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Research Article

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Protective efficacy of Emodin on the expression of inflammatory and angiogenic markers in experimental oral carcinogenesis

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ABSTRACT

The major aim of the present study is to focus the anti-inflammatory and anti-angiogenic potential of Emodin in 7, 12-dimethylbenz[a]anthracene (DMBA) induced oral carcinogenesis in the golden Syrian hamsters. Oral squamous cell carcinoma was induced in the buccal pouches of hamsters using the organ and site specific carcinogen, DMBA. Tumor incidence in DMBA alone and DMBA + Emodin treated hamsters was found to be 100% and 0% respectively. A mild to moderate preneoplastic lesions was, however, observed in the buccal mucosa of DMBA + Emodin treated hamsters. Emodin suppressed the formation of tumors by down-regulating the expression of inflammatory (NFκB, COX-2, iNOS, IL-6 and IL-10) and angiogenic (VEGF) markers in DMBA treated hamsters. To conclude, Emodin has exerted a potent anti-inflammatory and anti-angiogenic properties during DMBA induced oral carcinogenesis.

Key words: Inflammation, Angiogenesis, Oral carcinoma, Emodin, Hamsters.

INTRODUCTION

Cancer of the oral cavity affects the life quality of the patients and also threatens their survival outcome. It arises mainly due to ill-habits such as chewing of tobacco, betel nut, and areca nut, smoking cigars, bidis and cigarette and alcohol abuse [1]. The prevalence of oral cancer is steeply increasing worldwide, most commonly in developing countries, including India [2]. Abnormalities in the various molecular pathways, including inflammatory and angiogenic pathways has been reported in oral carcinogenesis [3]. Although several experimental models are employed to study the chemopreventive, biochemical and molecular efficacy of natural products in oral carcinogenesis, DMBA-induced oral carcinogenesis is the most preferred model due to its histopathological similarities with human oral tumors [4].

Emodin (Fig. 1) is one of the natural products widely used in Traditional Chinese medicine to treat various disorders [5]. It is abundantly present in the plants *Rheum palmatum*, *Kalimeris indica*, and *Ventilago madraspatana* [6-8]. The pharmacological and biochemical effects of Emodin have been well documented. It has been reported that Emodin significantly reduced the blood sugar level in experimental animal model [9]. Bhadauria [10] reported the hepatoprotective effect of Emodin in animal model. The antioxidant property of Emodin has also been documented [11]. *In vitro* studies explored the cytotoxic potential of Emodin in various cancer cell lines [12]. The objective of this study is to reveal the anti-inflammatory and anti-angiogenic properties of Emodin in DMBA induced oral carcinogenesis in golden Syrian hamsters.

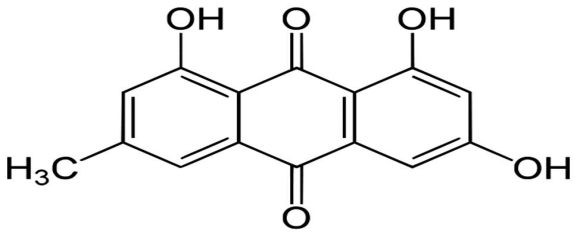

Fig. 1: The molecular structure of Emodin

EXPERIMENTAL SECTION

To explore the anti-angiogenic and anti-inflammatory potential of Emodin, the present study has utilized hamster buccal pouch carcinogenesis induced by DMBA as an experimental animal model. Golden Syrian hamsters, (Source: National Institute of Nutrition, Hyderabad) were divided into four groups and all the animals were maintained in the Annamalai University Animal House as per the ethical principles. All the four groups of animals received adequate pellet diet and water *ad* libitum. The animals were sacrificed (cervical dislocation) at the end of the experimental period and the buccal mucosa was excised and subjected to Western blotting and immunohistochemistry as described earlier [13-14]. The experimental design of the present study is depicted in fig. 2.

