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ORIGINAL ARTICLE

Tumor Preventive Efficacy of Emodin in 7,12-Dimethylbenz[a] Anthracene-Induced Oral Carcinogenesis: a Histopathological and Biochemical Approach

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Abstract The aim of the present study is to focus the chemopreventive potential of Emodin during 7,12-dimethylbenz [a]anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Tumors were developed in the buccal pouches of golden Syrian hamsters by painting with 0.5% DMBA thrice a week for 14 weeks. The status of lipid peroxidation, antioxidants and detoxification agents were utilized as biochemical endpoints and the expression pattern of apoptotic proteins was employed as molecular endpoints in addition to the histopathological studies, to substantiate the anticancer potential of Emodin. Hamsters treated with DMBA + Emodin revealed mild to moderate precancerous lesions such as hyperplasia and dysplasia whereas 100% tumor formation was noticed in hamsters treated with DMBA alone. Also, Emodin treatment modulated the status of lipid peroxidation, antioxidants, phase I and II detoxification agents and apoptotic proteins in favor of the inhibition/reversal/suppression of the oral tumorigenesis in DMBA treated hamsters. The present study thus concludes that the chemopreventive potential of Emodin relies on its pro-apoptotic and antioxidant efficacy during DMBA induced hamster buccal pouch carcinogenesis.

Keywords Oral cancer · Emodin · Lipid peroxidation · Antioxidants · Apoptosis · Detoxification agents

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Introduction

Cancer of the oral cavity emerges as a life threatening health burden worldwide. The global annual incidence of this cancer is sharply increasing and accounts for 4–5% of all cancers in the Western countries and constitutes about 40–50% of all malignant cancers in the developing nations especially in India, Pakistan, Bangladesh and Sri Lanka [1, 2]. The most prominent etiological factors of oral carcinoma include tobacco and betel quid chewing, tobacco cigarette and bidi smoking and alcohol consumption [3]. Apart from these risk factors, viruses such as HPV and EBV also play a significant causative role [4]. Mounting evidences clearly pointed out that the late diagnosis of oral cancer is the single most responsible factor for the increase in annual incidence, as well as a poor survival outcome of cancer patients despite recent advancement in the treatment strategy [5].

Biomarkers status in the tissues or blood could play a pivotal and significant role in the diagnosis of cancer as well as to plan the treatment options. Abnormal levels or expression of bunch of biochemical / molecular markers has been reported in experimental and human carcinogenesis [6, 7]. Earlier studies from our laboratory explored the abnormal status of a spectrum of biomarkers in oral carcinoma [8–10]. The present study has utilized the status of lipid peroxidation, antioxidants, phase I and II detoxification agents and apoptotic proteins to validate the chemopreventive potential of Emodin in 7,12-dimethylbenz[a]anthracene induced hamster buccal pouch carcinogenesis

Chemoprevention research is widely utilized as a promising strategy in the experimental oncology to focus or explore the anticancer potential of medicinal plants, their bioactive phytoconstituents and /or synthetic entities. To scientifically validate the anticancer potential of the phytoconstituents in oral carcinogenesis,

researchers employed 7,12-dimethylbenz[a]anthracene induced oral carcinogenesis in golden Syrian hamsters as a preferred experimental cancer model. Oral tumors developed from this experimental model showed close morphological and histopathological similarities with the human oral tumors [11]. Many naturally occurring substances were tested for their anticancer activity using experimental animal models, which resulted in the present availability of some 30 effective anticancer drugs.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a naturally occurring anthraquinone present in the roots and bark of numerous plants of the genus Rhamnus. Emodin occurs primarily as a mixture of two glycosides: the 3-O-glycoside of L-rhamnose (frangulin A) and the 3-O-glycoside of D-apiofuranose (frangulin B). Diverse pharmaceutical studies have pointed out that Emodin has diverse biological effects such as anticancer, antimicrobial, and anti-inflammatory effects [12–14]. Emodin, an inhibitor of protein tyrosine kinase, also possesses antiviral and immunosuppressive properties [15]. Animal experiments have reported its hypoglycemic, anti-inflammatory, antidiabetic and hepatoprotective properties too [16–18]. There are however, no scientific studies on the anticancer potential of Emodin in DMBA induced hamster buccal pouch carcinogenesis. To the best of our knowledge, this is the first scientific report, which explores the chemopreventive potential of Emodin in 7,12-dimethylbenz[a]anthracene induced hamster buccal pouch carcinogenesis

Materials and Methods

Animals

Forty golden Syrian hamsters (male, 7–8 weeks old, 80-120 g) were procured from National Institute of Nutrition (NIN), Hyderabad, India. The animals were maintained as per the ethical principles of Annamalai University Institution Ethical Committee (Registration Number 160/1999/ CPCSEA) in the Central Animal House, Annamalai University. Animals were subdivided into various groups and maintained separately in the polypropylene animal cages. The pellet diet and water were provided to all the experimental groups ad libitum.

Experimental Design

Experimental hamsters that received liquid paraffin alone three times a week for 14 weeks on the left buccal pouches and hamsters received Emodin alone (50 mg/kg b.w, three times a week for 14 weeks) served as group I and IV respectively. Hamsters that received topical application of DMBA (0.5% in liquid paraffin) on their left buccal pouches (three times a week for 14 weeks) were categorized as group II. Hamsters that received Emodin orally (50 mg/kg b.w, three times a week for 14 weeks) in addition to DMBA treatment served as group III. No.4 paint brush that delivers approximately 0.4 mg per application was used for the topical application of DMBA. The experimental animals were sacrificed at the end of experimental period and the status of biomarkers was assayed in the plasma, liver and buccal mucosa of experimental hamsters.

Tumor induction.

Topical application of 0.5% DMBA in liquid paraffin, three times a week for 14 weeks, on the buccal pouches of golden Syrian hamsters developed oral tumors that were confirmed histopathologically as well differentiated squamous cell carcinoma by the Oral Pathologist, Department of Oral Pathology, Annamalai University.

