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ORIGINAL ARTICLE

# Molecular Effect of Betanin on the Molecular Expression Pattern of Cell Proliferative and Inflammatory Signalling Pathways in DMBA-Induced Oral Carcinogenesis in Hamsters

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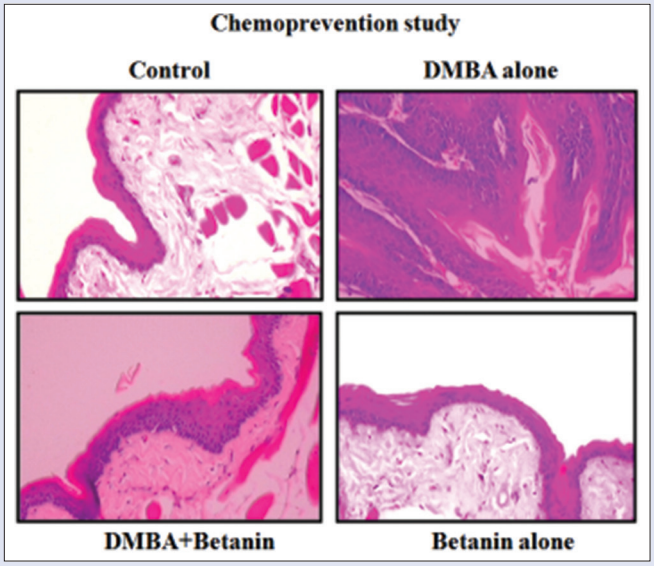
## ABSTRACT

**Background:** Cancer development is a sequential process as a result of various cellular adaptation events. Oral cancer (squamous cell carcinoma) is the most predominant variety of head and neck cancer. Betanin (BTN) is isolated from beetroot extracts and is a highly bioavailable antioxidant. BTN exerts a chemopreventive and cytotoxic activity on numerous cancer cells. However, precise identification of the molecules responsible for this tumor-inhibitory effect is pending. This study aimed to understand the molecular mechanisms underlying the chemopreventive effects of BTN in 7,12-dimethylbenz (a) anthracene (DMBA)-induced oral cancer in experimental hamsters. **Materials and Methods:** The interactions of BTN with antioxidant enzymes, lipid peroxidation, apoptosis, and inflammatory markers in the presence of DMBA were investigated in male golden Syrian hamsters. **Results:** Oral supplementation of BTN treatment (50 mg/kg BW) daily to oral tumor-bearing rats successfully prevented DMBA-induced oral carcinogenesis. Furthermore, BTN administration significantly prevented weight loss and reduced the tumor occurrence, burden, volume, and biochemical parameters such as TBARS, LOOH, SOD, CAT, GPx, GSH, and vitamins E and C. The histological analysis and expression pattern of molecular markers (increased apoptosis (caspase-3 and 9), proliferative markers (PCNA and Cyclin-D1), and inflammatory markers (TNF-α and COX-2) investigated in hamsters' buccal mucosa tissues revealed a significant anti-tumorigenic nature of BTN. **Conclusion:** The findings of this study show that BTN markedly reduces DMBA-induced oral cancer in hamsters.

**Key words:** Apoptosis, betanin, cell proliferative, DMBA, inflammatory, oral cancer

## SUMMARY

- Effect of BTN on inhibits tumor formation of DMBA-induced oral tumor hamsters.
- Effect of BTN on increased inflammatory protein expressions of oral squamous cell carcinoma hamsters.




**Abbreviations used:** BTN: Betanin; CAT: Catalase; CD: Conjugated dienes; COX-2: Cyclooxygenase-2; DMBA: 7,12-dimethylbenz (a) anthracene; GPx: Glutathione peroxidase; GSH: Reduced glutathione; GST: Glutathione S-transferase; IHC: Immunohistochemical; LOOH: Lipid hydroperoxide; PCNA: Proliferating cell nuclear antigen; PUFA: Polyunsaturated fatty acids ROS: Reactive oxygen species; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances.