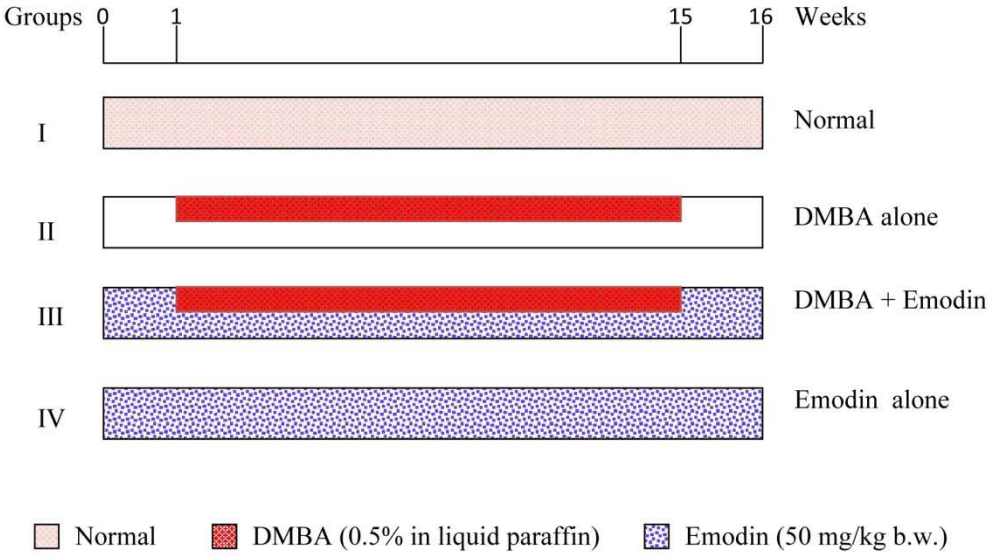

Fig. 2: The experimental design

Western blotting

Briefly, the protein bands obtained after the separation of proteins (PAGE) were treated with corresponding primary antibodies (VEGF, iNOS, IL-6 and IL-10: Cell Signaling Technology, Danvers, MA, USA). It was then incubated with horseradish peroxidase labeled secondary antibodies, followed by the enzyme substrate diaminobenzidine. The bands were scanned and analyzed densitometrically (Bio-Rad Image Lab™ software version 4.1 software).

Immunohistochemistry

After the routine procedure, the slides containing tissue sections were exposed to their corresponding primary antibodies (NFκB, COX-2: Dako, Carprinteria, CA, USA). The slides were then treated with secondary antibodies (horse radish peroxidase labeled), followed by incubation with the enzyme substrate, diaminobenzidine. The expression of the markers was examined under the microscope (Nikon Eclipse TS100 Microscope), after counter staining with hematoxylin.

RESULTS AND DISCUSSION

The status of inflammatory (NFκB, COX-2, iNOS, IL-6 and IL-10) and angiogenic (VEGF) markers expression in the buccal mucosa of golden Syrian hamsters was utilized to assess the anti-inflammatory and anti-angiogenic potential of Emodin in the DMBA treated hamsters (Figs. 3-5). Western blotting (VEGF, iNOS, IL-6 and IL-10) and immunohistochemistry (NFκB and COX-2) were employed to assess the expression pattern of the molecular

markers. Tumor bearing hamsters' (DMBA alone treated) buccal mucosa explored up regulation/over expression of the above markers as compared to control hamsters. Oral administration of Emodin modulated the expression of all the above mentioned markers towards their normal expression pattern (i.e. down-regulated the expression) in the DMBA treated hamsters.