Biochemical Markers

The specific and sensitive colorimetric methods were used to analyze the concentrations of the biochemical parameters in the plasma and tissues. The status of lipid peroxidation and antioxidants was analyzed according to the methods of Yagi [19] (plasma TBARS), Ohkawa et al., [20] (tissue TBARS), Desai [21] (plasma vitamin E) and Palan et al., [22] (tissue vitamin E), Beutler and Kelley [23] (GSH), Omaye et al., [24] (Vitamin C), Kakkar et al., [25] (Superoxide dismutase), Sinha [26] (Catalase), and Rotruck et al., [27] (Glutathione peroxidase). The status of phase I and II detoxification agents was measured according to the methods of Omura and Sato [28] (Cytochrome P_{450} and Cytochrome b_5), Tietze [29] (oxidized glutathione), Habig et al., [30] (Glutathione-S-transferase], Carlberg and Mannervik [31] (Glutathione reductase) and Ernster [32] (DT-Diaphorase).

Western Blotting

Buccal mucosa tissue samples excised from the experimental hamsters were subjected to protein quantification according to the method of Barfold et al., [33]. Proteins in the tissue extracts were then separated using polyacrylamide gel electrophoresis. The separated proteins were then transferred onto PVDF membrane using electroblotting. The blots were incubated with respective primary antibodies (p53, Bcl-xL, BAD: Cell Signaling Technology, Danvers, MA, USA), followed by incubation with secondary antibodies conjugated with horse radish peroxidase (Santa Cruz Biotechnology, USA). After a specified time period, the immune complex was treated with diaminobenzidine, the substrate of the enzyme. The bands were then scanned and the protein concentration was quantified densitometrically using Bio-Rad Image Lab™ software version 4.1 software.

Immunohistochemistry

Buccal mucosa tissue sections were incubated with respective primary antibodies (Bcl-2, Bax: Dako, Carprinteria, CA, USA) after routine procedure. The slides were then treated with horse radish peroxidase labelled secondary antibodies and incubated for 1 h at 37 °C. The substrate diaminobenzidine) was added to the immune complex and viewed under the microscope, when acceptable color intensity was attained.

Enzyme Linked Immunosorbant Assay

The activities of caspase 3 and 9 were measured in the buccal mucosa according to the instructions given in the user"s manual using ELISA. The caspase 3 and 9 assays are based on spectrophotometric detection of the chromophore-p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA and LEHD-pNA, respectively, at 405 nm in a microtitre plate reader

Statistical Analysis

The values are expressed as mean ± SD. The statistical significance was analyzed using One way analysis of variance followed by Duncan"s Multiple Range Test. The *p* value less than 0.05 betweeen two groups are considered as statistically significant.

Results

Tumor Incidence and Histopathological Changes

Histopathologically confirmed well differentiated squmous cell carcinoma was noticed in the buccal mucosa of hamsters treated with DMBA alone and the tumor incidence was 100% in these experimental animals. The tumor volume and burden in the hamsters treated with DMBA alone was also higher and is given in Table 1. Though hamsters treated with DMBA + Emodin did not exhibit any tumor formation on their buccal pouches (Table 2), hyperplasia, hyperkeratosis and dysplasia were noticed in all the animals (Figs. 1 and 2).

Phase I and Phase II Detoxification Agents in Liver and Buccal Mucosa

Imbalance in the status of Phase I (cytochrome b_5, P_{450}) and II (Glutathione S-transferase, Glutathione reductase) detoxification agents were noticed in the liver and buccal mucosa of the experimental hamsters (Figs. 3 and 4). While Phase I detoxification agents, that are involved in the metabolic activation of carcinogens, were increased in both liver and buccal mucosa, the phase II detoxification agents, that are involved in the excretion of carcinogenic metabolites, were decreased in the liver and increased in the buccal mucosa. Emodin administration orally to hamsters treated with DMBA bring back the status of detoxification agents in the liver and buccal mucosa.

TBARS and Antioxidants in Plasma and Buccal Mucosa

An inverse correlation was observed in the status of lipid peroxidation by-products (TBARS) between the plasma and buccal mucosa of hamsters treated with DMBA alone. While plasma TBARS was increased , the levels of buccal mucosa TBARS were declined as compared to the control hamsters (Figs. 5 and 6). Antioxidants also revealed the disturbed pattern in the hamsters treated with DMBA alone. While plasma enzymatic and non-enzymatic antioxidants were extensively reduced, SOD and CAT activities were decreased and vitamin E,GSH and GPx were increased in the buccal mucosa of hamsters treated with DMBA alone. Oral administration of Emodin re-established the status of lipid peroxidation by-products and antioxidants in the plasma and buccal mucosa of hamsters treated with DMBA.

Apoptotic Markers

The expression pattern of apoptotic markers [(p53, Bcl-xL and BAD (Figs. 7 and 8), Bcl-2 and Bax (Fig. 9) and caspase 3 and 9 (Fig. 10)] were analysed in the buccal mucosa of control and experimental hamsters in each group. Higher expression of Bcl-2, Bcl-xL, and lower expression of p53, Bax, BAD, caspase 3 and 9 were noticed in the buccal mucosa of hamsters treated with DMBA alone. Oral administration of Emodin at a dose of 50 mg/kg b.w to DMBA treated hamsters modulated the above said apoptotic markers towards the suppression of oral carcinogenesis (ie bring back the status).

