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Original Article

Modulating Effect of *Enicostemma littorale* on the Expression Pattern of Apoptotic, Cell Proliferative, Inflammatory and Angiogenic Markers During 7, 12-Dimethylbenz (a) Anthracene Induced Hamster Buccal Pouch Carcinogenesis

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ABSTRACT

Enicostemma littorale leaves are traditionally used for the treatment of several diseases, including inflammation and cancer. This study has taken effort to explore the antitumor initiating potential of *E. littorale* leaves (ElELet) by analyzing the expression pattern of apoptotic (p53, Bcl-2 and Bcl-2 associated X-protein), cell-proliferative (cyclin D1 and proliferating cell nuclear antigen), angiogenic (vascular endothelial growth factor), invasive (matrix metalloproteinase-2 and 9), and inflammatory (NF-κB and cyclooxygenase-2) markers during 7, 12-dimethylbenz (a) anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Oral tumors were induced in the buccal pouches of hamsters using the potent site and organ specific carcinogen, DMBA. DMBA application 3 times a week for 14 weeks resulted in tumor formation in the buccal pouches. Hundred percent tumor formations with dysregulation in the expression pattern of apoptotic, cell proliferative, inflammatory, angiogenic, and invasive markers were observed in the buccal pouches of hamsters treated with DMBA alone. ElELet at a dose of 250 mg/kg body weight orally to DMBA treated hamsters significantly prevented the tumor formation as well as corrected the abnormalities in the expression pattern of above mentioned molecular markers. ElELet thus modulated the expression pattern of all the above mentioned molecular markers in favor of the suppression of cell proliferation occurring in DMBA induced hamster buccal pouch carcinogenesis.

Key words: Angiogenesis, apoptosis, cell proliferation, *Enicostemma littorale,* inflammation, oral cancer

INTRODUCTION

The term "oral cancer" refers to a malignant neoplasm of the oral cavity, which includes cancer of the tongue, cheek, lower and upper gingival, floor of the mouth, lip and palates. Though oral cancer incidence shows a male preponderance in men in many countries, both men and women are equally affected in India. Cancer of the oral cavity accounts for around 40–50% of all malignancies in India. Excellent efforts have been taken in India to reduce

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the oral cancer incidence in Indian populations by creating social awareness of oral cancer through prevention program as well as by restricting the sale and use of tobacco, the major risk factors of oral cancer.[1,2]

7, 12-dimethylbenz (a) anthracene (DMBA), a well-known site-specific procarcinogen, is widely and commonly used to induce oral tumors in the buccal pouch (a pocket like anatomy) of golden Syrian hamsters. Topical application of DMBA 3 times a week for 14 weeks developed oral squamous cell carcinoma in the buccal pouches of hamsters. DMBA induced oral tumors mimic morphological, histological, biochemical, and molecular alterations of human oral cavity tumor. Chronic inflammation, sequential morphological and histopathological alterations, extensive DNA damage and over production of reactive oxygen species are the documented mechanisms for DMBA induced oral carcinogenesis.[3,4]

Genetic instability has been regarded as a predisposing factor for neoplastic transformation. Recent advances in molecular biology technique provide an insight into the process of neoplastic transformation and its promotion and progression. Oral carcinogenesis occurs due to multiple alterations in genes that control cell proliferation and differentiation. Accumulation and series of mutations in the DNA structure results in malignant transformation. Such abnormalities may also cause defects in cell differentiation, cell proliferation and apoptosis. Mounting evidences illustrated clearly that neoplasm arises due to defect in apoptotic, cell proliferative and angiogenic pathways.[5,6]

Recent cancer chemoprevention studies utilized apoptotic proteins as a target and plausible strategy to focus the anticancer potential of the test compound. p53 has multiple biological activities and plays an important role in the regulation of cell cycle. For its crucial role in apoptosis, cell-cycle, and cell proliferation, p53 is recognized as a molecular policeman and guardian of the genome. p53 mutation was associated with tobacco smoking and alcohol drinking.[7] Researchers suggested to utilize an immunohistochemical evaluation of p53 to assess the prognosis of oral carcinoma. Wild type p53 has a short half-life and thus p53 detected in tumor tissues using immunohistochemistry is a mutant one.

Bcl-2 family proteins play an important role in the cell death regulation. While some of the members of Bcl-2 gene family mediate anti-apoptotic process, others are involved in the pro-apoptotic process. Mitochondrial apoptosis is regulated by Bcl-2 family proteins. Extensive studies on Bcl-2 expression suggested that Bcl-2 can be used as a diagnostic and prognostic marker in carcinogenesis.[5,6] Bcl-2 expression was abnormal in several cancers, including oral carcinoma.[8] Bcl-2 associated X-protein (Bax) belongs to the Bcl-2 family, stimulates the mitochondrial outer membrane permeabilization to effectively induce apoptosis. Bax has a crucial role in the regulation of apoptosis. Dysregulation of Bax expression has been reported in several precancerous and cancerous lesions of various cancers including oral cancer.[9] Bax serves as a proapoptotic protein and is downregulated in various carcinogenesis.[10]

Proliferating cell nuclear antigen (PCNA), a 36 kd protein, has a crucial role in cell cycle regulation and its expression is associated with the proliferation and differentiation of cells. PCNA also has a role in the DNA repair mechanism where its involved in the excision and replacement of abnormal nucleotides. Profound studies pointed out dysregulation in G1/S checkpoint in various epithelial tumors and confer a growth advantage to the existing tumors.[11] Cyclin D1 has a vital role in the regulation of G1 to S transition in the cell cycle. It is a 45 kd protein located on chromosome 11q13. Baldin *et al*.,[12] reported that the nuclear protein cyclin D1 is required for the progression of cell cycle in the G1 phase. Zhao *et al*.,[13] reported that cyclin D1 expression correlates with the determined clinicopathological outcome and poor prognosis in oral carcinoma. Saawarn *et al.*[14] pointed out that cyclin D1 expression is significantly associated with cell differentiation, and its expression is increased with the increase in differentiation.

