Trop J Nat Prod Res, September 2020; 4(9):550-557 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Myrtenal Modulates the Immunoexpression of Cell Proliferative, Angiogenic and Invasive Markers in DMBA-Induced Hamster Oral Carcinogenesis

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ARTICLE INFO

Article history:
Received 06 August 2020
Revised 20 August 2020
Accepted 25 September 2020
Published online 03 October 2020

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ABSTRACT

Abnormal cell proliferation, invasion, metastasis and angiogenesis are the most prominent features of malignant tumours. The present study evaluated the modulating effect of myrtenal on the immunoexpression pattern of cell proliferative (PCNA and cyclin D1), angiogenic (VEGF) and invasive (MMP-2 and MMP-9) markers in 7,12-dimethylbenz(a)anthracene (DMBA)-induced experimental oral carcinogenesis in golden Syrian hamsters using immunohistochemical assay. Topical application (painting) of 0.5% DMBA (six hamsters), a site specific carcinogen, three times a week for 14 weeks resulted in the formation of tumors in the buccal pouches of golden Syrian hamsters , which was confirmed by histopathological studies. Buccal mucosa excised from the hamsters treated with DMBA alone (tumour-bearing hamsters) showed abnormal immunoexpression pattern of cell proliferative, angiogenic and invasive markers. Myrtenal administration (230 mg/kg b.w) orally to DMBA treated hamsters significantly downregulated the expression of the above said molecular markers in the chemopreventive phase and considerably decreased the expression pattern in the chemotherapeutic phase. The findings from this study, thus support the anti-cell proliferative, anti-angiogenic, and anti-invasive potential of myrtenal in DMBA-induced hamster buccal pouch carcinoma.

Keywords: Cancer, DMBA, hamsters, cell proliferation, angiogenesis, invasion.

Introduction

A spectrum of regulatory genes plays an important role in the regulation of cell cycle and during its transition from one phase to another phase.[1,2] Two such important genes, PCNA and cyclin D1, play a prominent role in the regulation of G_1 and S phase of the cell cycle. Cyclin D1 plays a very important role in the progression of the cell cycle during $G_1 \rightarrow S$ phase transition.[3] Cyclin D1 stimulates cell proliferation via forming complex with CDK4/6.[4] Cyclin D1 expression during the cell cycle is extracellular dependent on mitogenic signaling and its abnormal or aberrant expression has been reported in various malignancies including oral carcinoma.[5] Cyclin D1 plays a vital role in both cell proliferation and differentiation in the oral tumor tissues.[6] It has been reported that cyclin D1 over expression occurs and progresses from the early stage of oral carcinogenesis. The expression pattern of cyclin D1 could be used to differentiate the histological grade of oral tumors.[7] Over expression of cyclin D1 is associated with malignant tumor progression.[8] The cyclin D1 expression has been focused as a predictive biomarker of the cancer of the tongue and floor of the mouth.[9] Khan *et al*.[10] suggested that cyclin D1 expression could be considered as a prognostic marker in oral carcinogenesis.

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Citation: Buddhan R, Manoharan S, Elanchezhiyan C, Muralinaidu R, Karthik M. Myrtenal Modulates the Immunoexpression of Cell Proliferative, Angiogenic and Invasive Markers in DMBA-Induced Hamster Oral Carcinogenesis. Trop J Nat Prod Res. 2020; 4(9):550-557. doi.org/10.26538/tjnpr/v4i9.10

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Proliferating cell nuclear antigen (PCNA), an important cell proliferation biomarker, has been found to have a vital role in various biological processes such as DNA synthesis and repair.[11] PCNA has been considered or suggested as a significant sensitive index of cell proliferation due to the fact that its expression increases at G_1 and S phases of the cell cycle.[12] A large number of studies reported that the expression of PCNA progresses from normal to precancerous and then to malignancy.[13] Due to the massive role of cyclin D1 and PCNA in the process of cell proliferation during normal and pathological conditions, the present study utilized the expression pattern of PCNA and cyclin D1 to assess the anti-cell proliferative potential of myrtenal during DMBA-induced oral carcinogenesis.

Angiogenesis is a complex phenomenon regulated by proangiogenic and antiangiogenic factors in the human body. Under normal physiological conditions, angiogenesis occurs during embryogenesis, menstrual cycle, wound healing and in pregnancy.[14,15] The various factors that are able to stimulate angiogenesis include hypoxia, hypertension, low blood sugar, chronic inflammation and mechanical stress.[16,17] Angiogenesis has been reported in several pathological illnesses such as tumor growth, tumor metastasis,[18] rheumatoid arthritis,[19] myocardial ischemia [20] and diabetic retinopathy.[21]

Invasion and metastasis are the major features of malignant tumors. Vascular endothelial growth factor (VEGF) has been considered as an important growth factor that plays vital role in normal and pathological angiogenesis.[22] Profound studies explored its pro-angiogenic activity, antiapoptotic efficacy, mitogenic activity on endothelial cells, enhancing the vascular permeability and in the stimulation of cell migration. Targeting VEGF could help to identify the new anticancer as well as anticancer therapies for cancer. Srivastava *et al*.[23] have shown higher expression of VEGF in oral carcinoma using immunohistochemistry.

