

# Usnic acid attenuates 7,12-dimethylbenz[a] anthracene (DMBA) induced oral carcinogenesis through inhibiting oxidative stress, inflammation, and cell proliferation in male golden Syrian hamster model

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## Abstract

In this study, we investigated the chemopreventive efficacy of usnic acid (UA), an effective secondary metabolite component of lichens, against 7,12-dimethylbenz[a] anthracene (DMBA)-induced oral squamous cell carcinoma (OSCC) in the hamster model. Initially, the buccal pouch carcinogenesis was induced by administering 0.5% DMBA to the HBP (hamster buccal pouch) region about three times a week until the 10th week. Then, UA was orally treated with different concentrations (25, 50, 100 mg/kg b.wt) on alternative days of DMBA exposure, and the experimental process ended in the 16th week. After animal experimentation, we observed 100% tumor incidence with well-differentiated OSCC, dysplasia, and hyperplasia lesions in the DMBA-induced HBP region. Furthermore, the UA treatment of DMBA-induced hamster effectively inhibited tumor growth. In addition, UA upregulated antioxidant levels, interfered with the elevated lipid peroxidation by-product of thiobarbituric acid reactive substances, and changed the activities of the liver detoxification enzyme (Phase I and II) in DMBA-induced hamsters. Furthermore, immunohistochemical staining of inflammatory markers (iNOS and COX-2) and proliferative cell markers (cyclin-D1 and PCNA) were upregulated in the buccal pouch part of hamster animals induced with DMBA. Notably, the oral administration of UA significantly suppressed these markers during DMBA-induced hamsters. Collectively, our findings revealed that UA exhibits antioxidant, anti-inflammatory, antitumor, and apoptosis-inducing characteristics, demonstrating UA's protective properties against DMBA-induced HBP carcinogenesis.

## KEYWORDS

antioxidants, antitumor, DMBA, OSCC, usnic acid

## 1 | INTRODUCTION

Cancer, a foremost life threat to public health, is categorized by quick, uncontrollable cell division, invasion, and metastasis.<sup>[1]</sup> The GLOBOCAN 2020 worldwide cancers estimated over 19.3 million new

cancer cases and 10 million mortalities yearly from cancer. A population-based registry data from the National Cancer Registry Program, specify that the oral cavities are the leading sites of cancer, followed by the lungs and stomach amongst men and cervix, breast, and oral cavities, especially women.

In oral carcinoma, 377,713 new cases were reported and 207,252 death cases were registered yearly. In the Asian subcontinent, oral cancer accounts for over 121,096 cancer deaths.<sup>[2,3]</sup> Oral squamous cell carcinoma (OSCC) is more prevalent in India, with approximately 70% of cases reported in advanced stages (Stage III–IV).<sup>[4]</sup> Conventional oral cancer treatment methods such as surgical resection, radiation therapy, and chemotherapy have generally been successful in recent years. However, despite implementing intensive therapeutic strategies and supportive care, the average survival rate of oral cancer individuals ranges from 9 to 12 months.<sup>[5,6]</sup> A growing body of literature suggests that OSCC has highly resistant effects to all these treatments because of excessive DNA repair mechanisms, which enhance genomic instability to tolerate DNA damage and promote chemotherapy resistance.<sup>[7]</sup> Therefore, it is crucial to investigate alternative therapeutic options for treating OSCC.

Chemoprevention is a tumor control strategy that entails administering naturally occurring substances with low toxicity to prevent, slow down or reverse the illness altogether.<sup>[8]</sup> Phytochemicals and their derivatives are promising options for improving treatment efficiency in oral cancer patients and reducing adverse reactions. These phytochemicals are abundantly present in plants, fungi, algae, and lichens. It has a wide range of biotic and pharmacologic effects, such as antioxidant, immunomodulatory, anti-inflammation, antiangiogenic, and proapoptotic.<sup>[9,10]</sup> Usnic acid (UA) is the most prevalent secondary metabolite in lichens. It is the best secondary metabolite among medicinal lichens and has promising outcomes in cancer disease management. It is effective for several biological processes, including antibacterial, antifungal, antiviral, antioxidant, cytotoxic, anti-inflammatory, and anticancer properties.<sup>[11–17]</sup>

Moreover, studies have indicated that the anticancer effect of UA was through the signal transducer and activator of transcription 3 (STAT3) and RAS signal pathways cooperatively, thereby inhibiting tumor proliferation, angiogenesis, migration, and invasion.<sup>[18]</sup> Other studies have shown that UA inhibited angiogenesis and Bcap-37 tumor growth via vascular endothelial growth factor receptor (VEGFR2)-mediated AKT and extracellular signal-regulated protein kinases 1 (ERK1/2) signaling pathways in endothelial cells.<sup>[19]</sup> Furthermore, the antiproliferative properties of UA in many cancer cell line studies have recently been demonstrated in numerous research.<sup>[20–25]</sup> Therefore, UA is a potential candidate for cancer management; interestingly, it does not affect normal cells.<sup>[26]</sup> Polycyclic aromatic hydrocarbon of 7,12-dimethylbenz(a)-anthracene (DMBA) is extensively practiced in the experimental induction of site-specific cancer progression. Oxidative damage occurs in the metabolic activation of cancer-causing hydrocarbons, which is associated with the genotoxicity that is frequently developed by the fabrication of ROS (reactive oxygen species) and unpaired radicals.<sup>[27]</sup> Thus, it is considered an ideal animal model for studying the chemoprevention of experimental oral carcinogenesis in the male golden Syrian hamster. Furthermore, substantial evidence suggests that DMBA-induced oral cancers and human oral malignancies probably have similar

histological, morphological, biochemical, and molecular features.<sup>[28]</sup> Therefore, it is proposed to use this experimental oral cancer model to explore the potential of natural products and their bioactive components to prevent tumors.<sup>[29]</sup> However, to our knowledge, the chemopreventive properties of UA on DMBA-induced hamster buccal pouch (HBP) carcinogenesis have never been investigated. In this present study, we explored the anticarcinogenic ability of UA in the DMBA-painted HBP carcinogenesis model by assessing the level of antioxidants, lipid peroxidation (LPO), detoxification marker of Phase I and II agent, and histological findings. We also employed immunohistochemistry (IHC) to examine the expression pattern in the inflammatory markers (iNOS and COX-2) and proliferative cell markers (cyclin-D1 and PCNA) in HBP tissue. Our study indicates that UA could be a potential candidate for treating human OSCC patients.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

DMBA and UA were procured from Sigma-Aldrich Chemicals. Heparin, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), trichloroacetic acid, nitroblue tetrazolium, 1-chloro 2,4-dinitrobenzene (CDNB), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), thiobarbituric acid (TBA), dimethyl sulfoxide, and phenazine methosulphate were obtained from HiMedia Lab. Anti-PCNA, anti-cyclin-D1, anti-COX-2, and anti-iNOS antibodies were obtained from Santa Cruz Biotechnology Inc. The all-other supplementary reagents and solvents were obtained from NICE and SRL Chemicals.