Discussion

In vivo animal model could provide useful information not only about the distinct phases of carcinogenesis but also helpful to plan the treatment strategy, to investigate the new diagnostic tumor markers and to identify the new biochemical and molecular targets of the carcinogenic process [34]. Oral cavity, due to its easy accessibility, is utilized as the major target for chemoprevention studies. The development of precancerous and cancerous lesions could be easily and sequentially examined in the oral cavity. Extensive studies highlighted the chemopreventive effects of a diverse natural products or synthetic entities [35, 36]. Though several mechanisms were pointed out for the chemopreventive potential of the natural products, the anti-lipid peroxidative and pro-apoptotic properties were documented as a major mechanism. The present study was designed to explore the anticancer efficacy of Emodin in

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

Parameters	Group I Vehicle treated control	Group II DMBA Alone	Group III DMBA + Emodin	Group IV Emodin alone
Tumor incidence (oral squamous cell carcinoma)	0	100%	0	0
Total number of tumors /animals	0	33/10	0	0
Tumor volume (mm^3)/animals	0	394.38 ± 36.18	0	0
Tumor burden(mm^3)/animals	0	1301.45 ± 119.56	0	0

Tumor volume was measured using the formula, v = 4/3 [D1/2] [D2/2] [D3/2] where D1,D2 and D3 are the three diameters of the tumor

Tumor burden was calculated by multiplying tumor volume and the number of tumors / animals

DMBA induced oral cancer in the golden Syrian hamsters by utilizing the status of lipid peroxidation, antioxidants and detoxification agents are utilized as biochemical end points and apoptotic markers (p53, Bcl-xL, BAD, Bcl-2, Bax, caspase 3 and 9) are utilized as molecular end points. Hamsters treated with DMBA alone exhibited histopathological abnormalities such as severe hyperkeratosis, hyperplasia, dysplasia and well differentiated squmamous cell carcinoma, which closely mimic human oral cancer lesions. Though Emodin completely prevented or inhibited the tumor formation in hamsters treated with DMBA, the present study noticed mild to moderate dysplastic lesions in 30% of the hamsters, which indicates that Emodin significantly delayed the tumor formation in these animals.

Reactive oxygen species (ROS) cause adverse effects on physiological, biochemical and molecular pathways if they are excessively generated in a system. Reactive oxygen species induced lipid peroxidation is a major contributor of oxidative DNA damage, which in turn contributes to neoplastic transformation [37]. Abnormalities in ROS generation have been well documented in more than 50 pathological diseases, including cancer [37, 38]. To counteract the effects of ROS, human contains an array of complex and complicated endogenous enzymatic antioxidants (glutathione peroxidase, superoxide dismutase and catalase) and non-enzymatic antioxidants (Vitamin E, C and reduced glutathione) defense mechanism. An imbalance in oxidant and antioxidant mechanism could thus lead to carcinogenesis.

Plasma lipid peroxidation by-products and antioxidant status are the indicators of the oxidative stress of the system. Extensive studies documented that tumors and other damaged tissues due to carcinogenesis generated excessive ROS, which consequently enter into circulation [37–39]. Vitamin E, C and reduced glutathione, alone or in combination, play a pivotal role in scavenging excessive ROS from the body. The synergistic role of these antioxidants in the prevention of carcinogenesis has been well reported [39]. Lowered levels of vitamin E and reduced glutathione content were documented in various carcinogenesis [37, 40]. Vitamin E and glutathione inhibited various cancers, including oral cancer [41, 42].

Table 2 Histopathological changes in the buccal pouch of hamsters in control and experimental animals in each group

Groups	Parameters	Hyperkeratosis	Hyperplasia	Dysplasia	Squamous cell carcinoma
I	Vehicle treated Control	Absent	Absent	Absent	Absent
II	DMBA alone	Severe [10/10]	Severe [10/10]	Severe [10/10]	100% [10/10]
III	DMBA + Emodin (pre-initiation)	Severe [4/10]	Moderate to severe [3/10]	Mild to moderate [3/10]	Absent
IV	Emodin alone	Absent	Absent	Absent	Absent

Buccal mucosa tissues from hamsters treated with DMBA alone (Tumor bearing hamsters) showed sheets of dysplastic epithelium invading into connective tissues, epithelial island formation and individual cell keratinisation along with the keratin pearl formation

Buccal mucosa tissues from hamsters treated with DMBA + Emodin exhibited hyperplastic epithelium with hyperkeratinisation, basal cell hyperplasia and dysplasia in certain areas

Buccal mucosa tissues from control hamsters and hamsters treated with Emodin alone exhibited 2–3 layers of stratified squamous epithelium, basal layer cells with vesicular nucleus and dense and fibrous underlying connective tissues

Hyperkeratosis: Thickening of the outermost layer of the epidermis. It affects the keratin layer of the oral mucosa

Hyperplasia: An increase in the number of cells in a tissue or an organ. Though hyperplastic cells appear normal under a microscope, they may become cancerous cells

Dysplasia: The enlargement of a tissue or organ by the proliferation of cells. These cells look abnormal under a microscope but are not cancer cells

Carcinoma: A type of cancerous tissue growth that develops from the epithelial cells

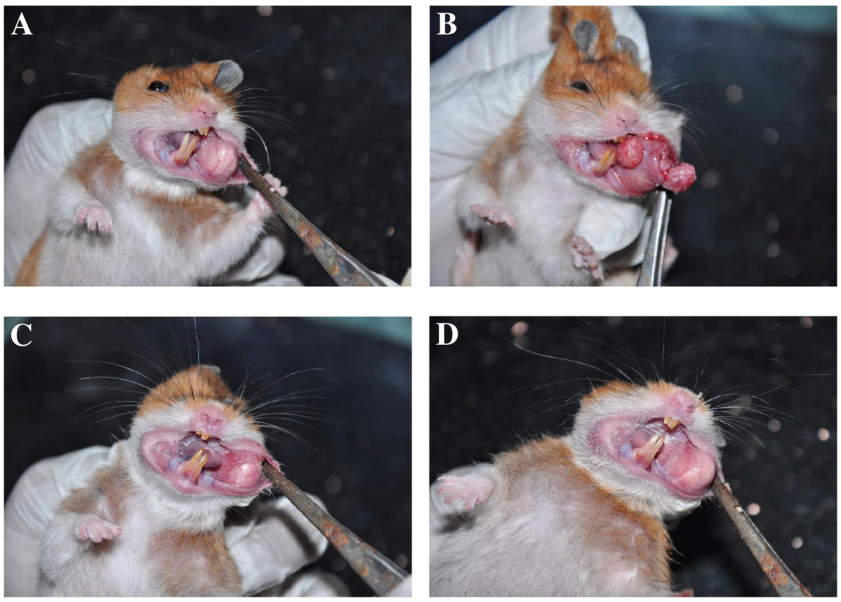

Fig. 1 Gross appearance of buccal mucosa in control and experimental animals in each group. A and D. Photograph exhibits buccal pouch mucosa without any abnormal lesions [Liquid paraffin alone treated and Emodin alone treated hamsters respectively]. B. Photograph shows well developed tumor formation in the buccal pouch of hamsters treated with DMBA alone. C. Photograph shows precancerous lesions in hamster treated with DMBA+ Emodin

Vitamin E and C are utilized as a nutrient by the tumor cells for their abnormal and rapid growth [41–43]. Increased levels of plasma TBARS might be due to lowered enzymatic antioxidant activities and reduced levels of non-enzymatic antioxidants.