NF-κB, an important transcription factor, has been implicated in various cellular processes, especially in inflammatory reactions, cell proliferation and apoptotic inhibition. NF-κB also regulates genes that have a prominent role in cell adhesion, proliferation, angiogenesis and immune response.[15] NF-κB has a pivotal role in the pathogenesis of several disorders including inflammation and carcinogenesis. Druzgal *et al.*[16] pointed out that the serum levels of NF-κB could indicate the prognosis as well as treatment options for carcinogenesis.

Prostaglandin synthesis from archidonic acid is regulated by the key enzyme, cyclooxygenase-2 (COX-2). Though COX-2 is undetectable in normal tissues, its activity is enormously enhanced in malignant tissues. COX-2 activity is stimulated by various stimuli, including carcinogens, inflammatory agents and tumor promoters. It has been reported that upregulation of COX-2 in tumor tissues accounts for increased levels of prostaglandins, which have a pivotal role in apoptosis, cell proliferation and angiogenesis.[17] Tumors expressed abnormal levels of COX-2 and thus abnormal COX-2 activity is associated with poor prognosis.[18]

Angiogenesis is one of the essential features of tumoriogenesis, due to its vital role in the tumor growth by facilitating nutritional transport from the circulation. Tumor progressing and invade to adjacent tissues and other parts of the body through angiogenesis. Vascular endothelial growth factor (VEGF), a dimeric and heparin binding

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glycoproteins, plays important role in angiogenesis and immune cells recruitment in the tumor microenvironment. VEGF also plays a vital role in cellular proliferation, vascular permeability, endothelial cell migration and apoptotic inhibition.[10] Recent studies have highlighted the significance of VEGF and VEGF receptors in various carcinogenesis.[19] VEGF was overexpressed in various malignancies and its abnormal expression is associated with poor prognosis of cancer.[20] A positive correlation between VEGF expression and tumor size and stages was reported in various cancers.[21] VEGF can effectively induce angiogenesis in solid tumors such as oral, breast and ovarian cancers.[10,21,22]

Matrix metalloproteinases (MMPs) perform a vital role in the degradation of extracellular matrix and basement membrane components. MMPs also play a vital role in tumor invasion and metastasis. They are zinc-dependent endopeptidase and are categorized into various classes based on their substrate specificities. MMPs are pro-enzymes and thus need proteolytic cleavage for their activation. MMP-2 and 9 are closely associated with tumor progression, invasion and metastasis.[23] Patel *et al.*[24] suggested that the status of MMP-2 could be considered as a molecular marker to predict oral cancer metastasis and plasma MMP-2 and 9 activities could be considered for treatment monitoring in patients with oral cancer. MMP-9, also named as gelatinase B, has a crucial role in the pathogenesis of inflammation, angiogenesis, oncogenesis, tumor invasion and metastasis. Vilen *et al.*[25] suggested that MMP-9 play a dual role in angiogenesis depending on the circumstances related to types and stages of the tumors. Invasion and metastasis of cancer at an early stage can thus be prevented by suppressing the expression of MMPs. Extensive studies correlated MMP-2 and 9 expressions with tumor progression, lymph node metastasis, tumor invasion and metastasis and survival outcome of oral cancer.[23-25]

Enicostemma littorale has been recommended in Indian traditional medicines such as Ayurveda for the treatment of various illness, including inflammation, diabetes mellitus and cancer.[26] Experimental studies have also reported its antioxidant, hepatoprotective and anticancer potential.[27,28] We recently reported the chemopreventive potential as well as its protective effect on cell surface glycoconjugates in DMBA induced oral cancer.[29,30] The aim of this study is, therefore, to explore the molecular chemopreventive potential of *E. littorale* in DMBA induced hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

Animals

Forty male golden Syrian hamsters, 8 weeks old, weighing 80–120 g, were obtained from National Institute of Nutrition, Hyderabad, Telangana, India, and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, Tamil Nadu, India. The animals were housed in polypropylene cages and provided standard pellet diet and water *ad libitum*. The animals were maintained under controlled conditions of temperature and humidity with a 12 h light/dark cycle.

Plant material

E. littorale leaves were collected from Cuddalore District of Tamil Nadu, India. The taxonomic identification of the plant was compared with the existing herbarium in the Department of Botany, Annamalai University.

Preparation of the plant extract (chemoprevention study)

Five hundred grams of dried, finely powdered *E. littorale* leaves were soaked in 1500 mL of 95% ethanol overnight. The residue obtained after filtration was again resuspended in equal volume of 95% ethanol for 48 h and filtered again. The above two filtrates were mixed, and the solvents were evaporated in a rotavapor at 40–50% under reduced pressure. A semisolid material (9%) obtained was stored at 4°C until use. A known volume of the residual extract (250 mg/kg body weight) was suspended in distilled water and orally administered to the animals by gastric intubation using force feeding tube during the experimental period.