### 2.2 | DMBA preparation

Oral carcinogenesis was administered in the hamster animal buccal pouch using a no. 4 paint brush to apply for 0.5% DMBA added in liquid paraffin three times per week for 10 weeks.<sup>[30]</sup>

### 2.3 | Animal maintenance

The Institutional Animal Ethics Committee authorized the experimental animal methodology at Annamalai University, Annamalai Nagar, India (Reg.no: AU-IAEC/PR/1254/7/19), and hamster is one of which follows the guidelines and advice of the Annamalai University ethical committee for animal care and the Indian National Law on animal care and utilization. We employed 36 male golden Syrian hamsters, 56–70 days of age, that weighed 80–120 g. In Rajah Muthiah Medical College and Hospital, Annamalai University, animals were housed and maintained in Central Animal House. The animals were categorized into six groups in polypropylene cages, fed with a regular animal pellet diet, and had permission to access unlimited

amounts of disinfected water. The animals' temperature was kept at 27°C, followed by a 12 h light and dark cycle to control humidity (50%).

## 2.4 | Experimental study

Thirty-six male golden color Syrian hamster rats (*Mesocricetus auratus*) were separated into six groups of six animals in each group. Group I control animals received only liquid paraffin (vehicle) weekly for 16 weeks. DMBA in liquid paraffin at a concentration of 0.5% was painted to the left buccal pouch of (Group II–V) animals three times per week, which was continued for 10 weeks. Animals in (Group III–IV) were administered with UA orally at varying doses (25, 50, and 100 mg/kg b.wt), initiating 1 week of previous exposure to the carcinogen and continuing on alternate days to DMBA induction till the completion of the experimental study. A higher dose of UA (100 mg/kg b.wt/day) was orally administered to Group VI animals throughout the study period. All experimental hamsters were killed by cervical dislocation at the end of the 16-week experimental study. The control and experimental hamsters' plasma, hepatic tissue, and buccal pouch were subjected to biochemical assays, histological and immunohisto analysis for each group.

## 2.5 | Chemoprevention study

The tumors were measured macroscopically in all hamster's buccal pouches. The approach suggested by Manoharan et al.<sup>[31]</sup> was used to assess tumor weight. Using a vernier caliper, the width of every tumor was evaluated. The tumor volume was determined using the formula:  $V = (4/3) (D1/2) (D2/2) (D3/2)$ , in which D1 is tumor length, D2 is tumor width, and D3 is tumor height, in millimeters. Then, the formula measured tumor load or burden, and tumor volume was divided by the total number of tumors in each hamster.

## 2.6 | Sample preparation

The animal blood sample, HBP and liver tissue (250 mg) were sliced into small pieces and homogenized in the 10% appropriate buffer (pH-7.0) in cold conditions to give homogenate. The homogenates were centrifuged at 1000 rpm for 15 min at 0°C in the cold centrifuge. The supernatant was then extracted and used for various biochemical estimations.

## 2.7 | Biochemical analysis

The plasma, buccal mucosa, and hepatic tissue samples from each control group and experimental hamster were exposed to biochemical tests. In addition, the LPO by product, thiobarbituric acid reactive substances (TBARS), antioxidants (SOD, catalase [CAT],

glutathione peroxidase [GPx], glutathione [GSH], Vit-E, and Vit-C), Phase I enzyme (cytochrome P450 and b5), and Phase II enzyme (GR, Glutathione-S-transferase [GST], GSH, and DT-diaphorase) were assessed.

The status of LPO was evaluated based on the amount of TBARS. TBARS in plasma and buccal mucosa according to the estimation methods presented by Ohkawa et al.<sup>[32]</sup> SOD activity was measured using the Kakkar et al. method, which relies on a 50% suppression of phenazine methosulfate-nitroblue tetrazolium and NADH production.<sup>[33]</sup> The activity of catalase, generally known as CAT, was evaluated using Sinha's technique, which is based on the reduction of hydrogen peroxide into water molecules.<sup>[34]</sup> The method was developed by Desai et al., to measure Vit-E levels associated with the drop of 2,4-dinitrophenylhydrazine (DNPH).<sup>[35]</sup> In addition, plasma Vit-C status was measured using Omaye et al. techniques.<sup>[36]</sup> When treated with 2,4-dinitrophenylhydrazine, the resultant is dehydro-ascorbic acid, which was fabricated by copper oxidation of crucial Vit-C that generates a colorful end product as according to the Abdel-Wahab and Wessam method, which is related to the down-regulation of 5, 5' dithiobis 2-nitrobenzoic acid (DTNB) and the level of reduced GSH that was evaluated.<sup>[37]</sup> Rotruck and his team's standard method was used to measure the exercise of GPx.<sup>[38]</sup>

Omura and Sato's<sup>[39]</sup> method estimated the state of liver markers Cyt, P450, and b5 in hepatic and left buccal tissue. The formation of color following the reaction between decreased cytochrome p450 and carbon monoxide, which was used to assess cytochrome P450. The differential range between diminished and reacted cytochrome was utilized as an indicator to estimate cytochrome b5 levels, which were then analyzed.<sup>[40]</sup> Glutathione reductase (GR) activity was determined using the Raffa et al. method, and it is connected to the downregulation of GSH disulfide to its reduced form.<sup>[41]</sup> GST was evaluated using the Catterall et al. technique, which is related to the conjugation of GSH thiol cluster with 1-chloro, 2,4 dinitrobenzene (CDNB).<sup>[42]</sup> The action of DT-diaphorase (DTD) in the hepatic tissue was determined by the standard Ernster's method, which utilized NADPH as the donor of electrons and 2,6-dichlorophenol indophenol as an electron mediator.<sup>[43]</sup>

## 2.8 | Histological examination

A section of the HBP region was removed and secured in a buffered solution containing 10% formalin. The left buccal tissues were embedded in paraffin after fixing. Hematoxylin and eosin (H&E) were used to stain 4–5  $\mu\text{m}$  tissue slices, while a light microscope was used to investigate the section to assess any histological alterations.<sup>[44]</sup>

## 2.9 | IHC

HBP tissue sections (4–5  $\mu\text{m}$  thick) embedded in paraffin were removed and rehydrated by combining them in an ethanolic solution. The HBP samples were immersed in the general protein-blocking

solution at 37°C for 15 min to block the binding point. The buccal tissue was then treated with primary antibodies for PCNA, cyclin-D1, iNOS, and COX-2, which were used to color proteins in IHC. The bound antibodies were then examined using a 2° antibody coupled with 3,3'-diaminobenzidine substrate and horseradish peroxidase. After an intense color, the slides were cleaned, discolored with hematoxylin, and enclosed with mounting media. Finally, every sample on the glass slide was examined under a microscope, and the method standardized by Gomez et al. was applied to calculate the proportion of expressing cells.<sup>[45]</sup>

## 2.10 | Statistics

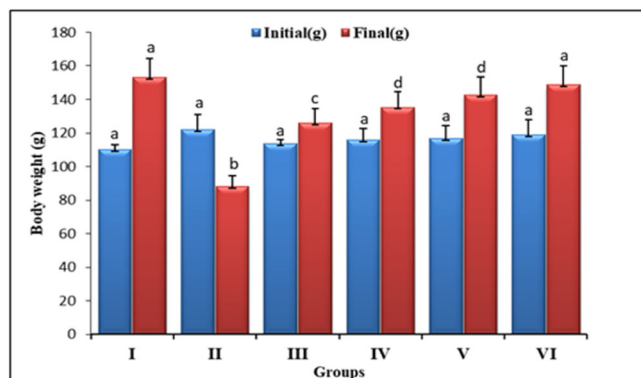
All the experimental outcomes are illustrated as mean ± standard deviation. A one-way variance analysis was employed for statistical comparisons, followed by Duncan's multiple range test. The differences were stated statistically, with significance at ( $p \leq 0.05$ ).