Oral tumor cells showed decreased susceptibility to oxidative stress due to their low PUFA content in the membrane. Reports however, showed higher expression of superoxide radicals and hydrogen peroxides in the oral tumor tissues [37, 43]. A decrease in SOD and CAT activities accompanied by increase in GSHPx activity was documented in the tumor tissues of oral cancers [38]. Our results confirm these findings. Abnormal content of reduced glutathione was shown in diverse solid tumors, including oral cancer [40]. An increase in the glutathione peroxidase activity could account for the increase in reduced glutathione content of tumor tissues. The present results suggest that tumor tissues might have sequestered nutrients such as vitamin C and E and antioxidants such as GSH to meet their nutritional demands as well as to reduce the oxidative stress.

Phase I detoxification agents metabolically activate the carcinogens into its ultimate carcinogenic metabolites. Phase II

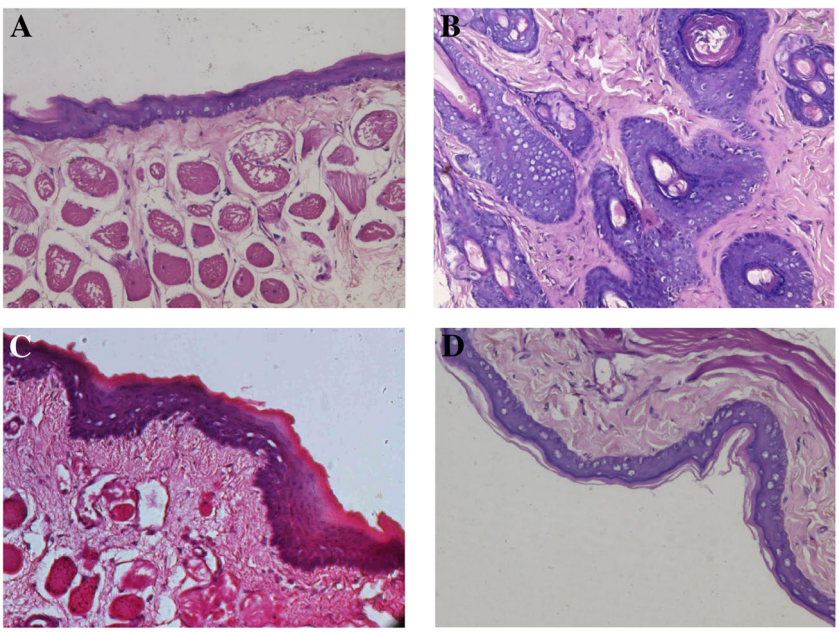

Fig. 2 Histopathological features observed in the buccal mucosa of control and experimental animals in each group. **a** and **d** Microphotographs shows well-defined stratified squamous epithelium [Liquid paraffin alone treated and Emodin alone treated hamsters respectively]. **b** Microphotograph reveals well differentiated squamous cell carcinoma with dysplastic epithelium and keratin pearls formation [DMBA alone treated hamsters]. **c** Microphotograph shows hyperplastic and dysplasic epithelium [DMBA+ Emodin treated hamsters]