Experimental protocol

The Institutional Animal Ethics Committee (Reg No: 160/1999/CPCSEA), Annamalai University, Annamalainagar, Tamil Nadu, India, approved the experimental design (Proposal No: 889 dated May 29, 2012). A total number of 40 hamsters were divided into four groups with 10 animals in each group. The Group I hamsters served as control and were painted with liquid paraffin alone 3 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 3 times a week for 14 weeks on their left buccal pouches. Group II hamsters received no other treatment. Group III hamsters were orally administered with ethanolic extract of ElELet at a dose of 250 mg/kg body weight on alternative days of DMBA painting. Group IV hamsters received oral administration of ElELet alone throughout the experimental period. The experiment was terminated at the of 16th week and all the animals were sacrificed by cervical dislocation.

Immunohistochemical staining

Paraffin embedded buccal mucosa tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. The tissue sections were then incubated

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with the respective primary antibodies (p53, Bcl-2, Bax, VEGF, PCNA and cyclin D1) followed by incubation with the secondary antibody conjugated with horseradish peroxidase. The immune complex was then treated with the substrate diaminobenzidine. The slides were then analyzed and scored for the positively stained cells under microscope. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium.

Each slide was microscopically analyzed and enumerated the percentage of the positively stained cells semi-quantitatively. The percentage of positive cells was scored according to the method of as follows: 3+ = stronger staining, more than 50% of cells were stained; 2+ = moderate staining, between 20% and 50% of the cells were stained; 1+ = weak staining, between 1% and 20% of cells were stained; 0 = negative, <1% of cell staining.[31]

Western blotting analysis

The protein bands (blot) obtained in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was transferred to polyvinylidene fluoride (PVDF) membrane. The PVDF membrane containing the blot was incubated with the respective primary antibodies (MMP-2, MMP-9, COX-2 and NF-κB) at 4°C over night. After incubation the membrane was washed with tris buffered saline with tween 20 and the blots were incubated with alkaline phosphatase-conjugated secondary antibody at room temperature. The protein bands were then developed using nitroblue tetrazolium/bromochloro-indolyl phosphate solution and the results were analyzed densitometrically using ImageJ software, developed by Wayne Rasband at the National Institutes of Health, U.S.A.[32]

Gas chromatograph-mass spectrometer analysis

Twenty-five grams of *E. littorale* powder soaked in 50 mL of distilled alcohol in a standard conical flask. This was kept in overnight and then the sample was filtered using Whatmann No. 1 filter paper. The filtrate was filtered with sodium sulfate to remove the sediments and traces of water in the filtrate. The filtrate was concentrated to 1 mL with the help of nitrogen flushing. The pure sample (2 µl) was then injected into the gas chromatograph-mass spectrometer (GC-MS) Instrument for further analysis.

GC-MS analysis of the ethanolic extract of *E. littorale* was performed using a Perkin Elmer GC Clarus 500 system comprising AOC-20i auto-sampler and a GC interfaced to a MS equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused capillary column (30 × 0.25 µm dF). For GC-MS detection, an electron ionization system was operated in the electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 2 µl was employed (split ratio of: 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed at 110°C (isothermal for 2 min), with an increase of 10°C/min to 200°C then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0–2 min and the total GC-MS running time was 36 min. The relative percentage amount each component was calculated by comparing its average peak area to the total areas. The mass detector used in this analysis was the Turbo-Mass Gold-Perkin-Elmer and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass version 5.2, developed by PerkinElmer, Inc., Connecticut, U.S.A.

Gas chromatograph program

Column: Elite - 5MS (5% diphenyl/95% dimethyl poly siloxane), 30 × 0.25 mm × 0.25 µm dF.

Equipment: GC Clarus 500 Perkin Elmer, carrier gas: 1 mL per min, split: 10:1, detector: Mass detector Turbo-Mass Gold-Perkin-Elmer, software: Turbo mass 5.2, developed by PerkinElmer, Inc., Connecticut, U.S.A. Sample injected: 2 µl.

Mass spectrometer program

Library used National Institute of Standard and Technology (NIST) version-year 2005, inlet line temperature 200°C, source temperature 200°C electron energy: 70 eV, mass scan (m/z): 45–450, solvent delay: 0–2 min, total running time: 36 min.[33]

Statistical analysis

The present study investigated the statistical significance between the experimental groups using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. The two different groups are considered statistically significant of the $P < 0.05$.

RESULTS

In the present study, overexpression of p53 (mutant), Bcl-2, NF-κB, COX-2, MMP-2 and 9, VEGF, cyclin D1 and PCNA and downregulation of Bax was noticed in the buccal mucosa of hamsters treated with DMBA alone (tumor bearing hamsters; Figures 1-8). The score of positively stained cells of p53, Bcl-2, Bax, VEGF, PCNA and cyclin D1 in control and experimental hamsters is shown in Table 1. *E. littorale* administration orally to DMBA treated hamsters revert the status of above molecular markers in favor of suppression of cell proliferation.

