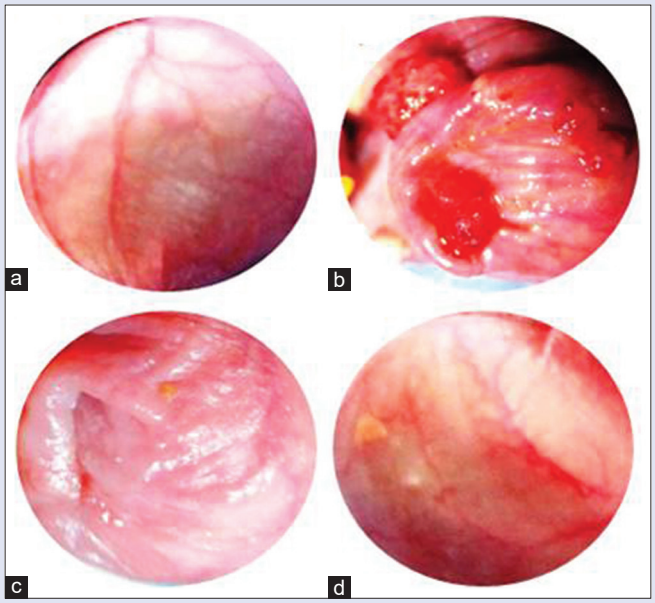

Figure 2: Photomicrograph showing the gross appearance of oral mucosa in the control and experimental hamsters. (a) control hamsters, (b) 7,12-dimethylbenz[a]anthracene painted hamsters shows exophytic and well differentiated squamous cell carcinogenesis, (c) 7,12-dimethylbenz[a]anthracene + D-carvone and (d) D-carvone-alone-treated hamsters revealed that the normal appearance of the oral mucosa

Effects of D-carvone on non-enzymatic antioxidant

Table 5 shows the non-enzymatic antioxidants statuses on plasma and oral pouch of normal and tested hamsters. Non-enzymatic antioxidants levels were significantly ($P < 0.05$) diminished in the plasma, while they were higher in the tumor tissues treated with DMBA alone painted hamsters. Supplementation with D-carvone to DMBA-painted hamsters showed extensively brought back the non-enzymatic antioxidants on together plasma and buucal tissues as compared to control and hamsters treatment with D-carvone alone.

Effect of D-carvone on xenobiotic-metabolizing enzymes

Table 6 depicts the effects of D-carvone on the actions by Phase I and II xenobiotic enzymes, on the hepatic tissues of normal and tested hamsters. The buccal tissues of DMBA painted hamsters showed that have significantly ($P < 0.05$) improved the levels of phase I enzymes and significantly ($P < 0.05$) diminished the levels of phase II enzymes compared to control hamsters. Supplementation of D-carvone with DMBA-treated hamsters notably brings back the activities of Phase I and II enzymes as close to the usual level.

Effect of D-carvone on Phase I and II detoxification agents in the oral tissues

Table 7 depicts the activities of Phase I and II xenobiotic-metabolizing enzymes of oral bouch tissues of the normal and tested hamsters. Activities of these phases I (Cyt p450 and b5) enzymes were significantly ($P < 0.05$) higher, while phase II (GST, GR, GSH, GSSG, and GSH/GSSG ratio were higher; GSSG was lower) enzymes in hamsters treated with DMBA alone painted as compared with control and hamsters. Supplementation of D-carvone to DMBA-treated hamsters notably ($P < 0.05$) restored the status of phase I and II enzyme as compared to control hamsers.