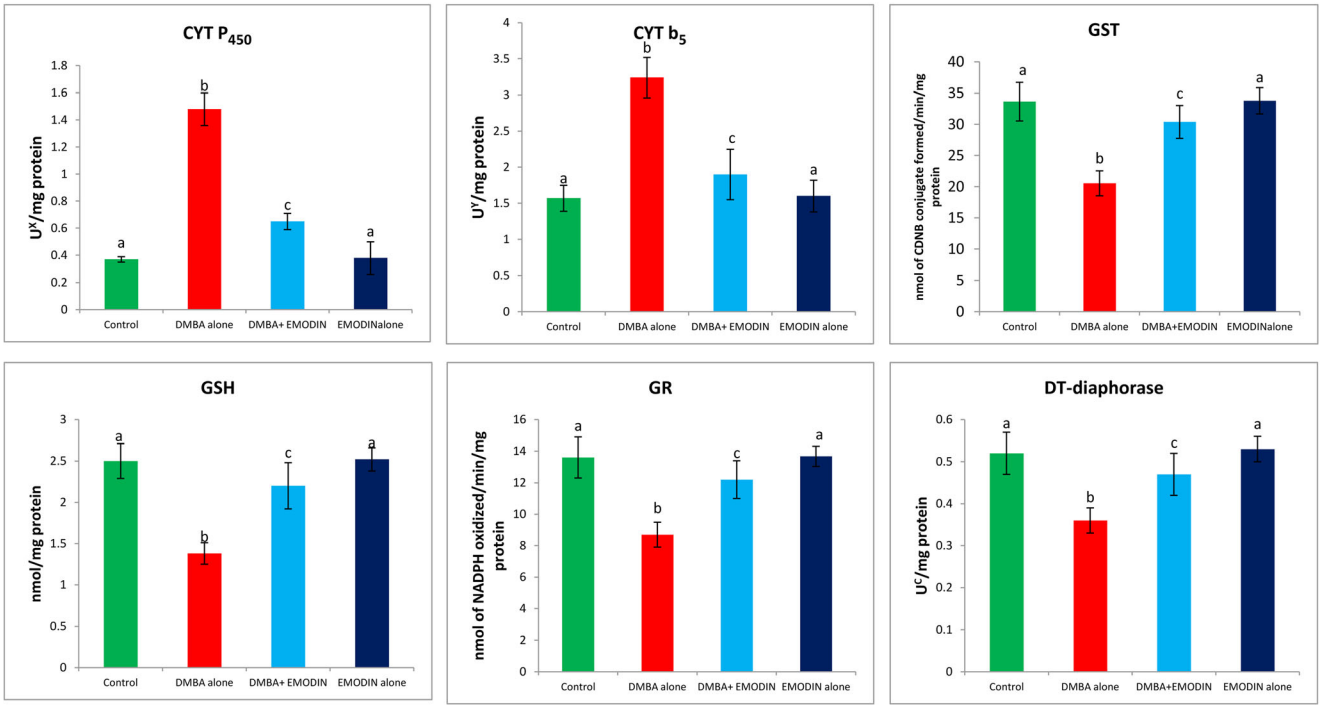

Fig. 3 Phase I and phase II detoxification agents in the liver of control and experimental hamsters in each group [n = 10]. Values are expressed as mean ± Standard deviation (S.D.) Values that do not share a common superscript between two groups differ significantly at $P < 0.05$ (DMRT). X — micromoles of cytochrome P_{450}; Y — micromoles of cytochrome b_5; C—micromoles of 2, 6-dichlorophenol reduced/min

detoxification agents effectively excrete these metabolites through urine via conjugation with reduced glutathione. Imbalance in the activities of phase I and phase II detoxification agents could therefore promote tumorigenesis [37, 38]. Recent chemoprevention studies pointed out impairment in the activities of phase I and II detoxification cascade

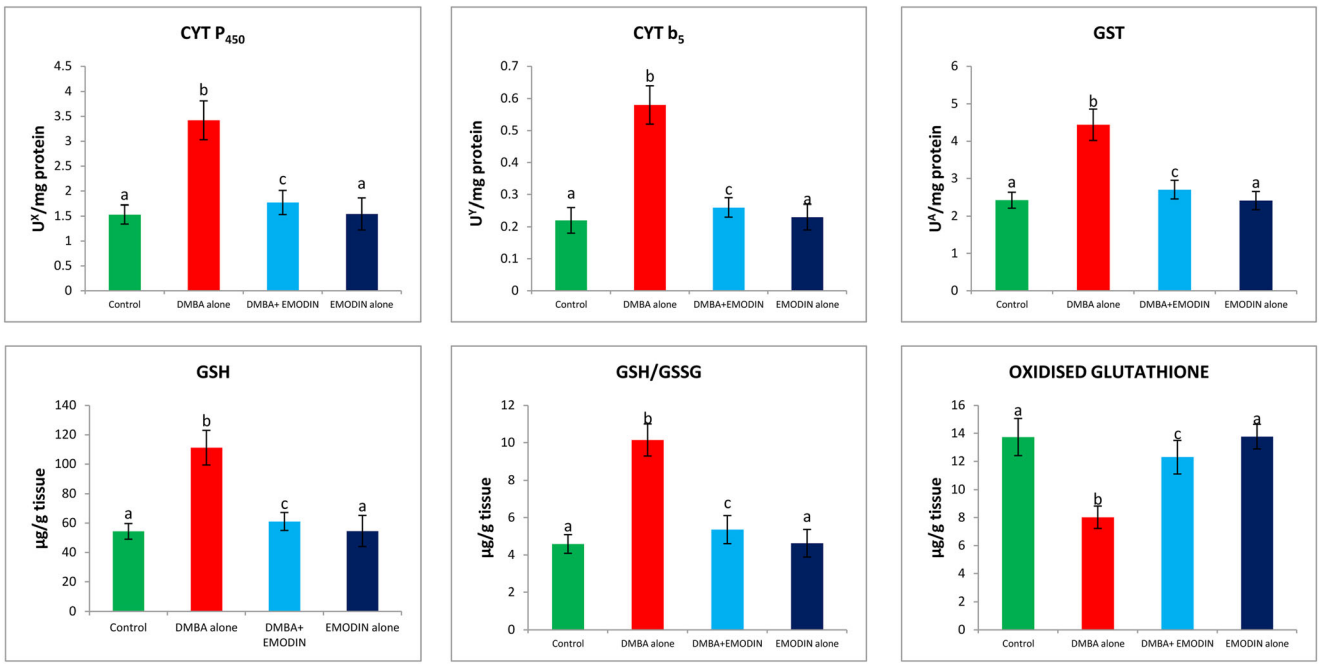

Fig. 4 Phase I and phase II detoxification agents in the buccal mucosa of control and experimental hamsters in each group [n = 10]. Values are expressed as mean ± Standard deviation (S. D) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at $p < 0.05$ (DMRT), X- micromoles of cytochrome p450; Y- micromoles of cytochrome b5; A – micromoles of 1- chloro 2,4 dinitrobenzene (CDNB) / reduced glutathione conjugate formed per minute