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## INTRODUCTION

Cancer is a multifactorial disease arising due to an insufficiency in the host's genetic makeup and response to exposure to environmental insult. The cellular adaptation process comprises evading apoptosis, uncontrolled cell division, and metastasis in physiological and pathological conditions.[1] Oral cancer, mostly malignant neoplasia, affects the oral cavity and has a predilection for lymph node metastasis. It is the fifth-most frequent cancer with significant public health implications worldwide. Oral cancer commonly progresses due to



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increased proliferation and culminates while displaying antigenic properties as a malignant tumor of the head and neck region or metastatic lesions.[2] While oral cancer is reportedly an inevitable occurrence, smoking and chronic alcohol intake comprise the most prominent risk factors in 90% of cases; having them together produces a pronounced synergic effect.[3] The incidence of oral cancer in India is extremely high and has been attributed to various etiological factors involved in exposure to pro-carcinogenic stimuli.[4] Recent studies state that the overall incidence rate of new oral cancer cases has been constantly increasing in men; surprisingly, it has slightly reduced in women in India.[5] The most common etiologic factors in many parts of the Western Pacific region, South-East Asia, and the Indian subcontinent are smoking through bidis, cigarettes, drinking alcohol, and chewing tobacco.[5]

7,12-dimethylbenz (a) anthracene (DMBA) exists in the environment as a byproduct of incomplete combustion of complex hydrocarbons and acts to stimulate the transcription promoter regions and monooxygenase in the microsomal fraction of the cell.[6] Further, thiol-epoxide is a toxic metabolite of DMBA that primarily binds to DNA, causing oxidative stress and leading to malignancy.[6] In studies based on hamsters with oral tumors, a scenario that closely resembles human oral carcinoma, DMBA-induced HBPCs is recommended as a significant oral cancer experiment to evaluate the anticancer potential of medicinal natural plant products, which can be pursued for future studies. The authors reported that the accumulation of reactive metabolite intermediates leads to an over-production of reactive oxygen species (ROS).[7]

Antioxidant enzymes act against ROS to inhibit the initiation and promotion of carcinogenesis. Interestingly, the process of malignant transformation of cells constantly decreases antioxidant activity, and eventually, cellular sensitivity to a carcinogen.[8] Superoxide dismutase (SOD) is the process of detoxification of $O_2$ – to $H_2O_2$, which ultimately protects cells from oxidative damage. On the contrary, catalase (CAT) is a heme protein responsible for the removal of $H_2O_2$, while GPx, present in the cytosol and mitochondrial matrix, and glutathione result in the degeneration of $H_2O_2$ to water ($H_2O$). A study reported that vitamin E and glutathione (GSH) were eliminated to function against the generated ROS and avert carcinogenesis. GSH, a critical cellular protecting agent, safeguards the cell against free radicals and peroxides with toxic compounds.[9] Phase I reactions include the use of the cytochrome P450 system found with the membrane of the endoplasmic reticulum. It generally provides functional polar groups to molecules that either facilitate excretion or further metabolism. Phase II reactions consist of adding hydrophilic groups to the original molecule, a toxic intermediate or a non-toxic metabolite formed in phase I, that requires further transformation to increase its polarity. The phase II conjugation reactions typically follow phase I activation, resulting in the transformation of xenobiotics into the water-soluble compound that is emitted in urine. A phase II reaction, such as glutathione-S-transferase (GST), favors the elimination of carcinogens. GST also functions as a detoxifier of the dihydrodiol and epoxide forms of polycyclic aromatic hydrocarbons.[10] Further, lipid peroxide (LPO) results from direct cellular damage; ROS interact with polyunsaturated fatty acids (PUFAs) and is easily attacked by free radicals, resulting in the formation of LPO and injuring the membrane components of the cell.[11]

Apoptosis is programmed cell death, which is exaggerated with progressing dysplasia and cancer. Thus, characterizing the apoptotic properties of a cell can help recognize tumor aggressiveness. A previous study had characterized apoptosis through biochemical and morphological change progression by activating several protein families leading to DNA fragmentation.[12] The number of apoptotic bodies in the tumor and dysplastic cells has been reported previously.[13] Cysteine-aspartic proteases (caspases) play an essential role in DNA fragmentation and chromatin condensation during the apoptotic cascade; in their inactive form, caspases remove the damaged cells via apoptosis.[14] Disruption of mitochondrial membrane induces the release of an apoptogenic factor, cytochrome c, which activates a family of caspases.[13] Further, proliferating cell nuclear antigen (PCNA), expressed in the G1 and S phases of the cell cycle, acts as a cofactor for DNA-polymerase δ, which is a critical regulatory mechanism in DNA replication. Oncoproteins can transcriptionally control the PCNA promoters. Cyclin-D1, one such oncoprotein, plays a leading role in cell cycle regulation; it is located on 11q13 in the human genome sequence and is present in abundance throughout the cell cycle. Deregulation of cyclin-D1 is the primary causative event in the carcinogenesis of many cancers. Cyclin D1 is involved in the G1 to S phase cell cycle transition.[15]

