

# Modulating Effect of Ferulic Acid on NF- $\kappa$ B, COX-2 and VEGF Expression Pattern During 7, 12-Dimethylbenz(a)anthracene Induced Oral Carcinogenesis

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**Abstract:** Ferulic acid, a natural antioxidant, has the potential to prevent inflammation and to modulate angiogenesis in both *in vivo* and *in vitro* models. The present study has investigated the modulating effect of ferulic acid on the expression pattern of COX-2, NF- $\kappa$ B and VEGF during 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Over expression of COX-2, NF- $\kappa$ B and VEGF was noticed in the oral tumor tissues of hamsters treated with DMBA. Oral administration of ferulic acid at a dose of 40 mg/kg body weight to hamsters treated with DMBA completely prevented the tumor formation and downregulated the expression of COX-2, NF- $\kappa$ B and VEGF during DMBA induced oral carcinogenesis. The present results suggest that ferulic acid might have suppressed oral tumor formation by down regulating the expression of COX-2, NF- $\kappa$ B and VEGF during DMBA induced oral carcinogenesis.

**Keywords:** Cyclooxygenase-2 (COX-2), Dimethylbenz(a)anthracene (DMBA), Nuclear Factor kappa B (NF- $\kappa$ B), Oral cancer and Vascular Endothelial Growth Factor (VEGF).

## INTRODUCTION

Oral carcinoma, a malignant cancer with poor prognosis and clinical outcome, is the fifth most frequent cancer worldwide. Though the oral cancer incidence is increasing worldwide highest incidences are reported every year from developing countries especially from India, where these forms of cancers account for 40-50% of all cancer [1]. Extensive use of tobacco, betel quid and alcohol consumption are recognized as major risk factors associated with the pathogenesis of oral cancers in high prevalence areas [2]. Late diagnosis and treatment delay are responsible for poor survival rate of oral cancer patients and the five year survival rate is thus still at 50% [3]. Oral cancer arises due to accumulation of multiple genetic abnormalities and the molecules that play a critical role in cell proliferation, inflammation and angiogenesis [4].

While cyclooxygenases play a vital role in the inflammatory process and cyclooxygenase-2 (COX-2) has been concerned in the progression of carcinogenesis and angiogenesis. The activity of COX-2 is provoked by tumor promoters, growth factors and cytokines during inflammatory and carcinogenic processes [5, 6]. NF- $\kappa$ B plays a key role in the regulation of tumor survival and progression, probably mediating inflammatory process.

NF- $\kappa$ B activity is turned on by numerous stimuli which include physical and chemical stresses, viral proteins chemotherapeutic agents and ionizing radiation. It has been reported that activated NF- $\kappa$ B is involved in the transcriptional regulation of over 400 genes implicated in inflammation carcinogenesis and cell survival [7]. Over expression of NF- $\kappa$ B and COX-2 was reported in several tumors including oral cancer [8-10].

Angiogenesis, the formation of new blood vessels from pre-existing capillaries, has been documented as a fundamental aspect in tumor growth and progression [11]. VEGF, a critical angiogenic factor, perform significant role in increasing vessel permeability and enhancing endothelial cell growth, proliferation, migration, and differentiation [12]. Over expression of VEGF has been reported in several malignancies including oral carcinoma [13-16]. VEGF is thus considered and utilized as a therapeutic target for various cancers including oral cancer [17].

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid), a phenolic compound is present in large quantities in various fruits and vegetables [18]. Ferulic acid possesses a wide range of therapeutic potential, which include antioxidant, anti-inflammatory, antiaging, neuroprotective, hepatoprotective and anticarcinogenic effects [19, 20]. Balakrishnan *et al.* (2007) have shown the antigenotoxic effect of ferulic acid in 7-12-dimethylbenz(a)anthracene induced genotoxicity has been reported [21]. Alias *et al.* reported the protective effect of ferulic acid on DMBA induced skin carcinogenesis in swiss albino mice [22].

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Previous studies from our laboratory have also shown the chemopreventive potential of ferulic acid against oral and mammary carcinogenesis [23, 24]. Recently we have shown the proapoptotic and anti-cell proliferative efficacy of ferulic acid against DMBA induced hamster buccal pouch carcinogenesis [25, 26]. The present study explores the anti-cell proliferative efficacy of ferulic acid against 7, 12-dimethylbenz(a)anthracene inducing hamsters buccal pouch carcinogenesis.