## 3 | RESULTS

### 3.1 | Measurement of body weight and tumor incidence

Figure 1 shows the changes in the body weight of different experimental hamsters. The untreated and experimental animals showed no significant differences. However, when DMBA-induced hamsters were compared to normal hamsters, their body weight was found to drop drastically. Furthermore, the oral administration of UA (50 and 100 mg/kg b.wt) efficiently increases the body weight of DMBA-exposed hamsters. The oral administration of UA alone has not affected the animal body mass of ordinary control hamsters.

Furthermore, the tumor incidence, burden, and volume found in the experimental hamsters are detailed in Table 1. In the current



**FIGURE 1** Outcome of UA on the animal body mass of the experimental and control hamster. The data are presented as means ± SD of six experimental animals for each group. Data that do not share a common superscript (a, b, c, d) differ significantly at  $p \leq 0.05$  (DMRT). DMRT, Duncan's multiple range test; UA, usnic acid.

investigation, we found that DMBA-induced HBP (Group II) had an incidence of tumors, a burden of tumors, and a volume of tumors of 100% greater than those found in untreated groups (Group I). On the other hand, oral administrations of UA on DMBA-induced hamsters (Groups III–V) demonstrated dramatically decreased tumor volume, incidence, and burden compared to experimental Group II animals treated with DMBA alone.

### 3.2 | Status of lipid by-product of TBARS and antioxidants levels in plasma

Figure 2A shows the plasma lipid by-product of TBARS and antioxidant levels in normal and experimental animals. The nonenzymatic and enzymatic antioxidant status in DMBA-treated experimental hamsters was significantly increased after oral administration of dissimilar doses of UA (25, 50, and 100 mg/kg b.wt). TBARS levels were significantly enhanced in tumor-bearing Group II hamsters; enzymatic and nonenzymatic antioxidant levels decreased simultaneously. In tumor-bearing hamsters, oral administration of three different dosages of UA dramatically improved the antioxidant levels (Groups III–V). The increased antioxidant properties were observed in hamsters that received the UA dosage of 50 and 100 mg/kg b.wt in Group IV and V animals. When comparing TBARS levels of both enzymatic, and nonenzymatic antioxidants in UA-administered Group VI hamsters with Group I control hamsters, they did not detect any significant differences.

### 3.3 | Status of lipid by-product of TBARS and antioxidants levels in buccal tissue

Figure 2B exhibits that the levels of TBARS and antioxidant status have altered in the buccal pouch of experimental Group II tumor-bearing hamsters compared to Group I hamsters; there was a reduction in the TBARS levels and changes in enzymatic and nonenzymatic antioxidant status (Vit-E, GSH, and GPx were raised; SOD and CAT were reduced). However, compared to the DMBA group, UA-treated animals (Groups III–V) significantly enhanced the activities of the previously mentioned enzyme parameters and decreased the TBARS level. The outcome was progressively noticeable when UA was administered at a dose of 50 and 100 mg/kg b.wt. The administration of UA alone to hamsters in Group VI revealed no substantial alteration in TBARS, nonenzymatic and enzymatic antioxidant levels compared to hamsters in Group I.

### 3.4 | Outcome of detoxification enzymes levels

Figures 3A,B illustrate the status of both detoxification agents, such as Phase I & II, in experimental and normal hamsters in the hepatic and buccal tissue. Increases were observed in the Phase I detoxification substance, such as cytochrome P450 and b5 in the liver and

**TABLE 1** Tumor incidence, number, volume, and burden in experimental and control groups ( $n = 6$ ).

Groups	Treatment	Tumor incidence (%)	Total number of tumors/animals	Tumor volume	Tumor burden (mm <sup>3</sup> )
I	Control	0	0	-	-
II	DMBA alone	100	19/6	458.61 ± 25.45 <sup>a</sup>	1451.95 ± 81.19 <sup>a</sup>
III	DMBA + UA (25 mg/kg b.wt)	17	5/6	85.10 ± 7.15 <sup>b</sup>	70.91 ± 5.43 <sup>b</sup>
IV	DMBA + UA (50 mg/kg b.wt)	0	0%	0	0
V	DMBA + UA (100 mg/kg b.wt)	0	0%	0	0
VI	UA (100 mg/kg b.wt)	0	0%	-	-

Note: The data are presented as means ± SD of six experimental animals for each group. Data that do not share a common superscript (a and b) differ significantly at  $p < 0.05$  (DMRT).

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; DMRT, Duncan's multiple range test; UA, usnic acid.

buccal tissues of tumor-bearing Group II hamsters compared to Group I control hamsters. On the other hand, there was a significant reduction in the Phase II detoxification enzyme levels (GR, GSH, GST, and DTD). UA orally administered in various doses of 25, 50, and 100 mg/kg b.wt, which suggestively reduced the production of Phase I cleansing metabolites and increased the activity of Phase II detoxification substances (GSH, GST, GR, and DTD) in (Groups III–V). Thus, UA at a concentration of 50 and 100 mg/kg b.wt was revealed to be the most effective as it improves the detoxification process in Group IV–V experimental hamsters. The UA alone-supplemented group (Group VI) and the control hamsters (Group I) showed no detectable altered observation.

### 3.5 | Histopathology assessment of buccal tissue

Histopathological analysis of buccal pouches from UA alone-treated and untreated control hamsters' tissue sections were stained with H&E depicted in Table 2 and Figure 4. The HBP of the UA and the untreated hamsters all had regular, integral, constant epithelial layers. No distinct histological alterations were seen in Groups I–VI animals. However, squamous cell carcinomas with well-differentiated OSCC and severe hyperkeratosis, hyperplasia, and whitish granular appearance were found in hamsters with tumors (Group II). Alternatively, tumor-bearing hamsters treated with UA effectively reduced the incidence of oral carcinogenesis and displayed mild preneoplastic to moderate hyperplasia and dysplasia alterations (Groups III–V).