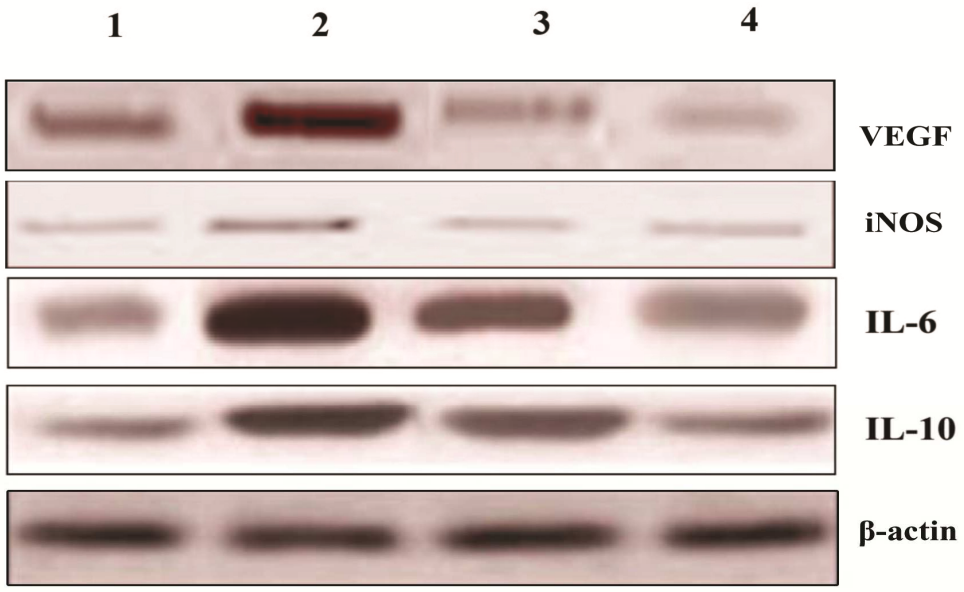

Fig. 3: Expression pattern of VEGF, iNOS, IL-6 and IL-10 in the buccal pouch tissues of control and experimental animals in each group
Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + Emodin, Lane 4: Emodin alone.

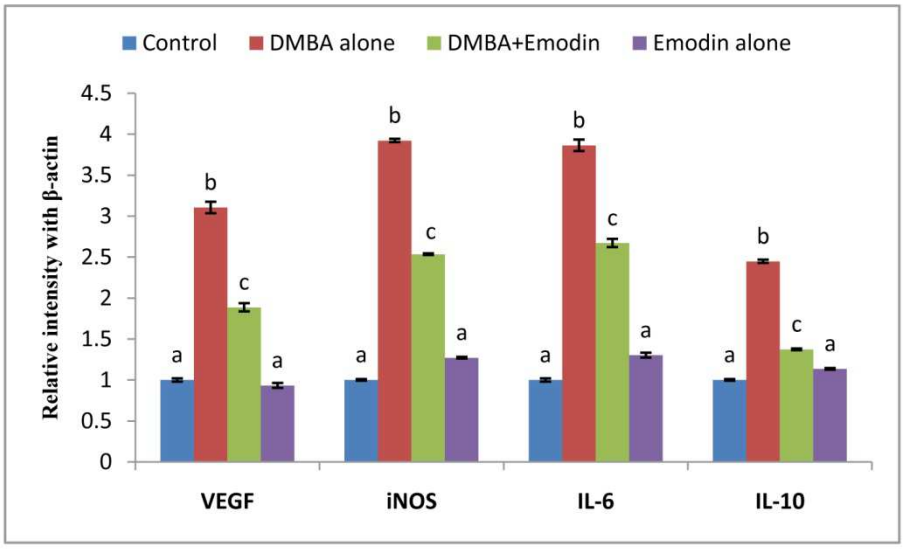

Fig. 4: Densitometric analysis of protein expression after normalization to β-actin in the buccal pouch tissues of control and experimental animals in each group
Data presented are the mean ±SD (n=10).
Common superscripts between two groups - not significant. Different superscripts between two groups - significant ($p<0.05$)

