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β-caryophyllene Modulate the Inflammatory and Apoptotic Signally Cascades to Alter the Cellular Response during DMBA Induced Experimental Oral Carcinogenesis; A Histological and In-silico Study

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Abstract

β-caryophyllene (BCP) is a more efficient pro-oxidant and anti-cancer property in our previous in-vitro studies. The motivation behind the present examination was to research the anticancer properties of BCP and its molecular mechanism on 7,12-dimethylbenz(a)anthracene (DMBA) treated hamsters. Hamsters were painted with 0.5% DMBA 3 times a week for 10 weeks to developed oral tumor and showed well progressed hyperplasia, dysplasia and differentiated Oral Squamous Cell Carcinoma (OSCC). DMBA alone treated hamster observed 100% tumor formation, elevated tumor incidence, volume and burden, lipid oxidation by-products, diminish antioxidant levels, body weight and imbalance of detoxification enzymes, along with up-regulation of inflammatory (NFκB, TNF-α, COX-2, iNOS, IL-6), mutant p53, anti-apoptotic (Bcl2) and down regulation of pro-apoptotic (Bax and caspase-9) markers expressions were observed. Oral pre-administration of BCP at different concentration (100, 200 and 400 mg/kg bw) to DMBA-treated hamsters for 14 weeks, completely prevent the OSCC and restored the above biochemical parameters to near normal level, while histological and western blotting investigation were positive support to the biochemical discoveries. These results indicated that BCP potentially inhibit the inflammatory, anti-apoptotic markers and up-regulate the pro-apoptotic markers. Based on our present finding BCP inhibit cancer cell progression and enhances the apoptosis in DMBA induced oral carcinogenesis. In-silico docking investigation was done to supplement the exploratory outcomes.

Keywords: Antioxidant, Apoptosis, β-caryophyllene, Detoxification Enzymes, DMBA, Oral Cancer

1. Introduction

Oral cancers are malignant growth of head and neck region, it could be occurring in the oral cavity (squamous cell carcinomas and salivary gland neoplasm). Recently, the WHO reported that 53000 Americans would be identified with oral cancer. Worldwide in this issue, the number of new cases is exceeding 640,000/year[1]. In 2018, oral cavity and lip cancer were together predicted at 354,864 new cases with death rates reaching 177,384 internationally[2], Based on Globocan 2018 data represents oral cancer is the second most common cancer in India and high rate of morbidity 1,19,992 and mortality 72,616. An advancement treatment for oral cancer are still unsatisfactory, the overall 5 year survival rate has not changed for past several decades, in which the patients are treated with chemotherapy and radiotherapy or both. The basic hazards of oral cancer are tobacco and tobacco-related products, betel nut chewing,

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heavy alcohol consumption and infectious viruses such as Human Papilloma virus, Human Herpes viruses and Epstein-Barr virus[3]. India is the second largest consumer of tobacco related products and nearly 300 million individuals live in below poverty, from these about 28.6% of the peoples consume tobacco related products, which are easily available and most affordable. Malignant growth research has given novel data about cellular and molecular processes in carcinogenesis. DMBA is a Polycyclic Aromatic Hydrocarbon (PAH) it have both initiating and cancer-promoting properties[4]. Oral cancer provoked by DMBA an organ specific carcinogen that develops via three phases specifically initiation, promotion and progression[5]. DMBA metabolism activated to form reactive metabolite, dihydrodiolepoxide, which further attach with A and G residues of DNA forming adducts, that may causes mutation to generating Reactive Oxygen Species (ROS) and inflammations. Prolonged and unrestrained activation of inflammation is a characteristic of many diseases including oral cancer[6].

Abundance ROS generations are fully toxic to cellular biomolecules such as proteins, lipids, DNA which may possibly to disturb the cellular antioxidant mechanism[7]. Free radicals may act an essential role in malignancy, due to Lipid Peroxidation (LPO) in membrane lipid (polyunsaturated fatty acid) are attacked by the excess generation of free radicals which can promotes progression of the carcinogenesis[8]. Antioxidant acts as to reduce the expression of free radicals in many in-vivo models[9,10]. Vitamin E is a main key antioxidant enzyme reduces the ROS levels there by diminishing the oxidative stress in tumor tissue[11]. The phase I and II detoxification enzymes are main drug-metabolising enzymes present in all eukaryotic cells. Phase I enzymes are super family of cytochrome P450-dependent monooxygenases, it converts procarcinogenic DMBA to highly reactive electrophilic intermediates, phase II enzymes play a vital role in inactivates or elimination the carcinogen into harmless agent[12].

Inflammation is a complex biological process, which comprises a wide spectrum of mediators, regulated by numerous signaling pathways and associated with a wide range of cellular responses[13]. NFkB is a transcription factor that has promotes the carcinogenesis by regulating the expression of genes that are involved in cell proliferation, metastasis and apoptosis[14]. Tumor necrosis factor alpha (TNF-α) is a multifunctional effects of pleiotropic cytokine playing an important role in apoptosis, inflammation, cell survival and neovascularization changes. In addition TNF-α promote the neoplastic cell growth and metastasis in several cancers[15]. Cyclooxygenase-2 (COX-2) and inducible Nitric Oxide Synthase (iNOS) is notable pro-inflammatory key mediator in pathogenesis of inflammatory diseases. Several molecular mechanisms of action have been involved in favorable effects of phytochemicals including anti-inflammatory through the reduction of pro-inflammatory cytokines such as NFκB, TNF-α, COX-2, iNOS, IL-6. BCP were found to be responsible for the distinct in-vivo anti-inflammatory properties of the essential oil from the leaves of Cordia verbenacea[16]. Apoptosis is a programmed cell death that is characterized morphologically by severe chromatin condensation and formation of apoptotic bodies[17]. Caspases, a group of cysteine proteases, are integrated as inactive pro-enzymes in normal cells. Caspase activation is modulating the different stimuli as well as DNA damage, oxidative stress and chemotherapeutic agents. Chemotherapeutic agents destroy the cancer cells by inducing the apoptosis especially by the activation of caspase cascade[18].

Terpenes have a place with the greatest class of secondary metabolites and fundamentally comprise of five carbon isoprene units. Terpenes are simple hydrocarbons, while terpenoids are altered class of terpenes with various functional groups and oxidized methyl group moved or removed at different positions. Sesquiterpenes is secondary metabolites consisting of three isoprene units ($C_{15}H_{24}$) and found in linear, cyclic, bi-cyclic and tri-cyclic forms[19]. Sesquiterpene has potent cytotoxicity against human KB, DLD-1, NCI-661 and Hela cancer cell lines[20]. A variety of sesquiterpene has documented to execute their anticancer ability to inhibition of inflammatory responses, prevention of metastasis and induction of apoptosis. Numerous terpenoids restrained diverse human cancer cells and are utilized as anticancer medication[21]. BCP (trans-(1R,9S)-8-Methylene-4,11,11trimethylbicyclo(7.2.0)undec-4-ene) is a natural bicyclic sesquiterpene, BCP present in many plants including: Clove, cinnamon, black pepper and copaiba balsam. Previous results were reported that BCP has several biological properties such as anti-oxidant, anti-inflammatory, neuroprotective[22], anticancer[23], anti-melanogenic[24], antidepressant, anxiolytic[25], anti-diabetic[26], cardioprotective effect by reducing the lipid content in the heart tissue[27] and it also used as cosmetics products[28]. In addition, these BCP is a good food additive agreed by FDA. Kim *et al.,* 2016[29] demonstrated that BCP inhibit the signal transducer and activator of transcription factor communications cascade and in this manner indicates the potential for the cancer treatment. BCP inhibit inflammatory process in bowel diseases[30,31]. BCP suppresses HFD-stimulated solid tumor development and lymph node metastasis using the *in vivo* model[32]. The in-silico study can be utilized to demonstrate the communication between a small molecule and a protein at the molecular level, which grants us to separate the conduct of minor particles inside the binding site of objective proteins also on elucidating basic biological processes[33]. In-silico docking examination

actualizes the quantitative forecasts of binding affinity, gave that ranking of docked chemical compounds depending on the binding scoring of the ligand-receptor complexes. Moreover, pharmacodynamics and pharmacokinetic properties have been studied through these methodologies[34].