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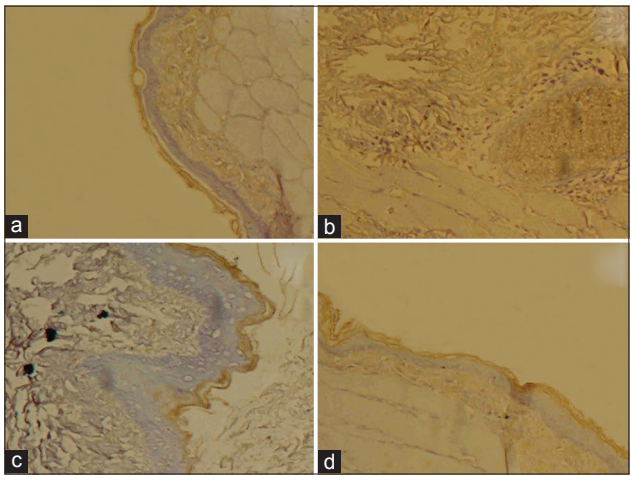


Figure 1: Immunoexpression pattern of P53 observed in the buccal mucosa of control and experimental hamsters in each group (×20). (a-d) Depict immunoexpression of P53 in control (a: expression not detectable), 7, 12-dimethylbenz(a)anthracene alone (b: Over expressed), 7, 12-dimethylbenz(a)anthracene + *Enicostemma littorale* (c: Down regulated) and *Enicostemma littorale* alone (d: Expression not detectable)

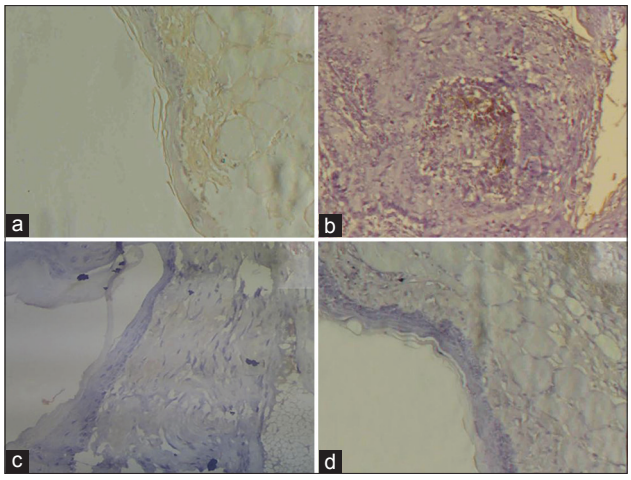


Figure 2: Immunoexpression pattern of Bcl-2 observed in the buccal mucosa of control and experimental hamsters in each group (×20). (a-d) Depict immunoexpression of Bcl-2 in control (a: Expression not detectable), 7, 12-dimethylbenz(a)anthracene alone (b: Over expression), 7, 12-dimethylbenz(a)anthracene + *Enicostemma littorale* (c: Down regulated) and *Enicostemma littorale* alone (d: Expression not detectable)

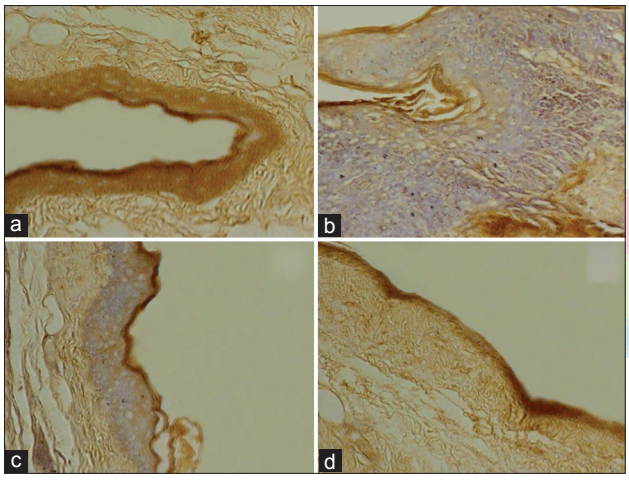


Figure 3: Immunoexpression pattern of Bax observed in the buccal mucosa of control and experimental hamsters in each group (×20). (a-d) Depict immunoexpression of Bax in control (a: Expression not detectable), 7, 12-dimethylbenz(a)anthracene alone (b: Nuclear expression negative), 7, 12-dimethylbenz(a)anthracene + *Enicostemma littorale* (c: Nuclear and cytoplasmic expression positive), d: Expression not detectable

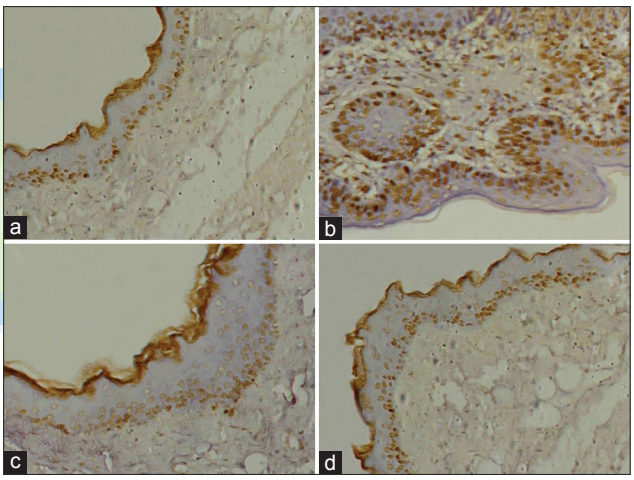


Figure 4: Immunoexpression pattern of proliferating cell nuclear antigen observed in the buccal mucosa of control and experimental hamsters in each group (×20). (a-d) Depict immunoexpression of proliferating cell nuclear antigen in control (a: Expression not detectable), 7, 12-dimethylbenz(a)anthracene alone (b: Over expressed), 7, 12-dimethylbenz(a)anthracene + *Enicostemma littorale*, (c: Down regulated), *Enicostemma littorale* alone (d: Expression not detectable)

The present study utilized GC Clarus 500 Perkin Elmer GC-MS. The GC-MS analysis showed the presence of 34 bioactive compounds [Table 2; Figure 9]. The database of the NIST is used to interpret the mass spectrum of the compounds. This database has more than 62,000 mass spectrum patterns. The structure and molecular weight of the test substance was ascertained using this database.