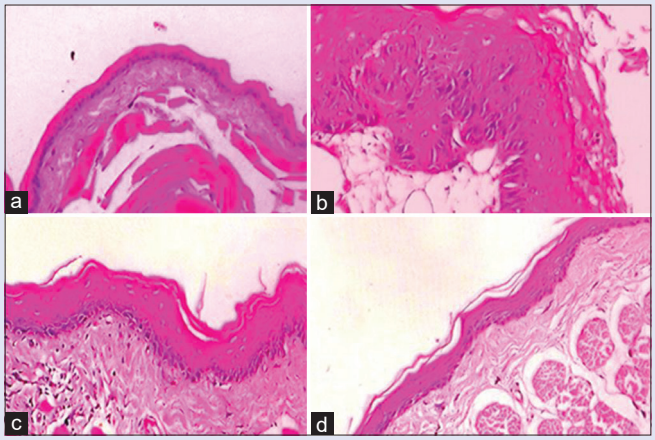

Figure 3: Histopathological findings in the buccal pouch mucosa of control and experimental hamsters (H and E staining). (a and d) Control and D-carvone 10 mg/kg bw, administrated hamsters showed normal squamous epithelium. (b) 7,12-dimethylbenz[a]anthracene alone-induced hamsters showed well differentiated squamous cell carcinogenesis exhibiting enlarged cells. (c) 7,12-Dimethylbenz[a] anthracene + D-carvone 10 mg/kg bw, hamsters showing cells with nuclear pleomorphic and architectural disarray

Table 2: Histopathological changes in the buccal mucosa of experimental hamsters

Groups/treatment	Group 1	Group 2	Group 3	Group 4
Keratosis	0	+++	++	0
Hyperplasia	0	+++	+	0
Dysplasia	0	+++	0	0
OSCC	0	+++	0	0

0: No change; +: Mild; ++: Moderate; +++: Severe. OSCC: Oral squamous cell carcinoma

Effects of D-carvone on immunohistochemistry expression in proliferating cell nuclear antigen and p53

Figures 4 and 5 and Table 8 summarize the represents IHC staining pattern of cell proliferation and apoptosis markers in normal and experimental hamsters. DMBA alone painted hamsters showed higher expression of PCNA and p53 proteins as compared to control hamsters. When supplemented with D-carvone to DMBA painted hamsters showed diminished expression of PCNA and p53 markers as compared with control hamsters.

DISCUSSION

Current research exploration was aimed to examine the cell proliferative and apoptotic effect of D-carvone on DMBA-induced hamster model in oral cancer. Tumor incidence, tumor volume, histopathological, and biochemical analysis were also evaluated in control and experimental hamsters. The results were showed that the D-carvone was the most biologically active agent, and there was a relatively large potency gap between the cellular toxic effects and induced apoptosis by D-carvone.

The ROS was helpful for oxidative damaging effect on molecule in living systems, if excess free radicals are not removed by the antioxidant method. These free radicals were unpaired, which is inducing the mutagenesis contribute into cancer development. Hence, a new focus on control OC is essentially needed.[34] Yu *et al.*[35] have found that D-carbon reduces *N*-nitrosodimethylamine-induced tumor formation and also pulmonary adenoma formation in a mice model. In the present study treatment with D-carvone diminished tumor development, tumor

Table 3: The levels of lipid peroxidation by-products in the plasma and oral mucosa of experimental hamsters

Groups/treatment	Group 1	Group 2	Group 3	Group 4
Plasma				
TBARS (nM/mL)	2.55±0.19^{a}	4.96±0.38^{b}	2.71±0.21^{c}	2.53±0.19^{a}
LHPO (mM/mL)	0.49±0.04^{a}	1.03±0.08^{b}	0.59±0.05^{c}	0.47±0.04^{a}
CD (µM/mL)	0.69±0.05^{a}	2.16±0.16^{b}	0.85±0.07^{c}	0.68±0.05^{a}
Oral mucosa				
TBARS (nM/100 mg protein)	63.09±4.80^{a}	38.97±2.97^{b}	60.35±4.62^{c}	65.04±4.95^{a}
LHPO (nM/100mg protein)	35.04±2.67^{a}	21.03±1.60^{b}	31.10±2.38^{c}	36.16±2.68^{a}
CD (mg/mg protein)	28.99±2.21^{a}	18.51±1.41^{b}	27.65±2.12^{c}	29.63±2.26^{a}