Cyclooxygenase-2 (COX-2) is an inductive isoenzyme that is stimulated by mitogens, cytokines, growth factors, and powerful pro-carcinogenic agents.[16] The widespread distribution of COX-2 activity is often linked to the appearance of different types of cancers and is correlated with a poor prognosis and survival. COX-2 is induced by several inflammatory mediators, including mitogens and tumor promoters, which are over-expressed in human oral cancer.[17]

Beetroots contain pigments together known as betanin (BTN), which organize a group of immensely bioavailable natural pigments antioxidant.[18] BTN possesses various properties such as antioxidant, anti-cancer, anti-inflammatory, diabetic, hypolipidemic, and hepatoprotective activities.[19] However, the apoptotic and anti-inflammatory underlying mechanism by which BTN remains to be elucidated. The present study aims to unravel the underlying molecular mechanisms corresponding to the chemopreventive, immunomodulating, and anti-cancer effects of BTN in DMBA-induced oral cancer in experimental hamsters.

## MATERIALS AND METHODS

### Study materials

#### *Chemicals used*

BTN (purity >98%) and DMBA (purity ≥95%) were obtained from Sigma-Aldrich, MO, USA. Antibodies and caspase-3 and 9 colorimetric kits were bought from Biovision (CA, USA).

#### *Animals*

Male golden Syrian hamsters weighing 80–120 g body weight (BW) were obtained from the Department Animal stock used for the study and maintained under laboratory conditions. They were kept in animal houses and fed on a pellet diet and water; necessary conditions for survival, that is, a temperature of 22°C–25°C with ≥45%–55% humidity, were maintained. The research was approved by the animal ethical committee of the School of Life Science and Technology, China (Approval No.: SLST20210753562). All rat treatments in the lab were performed as per the ethical guidelines recommended by IAEC.

#### *Preparation of BTN and DMBA induction*

BTN dissolved in phosphate buffer solution (PBS) using 5% dimethyl sulfoxide (DMSO) was given orally to the hamsters. Next, 0.5% DMBA solution and liquid paraffin were applied thrice a week on their left buccal pouches.[20]

### Plan for evaluation

About 24 hamsters were randomly divided into four groups, each with six hamsters: group I: control: DMSO; group II: DMBA alone;

group III: DMBA + BTN (50 mg/kg BW); group IV: BTN (50 mg/kg BW) alone.

The experiment was terminated at the end of the 14th week, and the hamsters were sacrificed by cervical dislocation. Then, they were washed, and the supernatant collected after centrifugation was used to determine biochemical parameters. The buccal mucosa was collected for histopathology and molecular research.

## Biochemical estimations

The plasma levels of LPO byproducts, such as lipid hydroperoxides (LOOH),[21] conjugated dienes (CD),[22] and thiobarbituric acid reactive substances (TBARS),[23] were determined as per the protocol, as were the levels of SOD,[24] glutathione peroxidase (GPx),[25] and the presence of CAT,[26] vitamin E,[27] and GSH[28] in tissues.

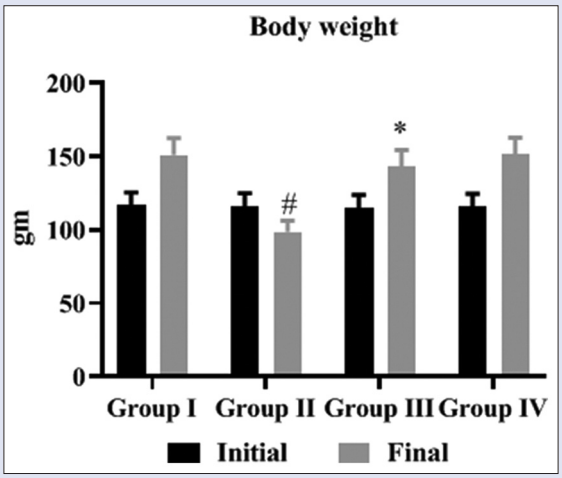


**Figure 1:** Effect of BTN on the bodyweight of experimental hamsters. Data are expressed as mean ± SD.*$P < 0.05$

### *Caspase activity*

Caspase activity was assessed using a caspase activity kit (Beyotime, Haimen, China) as per the manufacturer's instructions.