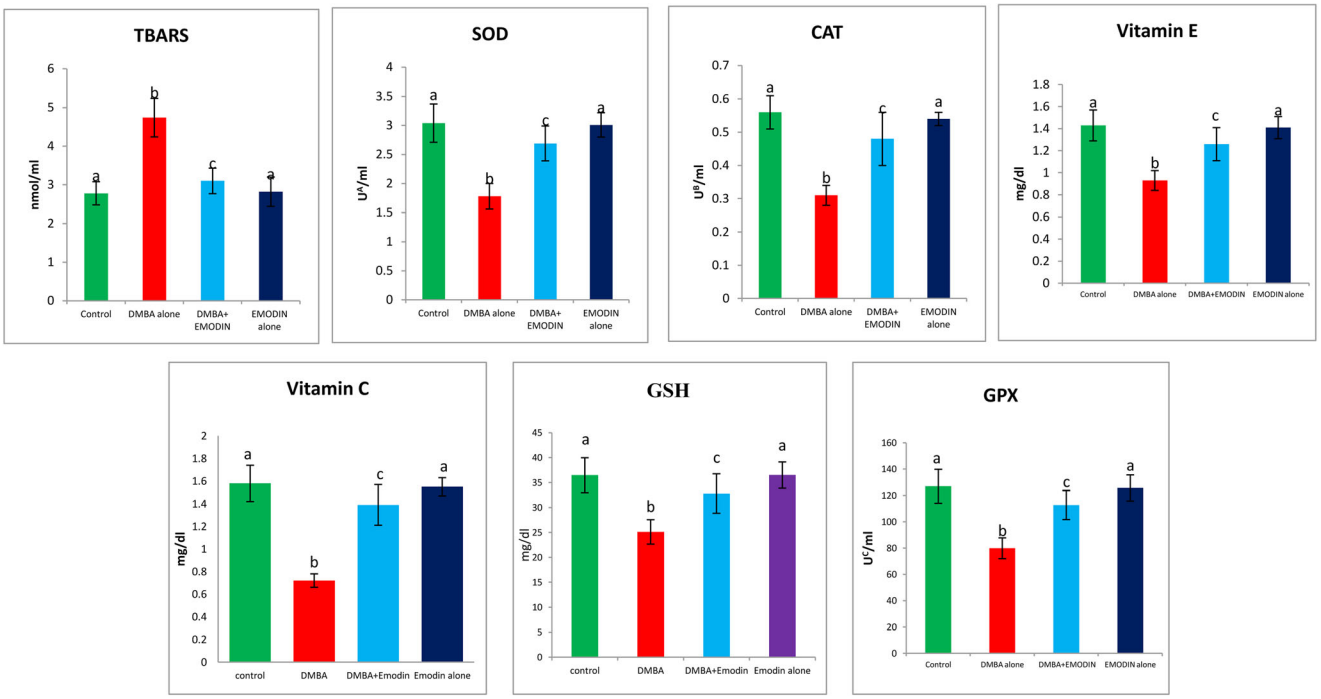

Fig. 5 TBARS and antioxidants in the plasma of control and experimental hamsters in each group [$n = 10$]. Values are expressed as mean ± standard deviation (S.D.) for ten hamsters in each group. Values that do not share a common superscript between two groups differ significantly at $P < 0.05$ (DMRT). A — the amount of enzyme required to inhibit 50% NBT reduction; B — micromoles of hydrogen peroxide utilized/s; C — micromoles of glutathione utilized/min

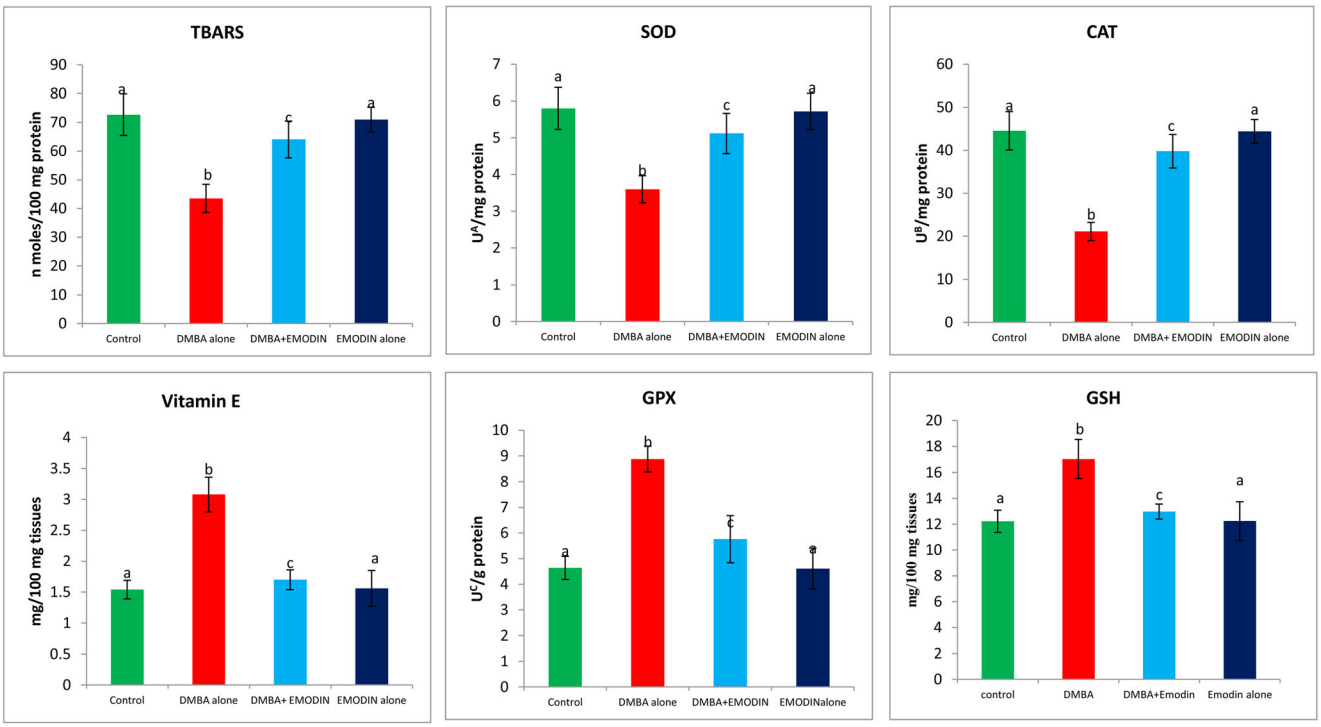

Fig. 6 TBARS and antioxidants in the buccal mucosa of control and experimental hamsters in each group [$n = 10$]. Values are expressed as mean + Standard deviation (S. D) for ten hamsters in each group. Values that do not share a common superscript between the groups differ significantly at $p < 0.05$ (DMRT), A – amount of enzyme required to inhibit 50% NBT reduction; B – micromoles of hydrogen peroxide utilized/s; C –micromoles of glutathione utilized/min