Results are expressed as mean±SD for six hamsters in each group. Data not sharing a common superscript (a-c) differ significantly at $P<0.05$ (DMRT). SD: Standard deviation; TBARS: Thiobarbituric acid-reactive species; CD: Conjugated dienes; LHPO: Lipid hydroperoxide; DMRT: Duncan's Multiple Range Test

Table 4: The levels of enzymatic antioxidant in the plasma and buccal mucosa of experimental hamsters

Groups/treatment	Group 1	Group 2	Group 3	Group 4
Plasma				
SOD (U*/mL)	2.53±0.19^{a}	1.06±0.08^{b}	2.47±0.19^{c}	2.59±0.20^{a}
CAT (U$^{\#}$/mL)	0.76±0.06^{a}	0.25±0.02^{b}	0.69±0.05^{c}	0.77±0.06^{a}
GPx (U$^{@}$/mL)	137.03±10.43^{a}	76.21±5.80^{b}	129.67±9.93^{c}	138.34±10.53^{a}
Oral mucosa				
SOD (U*/mg protein)	5.52±0.42^{a}	3.37±0.26^{b}	5.12±0.39^{c}	5.67±0.43^{a}
CAT (U$^{\#}$/mg protein)	37.92±2.89^{a}	21.97±1.67^{b}	35.10±2.69^{c}	38.70±2.95^{a}
GPx (U$^{@}$/mg protein)	8.01±0.61^{a}	19.63±1.49^{b}	7.04±0.54^{c}	7.98±0.64^{a}

U*: amount of enzyme to inhibit 50% NBT reduction. U$^{\#}$: µmole of H_2O_2 consumed/second. U$^{@}$: µmole of glutathione peroxidise consumed/min. Results are expressed as mean±SD for six hamsters in each group. Data not sharing a common superscript (a-c) differ significantly at $P<0.05$ (DMRT). SD: Standard deviation; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; DMRT: Duncan's Multiple Range Test

Table 5: The levels of non-enzymatic antioxidants in the plasma and buccal mucosa of experimental hamsters

Groups/treatment	Group 1	Group 2	Group 3	Group 4
Plasma				
Vitamin-E (mg/dL)	1.28±0.0^{a}	0.71±0.05^{b}	1.16±0.0^{c}	1.30±0.0^{a}
GSH (mg/dL)	22.97±1.75^{a}	09.91±0.75^{b}	20.93±1.60^{c}	23.54±1.79^{a}
Oral mucosa				
Vitamin-E (mg/100 mg tissue)	1.92±0.15^{a}	4.03±0.31^{b}	2.10±0.16^{c}	1.95±0.15^{a}
GSH (mg/100 mg tissue)	8.34±0.64^{a}	15.03±1.14^{b}	10.08±0.77^{c}	8.33±0.63^{a}

Results are expressed as mean±SD for six hamsters in each group. Data not sharing a common superscript (a-c) differ significantly at $P<0.05$ (DMRT). SD: Standard deviation; GSH: Glutathione; DMRT: Duncan's Multiple Range Test

Table 6: The levels of Phase I and Phase II detoxification enzymes in the liver of experimental hamsters

Group	Group 1	Group 2	Group 3	Group 4
Liver Phase I				
Cyt P_{450} (U^{x}/mg protein)	0.71±0.05^{a}	1.69±0.13^{b}	0.68±0.05^{c}	0.72±0.05^{a}
Cyt b_5 (U^{y}/mg protein)	1.32±0.10^{a}	2.39±0.18^{b}	1.38±0.11^{c}	1.27±0.10^{a}
Liver Phase II				
GSH (nmol/mg protein)	2.97±0.23^{a}	0.87±0.07^{b}	2.73±0.21^{c}	2.98±0.23^{a}
GST (U^{A}/mg protein)	38.03±2.90^{a}	12.93±0.98^{b}	32.10±2.46^{c}	39.05±2.97^{a}
GR (U^{B}/mg protein)	17.38±1.32^{a}	8.49±0.65^{b}	15.72±1.20^{c}	18.12±1.38^{a}
DTD (U^{C}/mg protein)	0.81±0.06^{a}	0.29±0.02^{b}	0.65±0.05^{c}	0.83±0.06^{a}