### 3.6 | The IHC expression level of PCNA, cyclin D1, iNOS, and COX-2

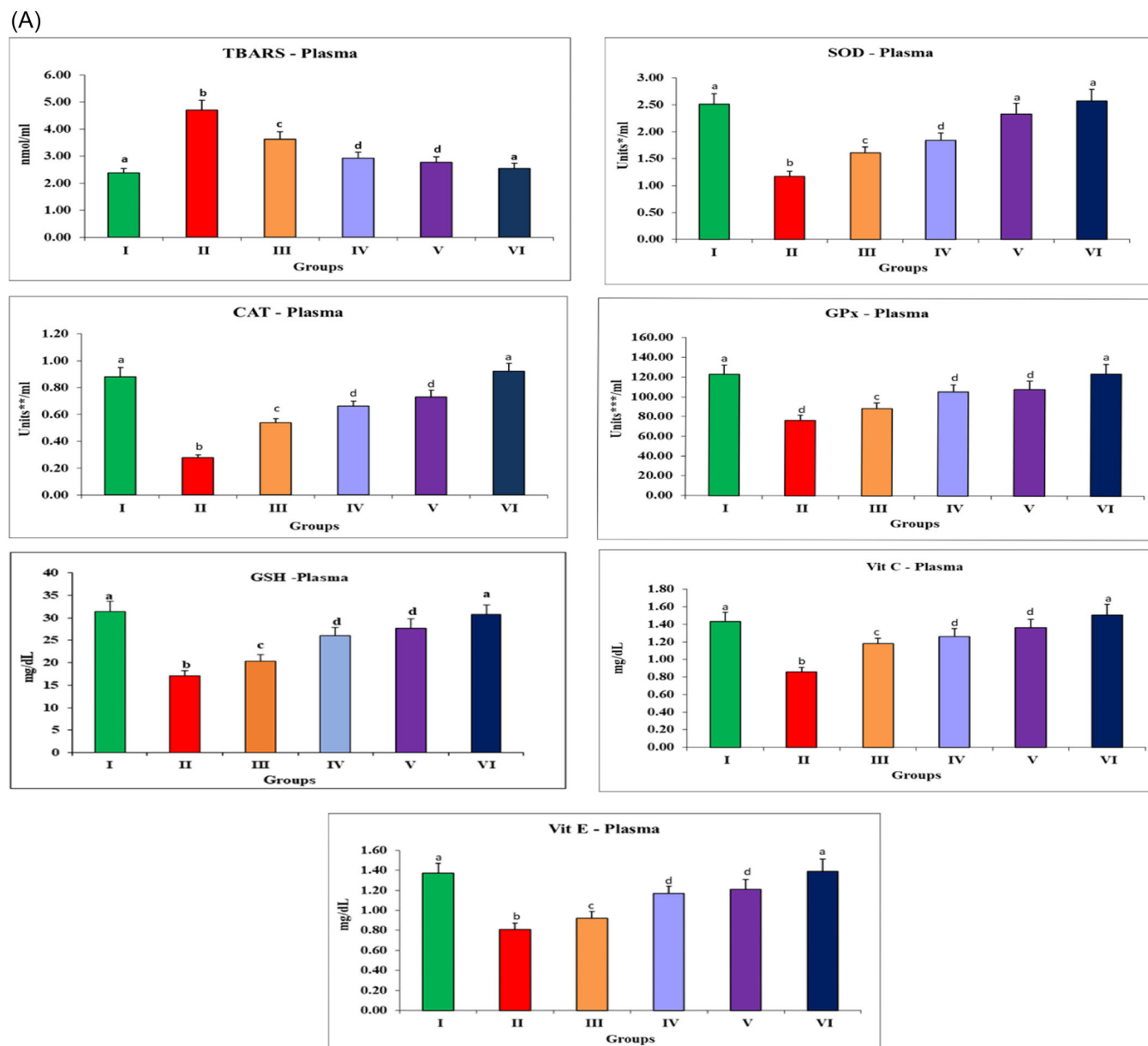
The IHC nuclear staining profile of cell proliferative (PCNA, cyclin D1), inflammatory biomarkers of (iNOS and COX-2), and proteins in the HBPs are present in Figure 5 compared to Group I untreated control hamsters. The expression of PCNA, cyclin D1, iNOS, and COX-2 was significantly increased in Group II of the tumor-bearing

hamsters. In Group III animals compared to Group II animals, there was only a slight upregulation of PCNA, cyclin D1, iNOS, and COX-2. In Group IV and V, hamsters showed no staining expression over cyclin D1, iNOS, PCNA, and COX-2. The same results were observed in Group I and VI hamsters, and we did not observe any significant PCNA, cyclin D1, iNOS, and COX-2 expression.

## 4 | DISCUSSION

OSCC is a prevalent malignant tumor with a poor perspective and several modifications and is a significant oncological issue worldwide. The prevalence of OSCC, recognized as a ubiquitous neoplastic condition with a high rate of disease condition and death rate, is a significant health threat in Asian countries.<sup>[46]</sup> Although the chemicals extracted from lichens typically do not function as medications, they serve as a starting point for synthesizing multiple potential targets. Anticancer properties can be found naturally in traditional Chinese medicinal herbs. Continuous research for novel chemopreventive drugs is a promising new approach to enhance cancer treatment.<sup>[47]</sup> UA-enriched extracts frequently treat several disorders in alternative medicine, especially those possessing several reported biological properties (Luzina et al.).<sup>[48]</sup> There is no effective evidence in the previous literature stating that UA has a chemopreventive impact on DMBA-induced oral carcinogenesis.

The present study reveals changes in histopathology, tumor volume, incidence, and burden in the experimental and control group. We observed a 100% neoplastic tumor formulation in DMBA-painted animals. On the other hand, tumor-bearing animals with UA treatment prevented tumor growth and showed mild to moderate preneoplastic symptoms, including dysplasia, keratosis, and hyperplasia. Furthermore, we determined the treatment of UA at 50 & 100 mg/kg b.wt, with optimum dose, resulting in a more dramatic suppression of uncontrolled cell growth than UA at 25 mg/kg b.wt (Group III). This shows the potential of UA to reverse the prevalent histological changes that appeared by DMBA painting, as well as investigate the anti-carcinogenic potential of these secondary metabolite compounds from lichens.



**FIGURE 2** Effect of UA on TBARS and antioxidant level in DMBA-induced HBP carcinogenesis model. (A and B) show the level of TBARS and antioxidants in each group's buccal tissue and plasma of the hamsters. The data are presented as means  $\pm$  SD of six experimental animals for each group. Data that do not share a common superscript (a, b, c, d) differ significantly at  $p \leq 0.05$  (DMRT). U\*, the amount of enzyme required to inhibit 50% NBT reduction; U\*\*, micromoles of hydrogen peroxide utilized/s; and U\*\*\*, micromoles of glutathione utilized/min. CAT, catalase; DMBA, 7,12-dimethylbenz[a]anthracene; DMRT, Duncan's multiple range test; GPx, glutathione peroxidase; GSH, glutathione; HBP, hamster buccal pouch; NBT, nitroblue tetrazolium; TBARS, thiobarbituric acid reactive substances; UA, usnic acid.

Excessive ROS production can significantly damage essential biomolecules, including lipids, proteins, and DNA, which can lead to the development of carcinogenesis. OSCC is one of many tumors whose progression is related to ROS that target oxidative stress.<sup>[49]</sup> In general, excessive ROS formation leads to increased LPO, which causes oxidative damage to cellular macromolecules.<sup>[50]</sup>

LPO by-products (TBARS) were found in plasma and tumor-bearing tissues at low and elevated levels, in which every cell from oxidative DNA damage caused by ROS.<sup>[51,52]</sup> However, we found that DMBA-painted hamsters' plasma levels of LPO by-products

were higher than those in the buccal mucosa. UA restored LPO by-products (TBARS) to near-normal levels in DMBA-induced HBP carcinogenesis, probably due to its antioxidant and antiproliferative effects.