### *Histopathology*

Tumor tissues were exclusively perfused with cold physiological saline set in 10% formalin. A microtome was used to slice sections of 3–5 μm in width; the specimen was stained with HE, followed by eosin, and examined under a light microscope.

### *Immunohistochemical (IHC) assay*

Dewaxed tissue embedded in paraffin was once again hydrated using graded ethanol solution, before being rehydrated with distilled water. Endogenous peroxidase was blocked by incubating 3% $H_2O_2$ in $CH_3OH$ for 10 min. The antigen was rescued by microwaving for 10 min in a citrate buffer solution and then washed with Tris-buffered saline. Tissue parts were incubated at 4°C overnight with the appropriate primary antibody (PCNA and Cyclin-D1, TNF-α, COX-2) (Biovision, CA, USA). The primary antibody was detected by incubating it for 30 min at room temperature with a secondary antibody conjugated to horseradish peroxidase (HRP). The antigen–antibody complex was distinguished with the help of 3,3-diaminobenzidine, an HRP substrate, after rinsing with Tris-buffered saline. Slides were then cleaned, counterstained using hematoxylin, and then covered using mounting medium until appropriate color intensity was achieved. Each slide was examined under a light microscope.

## Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) by using SPSS version 17.0. $P < 0.05$ was considered for statistical significance.

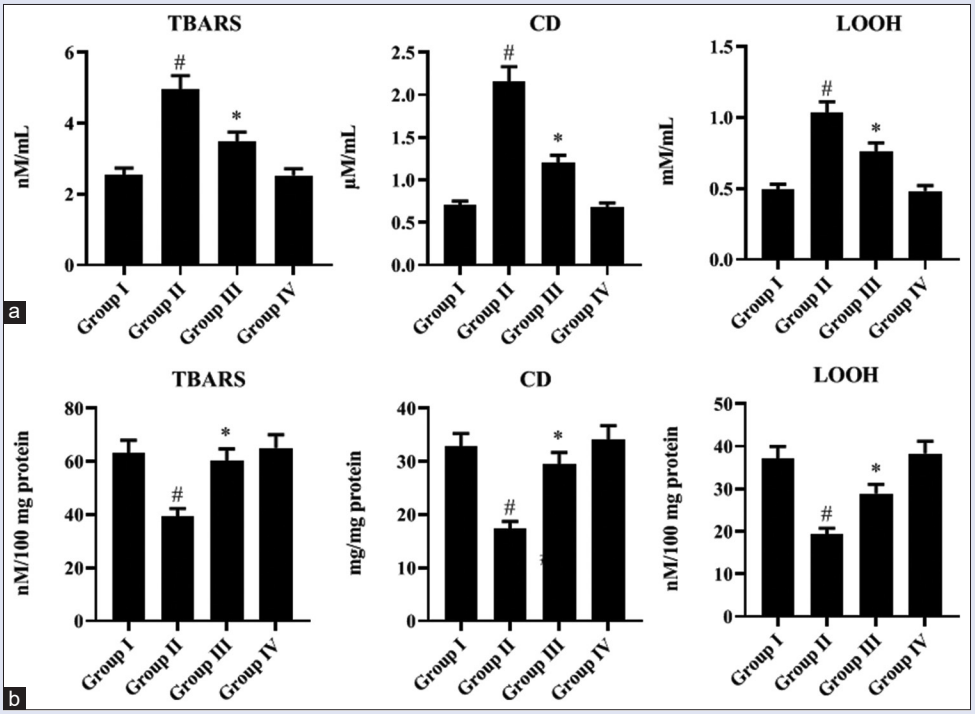


**Figure 2:** Effects of BTN on lipid peroxidation activity. (a) Plasma (b) Buccal mucosa. Data are expressed as mean ± SD. *$P < 0.05$