There were no reports on chemopreventive capability of BCP in DMBA-induced Hamster Buccal Pouch (HBP) carcinogenesis. Based on previous reports, we used 100, 200 and 400 mg/kg bw BCP to assess the chemopreventive capability of BCP in the well-established DMBA-induced OSCC by the analysis of detoxification phase I enzymes (Cyt P450 and b5), phase II (GST, GSH, GR, DTD and GSSG) enzymes, lipid peroxidation by-products (TBARS, CD and LOOH), enzymatic antioxidant (SOD, CAT, and GPx), non-enzymatic antioxidant (Vit-E and GSH) and histopathological pattern. We also researched the expression levels of inflammatory and apoptotic markers in hamster buccal tissue using western blot and molecular docking analysis.

2. Material and Methods

2.1 Chemicals

BCP (purity≥80%), DMBA (Purity≥95%), glutathione, reduced nicotinamide adenine dinucleotide and 1,1' ,3,3'-tetramethoxypropane were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Primary and secondary antibodies NFκB, TNF-α, COX-2, iNOS, IL-6, p53, Bax, Bcl2 and caspase-9 were purchased from Santa Cruz Biotechnology USA. Polyvinylidene Difluoride (PVDF) membrane (Bio-Rad) were purchased from Biovision, USA. Heparin, Thiobarbituric Acid (TBA), trichloroacetic acid, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis (2-nitro benzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), Nitro Blue Tetrazolium (NBT), Phenazine Methosulfate (PMS), cysteine hydrochloride and sodium meta arsenate, All other chemicals and solvents were of analytical grade purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India.

2.2 Animals

Thirty five male Syrian golden hamster (*Mesocricetus auratus*), animals 8–10 weeks old, weight 80–120 g, were obtained from National Institute of Nutrition (NIN), Hyderabad, India. Animals were maintained in Central Animal House, RMMC and H, Annamalai University, Chidambaram, Tamil Nadu, India. Polypropylene cages used to maintain the animals, under controlled temperature (27 ± 2^0C) and humidity (55 ± 5%) with a 12-h light/dark cycle. Feed and water provided ad libitum. The animals were maintained as per the guideline made by ethical committee of Annamalai University (Registration number 160/1999/CPCSEA Proposal no: 1149, Dated: 03.01.2017).

2.3 Induction of Cancer by using DMBA/ Preparation of BCP

Tumors were induced in the left buccal pouch of the hamster by painted with 0.5% DMBA in liquid paraffin 3 times a week for 10 weeks using a number four painting brush[35]. Preparation of BCP:BCP was dissolved in 1ml of corn oil and different concentration (100, 200 and 400 mg/kg bw), were orally administered by using the intragastric tube for a period of 14 weeks.

2.4 Experimental Design

A total of 35 hamsters 8 weeks old, weighing 80-120g were randomized into five groups (n = 7). The experimental protocol was shown in Figure 1. At the end of the investigational period, hamsters fasted overnight and sacrificed by cervical dislocation. Blood and buccal tissues were collected from hamsters of control experimental groups and processed for subsequent biochemical and molecular analysis. Macroscopically, Hamster Buccal Pouch (HBP) tumors were counted in each hamster. The diameter of each tumor was measured using a vernier caliper. The tumor volume was calculated as $V = \frac{4}{3}\pi \left(\frac{D1}{2}\right)\left(\frac{D2}{2}\right)\left(\frac{D3}{2}\right)$ where D1, D2 and D3 are the length, width and height respectively, of the tumor in millimeters (mm^3). Tumor burden was calculated by multiplying the tumor volume by the number of tumors per hamster[36]. We investigated biochemical characteristics using plasma, liver and buccal mucosa tissues.

2.5 Preparation of Blood and Tissue Sampling

Blood samples from the jugular vein were collected in heparinized tubes. Plasma was isolated by centrifugation at 1,000 x g for 15 min. The control and all experimental tumor tissues were washed with ice-cold saline and homogenized using the tris-HCL buffer, pH 7.4 were used for biochemical analysis.

2.6 Biochemical Analysis

The protein substance was assessed by the method of Lowry *et al.* 1951[37]. The level of detoxification enzymes such as cytochrome-p450 and b5, DT-diaphorase GST, GR, GSSG and GSH levels in liver and buccal mucosal tissue according to the methods of Omura and Sato 1964[38], Lind *et al.* 1990[39], Habig *et al.*, 1974[40], Carlberg and Mannervik, 1985[41], Anderson, 1985[42], respectively. LPO by-products Thiobarbituric Acid Reactive Substances (TBARS) levels

were estimated by methods of Ohkawa *et al.,* 1979[43], Lipid Hydroperoxides (LOOH) by the methods of Jiang *et al.*, 1992[44], and Conjucated Dienes (CD) by the methods of Rao and Recknagel, 1968[45]. The enzymatic antioxidants Superoxide Dismutase (SOD) were accessed by the methods of Kakkar *et al.,* 1984[46]. Glutathione Peroxidase (GPx) was estimated by the method of Rotruck *et al.,* 1973[47] and Catalase (CAT) as per method of Sinha, 1972[48] respectively. Non-enzymatic antioxidant Glutathione (GSH) and vit-E level in the plasma and buccal tissue were determined using the methods of Beutler and Kelly, 1963[49] Desai, 1984[50], and Palan *et al.,* 1991[51] respectively.