DISCUSSION

Early investigations of biochemical and molecular abnormalities could help to improve treatment strategy. A spectrum of molecular markers is involved in the initiation, promotion and progression of carcinogenesis. The status of tumor suppressor genes, oncogenes, and markers of cell proliferation, angiogenesis and inflammation could help in the diagnosis as well as the prognosis of oral cancer. In the present study, we have analysed the expression pattern of apoptotic (p53, Bcl-2 and Bax), cell proliferative (cyclin D1 and PCNA), inflammatory (NF-κB and COX-2) and angiogenic (VEGF) markers expression pattern during DMBA induced oral carcinogenesis. Hamsters treated with DMBA alone showed abnormal expression of above mentioned molecular markers in their buccal mucosa.

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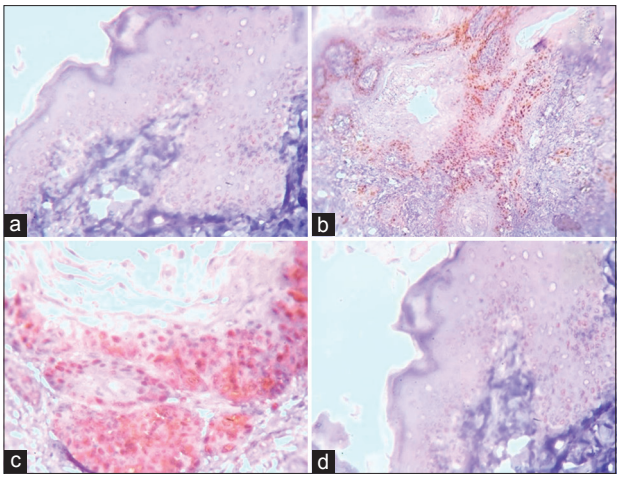


Figure 5: Immunoexpression pattern of cyclin D1 observed in the buccal mucosa of control and experimental hamsters in each group (20×). (a-d) Depict immunoexpression of cyclin D1 in control (a: Expression not detectable), 7, 12-dimethylbenz(a)anthracene alone (b: Over expressed), 7, 12-dimethylbenz(a)anthracene + *Enicostemma littorale* (c: Down regulated), *Enicostemma littorale* alone (d: Expression not detectable)

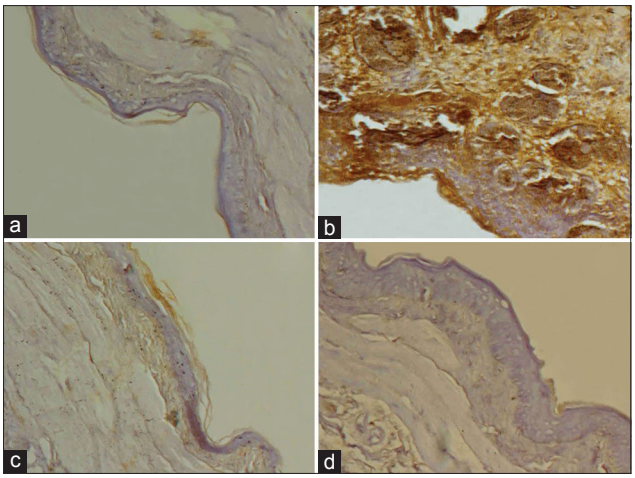


Figure 6: Immunoexpression pattern of vascular endothelial growth factor observed in the buccal mucosa of control and experimental hamsters in each group (×20). (a-d) Depict immunoexpression of vascular endothelial growth factor in control (a: Expression not detectable), 7, 12-dimethylbenz(a)anthracene alone (b: Over expressed), 7, 12-dimethylbenz(a)anthracene + *Enicostemma littorale* (c: Down regulated), *Enicostemma littorale* alone (d: Expression not detectable)

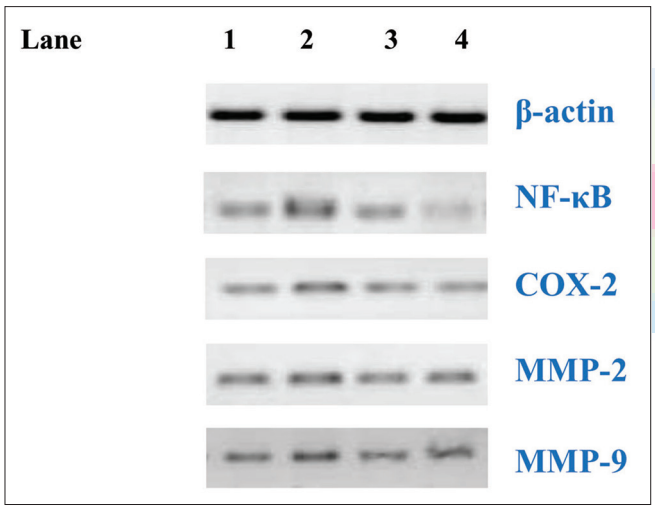


Figure 7: Expression pattern of NF-kB, cyclooxygenase-2, matrix metalloproteinase-2, matrix metalloproteinase-9 in control and experimental hamsters in each group. Lane 1: Control, Lane 2: 7, 12-dimethylbenz(a)anthracene alone, Lane 3: 7, 12-dimethylbenz(a) anthracene + *Enicostemma littorale*, Lane 4: *Enicostemma littorale* alone