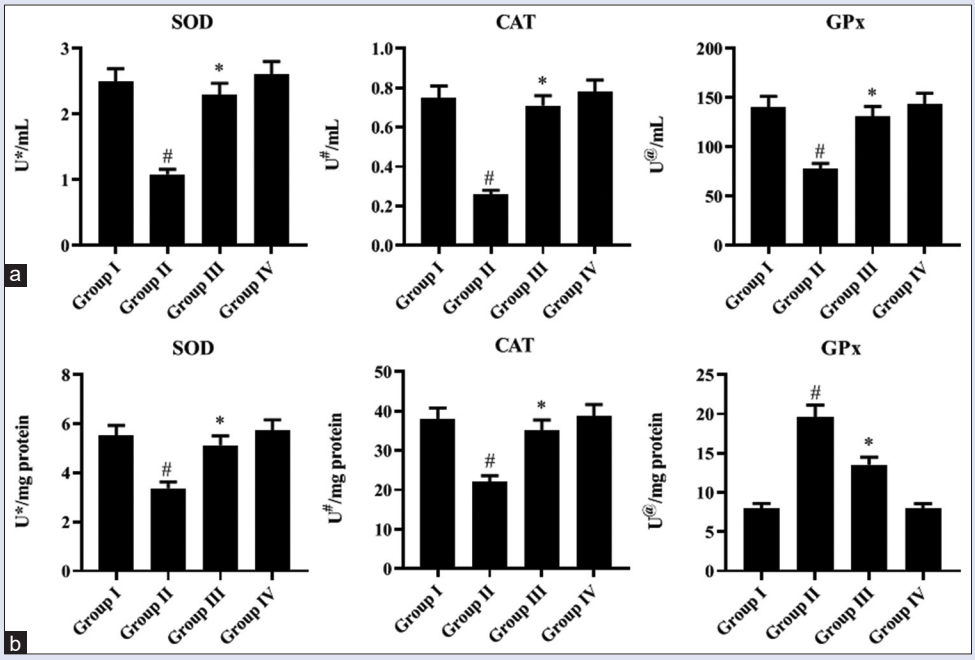


**Figure 3:** Effect of BTN on enzymatic antioxidants. (a) Plasma (b) Buccal mucosa. Data are expressed as mean ± SD. *$P < 0.05$

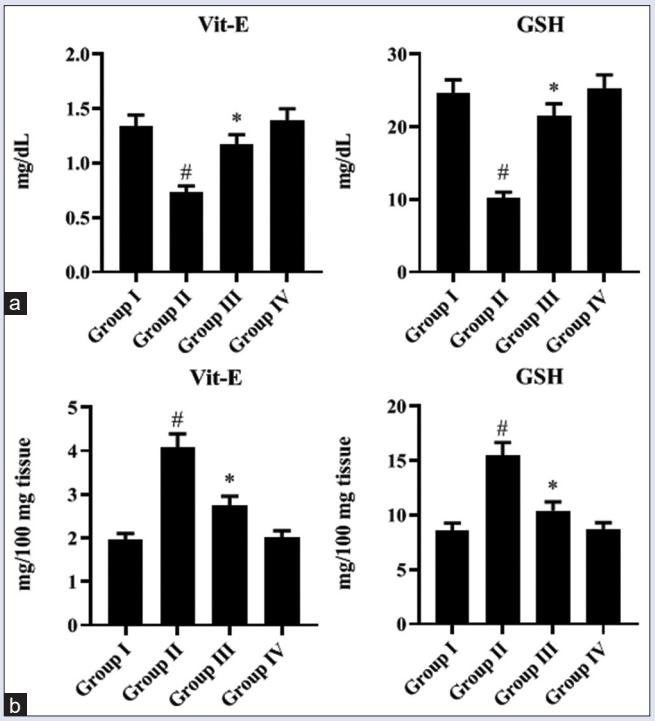


**Figure 4:** Effect of BTN on non-enzymatic antioxidants. (a) Plasma (b) Buccal mucosa. Data are expressed as mean ± SD. *$P < 0.05$

# RESULTS

## Effect of BTN on body weight

Figure 1 depicts the change in body weight of the hamsters from the baseline value. The initial findings for the experimental hamsters were identical across all subgroups. In contrast, at the end of the study, the BW of group II hamsters was significantly reduced as compared to group I ($P < 0.05$). However, simultaneous treatment with BTN (50 mg/kg BW) regulated the BW of group III hamsters significantly. Lastly, the BW of hamsters in group IV was not disturbed.