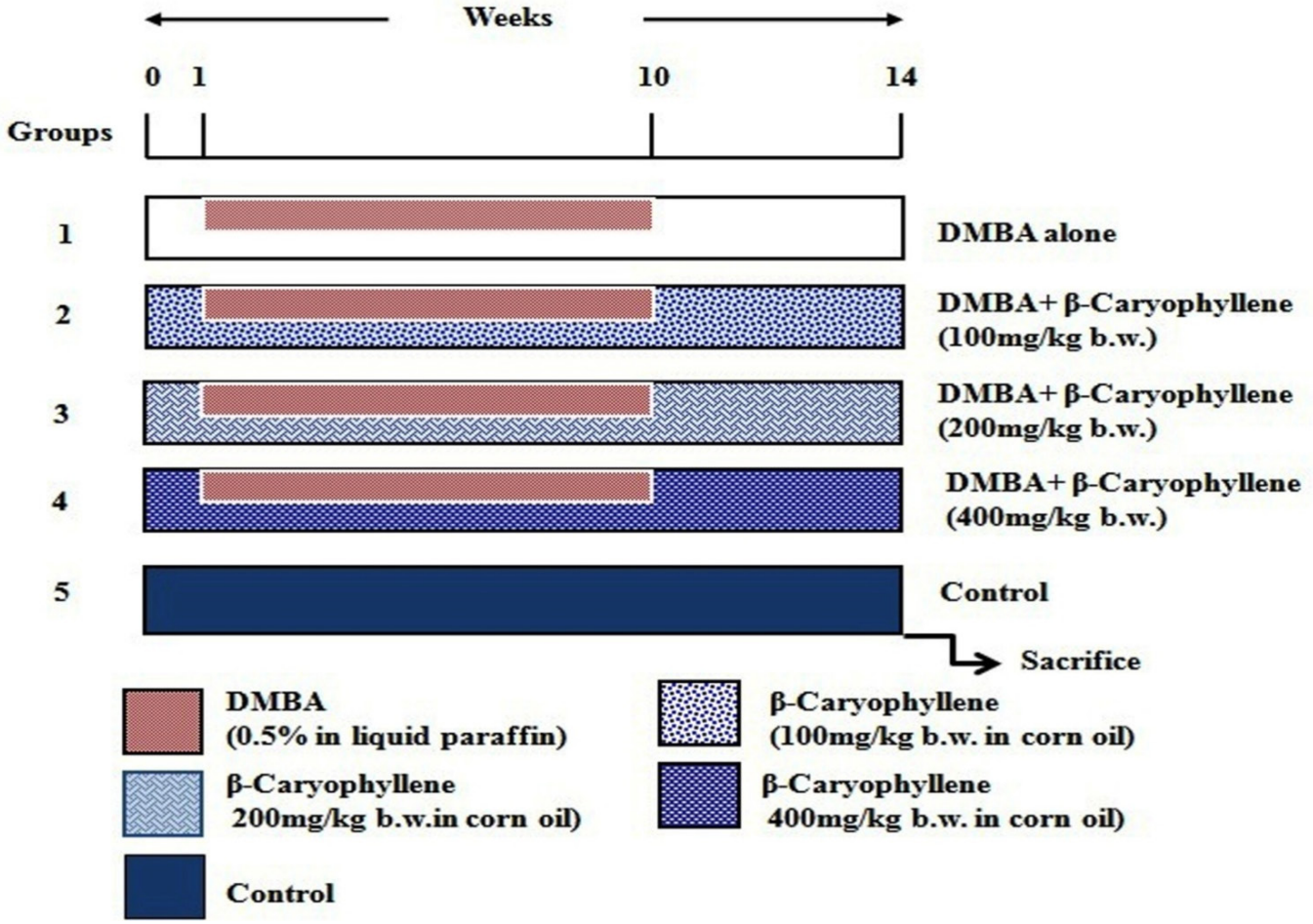


Figure 1. Schematic diagram of the experimental design

2.7 Histological Study

Briefly, buccal tissue sections were fixed in phosphate-buffered formalin solution for 48 h. After dehydration in increasing concentration of alcohol and cleared twice in xylene, then the tissues were embedded in paraffin and cut into sections 2–3 µm using a rotary microtome and mounted on clean glass slides. The slides were stained with hematoxylin-eosin dye, and finally observed at 40x magnification under the light microscope[52].

2.8 Western Blotting

The buccal tissues were homogenized with an appropriate buffer. The homogenate was centrifuged at 12,000 rpm for 15 min at 4^{0}C. The protein content was measured by the method of Lowry *et al.,* 1951[37]. Use bovine serum albumin as the standard. The protein was subjected 10% SDS-PAGE and transferred to PVDF membranes for the measurement of inflammatory NFκB, TNF-α, COX-2, iNOS, IL-6 and apoptotic markers mutant-p53, Bax, Bcl-2 and Caspase 9 and β-actin used as a control. Membranes were cleaned

with Tris-buffered Saline (TBS) containing 0.05% Tween 20 (TBST) and were then blocked for 1 h in TBS containing 3% BSA. The membranes were then washed with TBST and then incubated overnight at 4^0C with the primary antibodies with a dilution of 1:1000. Membranes were washed with TBST and treated with HRP-conjugated secondary antibodies for 60 min at RT. The PVDF membranes were then washed with TBST thrice with 10 min interval and the developed bands were detected using a Chemiluminescence (ECL) substrate. The images were acquired by Image Studio software.

2.9 Protein Dataset

Molecular docking studies were carried out using autodock vina, 3D structure was visualized by Discover Studio Version 4.5 (Biovia Dassault system, Inc. USA) this tool to predict the preferred binding mode and binding sites of BCP with p53, Bax and Bcl2. Protein Data Bank (PDB) provides the protein structural information by method of X-ray crystallographic and NMR. To remove the water, interacting heavy atoms, metal ions from the protein structure and added with hydrogen atoms before the docking analysis. COX-2: PDB ID: 3NTG, Resolution: 2.19 Å, R-Value Free: 0.255, R-value Work: 0.206, p53- PDB ID:1YCS Resolution: 2.2 Å, R-Value Free: 0.286, R-value Work: 0.205, BCl2-PDB ID:5JSN Resolution: 2.1 Å, R-Value Free: 0.205, R-value Work: 0.159, BAX- PDB ID: 2K7W proteins were retrieving from PDB. Ligand Preparation: Drug compounds BCP were obtained from the PubChem website https://pubchem.ncbi.nlm.nih.gov/.ChemSketch (http://www.acdlabs.com) was used to construct the structure of the ligand and to determine the basic properties.

2.10 Statistical Analysis

The values are expressed as means ± SD. Comparisons were performed using one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT), using SPSS version 17.0 and statistical significance was set at $p \leq 0.05$.

3. Results

3.1 Measurement of Animal Body Weight

Figure 2 illustrates the changes between the initial and final stages of animal body weights in control and all the experimental groups. At the end of experimental period body weights was significantly diminished in DMBA alone treated Group 1 hamsters as compared with control group, while oral administration of BCP at dose dependent manner (100, 200 and 400 mg/kg b.w.) exhibit a significant improvement in the body weight of DMBA treated hamsters. In particular, BCP (200 and 400 mg/kg b.w.) treated hamster showed the significantly increased body weights were observed, when compared with DMBA alone treated groups.

3.2 Tumor Incidence, Volume and Burden

Table 1 represents the effect of BCP on tumor incidence, volume and burden in DMBA induced oral cancer. We have observed 100% tumor development with mean tumor volume (282.14 mm^3) and tumor burden (1692.84 mm^3) in DMBA alone treated Group 1 hamsters. Oral administration of BCP to DMBA painted Groups 3 and 4 hamsters for 14 weeks completely prevented the OSCC, Group 2 exhibits significantly suppress the tumor size; no tumors were found in the control Group 5.

3.3 Effect of BCP on Detoxification Enzymes in the Liver

Figure 3 depicts the status of phase I (Cyt P450 and b5) and phase II (GST, GSH, GR and DTD) detoxification enzymes in the liver of control and all the experimental groups. The levels of phase I enzymes was enhanced, whereas phase II enzymes was significantly ($p<0.05$) diminished in hamsters painted with DMBA alone (Group 1) as compared with control hamsters. Oral administration of BCP at 100 mg/kg b.w., to DMBA-treated Group 2 slightly ($p<0.05$) brought back the phase I and II enzymes and then BCP 200 mg/kg b.w. to DMBA-painted Group 3 significantly ($p<0.05$) restored the levels of phase I and II detoxification enzymes to near normal range. Group 4 (400 mg/kg b.w.) hamsters showed no significant difference in the status of phase I and II detoxification enzymes as compared with the control Group 5.

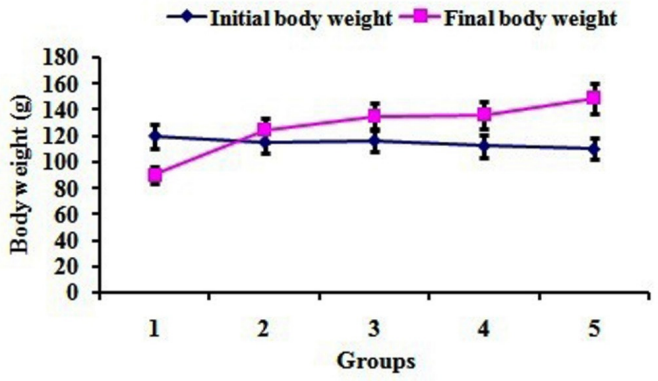


Figure 2. Initial and final body weights of control and experimental hamster groups. Values are means ± SD. Values not sharing a given superscript differ significantly ($p<.05$).