U^{x}: µmoles of cytochrome P450; U^{y}: µmoles of cytochrome b5; U^{A}: µmoles of 1-chloro-2, 4-dinitro benzene reduced glutathione conjugate formed/minute; U^{B}: µmoles of NADPH oxidized/ hour; U^{C}: µmoles of 2,6-dichloro indophenols reduced/ minute. Results are expressed as mean±SD for six hamsters in each group. Data not sharing a common superscript (a-c) differ significantly at $P<0.05$ (DMRT). SD: Standard deviation; GSH: Glutathione; GST: Glutathione-S-transferase; GR: Glutathione reductase; DTD: DT-diaphorase; DMRT: Duncan's Multiple Range Test

incidence and burden in DMBA-treated HOC than D-carvone, due to it does not disrupt the normal tissues, and therefore, it was considered as non-toxic.

The histopathological pattern of D-carvone administered with DMBA painted hamsters moderately affects oral carcinoma, which DMBA can stimulate histologic modification. Well-modified pattern of tumor tissues with keratin and epithelial pearls, architectural disarray was observed in DMBA-induced tumor hamsters. Treatment with D-carvone has greatly conserved the normal architectural in the buccal cells of D-carbon alone.

The potentiality of D-carvone to restore the normal histological changes appeared by DMBA induction, examined the anti-carcinogenic potential of this monoterpene.

Previously ROS synthesis, it acts in response with lipid bilayers causing LPO resulting in the formation of ROS substance.[36] Our results demonstrated that the DMBA induced HBPC showed that at low levels of LPO as compared to normal hamsters. Cancer cell proliferation levels were low. Administration of D-carvone to DMBA-induced hamster has restored the status of TBARS, CD, and LOOH as close to the normal

levels than D-carvone alone, which is due to the anti-lipid peroxidative activity of D-carvone.

Natural antioxidant enzymes are a major defense mechanism that protects the cells from toxic ROS and mutagens. Along with these, SOD combines with CAT to ultimate free radicals may result ameliorate oxidative injury. GPX catalyzes the reduction of peroxides which converted into non-toxic products and scavenger's lipid peroxides using GSH regenerating enzymes to applicable to balance cellular GSH status through catalyzing the GSSG to the decreased appearance, thus changing the GSH/GSSH ratio. GSH is a powerful nucleophile due to its redox and detoxification process.[13,37]

In our study were suggested that the chemopreventive potential of D-carvone also has attributed to its antioxidants property and anticancer activity from most of the biological activities. In buccal mucosa, D-carvone brought back the status and the susceptibility of LPO, enhanced the GPX function and GSH/GSSG ratio, while in the hepatic tissue of the LPO was diminished, in elevation of antioxidants.

DMBA is activated during metabolism by the actions of cyt p450 and epoxidation via Cyp-b5 isoforms to develop DMBA-3,4-dihydrodiol-1,2-epoxide, active carcinogen. GST and GR can detoxify the pro-carcinogen metabolites. Phase II biotransformation enzymes is GST, earlier process in conjoining with functionalized P450 metabolites by GSH, so that it favoring carcinogen removal from disease condition.[38] DTD is an flavoprotein, it facilitates to reduce the two-electron of Quinones, hence avoiding the generation of semi-quinones.[39] Phase II biotransformation enzymes are normally stimulated concurrently with Phase I enzymes. Phase II biotransformation enzymes are normally stimulated concurrently with Phase I enzymes. Additional, pro-carcinogen were normally activated transcription of DTD, Cyt-P450, and GST by liganding to the xenobiotic reactive element gives on the promoter region of those particular genes[40,41] when enhanced levels of carcinogen-metabolizing enzymes, it corroborates and confirms development of these enzymes resulted in the cancer development and might be in control of driving normal cells to malignant cells. In dissimilarity to the HCP, in the hepatic of oral tumor-induced hamsters, the improvement in Cyt-P450 and LPO was conveyed by compromised antioxidant and Phase II xenobiotic enzymes.