## Effect of BTN on tumor burden, incidence, and volume

Table 1 presents the results for the tumor occurrence, burden, and volume in the control and experimental group hamsters. We observed that the group II hamsters experienced 100% tumor development with a mean tumor volume of 183.58 ± 14.05 $mm^3$ and a tumor burden of 1468.64 ± 112.47 $mm^3$ (assessed using the magnitude of premalignant lesions and squamous cell carcinoma (SCC)). On the contrary, simultaneous treatment with BTN (50 mg/kg BW) prohibited the development of SCC significantly ($P < 0.05$). In the hamster treated with BTN (50 mg/kg BW) alone, no tumor growth was observed.

## Effect of BTN on LPO activity

The amount of LPO byproducts, such as TBARS, LOOH, and CD, present in the plasma and oral mucosa of the control and the experimental hamsters are shown in Figures 2a and b. LPO byproduct levels were slightly higher in the plasma of tumor-bearing hamsters but lower in the buccal mucosa. The LPO byproducts in group II DMBA-applied hamsters were substantially reduced after oral administration of BTN (50 mg/kg BW). The level of LPO byproducts in group IV revealed no noticeable variations ($P < 0.05$) from the control-group hamsters.

## Effect of BTN on enzymatic antioxidant levels

Figure 3a shows that antioxidant levels in plasma were reduced in the tumor-bearing group II hamsters, and the buccal mucosa [Figure 3b] of group II hamster was different (SOD, and CAT were reduced; GPx were improved). Oral supplementation of BTN (50 mg/kg BW) to DMBA-applied group III hamsters appeared to have reversed these antioxidant changes to a near-normal range, whereas the antioxidant status of group IV hamsters did not differ notably from that of the control hamsters.

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

| Groups/Treatment | Control | DMBA | DMBA + Betanin (50 mg/kg BW) | Betanin-alone (50 mg/kg BW) |
|---|---|---|---|---|
| Tumor incidence | 0 | 100% | 0 | 0 |
| Total number of tumor/hamsters | 0 | 8±0.71 | 0 | 0 |
| Total volume ($mm^3$)/hamsters | 0 | 183.58±14.05 | 0 | 0 |
| Tumor burden ($mm^3$)/hamsters | 0 | 1468.64±112.47 | 0 | - |

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

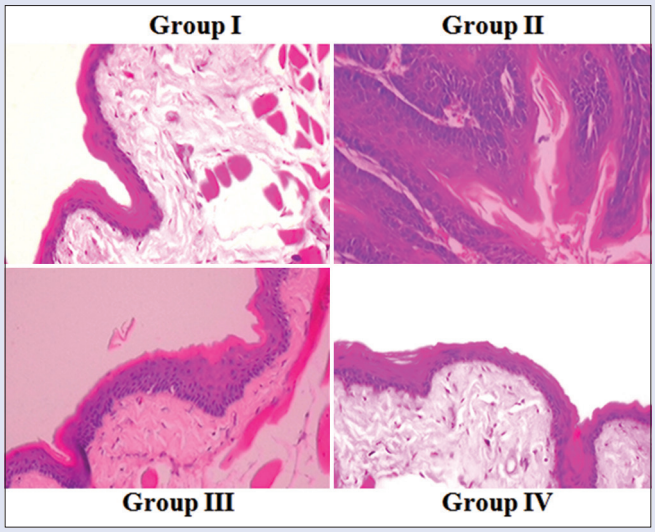


**Figure 5:** Effect of BTN on histopathology of buccal mucosa

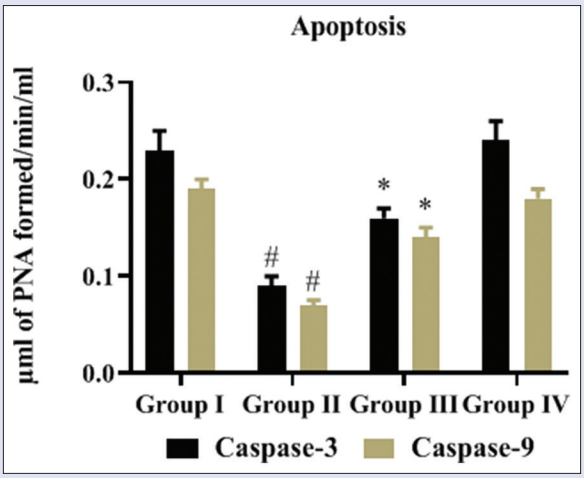


**Figure 6:** Effect of BTN on caspase-3 and -9. Data are expressed as mean ± SD. *$P < 0.05$

## Effect of BTN on non-enzymatic antioxidants

Figure 4a shows that the plasma non-enzymatic antioxidant levels were reduced in the tumor-bearing group II hamsters; buccal mucosa [Figure 4b] of the group I hamsters was different (GSH and vitamin E were improved) after the study. Oral supplementation of BTN (50 mg/kg BW) to the group III hamsters seemed to have reversed these antioxidant changes to a near-normal range. Lastly, the antioxidant status of BTN-alone (50 mg/kg BW) hamsters was equal to that of the control group.