Table 1. Tumor incidence, tumor number, tumor volume and tumor burden in control and experimental hamsters in each group

	DMBA alone	DMBA + BCP (100 mg/kg b.w.)	DMBA + BCP (200 mg/kg b.w.)	DMBA+BCP (400 mg/kg b.w.)	Control
Tumor incidence (oral squamous cell carcinoma)	100%	18.38%	0%	0%	0%
Total number of tumors /hamsters	6 ± 0.89	3 ± 0.23	0	0	0
Tumor volume (mm^3)	282.14 ± 21.49	21.56 ± 1.65	0	0	0
Tumor burden (mm^3)	1692.84 ± 96.78	64.68 ± 4.93	0	0	0

Values are expressed as mean ± SD for seven hamsters in each group. 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^3) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/hamsters.

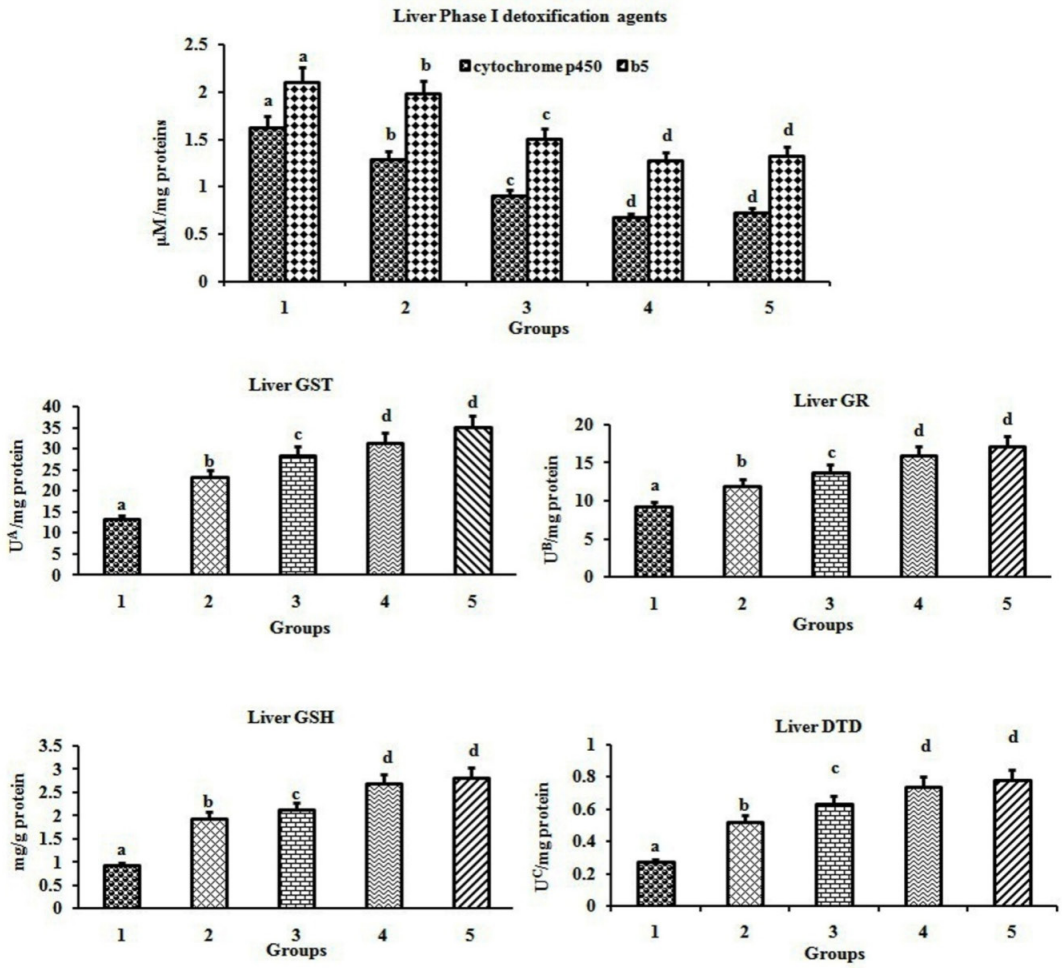


Figure 3. Activities of phase I and II detoxification enzymes in the liver of hamsters in each group. Values are means± SD. U^A: micromoles of 1-chloro-2,4-dinitro benzene reduced glutathione conjugate formed/minute; U^B: micromoles of NADPH oxidized/hour; U^C: micromoles of 2,6-dichloro indophenols reduced/minute. Values are expressed as mean ± SD for seven animals in each group. Values not sharing a common superscript differ significantly at $p<.05$.

3.4 Effect of BCP on Detoxification Enzymes in the Buccal Mucosa

Figure 4 depicts the levels of phase I and II detoxification enzymes in the buccal tissue of control and all the experimental groups. The levels of phase I (Cyt-p450 and b5) detoxification enzymes were significantly ($p< 0.05$) elevated, whereas phase II enzymes were altered (GST, GR, GSH, GSSG, GSH/GSSG ratio were elevated and GSSG levels were diminished) in tumor-bearing hamsters. Oral administration of BCP at 100 mg/kg b.w., to DMBA-painted Group 2 slightly ($p < 0.05$) brought back the phase I and II enzymes, BCP (200 mg/kg bw) to DMBA painted hamsters significantly ($p< 0.05$) restored the status of detoxification enzymes to near normal range (group 3). Group 4 (400 mg/kg bw) confirmed no significant difference in phase I and II detoxification enzymes when compared with control Group 5.