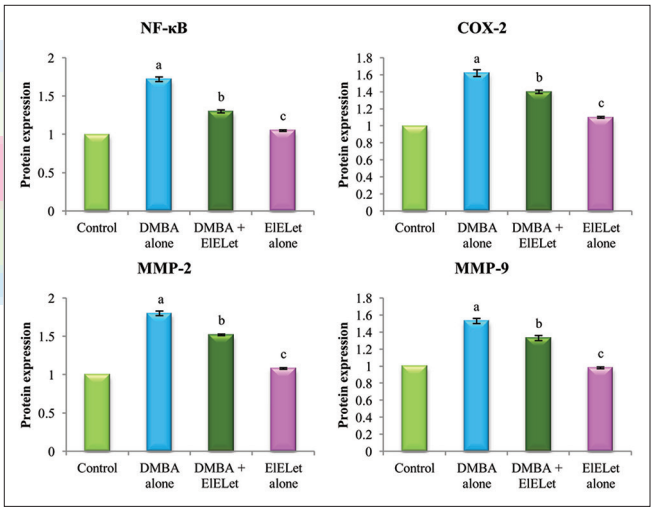


Figure 8: Densitometric analysis of NF-kB, cyclooxygenase-2, matrix metalloproteinase-2, matrix metalloproteinase-9 in control and experimental hamsters in each group. Values are expressed as mean ± standard deviation for 10 hamsters in each group. Values that do not share a common superscript in the same column differ significantly at $P < 0.05$ (Duncan's Multiple Range Test)

p53 has a crucial role in the maintenance of cellular integrity. A large number of investigations documented p53 alterations in the early stages of oral carcinogenesis itself. Mutant p53 overexpression was documented in 27–35% of oral premalignancy and around 33–100% in squamous cell carcinoma.[34] Vora *et al.*,[34] suggested that a person who has higher p53 expression is more prone to cancer development. Cutilli *et al.*,[35] reported that tumors exhibit resistance toward chemotherapy if they have higher p53 expression. Though the mechanism of p53 role in oral carcinogenesis is poorly understood, p53 mutations are shown in more than 50% of oral cancer patients. The results of the present study also confirmed over expression of mutant p53 during oral carcinogenesis.

Upregulation of Bcl-2 accompanied by downregulation of Bax could help to inhibit apoptotic process and thereby facilitates cell survival. Any agents that prevent or inhibit abnormal expression of Bcl-2 could thus emerge as a therapeutic anticancer agent. Tarquinio *et al.*,[36] have shown over expression of Bcl-2 in the endothelial cells of patients with oral squamous cell carcinoma. They suggested Bcl-2 as a proangiogenic signaling molecule and play a

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pivotal role in tumor growth and angiogenesis. Inverse association between Bcl-2 and Bax in carcinogenesis could help the tumors to progress further.[37] Our results lend credibility to these observations.

Proliferating cellular nuclear antigen interacts with various cellular proteins that have role in cell cycle. Abnormal cell proliferation accompanied by evasion of apoptosis is a common phenomenon observed in oral carcinogenesis.[38] PCNA expression was increased both in precancerous lesions and in oral malignancy.[6] Enormous cancer studies demonstrated abnormal expression of PCNA in tumors using immunohistochemistry or western blotting.[6,10] Our results corroborate these observations.

Cyclin D1, a protooncogene and cell-cycle regulator, is involved in the control of restriction point in the G1 phase. Abid and Merza,[39] showed cyclin D1 overexpression using immunohistochemistry in 88% oral cancer patients. The cyclin D1 expression has been correlated with histological grading in tumor staging in various types of cancers including oral carcinoma.[40] It has been pointed out that the enhanced risk of recurrence, poor prognosis and lymph node metastasis are associated with cyclin D1 overexpression.[41] Increase in cyclin D1 expression could cause G1 phase shortening and thereby leads to abnormal cell proliferation.[42] Das *et al.*,[43] have shown overexpression of cyclin D1 in patients with tobacco related oral carcinoma. The results of the present study support these findings.

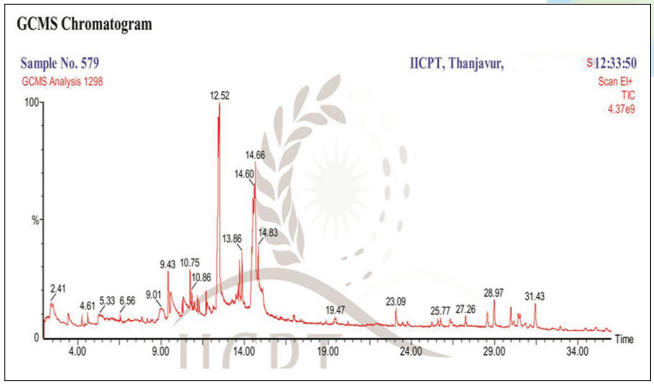


Figure 9: The gas chromatograph interfaced to a mass spectrometer chromatogram of investigated compounds from *Enicostemma littorale*