Antioxidants are appropriate in the defense against ROS-mediated oxidative damage.<sup>[53]</sup> Several types of carcinogenesis have been linked to the reduced level of enzymatic and nonenzymatic antioxidant substances that can prevent cell and tissue damage from increased LPO and excessive ROS generation.<sup>[54]</sup> Different tumor tissues have demonstrated excessive

(B)

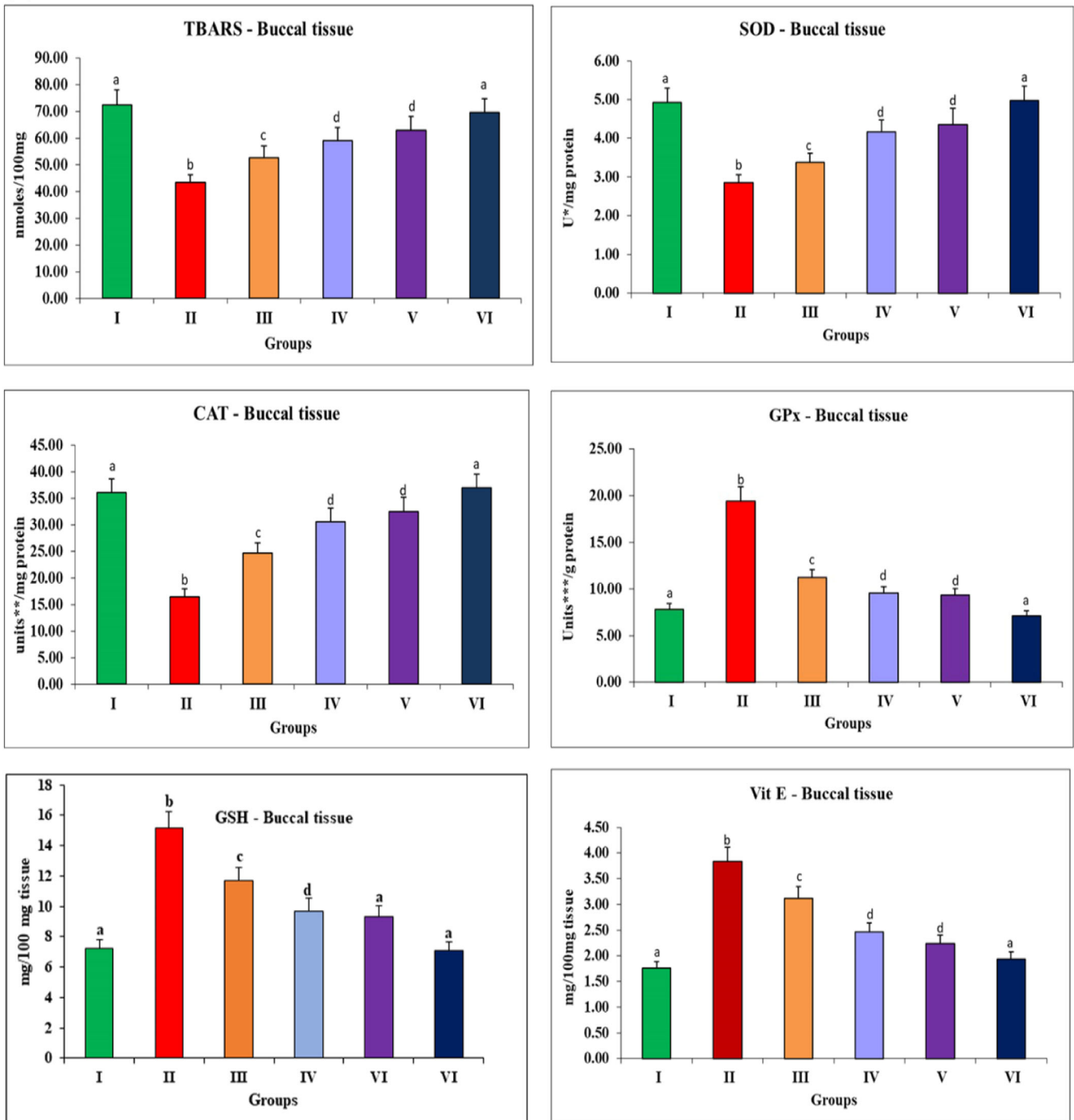


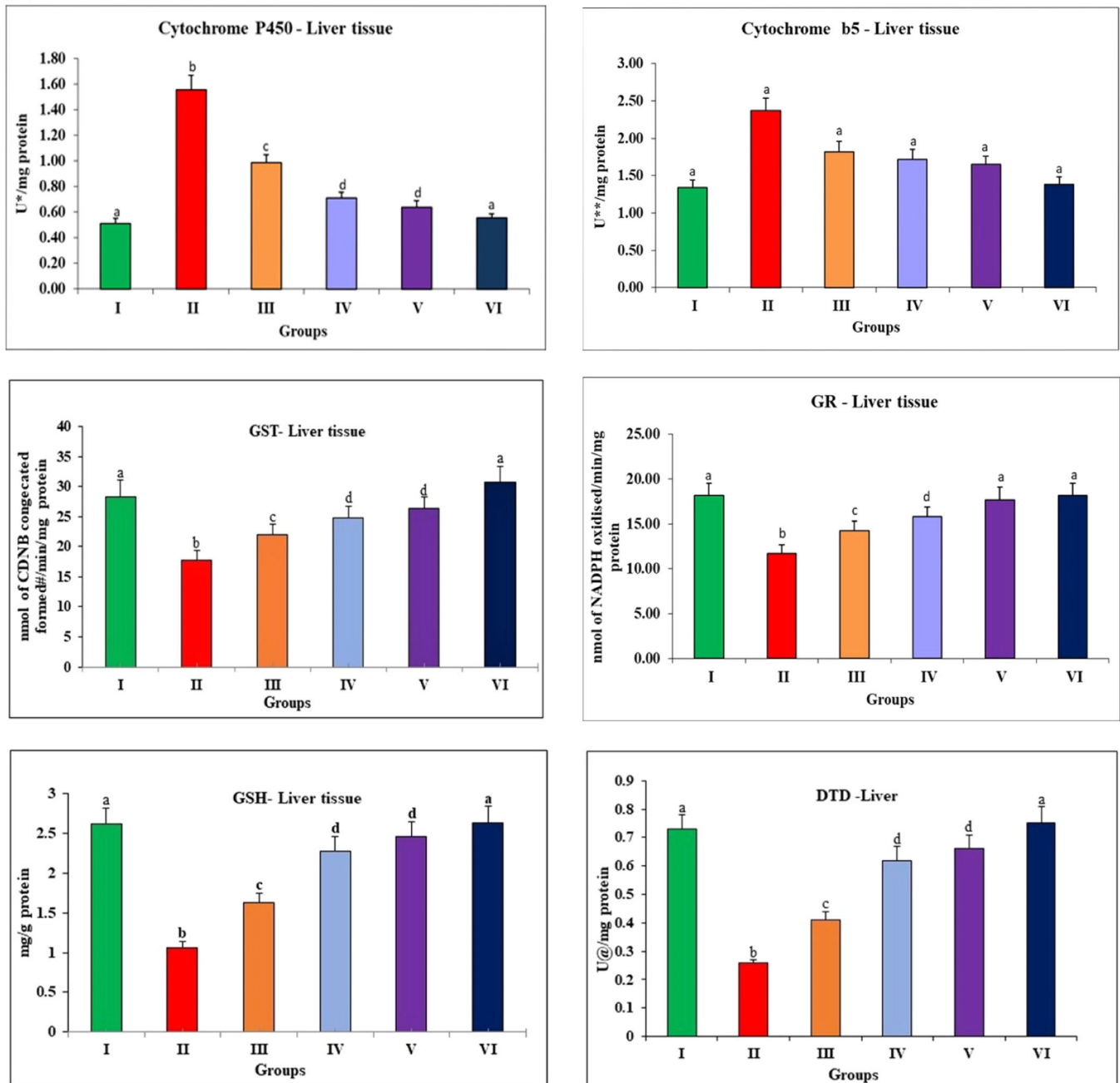
FIGURE 2 (Continued)