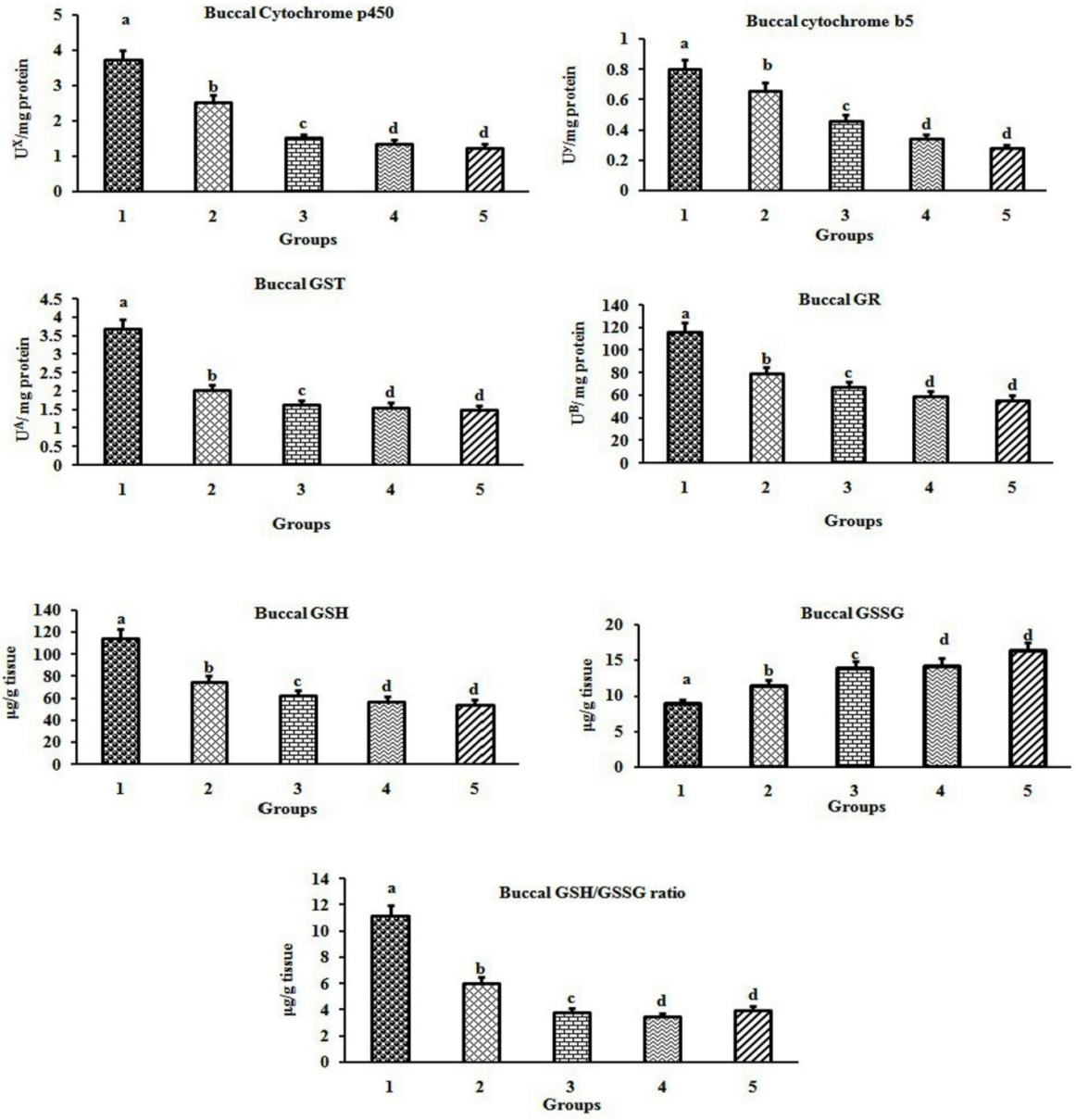


Figure 4. Activities of phase I and II detoxification enzymes in the buccal tissues of hamsters in each group. ($p<0.05$) U^x: micromoles of cytochrome p450; U^y: micromoles of cytochrome b5; U^A: micromoles of 1-chloro-2, 4-dinitro benzene reduced glutathione conjugate formed/minute. U^B: µmoles of NADPH oxidized/hour. Values are expressed as mean ± SD for seven animals in each group. Values not sharing a given superscript differ significantly $p<.05$.

3.5 Lipid Peroxidation by Products

Figure 5 illustrates the status of LPO by-products (TBARS, CD and LOOH) in the plasma and buccal tissue of control and all the experimental hamsters. The levels of LPO by-products were significantly ($p<0.05$) raised in the plasma, while diminished in the buccal tissues of tumor-bearing Group 1 hamster. Oral administration of BCP at 100 mg/kg b.w., to DMBA-painted Group 2 slightly reversed ($p < 0.05$) the LPO by-products. BCP (200 mg/kg b.w.) to DMBA painted Group 3 hamster significantly ($p<0.05$) LPO by-products returned to the near-normal level. Group 4 (400 mg/kg b.w.) showed no significant differences in LPO by-products as compared with the control group.

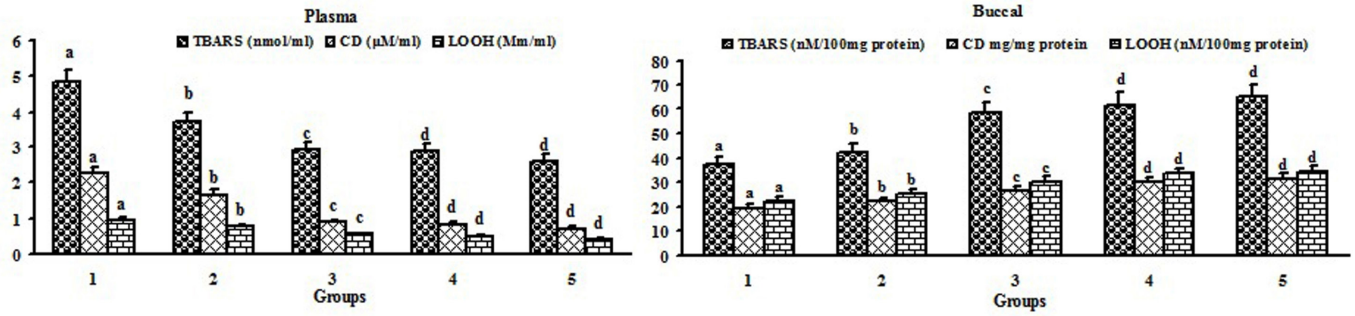


Figure 5. Statuses of LPO byproducts (TBARS, CD and LOOH) in the plasma and buccal mucosa tissue in control and experimental hamsters in each group. Values are means ± SD. Values not sharing a given superscript differ significantly ($p<.05$).

3.6 Antioxidant Status

Figure 6 a and b showed the status of enzymatic (SOD, CAT, GPx) non-enzymatic antioxidants (GSH and vitamin E) in the plasma and buccal tissue of control and all the experimental groups. Status of antioxidants was significantly ($p<0.05$) diminished in tumor-bearing hamsters, as compared with the control Group 5. Oral administration of BCP at 100 mg/kg b.w., to DMBA-painted Group 2 showed mildly reversed ($p<0.05$) the plasma antioxidants, BCP (200 mg/kg b.w.) to DMBA painted hamsters the status of plasma antioxidants levels were turned to near-normal range, Group 4 (400 mg/kg b.w.) showed no significant differences in antioxidants as compared with the control Group 5. Figure 6b disturbances in antioxidant status GPx, GSH, and vitamin E were enhanced; SOD and CAT were reduced in tumor-bearing hamsters when compared with the control group. Oral administration of BCP at 100 mg/kg b.w., to DMBA-painted Group 2 showed mildly inverted ($p<0.05$) the antioxidants status, BCP (200 mg/kg bw) to DMBA-treated hamsters restored the antioxidants levels in the buccal tissue. Group 4 (200 mg/kg bw) showed no vital distinction in antioxidant levels (enzymatic and non-enzymatic) as compared with the control group.

3.7 Histopathology

Figure 7 a and b showed the histological changes observed in control and all the experimental groups. DMBA alone painted Group 1 showed hyperkeratosis, epithelial hyperplasia, dysplasia and well-differentiated OSCC. Hyperkeratosis and dysplasia were exhibited in BCP treated Group 2 hamster. Hyperkeratosis and epithelial hyperplasia were seen in Groups 3 and 4 hamsters. Normal cellular architecture with no sign of cancer cell proliferation was observed in the control Group 5.

3.8 Western Blotting

To further, define the impact of BCP on inflammatory and apoptotic markers in control and all the experimental groups. The protein levels of inflammatory NFκB, TNF-α, COX-2, iNOS, IL-6 and apoptotic markers p53, Bcl-2, Bax, caspase-9 expression were analyzed by Western blot. As showed in (Figures 8, 9 a and b) respectively. Inflammatory NFκB, TNF-α, COX-2, iNOS, IL-6 were up-regulated in DMBA alone treated group; whereas BCP treated with dose dependent manner in experimental group the above marker levels were significantly down regulated compared with control group. Apoptotic marker mutant p53 and Bcl-2 expression were up-regulated in tumor bearing hamster, in contrast the Bax and caspase-9. When treated with different concentration of BCP to the control and DMBA treated groups exhibits mutant p53 and Bcl-2 expression was down-regulated; Bax and caspase-9 expression were up-regulated.