A number of chemopreventive agents authority of Phase I and II carcinogen-metabolizing enzymes, thus block a cancer-beginning

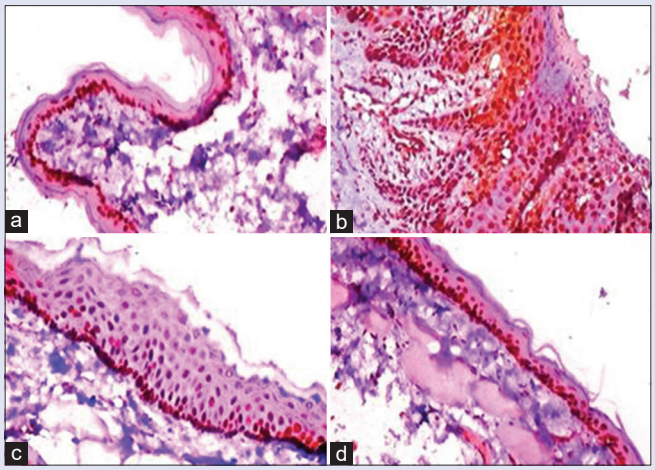

Figure 4: Representative photomicrograph showing immunohistochemical staining for proliferating cell nuclear antigen expression observed control and experimental hamsters. (a and d) Control and D-carvone alone hamsters showing expression not detectable. (b) 7,12-Dimethylbenz[a]anthracene alone hamsters shows over expression. (c) 7,12-Dimethylbenz[a]anthracene + D-carvone 10 mg/kg bw, hamsters shows decreased expression of the Proliferating cell nuclear antigen protein

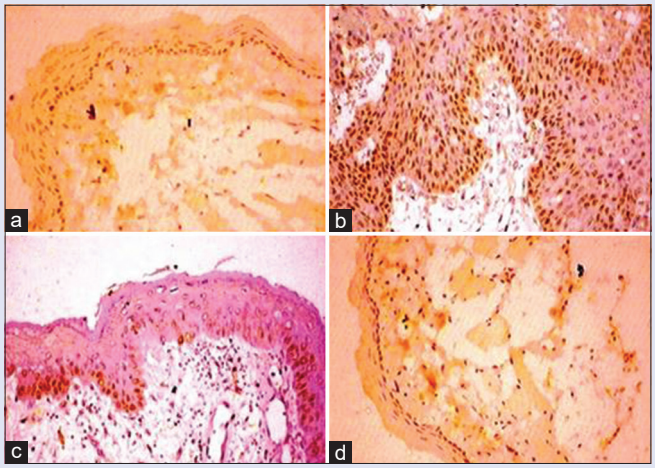

Figure 5: IHC staining for p53 protein expression observed control and experimental hamsters. (a and d) Control and D-carvone alone hamsters showing the absence of p53 protein expression. (b) 7,12-dimethylbenz[a] anthracene alone hamsters shows over expression of p53 protein. (c) 7,12-dimethylbenz[a]anthracene + D-carvone hamsters show decreased expression of p53 protein

Table 7: The levels of Phase I and Phase II detoxification enzymes in the buccal mucosa of experimental hamsters