$H_2O_2$  production.<sup>[55]</sup> Antioxidant enzyme activity is in line with SOD and CAT to resolve ROS. SOD is the potent antioxidant enzyme defense mechanism by reducing superoxide-free radicals into molecule-level oxygen and hydrogen peroxide.<sup>[56]</sup> In addition to protecting cells from oxidative damage brought on by ROS, secondary enzymes CAT and GPx convert  $H_2O_2$  to  $H_2O$ .<sup>[57]</sup> Thomas and Sethupathy report that tumor tissues have been possessed to have decreased levels of SOD and CAT in vivo

models. GSH and GPx possess antioxidant properties and are regarded as indicators of chemoprevention.<sup>[58]</sup>

In tumor tissue, increased levels of GPx, GSH, and Vit-E have been found, although their levels in the blood are low.<sup>[59]</sup> The oxidative stress-related data revealed a decrease in antioxidant enzyme activity. From these results, we suggested that the current investigation found a substantially increase of Vit-E and GSH in buccal tissue and reduced plasma tumor-bearing hamsters. The

(A)



**FIGURE 3** Effect of UA on detoxification enzyme status in DMBA-induced HBP carcinogenesis. (A and B) show the level of detoxification enzymes (Phase I & II) in the hepatic and buccal tissue of hamsters in every group. The data are presented as means  $\pm$  SD of six experimental animals for each group. Data that do not share a common superscript (a, b, c, d) differ significantly at  $p \leq 0.05$  (DMRT). U\*, micromoles of cytochrome P450, U\*\*, micromoles of cytochrome b5, #, micromoles of 1-chloro 2,4 dinitrobenzene (CDNB)-reduced glutathione conjugate/min; @, micromoles of NADPH oxidized with DTD/h. DMBA, 7,12-dimethylbenz[a]anthracene; DMRT, Duncan's multiple range test; GSH, glutathione; HBP, hamster buccal pouch; UA, usnic acid.

previous report showed that the administration of UA decreased the level of LPO and increased the level of SOD, GPx, and GSH in rat stomach tissue.<sup>[60]</sup>

The outcome of the present study shows these biochemical findings revealed the efficacy of UA, a secondary metabolite compound, in tumor-bearing hamsters; thus, we suggest that UA administration

enhanced the enzymatic and nonenzymatic antioxidant levels in buccal tissue and plasma or serum, indicating its antioxidant effects. The liver is a crucial detoxifying organ that can eliminate toxic substances through the modulation of Phase I detoxification enzymes and prevent cellular damage effectively by stimulating Phase II enzyme activity through a single mechanism.<sup>[61]</sup> The bioactivities of DMBA enhance the Phase I

(B)

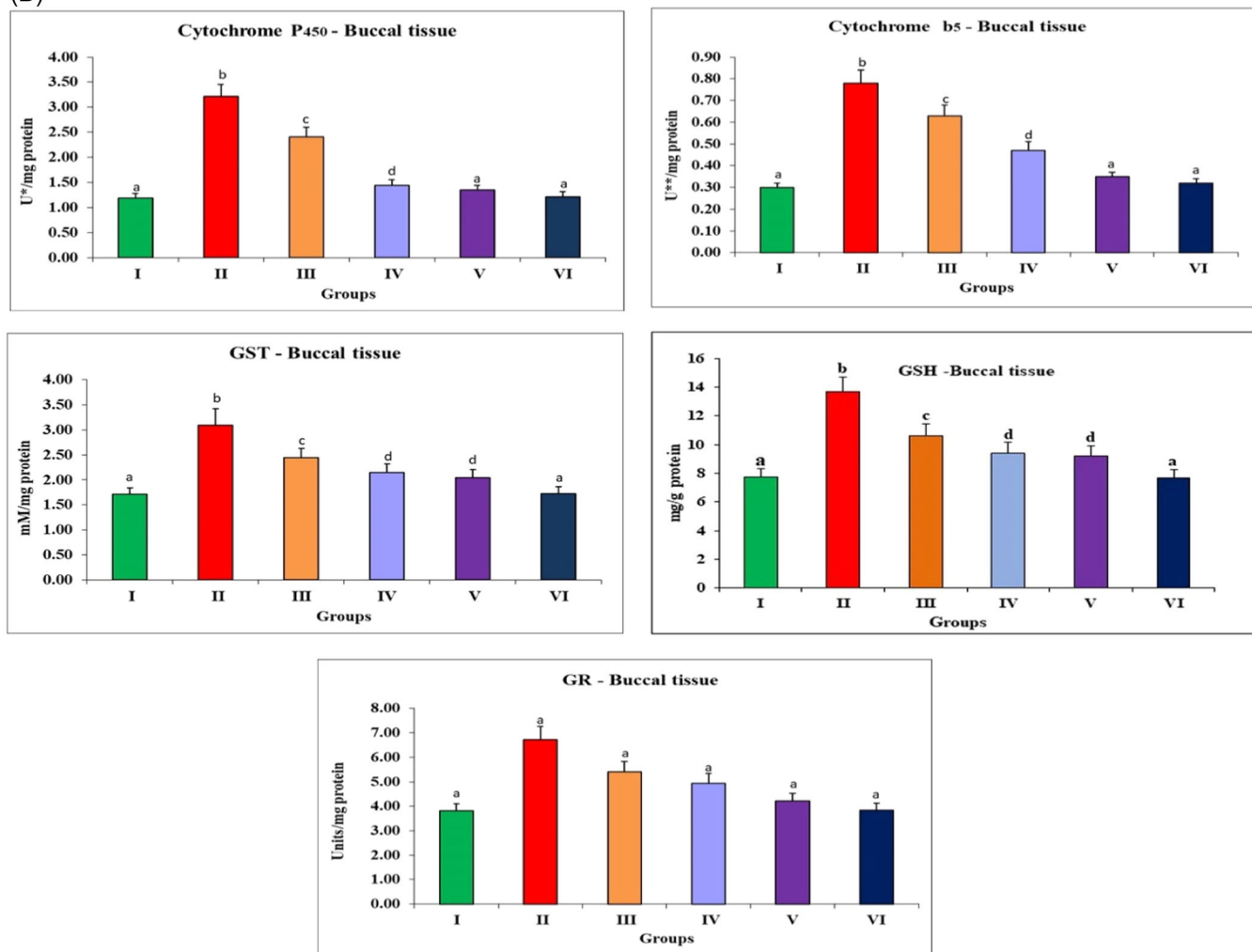


FIGURE 3 (Continued)