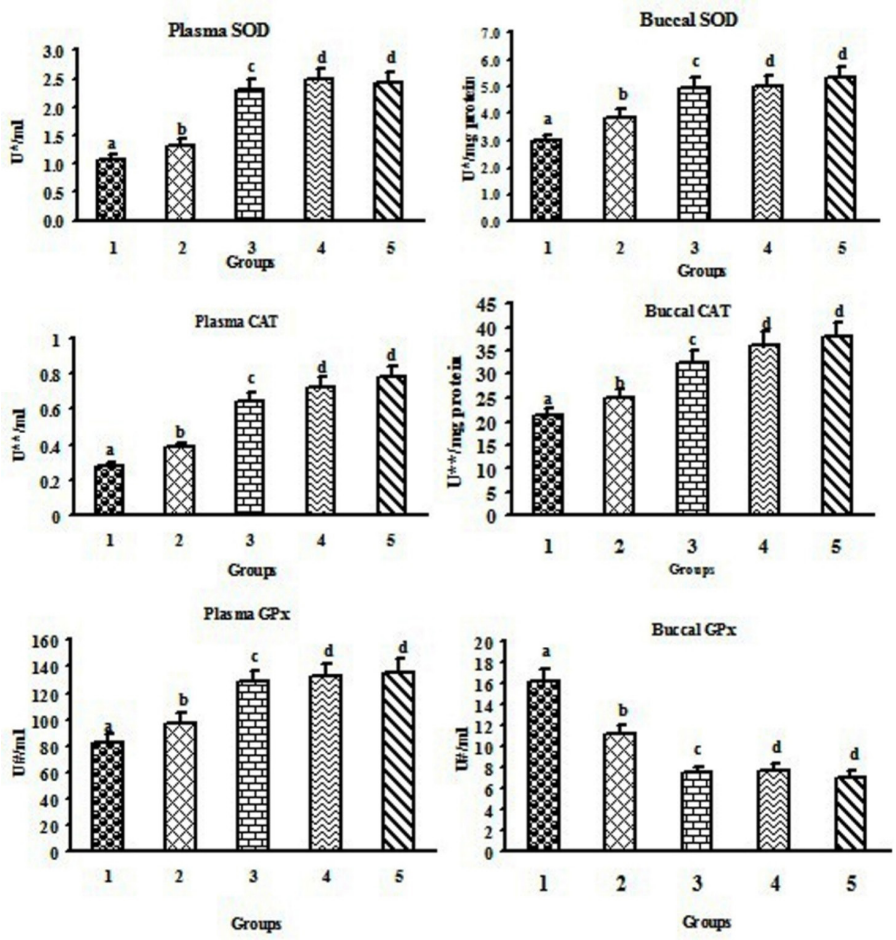


Figure 6(a)

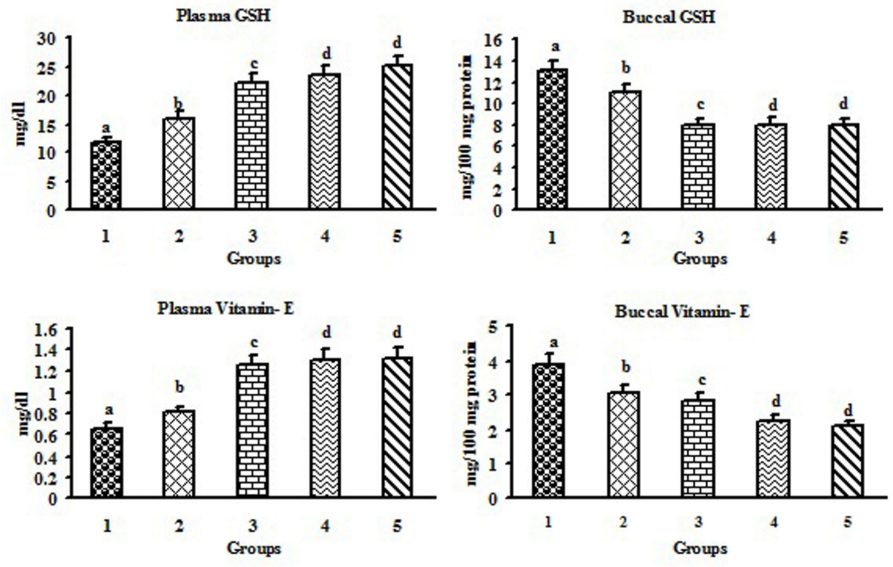


Figure 6(b)

Figure 6 (a and b) Status of enzymatic and non-enzymatic antioxidants in the plasma and buccal mucosa tissue of hamsters control and experimental in each group. Values are means ± SD. Values not sharing a given superscript differ significantly ($p<.05$).

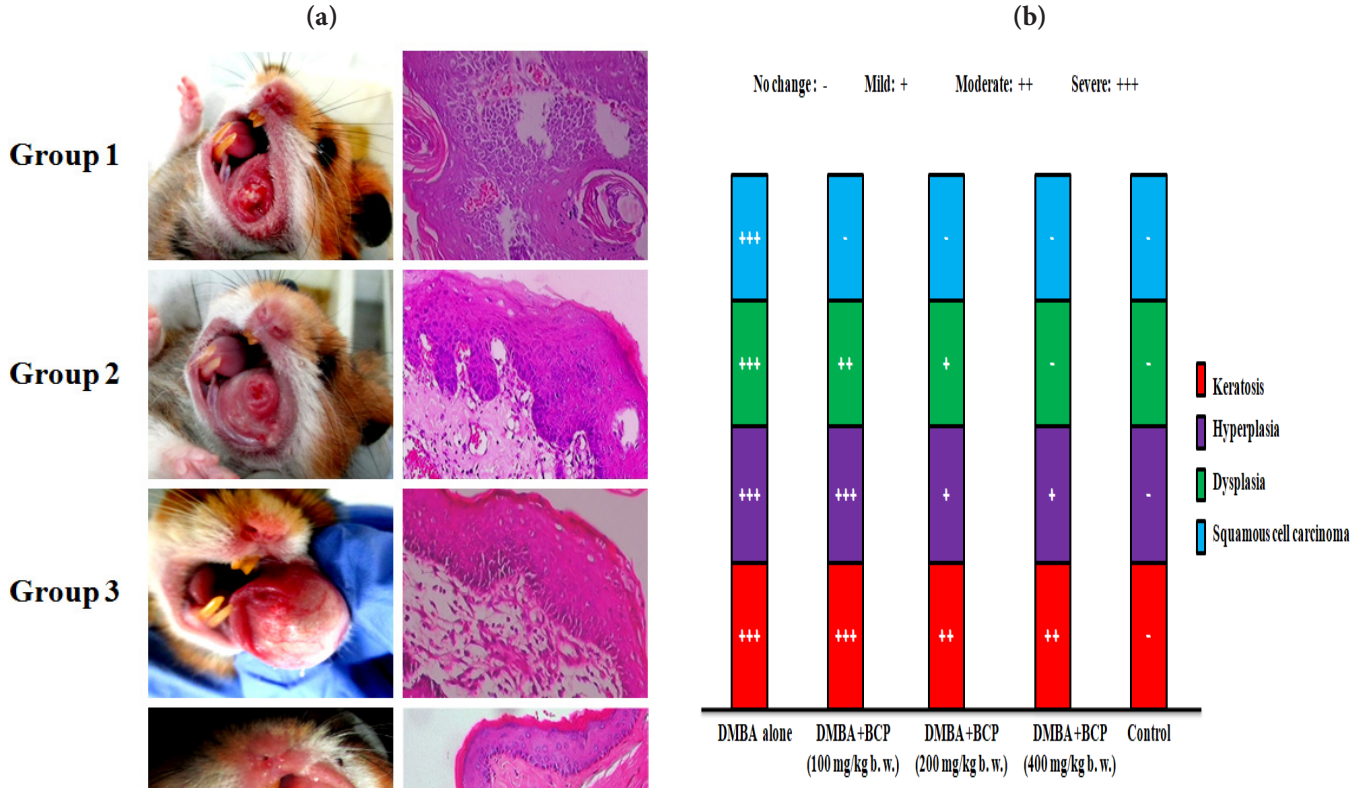


Figure 7. (a and b) Showed histopathological observation in control and treatment hamsters in each groups, A) showed cross appearance of the in each groups, B) DMBA alone treated Group 1 showed Well differentiated OSCC exhibiting epithelial and keratin pearls. BCP treated Group 2 showed epithelial hyperkeratosis and dysplasia. Buccal pouch epithelium from Groups 3 and 4 hamsters showed moderate keratosis and hyperplasia. Group 5 exhibited normal architecture with no signs of cell proliferation (40x).