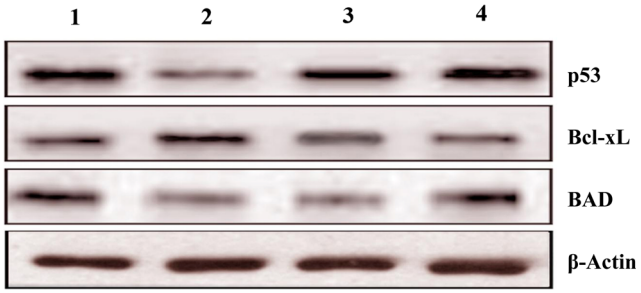

Fig. 7 Expression pattern of p53, Bcl-xL and BAD in the buccal pouch tissues of control and experimental animals. (**a**) Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Emodin, Lane 4: Emodin alone

in the liver and tumor tissues of tumor bearing hamsters, which was restored after treatment with the natural products [37, 40]. The present study provided additional support to these findings. Frequent topical application of the carcinogen on the buccal mucosa could account for the enhanced activities of both phase I and II detoxification agents in tumor bearing hamsters. In contrast, liver, is the major detoxifying organ, is unable to excrete the continuously accumulated carcinogenic metabolites produced from the metabolic activation of DMBA by the action of phase I detoxification agents. This might be the reason for the decreased activities of liver phase II detoxification agents. Emodin administration orally at a dose of 50 mg/kg bw to hamsters treated with DMBA, not only prevented the formation of tumors, but also enhanced the antioxidant defense system and modulated the detoxification agents towards the suppression of DMBA mediated oral carcinogenesis.

Immunohistochemistry and western blotting in addition to histopathological examinations are serving as a useful strategy for therapeutic interventions. Immunohistochemistry plays a critical role in the investigation of expression of molecular markers in the tumor tissues and is easier to perform as well

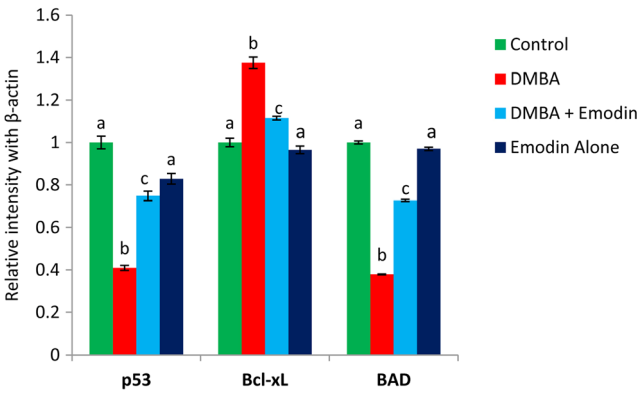

Fig. 8 Densitometric analysis of p53, Bcl-xL and BAD values are normalized with β-actin. Data presented are the mean ± SD (n = 10). Common superscripts between two groups - not significant. Different superscripts between two groups - significant $p < 0.05$

as less expensive. p53 is one of the most common tumor suppressor genes and plays a vital role in several cellular pathways including, apoptosis, cell cycle regulation and signal transduction. The cell signalling pathways triggered by cellular stresses are critically regulated by p53. p53 upregulates Bax and downregulates Bcl-2 during apoptosis [44]. Around 50% of human tumors revealed mutations or deletions in the gene encoding p53 protein [45]. A large number of studies pointed out downregulation of p53 and Bax and upregulation of Bcl-2 in hamsters bearing oral tumors [35, 36]. Loss of p53 gene is recognized as a common characteristic feature in the poorly differentiated squamous cell carcinoma [46]. In contrast to accumulation of mutant p53 proteins in immunohistochemsitry studies, the present study observed a reduction in wild type p53 protein expression in the tumor tissues. Caspases, especially caspase-3 and 9 perform a pivotal role in stimulating the signals of apoptosis by mediating Bcl-2 cleavage. Lowered activities of caspase 3 and 9 have been reported in various cancers, including oral cancer [37, 46, 47]. Our results are in line with these findings.

Apoptotic markers such as Bax deserve vast attention due to its key role in the apoptotic process. It has been reported that the pro-apoptotic proteins such as Bax are the major transcriptional targets of p53 [44, 46]. Bose et al., [48] pointed out Bax as an independent prognostic molecular marker in oral carcinoma and its expression was associated with disease-specific survival. It has been reported that the Bax expression was exclusively cytoplasmic in oral squamous cell carcinoma [46, 48]. While Bcl-2 is involved in the activation of apototic pathway, Bax inhibits the pathway. Bcl-2 protects the cells that are prone to apoptosis by enhancing their survival. Higher expression of Bcl-2 has been reported in malignant salivary gland tumors [49]. Overexpression of Bcl-2 has been reported in oral dysplastic epithelium [50]. Rahmani et al., [51] suggested that Bcl-2 was overexpressed in oral squamous cell carcinoma, which was due to loss of PTEN activity accompanied by Akt activation. An imbalance in Bcl-2/Bax ratio is a common observation noticed in several types of tumors, including oral tumors [46, 47]. The present study observed higher expression of Bcl-2 and lowered expression of Bax in the buccal mucosa of all the hamsters treated DMBA alone.