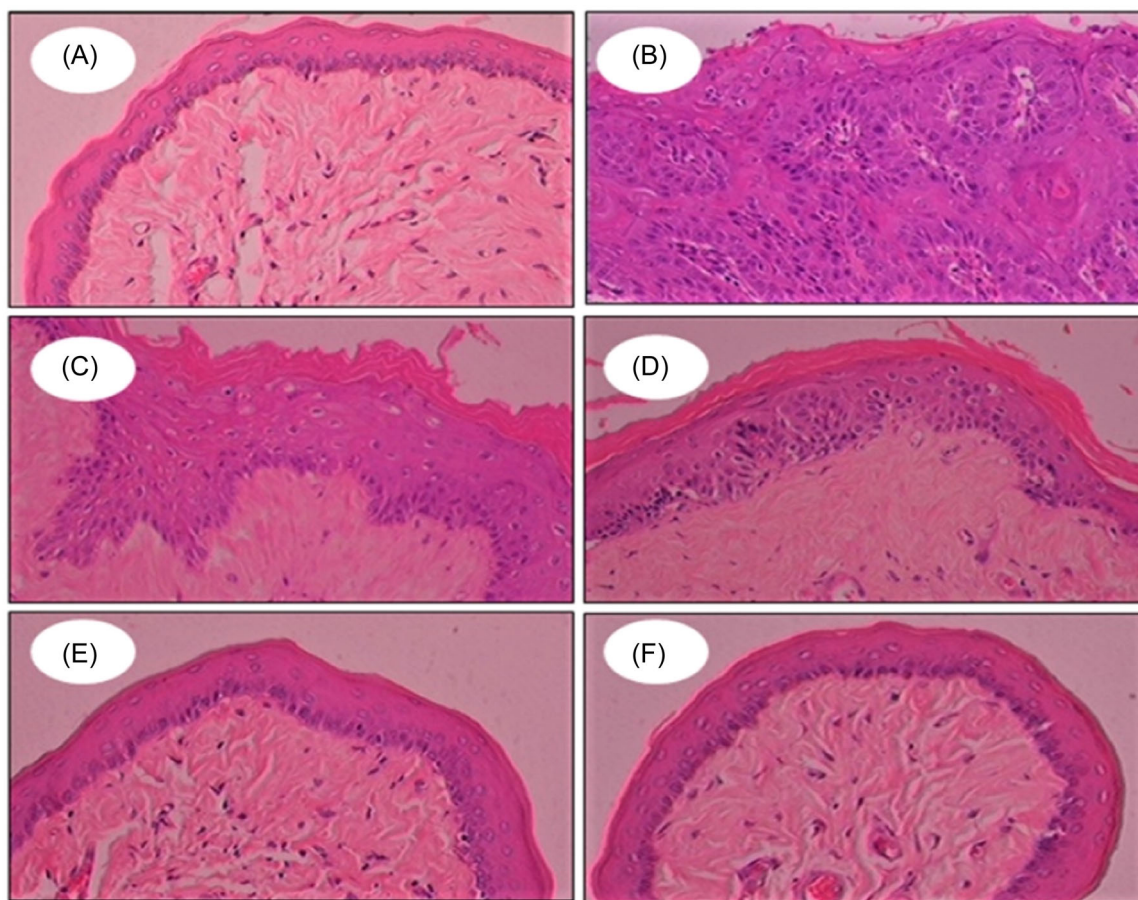
TABLE 2 Histopathological changes in the buccal mucosa of vehicle control and experimental hamsters in each group.

Groups	Treatments	Keratosis	Hyperplasia	Dysplasia	SCC
I	Control	-	-	-	-
II	DMBA alone	+++	+++	+++	+++
III	DMBA + UA (25 mg/kg b.wt)	+++	++	++	-
IV	DMBA + UA (50 mg/kg b.wt)	++	-	-	-
V	DMBA + UA (100 mg/kg b.wt)	+	+	-	-
VI	UA alone (100 mg/kg b.wt)	-	-	-	-

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; UA, usnic acid.  
(-), absent; (+), mild; (++) , moderate; (+++) , severe.

detoxifying enzymes (Cyt-p450 and b5) and reduce the Phase II detoxifying enzymes (GST, GR, GSH, and DTD) in the DMBA-treated hamster.<sup>[62]</sup> As a result of these enzymes' metabolic activation of DMBA, dihydrodiol epoxide is produced. This compound binds to the purine bases in DNA to form covalent adducts, which lead to mutation and cancer development.<sup>[63]</sup>

According to a recent study by Ramachandran et al., the chemical carcinogen DMBA-induced tumor-bearing hamsters with the enhanced Phase I and the reduced levels of Phase II enzyme were observed.<sup>[64]</sup> In addition, numerous secondary metabolic compounds can modulate xenobiotics' metabolism by inhibiting or inducing detoxification mechanisms.<sup>[65]</sup>



**FIGURE 4** Effect of UA on histological examination in DMBA-induced hamster HBP carcinogenesis model in each group. Well-differentiated buccal epithelial tissue from Groups I and IV showed typical architecture and no indications of cell growth (A, F). (B) In Group II hamsters, squamous cell carcinoma is well differentiated and keratin pearls. (C) The buccal epithelial layer of Group III hamsters exhibited a dysplastic epithelial layer. (D, E) Hamster buccal epithelial layer from Groups IV and V showing mild hyperplasia, keratosis, and hyperkeratosis (H&E x400). DMBA, 7,12-dimethylbenz[a]anthracene; HBP, hamster buccal pouch; H&E, hematoxylin and eosin; UA, usnic acid.