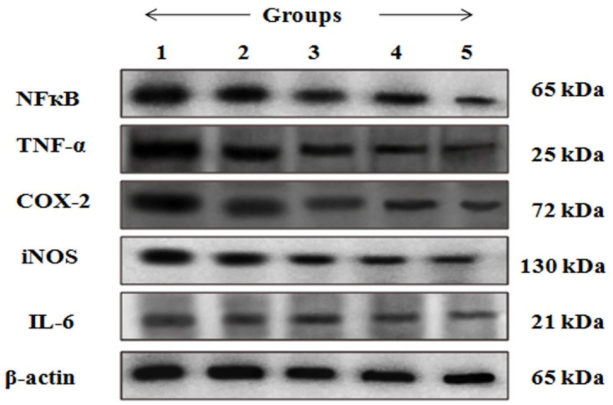


Figure (8a)

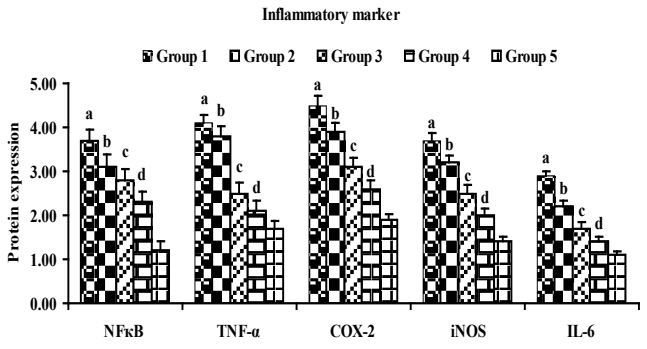


Figure (8b)

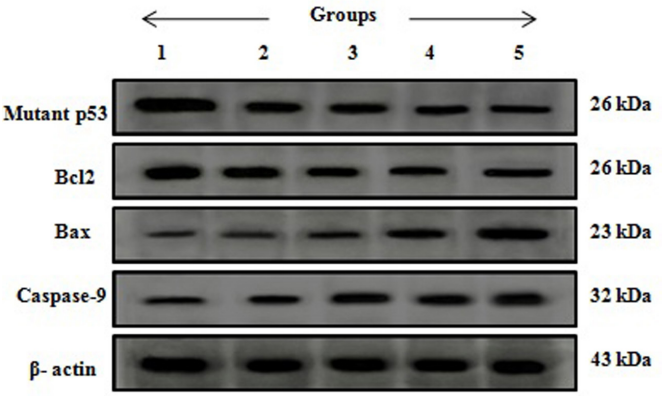


Figure (9a)

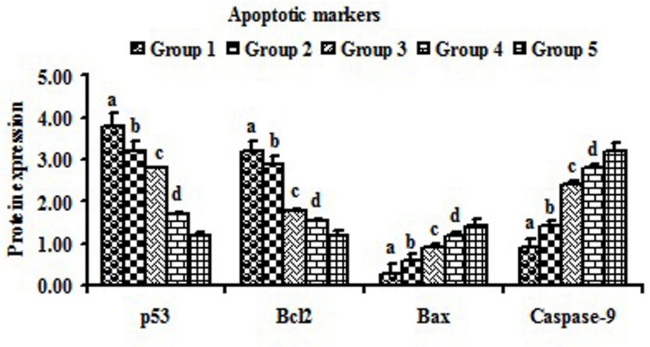


Figure (9b)

Figure 8, 9. (a and b) Shows the band intensities were quantified by densitometry and normalized to respective β-actin loading control. (B) The representative graph shows the relative protein expression of fold changes in Western blots. Values are expressed as mean ± SD for three experiments. Values that do not share a common superscript letter (a, b, c, d) between control groups differ significantly at p<.05 (One way ANOVA followed by DMRT).

3.9 Molecular Docking Analysis

Figure 10 showed the corresponding amino acids are involved in hydrogen bond formation between targeted protein and bioactive compound. The BCP was bound to respective amino acids of Cox-2 3ntg, p53 1ycs, Bax 2k7w, Bcl2 5jsn protein. The pink color was denoted that the alkyl and Pi-alkyl group Cox-2: PHE 186, PHE 381, LEU 377, VAL 281, VAL 430, PHE 390, LEU 280, LEU 394 and molecular docking energy Kcal/mol-12.1385. p53: LEU 264, ARG 267, PRO 98, ILE 254, MET 160 and molecular docking energy Kcal/ mol -9.26866. Bax: CYS 62, LEU 161, PHE 165, LEU 59, LEU 63, VAL 111, VAL 173, ILE 66 and molecular docking energy Kcal/ mol -10.4493. Bcl2: PHE 112, TYR 108, PHE 104, PHE 153, LEU 137, ALA 149 and molecular docking energy Kcal/mol -10.9188. When compared to Cox-2 and p53 protein molecules communication between BCP the high-level binding energies score was observed, whereas apoptotic protein Bax and Bcl2 binding interaction too high. BCP could be interacting with Bax and Bcl2 proteins; it can be developed high conformation changes were seen in the protein-ligand complex. The present examination was denoted that BCP has a great inhibitory action on p53 and Bcl2, it can be useful in cancer treatment. This computational technique supported and helps to recognize the more potent inhibitors through ligand-receptor interaction.

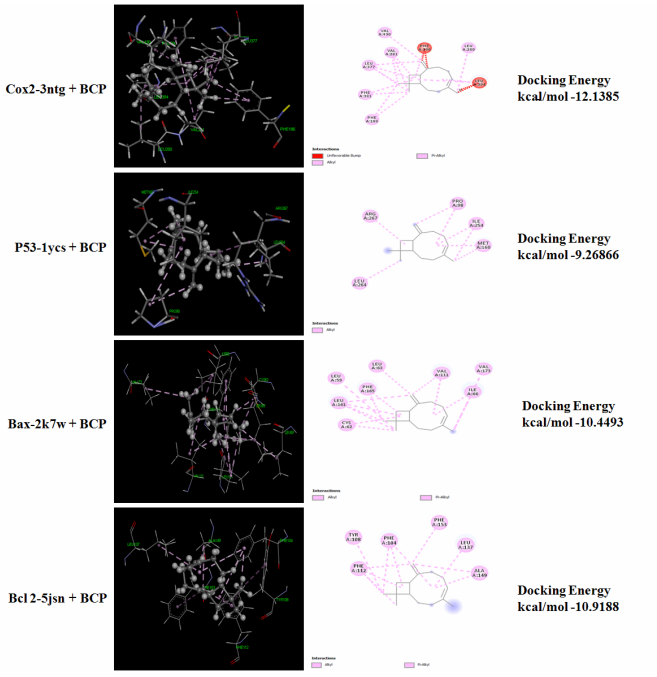


Figure 10. Molecular docked structures of different ligand-protein complexes: Interaction of BCP with inflammatory Cox-2 and pro/anti-apoptotic protein p53, Bax and Bcl_2 respectively.

4. Discussions

Oral carcinogenesis is a very complex multifactorial process that happens when epithelial cells are influenced by different genetic modification. OSCC have been etiologically and epidemiologically coupled with cancer causing hazards PAHs present in tobacco and tobacco-related product in very high concentration, which induce 75% of oral cancer, and it associated with heavy alcohol consumption[53]. Several PAHs stimulated the gene transcription of detoxification enzymes to the metabolite benz[a]anthracene derivatives and thereby promoting the DNA adducts formation[54]. Oral cancer progression leads to histopathological changes during DMBA-induced HBP carcinogenesis which is similar that of our pervious results[55]. Our present study, DMBA alone painted group for about 2-4 weeks and 4-8 weeks observed white (leukoplakia) and red patches (erytroplakia) on their left buccal pouch respectively. These results are concordant with velu *et al.* 2017[56]. Chemopreventive agents are shown to restrain the growth of tumors in the HBP administered once before, during or after initiation by carcinogen[57].