## ANIMALS

Male golden Syrian hamsters, eight weeks old, weighing 80-120g (information provided by the breeder) were purchased from National Institute of Nutrition, Hyderabad and housed in polypropylene cages at room temperature ( $27\pm 2^{\circ}\text{C}$ ) with relative humidity  $55\pm 5\%$ , in the Central Animal House, Annamalai University. The animals were provided with standard pellet diet and water *ad libitum*. Annamalai University Animal Ethical Committee (Register number 160/1999/CPCSEA), Annamalainagar, India approved the experimental design (proposed no.872, dated 29.05.2012).

## TUMOR INDUCTION

Topical application of 0.5% DMBA in liquid paraffin three times a week for 14 weeks developed oral squamous cell carcinoma in the buccal pouch of golden Syrian hamsters.

## EXPERIMENTAL DESIGN

Forty golden Syrian hamsters were categorized into four groups of ten hamsters in each. The experimental hamsters were allowed to acclimatize the new environment for about one week before the experimental work was started. Group I hamsters served as control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Groups II and III hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group II hamsters received no further treatment. Group III hamsters received oral administration of ferulic acid at a dose of 40mg/kg bw, starting one week before the exposure to the DMBA and continued on days alternate to DMBA painting until one week after the final exposure of the DMBA. Group IV hamsters received oral administration of ferulic acid alone throughout the experimental period. The experiment was terminated at the end of 16<sup>th</sup> week and all animals were sacrificed by cervical dislocation. Animals were categorized

based on their age and body weight. All the animals are inbred, and may not cause difference in results.

## IMMUNOHISTOCHEMICAL EXPRESSION OF VEGF

The antigen that was retrieved from buccal mucosa tissue section was incubated with the respective primary antibody specific to VEGF over night at  $4^{\circ}\text{C}$ . The immune complex a subsequently incubated with the secondary antibody conjugated with horseradish peroxidase for 30 minutes at room temperature. The antigen-antibody complex was than detected using 3, 3'-diaminobenzidine, the substrate of horseradish peroxidase. As soon as acceptable color intensity was attained, the slides were counter stained with hematoxylin [27].

## COX-2 ACTIVITY BY ELISA

Buccal mucosa COX-2 activity was assayed colorimetrically by monitoring the appearance of oxidized N, N, N', N'-tetramethyl-P-phenylenediamine (TMPD) at 590nm using ELISA kit for Cyman's COX activity assay kit for COX-2 [28].

## EXPRESSION OF NF- $\kappa$ B USING REAL-TIME PCR

The total RNA was isolated from the buccal mucosa and reverse transcribed to cDNA with random primers (Table 1) using high cDNA. Reverse Transcriptase kit-cDNA was amplified using a thermal cycler to measure the expression pattern of NF- $\kappa$ B, with  $\beta$ -actin as a control, with SYBRA green fluorophore following the manufacture's recommended amplification procedure. The relative quantification of NF- $\kappa$ B was measured using threshold cycle (CT) method [29].

## STATISTICAL ANALYSIS

The data is expressed as mean  $\pm$  SD. One way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) was used to performed statistical comparison between the groups. The obtained results were considered statistically significant if the P values were less than 0.05.

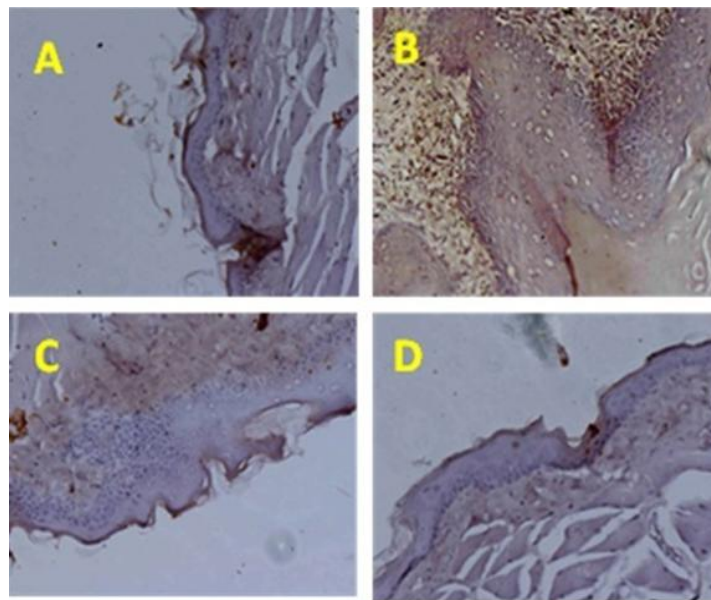
## RESULTS

### The Immunoexpression Pattern of Angiogenic Marker

The VEGF expression pattern of control and experimental hamsters in each group was depicted in Fig. (1). DMBA alone treated hamsters revealed over