## Effect of BTN on the histopathology of buccal mucosa

Figure 5 (groups I–IV) shows well-differentiated SCC with epithelial and keratin pearls in the buccal mucosa of group II hamsters. In the DMBA + BTN-treated hamsters, mild to moderate hyperplasia and keratosis were observed (group III). The cellular architecture of the BTN alone (group IV) and normal control (group I) hamsters was normal, with no indications of cell proliferation.

## Effect of BTN on caspases activity

Figure 6 depicts the status of caspase-3 and 9 in the experimental animals induced with DMBA. The levels of caspase-3 and 9 drastically reduced in the group II animals treated with DMBA alone, whereas the caspase levels were substantially increased in the DMBA + BTN-treated groups. No statistically significant difference was observed between the BTN-alone and control groups hamsters.

## Effect of BTN on the expression of inflammatory markers

Figure 7 shows the IHC expression patterns of inflammatory markers (Cyclin-D1, TNF-α, PCNA, and COX-2) in the experimental groups. In animals treated with DMBA alone, an increased expression of inflammatory markers was observed compared to the control group. Further, when the DMBA-painted hamsters were given BTN, their expression was reduced as compared to the control group. In comparison to control hamsters, the group IV hamsters had a relatively regular expression of the abovementioned markers.

# DISCUSSION

Chemoprevention is a crucial strategy for slowing cancer progression; therefore, there is a need to explore natural products that can act as chemopreventive agents. In most cases, including modern medicine, active drugs against disease-causing agents are derived from natural products.[29] The most prevalent strategies worldwide for cancer treatment include chemotherapy, radiotherapy, and surgery; however, they are related to severe side effects. On the contrary, compounds obtained naturally, especially those derived from plants substances, are known to have anticancer behavior, with the majority of them able to target the molecular foci of cancer.[30]

LPO is a cellular event that occurs when ROS interact with the PUFA portion of the cell membrane, which causes cellular necrosis and inflammation. LPO and cell proliferation have an opposite association in most cases[31]; LPO levels in tumor cells are substantially lower than in their normal counterparts. Furthermore, tumor cells proliferate faster when LPO levels are low, and LPO is less severe in malignant tissues because they are less vulnerable and immune to free radical attack. Additionally, several reports have described a link between reduced PUFA content and improvements in antioxidant status and detoxification agents, as well as lower LPO levels in the tumor tissue of DMBA-treated experimental animals. Also, a rise in GPx and GSH levels in tumor cells is linked to a substantial decrease in LPO and increased cell proliferation. In our study, we observed that the LPO byproduct levels were regulated to the levels of control groups by the effects of BTN.