The main sources of chemopreventive agents are found in essential oils which include sesquiterpenes. Sesquiterpenes has possess numerous biological properties which including detoxification of toxic metabolites, stimulation of phase II detoxification enzymes, protease inhibition, scavenging of ROS, inhibition of cancer cell progression, angiogenesis, mutant p53 expression and DNA adduct formation[58]. However this offering pathways the way to deal with new drug for the treatment of cancer. Currently, agents that induce of phase II detoxification enzymes are active against several cancers[59]. Zheng *et al.,* 1992[60] demonstrated that BCP showed significantly induces the phase II detoxifying enzymes (GST) in the mouse induced by chemical carcinogenesis. Our present data reveals that BCP role as 'dual-acting agents' by decreased the phase I agent and increased phase II detoxification enzymes there by reducing the carcinogen content in the host. These result in lines with Di Sotto *et al.,* 2010[61]. Thus, sesquiterpenes BCP shows promising agent against potent carcinogen (or) act as anti-cancer drugs. ROS and LPO by-products cause oxidative stress leads to deleterious effect in the depletion of the endogenous antioxidants systems such as SOD, CAT, GPx, GSH. LPO by-products are formed by the free radical attack on lipids layer, producing large amounts of reactive species, which also a major factor involved in tumor initiation and progression[62,63]. SOD and CAT act interactive manner with antioxidant enzymes to shield against ROS. SOD is the most significant defense enzyme against free radical harm by converting superoxide radicals to molecular O_2 and H_2O_2. Secondary enzymes, CAT and GPx, convert H_2O_2 to H_2O that additionally protects cells from oxidative damage[64]. GSH is the most plentiful intracellular low molecular weight thiol and acting an important role in metabolic defensive functions, including H_2O_2 reduction, drug metabolizing process, and free radical scavenging[65]. Vitamine E is a fat-soluble vitamin that has strong antioxidant properties. The main role is to shield against LPO[66]. They respond more quickly than PUFA with trap peroxyl radicals and consequently act to breakdown the chain reaction of LPO. It not only prevents the initial formation of carbon centred radicals in a lipid rich environment, but also reduces the generation of secondary free radicals[67]. Epidemiological examination demonstrated that individuals who eat a diet rich in Vitamin E present a low risk of many types cancer[68]. Several reports are documented that BCP have antioxidant capability such as reduces systemic inflammation and oxidative stress in in-vivo study[69–71]. Pant *et al.,* 2014[72] suggested that in-vitro study of BCP was able to reduce the mitochondrial oxidative stress and balancing the intracellular ROS thereby maintaining cellular redox homeostasis. Our outcomes also substantiate with this finding.

NF-κB also acts in the organized cell cycle process, which is a basic component in deciding the level of cell apoptosis and proliferation. At last, irregularities in the NF-κB pathway are also frequently found in a many types of human cancer. Previous report suggested that BCP has been suppressing the NF-κB and iNOS activation through attenuation of the oxidative stress[73]. In vitro study results proposed that BCP was found to be instigating G2/M phase cell cycle arrest in leukemia cancer cells (RAW 264.7), it could suppress the cancer progression by repressing different intracellular signaling cascades such as cell survival, inflammatory and apoptotic pathways[74]. Tumor cell produce own cytokines on their microenvironment which activate the formation of inducible forms of Cox-2. Therefore, higher expression of Cox-2 prompts the way to development of many types of cancer which is due to sustained cell proliferation, activation of pro-angiogenic markers, invasion and metastasis. In vitro study proved that BCP treated with lymphoma and neuroblastoma cells down regulate the Cox-2 expression[75]. Kuwahata *et al.,* 2012[76] demonstrated that the agonist effect of BCP in relation to CB2 receptors is the main mechanism responsible for inhibiting the TNF–α and IL-6 inflammatory signaling pathways. In addition, BCP suppress the tumor growth and metastasis of melanoma cells in a diet-induced obesity mouse model[32]. The inhibition of NF-κB, TNF-α, COX-2, iNOS and IL-6 expression could attenuate the inflammatory signaling pathway. Our pervious in vitro study suggested that oral cancer KB cells treatment with BCP significantly repress the inflammatory gene such as NFκB, TNF-α, COX-2, iNOS and IL-6 expression[77]. The BCP has been showed anti-inflammatory activity against

the carrageenan-induced paw edema model[78]. Di Sotto *et al.,* 2010[61] suggested that oral administration of BCP at 300 mg/kg/day significantly reduce the inflammation of the colon in mice.

Several anticancer drugs induce tumor cell apoptosis that could be a noticeable approach for cancer therapy. p53 is an eminent tumor suppressor phosphoprotein that can inhibit cells from becoming malignant via initiation of cell cycle arrest, it is an important regulator of apoptosis and carcinogenesis[79–81]. Mutations of p53 gene, the most chronic alterations in human OSCC and HBP carcinomas, incited conformational changes that prolong the half-life of the p53 protein enabling immunolocalisation in the nuclei of cancerous cells. Over-expression of p53 in HBP carcinomas observed its characteristic of augmented neoplastic cell multiplication and is in line with similar discoveries in both human and animal cancer[82]. Amiel *et al.,* 2012[83] suggested that BCP shows antiproliferative activity and apoptosis-inducing agent against neoplastic cells.

Profound studies reported that deregulation of Bcl-2/Bax ratio in leukemia, colon, lung, skin and oral carcinogenesis[84,85]. We observed that BCP pre-treatment with DMBA-induce oral cancer in hamster showed the down-regulate expression of the p53, Bcl-2 and up-regulate the Bax expression, which could be responsible for the drug-induces apoptotic processes and thereby reducing the cancer cell survival. Caspase-9 plays a vital role in initiating apoptosis execution in cells that need to be removing damaged cells to suppress uncontrolled cell proliferation.[86] Physiological conditions such as, loss of cytochrome c from the mitochondria (cytochrome-c/dATP) modulate the formation of apoptosomes complex is essential for caspase-9 activation. Apoptosomes complex binds multiple procaspase-9 and it was suggested that locally accumulating procaspase-9 may promote its dimerisation and caspase 3 activation[87]. Decreased caspase-9 expressions were associated with the absence of apoptotic machinery was impaired in DMBA alone treated hamsters[6]. In vitro study suggested that neuroblastoma cells were treated with BCP significantly up-regulate the pro-apoptotic caspases-9 expression[75,83]. In our results also agree with earlier reports, which indicated that BCP have apoptosis inducing properties. The in-silico docking examinations were ranked by their docking scores and binding affinity of the compound[88]. The protein-ligand composites were analyzed to recognize the interactions between protein bases and bound ligands. The examination of the protein-ligand complexes discovered binding site residue, including amino acid residues, water and metal atoms[89]. The prediction of ADMET properties assumes a vital role in the drug discovery process. Medications for which the ADMET were not decided brought about practically 60% disappointments of all medications in the clinical stages[90]. BCP were stronger and higher binding affinity to Cox-2, p53, Bcl2 and Bax proteins. Based on the in-silico study results gave the BCP potentially induce the apoptosis in cancer cells. Overall, these finding suggested that BCP could suppress the cancer cell progression and promote the apoptosis in DMBA-treated hamster.

5. Conclusions

In conclusion, The possible mechanisms for the protective effects of BCP includes enhancing antioxidant enzymes, altered levels of detoxifying enzymes and significantly reverse the status of LPO by-products and modulate histopathological progress in the DMBA-induced oral cancer. Oral administrations of BCP doses of 200 and 400 mg/kg bw significantly suppressed the formation of tumor in DMBA-induced hamsters through inhibit the inflammatory and anti-apoptotic markers, whereas stimulate the pro-apoptotic gene. Moreover these results were consistent with in-silico analysis. From all these results observation we concluded that BCP may prevents the DMBA induced oral cancer.

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7. Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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