**Table 1. List of primers used for Real-time PCR.**

Genes	Primers	Sequences
$\beta$ -actin	Forward	5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3'
	Reverse	5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3'
NF- $\kappa$ B	Forward	5'-ATGGACGATCTGTTTCCCCT-3'
	Reverse	5'-CGGTTTACTCGGCAGATCTT-3'



A and D - Control and ferulic acid alone, B - DMBA alone, C - DMBA + ferulic acid.

**Fig. (1).** Immunoexpression pattern of VEGF protein observed in the buccal mucosa of control and experimental hamsters in each group.

expression of VEGF in their buccal mucosa. Ferulic acid administration to the hamsters treated with DMBA remarkably decreased the expression of VEGF in their buccal mucosa. Ferulic acid alone treated hamsters exhibited expression similar to that of control hamsters.

**Activities of COX-2 by Enzyme Linked Immunosorbent Assay [ELISA]**

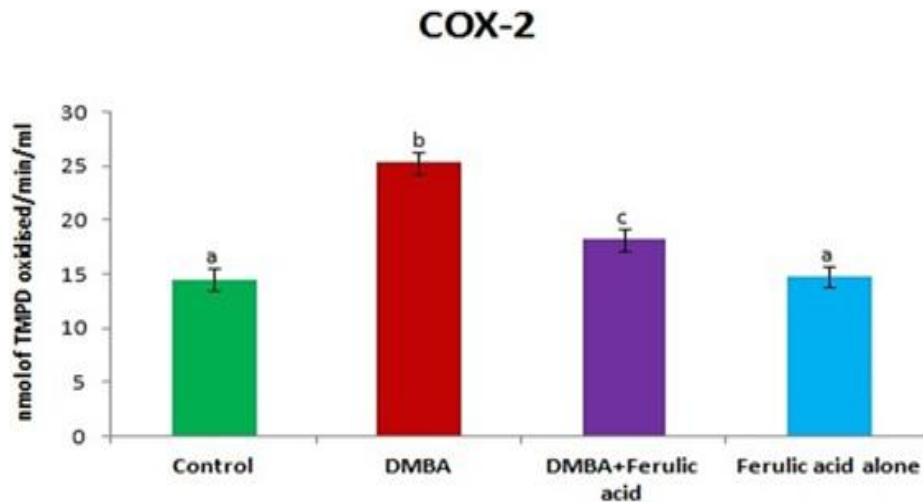
The status of COX-2 in control and experimental hamsters in each group is shown in Fig (2). COX-2 activity was increased in hamsters treated with DMBA alone. Oral administration of ferulic acid at a dose of 40mg/kg bw hamsters treated with DMBA restored the COX-2 activity to near normal range.

**Fold Increase in the NF- $\kappa$ B mRNA Expression Pattern Using Real – time PCR**

The primer melting curve and fold increase in the NF- $\kappa$ B mRNA expression pattern of control and experimental hamsters in each group is depicted in Figs. (3 & 4). Hamsters treated with DMBA alone showed over expression of NF- $\kappa$ B mRNA, which was brought back to near normal pattern in DMBA+ ferulic acid treated hamsters. Similar NF- $\kappa$ B mRNA expression pattern was observed in control hamsters and hamsters treated with ferulic acid alone.