Bcl-2 related gene long isoform (Bcl-xL) plays a critical role in the preservation of mitochondrial membrane integrity by interfering directly with the Bax. Tumors that express Bcl-xL abnormally were suggested to have a worse prognosis [52]. BAD, a member of Bcl-2 family plays a crucial role in the regulation of apoptosis. BAD promotes apoptosis by inhibiting the functions of Bcl-2 and Bcl-xL [53]. The development of B-cell lymphoma has been reported in BAD- deficient mice [53]. BAD, in addition to apoptosis regulation, plays a critical role in cell proliferation and tumor progression

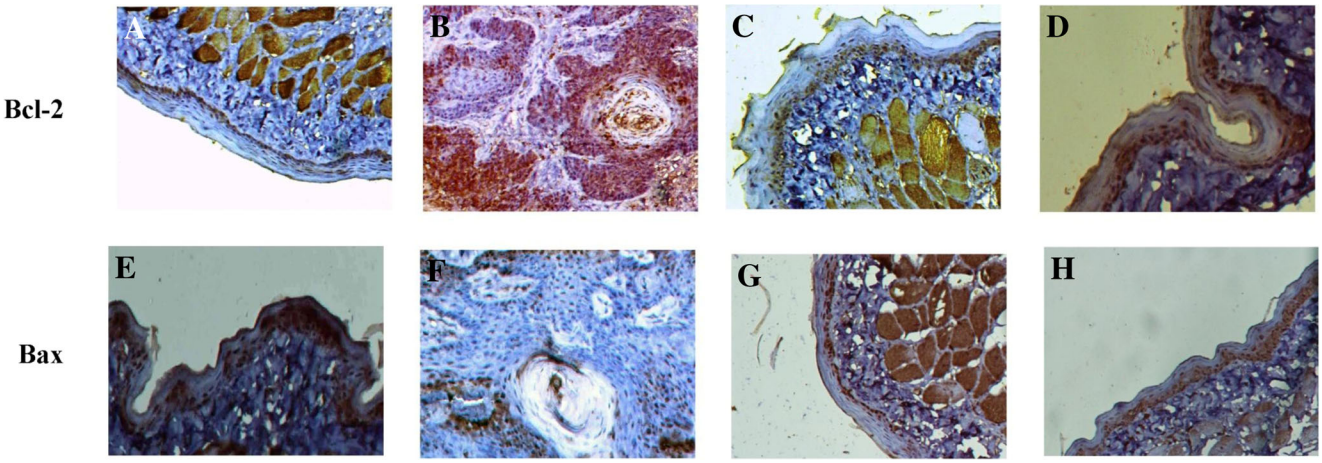

Fig. 9 Immunoexpression Pattern of Bcl-2 and Bax proteins observed in the buccal mucosa of control and experimental hamsters in each group. Bcl-2: **a** and **d** Control and Emodin alone (expression not detectable); **b** DMBA alone (over expression); **c** DMBA + Emodin (down regulated). Bax: **e** and **h** Control and Emodin alone (nuclear expression positive), **f** DMBA alone (nuclear expression negative), **g** DMBA + Emodin (nuclear and cytoplasmic expression positive)

[52, 53]. Yancey et al., [54] pointed out that BAD dephosphorylation in prostate cancer cells induced rapid apoptosis. Jiang et al., [55] reported that overexpression of BAD stimulated apoptosis and has negative impact on tumor cell proliferation and progression. Higher expression of proapototic (p53, Bax and BAD) proteins accompanied by decreased expression of the antiapoptotic protein (Bcl-2) in DMBA + Emodin treated hamsters would clearly imply that Emodin might have modulated the apoptotic pathway towards the inhibition of oral tumor formation in the buccal pouches of golden Syrian hamsters. Moreover, the proteolytic network caspases were also modulated by Emodin towards inhibition of cell proliferation as evidenced by increased expression of caspase 3 and 9 in DMBA + Emodin treated hamsters.

The present study thus explores the chemopreventive potential of Emodin in DMBA induced hamster buccal pouch carcinogenesis. The antioxidant and pro-apoptotic efficacy of Emodin might have contributed to the prevention or suppression of DMBA induced oral carcinogenesis. The chemopreventive potential of Emodin might be due to the 1st,6th and 8th phenolic hydroxyl groups present on its molecular structure.

Conclusion

An interesting observation noticed in the present study is the ability of Emodin to completely inhibit the formation of DMBA-induced tumors in the buccal pouches of golden Syrian hamsters. However, the present study noticed severe hyperplasia and mild to moderate dysplasia in DMBA + Emodin treated animals, which suggest that Emodin might have significantly delayed or suppressed the tumor formation. Further studies are warranted to confirm the Emodin efficacy by extending the duration of the experimental period.

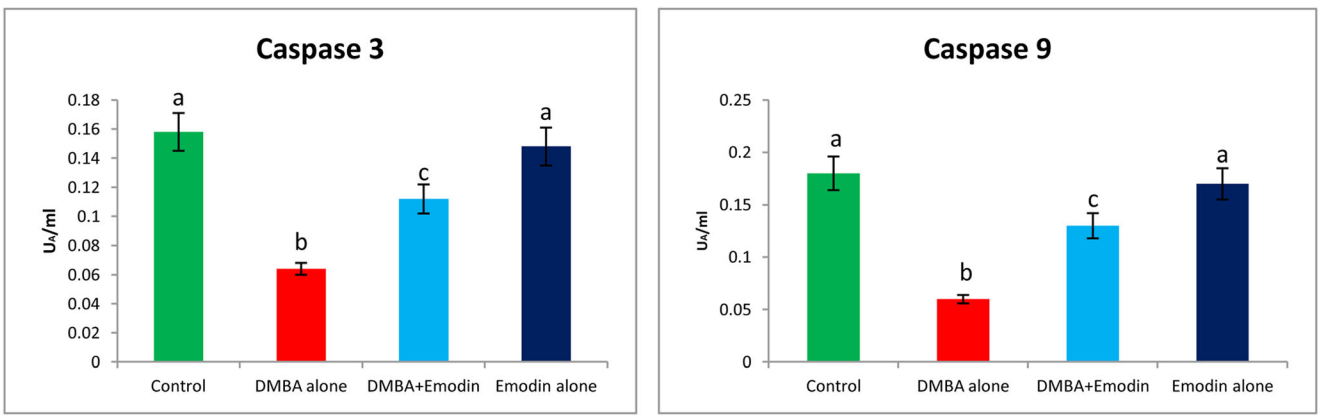

Fig. 10 Buccal mucosa Caspase-3 and 9 activities in control and experimental hamsters in each group (n = 10). Values are expressed as mean ± SD. Values that do not share a common superscript letter between groups differ significantly at $p < 0.05$

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