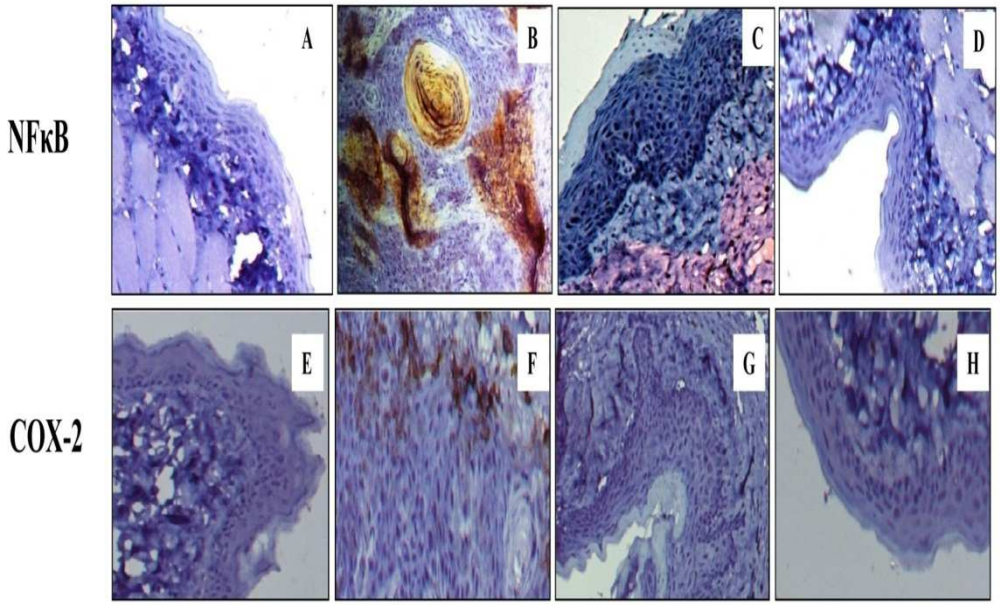

Fig. 5: Immunoexpression pattern of NF-κB and COX-2 proteins observed in the buccal mucosa of control and experimental hamsters in each group

NFκB: A and D *- Control and Emodin alone (expression not detectable),* ***B*** *- DMBA alone (over expression),* ***C*** *- DMBA + Emodin (down-regulated)*

COX-2: E and H *- Control and Emodin alone (expression not detectable);* ***F*** *- DMBA alone (over expression);* ***G*** *– DMBA + Emodin (down-regulated)*

Inflammation plays a vital role in the process of neoplastic transformation and up regulation of inflammatory markers favors tumor promotion, progression and metastasis [15]. Though there are several types of inflammatory mediators, NFκB, COX-2 and iNOS play critical and crucial role in the inflammatory processes. A multiple molecular markers including, NFκB, COX-2 and iNOS has been utilized as a molecular prognostic markers of oral carcinogenesis [16]. Inflammation is associated with all the three distinct phases (initiation, promotion, progression) of carcinogenesis [17]. Profound studies pointed out that DMBA mediates oral carcinogenesis via inducing chronic inflammation in the buccal mucosa [18].

NFκB, a redox sensitive transcription factor, plays an important role in the regulation of several genes, including genes involved in the inflammation, cell adhesion and proliferation [19]. NFκB has been documented to play a pivotal role in the regulation/transactivation of multiple genes including, COX-2, TNFα, iNOS, Bcl-2 and VEGF [20]. Activation of NFκB and COX-2 is associated with inflammation, apoptosis, angiogenesis and tumorigenesis [21]. Extensive studies reported that NFκB serve as an anti-apoptotic protein in carcinogenesis [22]. Abnormal expression of NFκB in turn up regulates its downstream genes cyclooxygenease-2 (COX-2), iNOS and cytokines [23].

Cyclooxygenase plays a pivotal regulatory role in the formation of several important biological mediators, including thromboxane, prostaglandin and prostacyclins [24]. COX-2 is not only involved in the inflammatory cascade but also plays a critical role in the initiation and progression of various carcinogenesis, including oral cancer [25]. COX-2 proteins are the major contributing factors in the process of tumor associated inflammation [26]. COX-2 up regulation plays a crucial role in the angiogenic process and apoptotic inhibition [27]. Byatnal et al., [28] highlighted COX-2 as an imperative biomarker of oral carcinogenesis. They suggested that COX-2 over expression was associated with tumor grading and patients' survival outcome. Higher expression of COX-2 has been documented in the inflammatory and tumour tissues [29]. A positive correlation between COX-2 over expression and local recurrence of tumor has been shown [30]. Abnormal expression of COX-2 mediates tumor invasion by increasing the activity of matrix metalloproteinases [31]. Higher expression of COX-2 has been reported in several epithelial tumors [32]. Abnormal expression of COX-2 and NFκB in oral cancer tissues resulted in treatment resistance [33].

iNOS, one of the isoforms of nitric oxide synthase, plays a key role in the production of endogenous nitric oxide. iNOS expression is associated with all the three stages of malignancy [34]. Abnormal expression of iNOS has been pointed out in several malignant cancers, including head and neck carcinoma [35]. Connelly et al., [36] have shown higher expression of iNOS in oral carcinoma. iNOS expression has been connected with tumor staging and metastasis as well [37]. Yang et al., [38] suggested that the survival outcome of the oral cancer becomes shorter if

their tumors have abnormal iNOS expression. Elevated iNOS expression has been shown in human and animal oral tumor tissues [39]. Tumor promotion and progression occurs in parallel with iNOS expression. A large number of studies claimed iNOS expression as a target for cancer chemoprevention [40].