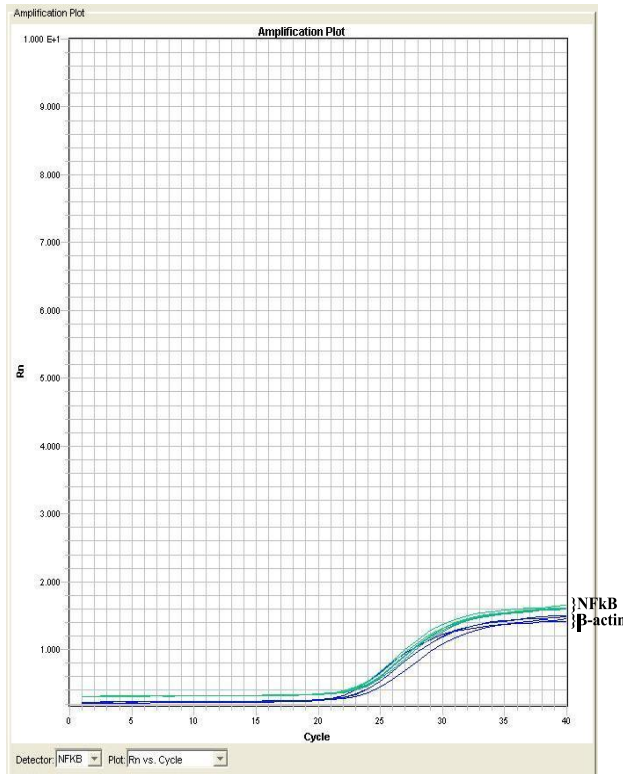
**DISCUSSION**

In the present study, we noticed upregulation of NF- $\kappa$ B, COX-2 and VEGF in the buccal mucosa of hamsters treated



Values are expressed as mean  $\pm$  SD for 10 hamsters in each group. Values that do not share a common superscript letter between groups differ significantly at  $p < 0.05$ . (Analysis of variance followed by DMRT)

**Fig. (2).** The status of COX-2 in control and experimental hamsters in each group.



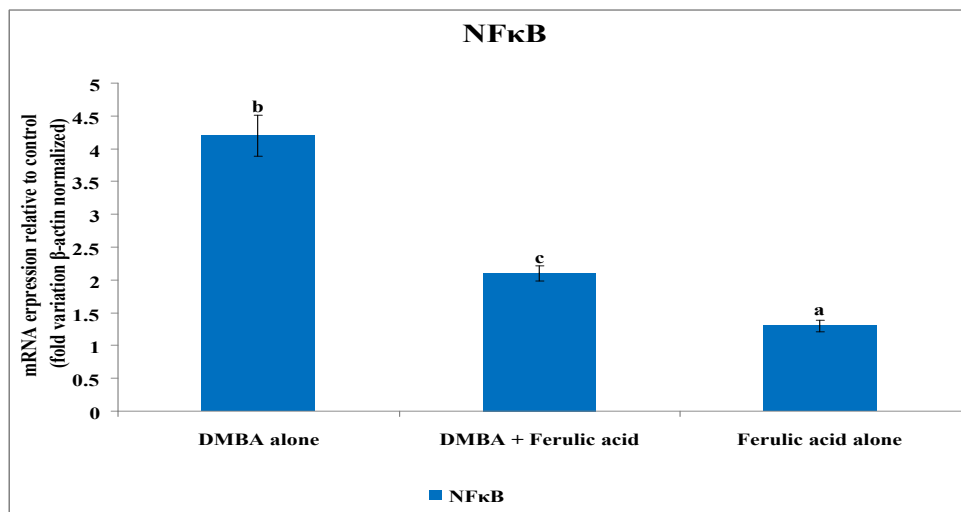
**Fig. (3).** RT-PCR primers melting curve for Nf-κB mRNA expression pattern of control and experimental hamsters in each group.

with DMBA alone. Oral administration of ferulic acid down regulated the above biomarkers in hamsters treated with DMBA. Diverse studies envisaged inflammation as a possible trigger of cancer development. In the present study, we investigated the expression of a panel of biomarkers (VEGF, COX-2, NF-κB) that are associated with

carcinogenesis. Mounting evidences also highlighted the significance of inflammation during various stages of carcinogenesis [30, 31]. A large number of studies have utilized NF-κB and COX-2 as a target for cancer prevention [32, 33]. Extensive studies pointed out that agents that can able to inhibit the expression of NF-κB and COX-2 could serve as an anticancer agent [34-36].