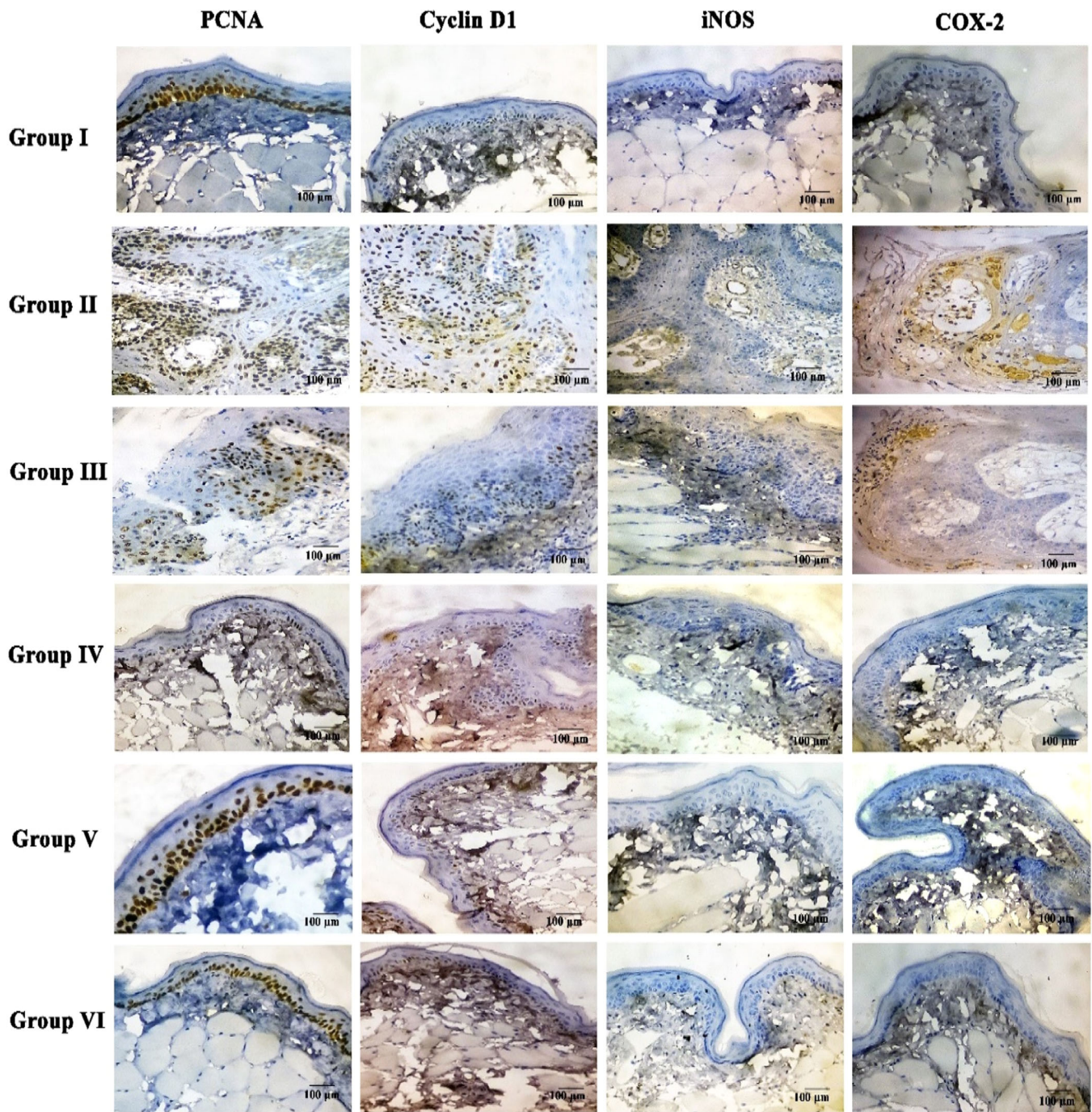
Our present study coincides with previous related studies by which detoxifying agents like Phase I and II (detox enzyme status) increased liver and buccal tissues that decreased correspondingly.<sup>[66]</sup> The administration of UA prevented oral carcinogenesis from being metabolically activated during DMBA-induced HBP carcinogenesis.

The greatest cancer cell proliferative indicators are PCNA and cyclin D1, which may be related to the knowledge that tumor cell formation and cell cycle development must be employed as marking indications for cancer detection.<sup>[67]</sup> Additionally, one of the exceptional nuclear proteins known as cyclin D1 is associated with the transition between the G1 phase and synthesis phases of the cell cycle.<sup>[68]</sup> In addition, the present investigation revealed enhanced proliferative cell biomarkers of cyclin D1 and PCNA expression in the hamster animal buccal pouch carcinogenesis model, which is reliable with the outcomes of DMBA-induced HBP carcinogenic experimental studies. Upregulation of COX-2 in different types of human cancer, chemopreventive effects on cancer development by potent inhibitors of COXs, and upregulation of COX-2 linked with enhanced angiogenesis and metastasis support their role in carcinogenesis.<sup>[69]</sup> Arachidonic Acid, which

COX-2 produced, is associated with various cellular mechanisms for the metabolism of PGE2 and is also significantly expressed in many inflammatory and malignant conditions.<sup>[70]</sup>

The present study, which followed the high variation of the malignant squamous cells found COX-2 expression as brown granular/diffuse cytoplasmic staining, primarily perinuclear, and it showed various staining intensities. This evidence is comparable to the previous findings.<sup>[71]</sup> In OSCC of the buccal tissue, a correlation was obtained between the intensity of COX-2 expression staining and the disease's advanced stage.<sup>[72]</sup> Increased iNOS expression might lead to excess nitric oxide (NO) production levels, which can increase harmful impacts such as inflammation, DNA damage induction, immune system inhibition, and tumor formation.<sup>[73]</sup> Geng et al. demonstrated that UA has an inhibitory effect on gastric cancers. They further hypothesized that UA prevented gastric cancers by inducing apoptosis and down-regulating Bcl-2 expression as an antiapoptotic protein, while upregulating proapoptotic protein Bax expression.<sup>[74]</sup>

In addition, UA has been suggested to induce cellular differentiation, apoptosis, cell proliferation suppression, and modulation of cell cycle progression previously.<sup>[75]</sup> In our present study, the oral



**FIGURE 5** Effect of UA on immunohistochemical markers of PCNA, iNOS, cyclin, and COX-2 expression pattern of each control and experimental hamsters' buccal mucosa (x400). Group I and IV hamsters showed no PCNA, iNOS, cyclin, and COX-2 expression pattern. DMBA-induced Group II hamsters displayed overexpression of PCNA, iNOS, cyclin, & COX-2. Group III hamsters showed slight expression of PCNA, iNOS, cyclin, and COX-2, while Group IV and V hamsters showed down expression of PCNA, iNOS, cyclin, and COX-2. DMBA, 7,12-dimethylbenz[a]anthracene; UA, usnic acid.

treatment of UA significantly enhanced the antioxidants and detoxification mechanism. It decreased the expression of PCNA, cyclin D1, iNOS, and COX-2, which favor in preventing cell proliferation and its processes. This study shows that UA (50 and 100 mg/kg b.wt) is more efficient at suppressing HBP oral carcinogenesis predicated on tumor burden, morphological, and biochemical marker alteration.

## 5 | CONCLUSION

Thus, we concluded that UA exhibited significant anti-neoplastic efficacy against DMBA-induced HBP carcinogenesis. This is accomplished by inhibiting LPO caused by oxidative stress and inducing antioxidants and enzymes by activating Phase I and Phase II

detoxification agent. Further, the immunohistology assessment does not depict any significant alterations in PCNA, cyclin D1, iNOS, and COX-2 expression. Therefore, UA might significantly prevent or suppress tumor growth and trigger apoptosis. The findings demonstrated the potential effect of UA as a chemoprotective agent against OSCC.

#### AUTHOR CONTRIBUTIONS

**Study concept and study design:** Suresh Kathiresan. **Data acquisition:** Theerthu Azhamuthu. **Animal study and biochemical assay:** Theerthu Azhamuthu. **Data analysis and interpretation:** Suresh Kathiresan and Theerthu Azhamuthu. **Statistical analysis:** Suresh Kathiresan, Theerthu Azhamuthu, and Ilanchitchenni Senkuttuvan. **Manuscript preparation:** Suresh Kathiresan. **Manuscript editing:** Suresh Kathiresan and Theerthu Azhamuthu. **Manuscript review:** Suresh Kathiresan and Theerthu Azhamuthu. **Manuscript revision:** Suresh Kathiresan, Theerthu Azhamuthu, Ilanchitchenni Senkuttuvan, Nihal Ahamed Abulkalam Asath, and Pugazhendhi Ravichandran. All authors were read and approved the current version of the research article to be published.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The data will be available from the authors on reasonable request.

#### ETHIC STATEMENT

This work was approved by the IAEC of Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, India. The ethical approval number is (AU-IAEC/PR/1254/7/19). The study was done on an experimental animal with no need to consent to participate.

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