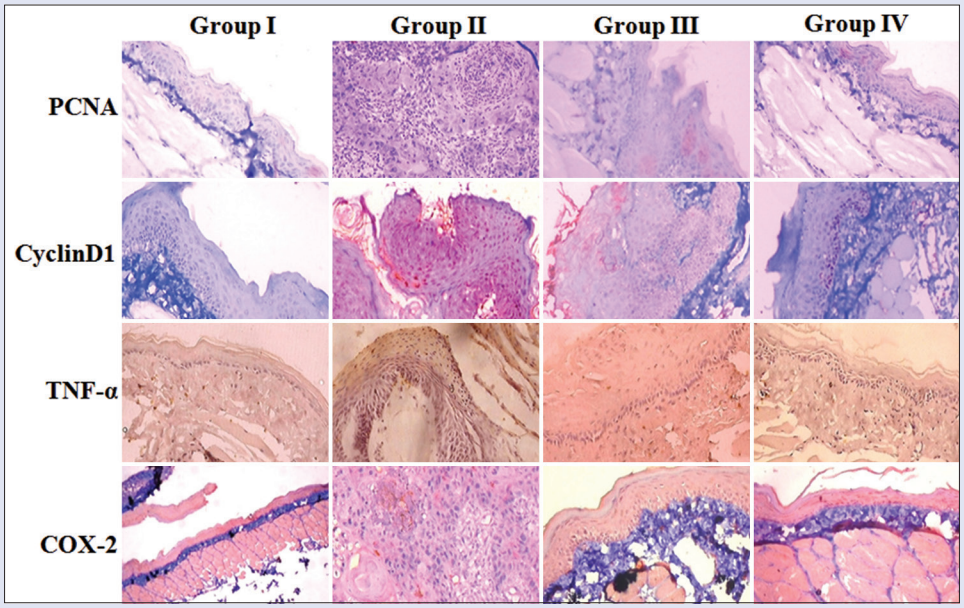


**Figure 7:** Effect of BTN on the expression of inflammatory markers

Chronic DMBA exposure lowers antioxidant activity in different tissues, both enzymatic and non-enzymatic, and reduced SOD and CAT activity in malignant cells facilitates invasion and metastasis.[32] We also obtained similar results in tumor-bearing hamsters. Respiratory epithelial cells contain both SOD and CAT, which protect against potentially toxic free radicals. Similarly, the tripeptide GSH is essential for protecting sulfhydryl groups from oxidation. Accordingly, deficiencies in the intracellular GSH and GPx activity have been related to the rate of cancer cell proliferation.[33] The overuse of antioxidants to remove free radicals by malignant cells may explain the lower levels of GSH and the GSH-based enzyme, GPx, in the plasma of malignancy-bearing animals.[34] Therefore, the adequate concentration of antioxidants (enzymatic and non-enzymatic) is essential for the proper regulation of free radicals, which play a crucial role in carcinogenesis. We observed that BTN substantially regulated the antioxidant levels, which were comparable to the regular levels in the control group.

The caspase cleavage cascade is a well-known process that starts with initiator caspase activation via intrinsic and extrinsic pathways. Caspase-9 is the key initiator caspase for the intrinsic pathway to induce cell death. Internal factors such as severe oxidative stress, genetic alteration, and hypoxia activate the intrinsic pathway.[35] It is known that caspases tend to regulate the apoptotic activity of cells. In our study, BTN increased the expression levels of caspases 3 and 9 in the DMBA + BTN-treated groups compared to the DMBA-alone group, exhibiting its apoptosis-inducing nature in DMBA-induced carcinogenic cells.

Cell proliferation is a biological process involved in oncogenesis that is critical to preserving and developing tissue homeostasis in all living organisms.[36] A previous report revealed that PCNA and retinoblastoma inhibition phosphorylation phosphoprotein bind to cyclin-D1 during the late G1/S step and inhibit the retinoma kinase. Because of this, the AP-1, c-fos, and c-dependent kinases (members of the cdc2 and cdkds families) are needed for the differentiation of cell cycle checkpoints during tumor initiation.[37] Over-expression of PCNA increases the percentage of S-phase cells, which in turn shortens the duration in which the overall cell proliferation induces the G1 phase arrest and decreases the G2/M-phase output.[38] In our study, PCNA over-expression was present in the hamsters with tumors, which concurs with the results of previous studies that describe its significance in tumor development. These results indicate that PCNA gene expression study is a reliable indicator of tumor-onset assessment. Other studies have also utilized the over-expression of PCNA as a predictor for oral cancer.[39]

Furthermore, the IHC analysis revealed that BTN reduced the expression levels of proliferative cell markers (PCNA and cyclin-D1) and inflammatory markers (TNF-α and COX-2) in animals with DMBA-induced oral carcinogenesis, corroborating its protective effects. Several phytochemicals with chemopreventive potential have been discovered in recent years that can work by inhibiting mutagenesis, inducing apoptosis, and modulating the activity of detoxification agents. Several studies have highlighted the various biological activities of BTN owing to its complex biochemical, genetic, and pharmacological functions.[40-43]

## CONCLUSION

In conclusion, BTN exerts its effects by regulating the level of antioxidants and LPO products. Additionally, it upregulates the caspase-3 and 9 expression and inhibits the expression of inflammatory markers (PCNA, cyclin-D1, TNF-α, and COX-2). Because BTN attenuates the DMBA-induced oral cancer in the hamsters, it can be used as a therapeutic candidate for oral cancer treatment.

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### Conflicts of interest

There are no conflicts of interest.

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