NF-κB acts as a connecting bridge between inflammation and tumoriogenesis. Abnormal NF-κB expression was documented in inflammation, diabetes, atherosclerosis and cancer.[44] NF-κB expression was higher in patients with oral sub mucous fibrosis.[45] NF-κB over expression not only mediates carcinogenesis, but also its invasion and progression. NF-κB could therefore be considered as a target for therapeutic intervention. Mounting studies documented overexpression of COX-2 in premalignant and malignant tissues of the oral cavity.[46] Studies have also demonstrated overexpression of COX-2 mRNA in oral precancerous and cancerous tissues.[10,46] COX-2 over expression in turn inhibits apoptosis, promotes cell proliferation and induces angiogenesis in tumor tissues.[47] COX-2 has been implicated in the pathogenesis in several types of carcinogenesis.[10,46,47] Our results lend credence to these observations.

An increase in vascularity has been demonstrated from normal to precancerous lesions and to cancerous lesions. Extensive studies reported increased levels of VEGF in the serum of cancer patients.[48] Aggarwal *et al.*,[49] suggested that serum VEGF status may be used as a prognostic and diagnostic marker for oral cancers. It has also been reported that VEGF is a promising target for the prevention of tobacco related oral cancer.[50] It has been pointed out that the suppression of angiogenesis by inactivating VEGF diminished the growth and progression of tumors.[51] The present results are in line with these findings.

Fan *et al.*,[52] showed a positive expression of MMP-2 and 9 in the cytoplasm of stromal cells and proliferating cells in tongue carcinogenesis. Increased expression of MMP-2 in patients with severe dysplasia has been reported.[53] MMP-9 is over expressed in oral cancer tissues and inflammatory tissues around carcinoma islands.[25] MMP-9 overexpression degrades Type IV collagen in the basement membrane of oral tumor tissues, thereby contributing to invasion and metastasis.[54] Solid tumors, even under hypoxia, can grow up to 1–2 mm^3 without vascularization. Bergers *et al.*,[55] reported that the angiogenic switch to release VEGF was triggered by MMP-9. Metastatic tumors over express VEGF and MMP-9 than nonmetastatic tumors.[56] It has been reported that MMP-9 expression in oral tumor tissues

Table 1: The score of positively stained cells of p53, Bcl-2, Bax, VEGF, PCNA and cyclin D1 in control and experimental hamsters in each group

Groups/markers	p53				Bcl-2				Bax				VEGF				PCNA				Cyclin D1			
	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+
Control	10	0	0	0	7	3	0	0	1	3	4	2	10	0	0	0	10	0	0	0	10	0	0	0
DMBA alone	0	1	3	6	0	1	2	7	6	3	1	0	0	2	3	5	0	1	3	6	0	2	3	5
DMBA + ElELet	0	7	2	1	2	5	3	0	3	3	4	0	6	3	1	0	0	6	3	1	0	4	3	3
ElELet alone	10	0	0	0	6	4	0	0	2	3	4	1	10	0	0	0	10	0	0	0	10	0	0	0

Values are given as number of hamsters (n=10). The percentage positive cells were scored as: 3+ = Strong staining, more than 50% of cells were stained, 2+ = Moderate staining, between 20% and 50% of cells were stained, 1+ = Week staining, between 1% and 20% of cells were stained, 0 = Negative, <1% of cell staining. VEGF = Vascular endothelial growth factor, PCNA = Proliferating cell nuclear antigen, DMBA = 7, 12-dimethylbenz (a) anthracene

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Table 2: Components identified in the *Enicostemma littorale* leaf powder (GC-MS study)