Group	Group 1	Group 2	Group 3	Group 4
Oral mucosa				
Cyt P_{450} (U^x/mg protein)	1.41±0.11^a	3.28±0.25^b	1.50±0.11^c	1.38±0.11^a
Cyt b_5 (U^y/mg protein)	0.43±0.03^a	0.67±0.05^b	0.47±0.04^c	0.42±0.03^a
Oral mucosa				
GST (U^A/mg protein)	1.50±0.11^a	3.36±0.26^b	1.59±0.12^c	1.48±0.11^a
GR (U^B/mg protein)	58.08±4.42^a	120.12±9.15^b	61.93±4.74^c	57.19±4.35^a
GSH (µg/g tissue)	60.92±4.64^a	111.55±8.49^b	63.61±4.87^c	58.08±4.42^a
GSSG (µg/g tissue)	14.28±1.09^a	9.04±0.69^b	12.86±0.98^c	13.62±1.04^a
GSH/GSSG (µg/g protein)	4.12±0.31^a	13.04±0.99^b	5.01±0.38^c	4.05±0.31^a

U^x: µmoles of cytochrome P450; U^y: µmoles of cytochrome b5; U^A: µmoles of 1-chloro-2, 4-dinitro benzene reduced glutathione conjugate formed/minute; U^B: µmoles of NADPH oxidized/ hour. Results are expressed as mean±SD for six hamsters in each group. Data not sharing a common superscript (a-c) differ significantly at $P<0.05$ (DMRT). SD: Standard deviation; GSH: Glutathione; GST: Glutathione-S-transferase; GR: Glutathione reductase; GSSG: Oxidized glutathione; DMRT: Duncan's multiple range test

Table 8: Immunohistochemistry scoring of proliferating cell nuclear antigen and p53 expressions examined in control and experimental hamsters

Groups/ treatment	PCNA				p53			
	0	1+	2+	3+	0	1+	2+	3+
Group 1	0	6	0	0	10	0	0	0
Group 2	0	0	2	4	0	0	1	5
Group 3	0	3	2	1	1	3	2	0
Group 4	0	6	0	0	3	3	0	0

Results are given as number of hamsters (n=6). The percentage of positive cells was scored as: 3+: Strong staining, >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, <1% of cell staining. PCNA: Proliferating cell nuclear antigen

phase.[42] Gopalakrishnan *et al*.[43] have assessed that D-carvone prevents DMBA-provoked skin tumorigenesis in the Swiss albino male mice. Further they have proved that D-carvone inhibits the tumor formation, which in turn decreases the level of Phase I and II substances. Finally, they explained the downregulation of apoptotic (p53 and Bcl-2) proteins. In our current study, the HPBC, role of D-carvone blocking mediator via repressing Phase I and inducing expression of Phase II enzyme levels. Numerous investigations have revealed dual action drugs are further believable as cancer chemoprotective drugs as they prevent cellular metabolic stimulation and encourage biotransformation and removal.[44] These studies have supported our study.

PCNA is a pivotal role in cell proliferation cancer markers, including OC.[45] Mutant p53 is a tumor suppressor marker which is related to the regulation of cell-cycle entry, which is detected or mutated in cancer cells.[46] Elevated regulation of PCNA can be seen on the buccal tissues of DMBA-induced hamsters. Mutant p53 is involved in ROS homeostasis by regulating the antioxidant defense mechanism against ROS-induced oxidative damage. IHC patterns of molecular markers in buccal tissue may help to measure the cell proliferation, early diagnosis of pre- and post-cancerous lesion in OC. Furthermore, in our study revealed upregulation of PCNA and mutant p53 markers expressions were observed in hamsters treated with DMBA alone, in contrast in D-carvone treated hamsters suppressed the expression pattern, so that the development of HBPC were inhibited through cell proliferation downregulation of PCNA and mutant p53 expressions.

CONCLUSION

From, our findings we concluded that D-carvone have an modulatory effect of DMBA-induced HBPC by brought back the levels of antioxidant, LPO, xenobiotic metabolizing enzymes by biochemical analysis results and inhibition cell proliferation and apoptotic markers expression by IHC analysis, which finally delayed the development of tumors. Accordingly, D-carvone shows a potential applicant to inhibit hamster oral mucosal carcinogenesis. In spite of this, future studies are needed to recognize the accurate function of D-carvone in a variety of molecular mechanisms, pertaining to the antitumor effects.

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Conflicts of interest

There are no conflicts of interest.

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