NF-κB regulates the survival and progression of tumor cell by continuously triggering inflammation. NF-κB and cyclooxygenase-2 activity is enhanced by several stimuli that are associated with inflammatory processes [37]. It has been reported that NF-κB inhibits apoptotic pathway by stimulating the expression of BCL-2 and caspases [38]. Upregulation of COX-2 expression is often a common phenomenon during inflammation and carcinogenesis [39]. COX-2 expression is associated with invasion and metastasis in patients with gastric and colon cancer [40, 41]. Over expression of COX-2 is not only associated with tumor progression and metastasis but also involved in tumor angiogenesis [42]. COX-2 maintains cell survival during carcinogenesis by modulating apoptotic proteins. Over expression of COX-2 and NF-κB were reported in both precancerous and cancerous lesions [43, 44].

Investigation of angiogenic inhibitor could play an important role in cancer therapy and improve clinical outcome. Angiogenesis, a fundamental phenomenon of tumorigenesis, is involved in the progression of tumors and metastasis [45]. Angiogenesis in tumor cells aid for its spread as well as to meet nutrient and oxygen demand for the growth. In recent years, researchers focused their attention to investigate the drugs to inhibit the expression of angiogenic factors, which could provide a valuable therapeutic target for cancer treatment [46, 47]. VEGF, a powerful angiogenic factor, has been implicated in the pathogenesis of neoangiogenesis in various malignancies including oral carcinoma. Over expression of VEGF was reported in epithelial tumors. Mounting evidences pointed out that over



Values are expressed as mean ± SD for 10 hamsters in each group values that do not share a common superscript letter between groups differ significantly at  $p < 0.05$  (DMRT)

**Fig. (4).** Fold increase in the mRNA expression pattern for NF-κB in hamsters treated with DMBA alone, DMBA + Ferulic acid and Ferulic acid alone.

expression of VEGF either in circulation or in tumor tissues is associated with poor prognosis as well as tumor progression of several cancers including oral cancer [48-50].

Medicinal plants or their active constituents that possess anti-inflammatory and anti-angiogenic activity could improve the therapeutic outcome in cancer patients. In the present study, ferulic acid administration at a dose of 40 mg/kg bw down regulated the expression of NF- $\kappa$ B, COX-2 and VEGF in hamsters treated with DMBA. Present results reveal that ferulic acid might have inhibited the inflammatory and angiogenic pathway during DMBA induced oral carcinogenesis. Previous studies from our laboratory demonstrated apoptotic and anticell proliferative potential of ferulic acid during DMBA induced oral carcinogenesis. Down regulation of NF- $\kappa$ B and COX-2 observed in the DMBA+ ferulic acid treated animals suggested that NF- $\kappa$ B and COX-2 inhibition might have enhanced the apoptotic potential of ferulic acid during oral carcinogenesis. Ferulic acid also prevented tumorigenesis in several types of experimental carcinogenesis including oral cancer.

Islam *et al.* [51] reported that phytosteryl ferulate significantly inhibited the expression of NF- $\kappa$ B in LPS - stimulated RAW 264.7 macrophages. Jung *et al.*, [52] suggested that dietary ferulic acid suppressed NF- $\kappa$ B activity through inhibition of NIK/IKK and MAPK *in vivo*. It has been reported that ferulic acid augmented angiogenesis *via* VEGF, PDGF and HIF-1 $\alpha$  in human umbilical vein endothelial cells [53, 54]. In contrast, we noticed a down regulation of VEGF in DMBA + ferulic acid treated animals. This is probably due to suppression of NF- $\kappa$ B and Cyclin D1 by ferulic acid during DMBA induced oral carcinogenesis. Srivastava *et al* [55, 56] explored glutathione peroxidase, glutathione-S-transferase and glutathione reductase as a major biomarkers in the diagnosis as well as therapeutics of oral cancer.

Our present study concludes that the anti-inflammatory and anti-angiogenic effect of ferulic acid might have served as an important phenomenon in the suppression of or delayed the formation of oral tumors in the buccal mucosa of hamsters treated with DMBA.

### Limitation of the Study

In the present study, we have not measured the ferulic acid content in the blood of experimental animals along with the parameters related to inflammation, and angiogenesis.

### Future Direction

The results of the present study highlight the significance of ferulic acid in therapeutic intervention methodology against oral carcinogenesis in which angiogenesis and inflammation play a key role. The present study may thus warrant to consider ferulic acid as a promising candidature for oral cancer treatment along with currently available therapeutic drugs. Efforts will therefore be taken up to explore the anti-inflammatory anti-angiogenic potential of ferulic acid in human carcinogenesis.

### CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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