RT	Name of the compound	Molecular formula	Molecular weight	Peak area
2.41	Butanoic acid, 3-hydroxy	$C_4H_8O_3$	104	2.28
3.46	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	1.89
4.27	2 (3H)-benzofuranone, hexahydro-3-methylene-	$C_9H_{12}O_2$	152	0.42
4.61	3,8-dioxatricyclo[5.1.0.0 (2,4)]octane, 4-ethenyl-	$C_8H_{10}O_2$	138	0.70
5.33	Ascaridole epoxide	$C_{10}H_{16}O_3$	184	1.96
6.56	6-acetyl-á-d-mannose	$C_8H_{14}O_7$	222	0.42
7.85	Octan-2-one, 3,6-dimethyl-	$C_{10}H_{20}O$	156	0.48
9.01	Desulphosinigrin	$C_{10}H_{17}NO_6S$	279	4.09
9.43	3-oxabicyclo[4.3.0]nonan-2-one, 8-isopropylidene-, cis-	$C_{11}H_{16}O_2$	180	3.01
9.58	Tetracyclo[4.4.1.1 (7,10).0 (2,5)]dodec-3-en-11-ol	$C_{12}H_{16}O$	176	4.86
10.35	L-serine, O-(phenylmethyl)-	$C_{10}H_{13}NO_3$	195	2.55
10.75	E-2-tetradecen-1-ol	$C_{14}H_{28}O$	212	1.32
10.86	1-hexadecanol, 2-methyl-	$C_{17}H_{36}O$	256	0.48
11.18	13-heptadecyn-1-ol	$C_{17}H_{32}O$	252	0.59
11.29	Estra-1,3,5 (10)-trien-17á-ol	$C_{18}H_{24}O$	256	0.60
11.71	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	0.59
12.52	n-hexadecanoic acid	$C_{16}H_{32}O_2$	256	18.28
13.71	[1,1'-bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	$C_{21}H_{38}O_2$	322	1.68
13.86	Phytol	$C_{20}H_{40}O$	296	2.11
14.66	9, 12-octadecadienoic acid (Z, Z)-	$C_{18}H_{32}O_2$	280	29.33
14.83	Octadecanoic acid	$C_{18}H_{36}O_2$	284	8.45
16.96	Octan-2-one, 3,6-dimethyl-	$C_{10}H_{20}O$	156	0.27
19.47	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	$C_{24}H_{46}O_2$	366	0.79
23.09	Squalene	$C_{30}H_{50}$	410	1.39
25.24	Z, Z, Z-4,6,9-nonadecatriene	$C_{19}H_{34}$	262	0.33
25.59	2H-pyran, 2-(7-heptadecynyloxy) tetrahydro-	$C_{22}H_{40}O_2$	336	0.48
25.77	Cholestan-3-ol, 2-methylene-, (3á,5à)-	$C_{28}H_{48}O$	400	0.47
26.53	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.65
27.26	Vitamin E	$C_{29}H_{50}O_2$	430	0.72
28.97	Cholestan-3-ol, 2-methylene-, (3á,5à)-	$C_{28}H_{48}O$	400	1.42
28.97	Stigmasterol	$C_{29}H_{48}O$	412	2.49
29.97	á-sitosterol	$C_{29}H_{50}O$	414	1.68
30.42	2,2,4-trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	$C_{30}H_{52}O$	428	0.95
31.43	Lupeol	$C_{30}H_{50}O$	426	2.27

GC-MS = Gas chromatography-mass spectrometry, RT = Retention time

facilitates the tumor cells to invade through extracellular matrix.[57] Drastic increase in MMP-9 expression was reported in breast, renal, colon and oral carcinoma.[58,59] Over expression of MMP-1, 2, 3 and 9 was reported in oral tumor tissues as compared to normal counterparts.[60] Lee *et al*.,[61] reported over expression of MMP-1, 2, 3, 8, 9 and 28 in oral cancer patients with betel quid chewing habits. We also noticed a similar pattern of results in hamsters bearing oral tumors.

Oral administration of *E. littorale* (ELELet) at a dose of 250 mg/kg body weight to DMBA treated hamsters significantly prevented the tumor formation[29] as well as modulated the expression of apoptotic (p53, Bcl-2 and Bax), cell proliferative (cyclin D1 and PCNA), inflammatory (NF-κB and COX-2), invasive (MMP-2 and 9) and angiogenic (VEGF), markers towards the inhibition or suppression of oral carcinogenesis. The present study thus indicates the presence of some potent anticarcinogenic principles in the leaves of *E. littorale*, which might have played the anticancer role. The present study has thus taken effort to investigate the presence of active components in the *E. littorale* leaves using GC-MS analysis.

Preliminary phytochemical studies have revealed the presence of flavonoids, alkaloids. Anthroquinones, tannins, sterols, swertiamarin and glycosides.[62] Reports also showed the presence of several minerals such as calcium, potassium and iron. *E. littorale* is rich in several amino acids, such as alanine, serine, tryptophan, glutamic acid, phenylalanine and ionone.[63] Ambikapathy *et al*.,[64] performed GC-MS analysis to determine the bioactive compounds of the whole plant (*E. littorale*) methanol extract. Vinotha *et al*.,[65] showed the presence of alkaloids, saponins, flavonoids, steroids, tannins, coumarins and quinines in the whole plant methanolic and ethanolic

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extract. Selvaraj *et al*.,[66] reported the presence of tannins, terpenoids and phenolic compound using qualitative analysis. Abirami and Gomathinayagam[67] reported the biochemical composition of *E. littorale* blume extracts. They suggested that *E. littorale* is rich in Vitamin E and C. Sathishkumar *et al*.,[68] suggested that the aerial part of *E. littorale* is rich in flavonoid, chlorophyll and proteins.

We carried out GC-MS analysis to investigate the bioactive components that are responsible for anticancer potential. GC-MS analysis revealed the presence of 34 bioactive components in the *E. littorale* leaf extract. Among 34 constituents, we noticed two important components squalene at retention time (RT) 23.09 and 1.39% peak area as well as lupeol at RT 31.43 and 2.27% peak area. Squalene and lupeol have diverse pharmacological properties including antioxidant, anticancer and tumor protective potential. Previous studies from our laboratory reported the chemopreventive potential as well as proapoptotic, anti-inflammatory and anti-angiogenic potential of lupeol in DMBA induced hamster buccal pouch carcinogenesis.[69,70] Manimaran *et al*.,[71] reported that lupeol showed potent anti-cellproliferative, anti-inflammatory and anti-angiogenic potential during DMBA induced hamster buccal pouch carcinogenesis. Rao *et al*.,[72] demonstrated the anticancer efficacy of squalene in azoxy methane induced rat colon carcinogenesis. It has also been demonstrated that squalene significantly prevented benzo (a) pyrene-induced skin carcinogenesis.[73] The presence of bioactive components such as lupeol and squalene justify the chemopreventive potential of *E. littorale* leaves in DMBA-induced hamster buccal pouch carcinogenesis.

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Conflicts of interest

There are no conflicts of interest.

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