Inflammatory cytokines and chemokines always occupy the surrounding of tumor cells to promote and progress the carcinogenesis [41]. Multiple biological activities of IL-6 includes, role in B- cell maturation, cell survival and proliferation and inhibition of apoptosis [42]. Growth factor role of IL-6 has been reported in various tumors [43]. Higher expression of IL-6 has been linked with poor prognosis in colon, mammary and lung cancer patients [44]. IL-6 serves a key role in cell-cell signaling messengers and in the activation of NFκB [45]. Rhodus et al., [46] showed over expression of IL-1, IL-6 and IL-8 in human oral cancer cell lines. IL-6 is one of the inflammatory cytokines responsible for the inflammation-driven oral carcinoma [43]. Tumor cells release IL-6, a pro-inflammatory cytokine, which play an important role in the chemoresistance [17]. IL-6 was found to be abnormally expressed in several cancers, including mammary, esophageal and oral cancers [47]. Gasche et al., [48] reported that IL-6 stimulated carcinogenesis in oral carcinoma cells by altering DNA methylation. An increased level of IL-6 has been documented in the blood and saliva of the oral cancer patients [49]. IL-10 has a crucial role in the abnormal proliferation of several tumors, including lung, gastric and skin carcinoma [50]. IL-10 promotes angiogenesis and inhibits apoptosis in lung tumors [51].

The formation of new blood vessels from the pre-existing blood vessel system is termed as angiogenesis [52]. It is an essential phenomenon to meet the nutritional and oxygen demand of the growing tumors [53]. Although more than 20 angiogenic stimulating factors are available, the most important one is Vascular Endothelial Growth Factor (VEGF) [54]. VEGF, a 46 K Da glycoprotein, plays a critical role in the stimulation of endothelial cell differentiation and proliferation [55]. Extensive studies reported VEGF over expression in several types of solid tumors, including oral carcinoma [56]. Kim et al., [57] pointed out that over expression of VEGF might have played a crucial role in the progression of oral cancer. Profound studies documented that the expression of VEGF increases with increase in micro blood vessel density in various tumors including oral cancer [58]. VEGF over expression has been associated with poor prognosis [59]. Henriques et al., [60] have shown over expression of VEGF in tongue carcinogenesis. A positive association between VEGF expression and tumor stage and lymph node metastasis has been shown in head and neck cancer [61]. VEGF over expression facilitated the progression of solid tumors and their metastasis [62].

Medicinal plants and their bioactive constituents play an important role in the prevention of carcinogenesis. Researchers explore the antitumor potential of the natural products using several molecular targets (signaling pathways) [63]. The present study utilizes inflammatory and angiogenic markers to test the efficacy of Emodin's anti-inflammatory and anti-angiogenic potential in experimental oral carcinogenesis. The modulating ability of Emodin on these markers' expression were tested using immunohistochemistry and western blotting. *In vitro* and *in vivo* anti-inflammatory potential of Emodin has been demonstrated by few studies [64]. Emodin's pleiotropic potential on various molecular signaling pathways including inflammation and cancer has been well documented [65]. Emodin down-regulated the expression of IL-6, IL-10, iNOS and VEGF (as evidenced by Western blotting) an.d significantly decreased the expression of NFκB and COX-2 (as evidenced by immunohistochemistry) in the buccal mucosa of tumor bearing hamsters (DMBA alone treated hamsters). The present finding thus reveal the anti-inflammatory and anti-angiogenic potential of Emodin in DMBA induced hamster buccal pouch carcinogenesis. Although the exact mechanism for the anti-inflammatory and anti-angiogenic property of Emodin is unclear, its effect on the inhibition of DMBA induced inflammation and carcinogenesis might have played a possible role.

CONCLUSION

The present study explores, for the first time, the anti-angiogenic and anti-inflammatory potential of Emodin in DMBA induced oral carcinogenesis, as evidenced by down-regulation of NFκB, COX-2, iNOS, IL-6, IL-10 in DMBA+Emodin treated hamsters. The tumor preventive potential of Emodin is partly due to its anti-angiogenic and anti-inflammatory properties during DMBA induced oral carcinogenesis.

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