Matrix metalloproteinases (MMPs), zinc dependent proteinases, play multiple roles in carcinogenesis, which include degradation of extracellular matrix, stimulation of cell proliferation, angiogenesis and

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metastasis.[24,25] Over expression of matrix metalloproteinases, contribute to tumor progression, invasion and metastasis. Farhadi and Mohamadi[26] highlighted the significance of MMPs 2 and 9 in the clinical outcome of oral squamous cell carcinoma. Massano *et al.*[27] suggested that MMP expression pattern should be studied in tumor tissues of all oral cancers. The present study thus evaluated the modulating effect of myrtenal on the immunoexpression pattern of VEGF and MMP-2 and 9 in DMBA-induced oral carcinogenesis in golden Syrian hamsters.

Myrtenal plays a vital role in the prevention of several illnesses. It is one of the major bioactive constituents of several medicinal plants' (cumin, pepper, eucalyptus and mint) essential oil. It is a bicyclic terpenoid and possesses several pharmacological and biochemical effects as well. Myrtenal showed anti-inflammatory, antidiabetic and antioxidant properties in experimental animals.[28-30] The antitumor potential of myrtenal against colon and liver cancers have been reported.[29,31] The neuromodulatory effect of myrtenal has also been reported in experimental animal models.[32] Recently, research from our laboratory explored the chemopreventive efficacy of myrtenal in DMBA-induced oral carcinoma and suggested its apoptotic potential as a possible mechanism for the prevention of oral tumor formation in golden Syrian hamsters.[33,34] To the best of our knowledge, there are no reports on the modulating effect of myrtenal on the expression pattern of PCNA, Cyclin D1, VEGF, MMP-2 and MMP-9 in experimental oral cancer. The present study explored the anti-cell proliferative, anti-angiogenic, and anti-invasive potential of myrtenal to validate the anticancer potential in DMBA induced hamster buccal pouch carcinoma.

Materials and Methods

Animals

Golden Syrian hamsters, the ideal experimental model for studying oral carcinogenesis, were purchased from National Institute of Nutrition, Hyderabad, India. Thirty male hamsters, 7-8 weeks old weighing 80-120 g were employed for the study. The animals were divided into five experimental groups each group containing six hamsters. The animals were maintained in the Annamalai University Central Animal House and the experimental protocol was followed as per the ethical principles and suggestions of the Annamalai University Institutional ethics committee principles (Proposal N0:1175).

Experimental Design

Hamsters that received the topical application of the liquid paraffin alone on their buccal pouches, three times a week for 14 weeks, were categorized as group I. Group II hamsters, served as tumor bearing hamsters, received topical application of 0.5% DMBA in liquid paraffin, three times a week for 14 weeks, in their buccal pouches. Hamsters that received topical application of DMBA and oral administration of myrtenal (230 mg/kg bw) on alternate days, three times a week for 14 weeks, served as group III. Group 1V hamsters received topical application of DMBA for 10 weeks (three times a week, alternate days), followed by oral administration of myrtenal for further eight weeks (three times a week). Hamsters that received oral administration of myrtenal alone throughout the experimental period served as group V. After the completion of the experimental protocol, the experimental animals were sacrificed by the procedure of cervical dislocation. Buccal pouches were then excised from the experimental animals and subjected to immunohistochemical studies to analyse the expression pattern of cell proliferative,angiogenic and invasive markers.

Immunohistochemical staining

The immunoexpression pattern of cell proliferative, invasive and angiogenic markers in the buccal mucosa of experimental hamsters was analysed by immunohistochemical staining.[34] Briefly, the antigen retrieved paraffin embedded tissue sections were first treated with the primary monoclonal antibodies corresponding to the cell proliferative markers (PCNA, cyclin D1), invasive markers (MMP2 and 9) and angiogenic marker (VEGF). The slides were subsequently treated with the horseradish peroxidase-labelled secondary antibodies and with the chromogenic substrate, diaminobenzidine (DAB). The slides were counterstained with hematoxylin and the immunoexpression pattern of the above said molecular markers were examined under the microscope.

Results and Discussion

Cyclin D1 and PCNA expression pattern in the buccal mucosa of control and experimental hamsters were analyzed using immunohistochemical techniques and the expression pattern is depicted in Figures 1 and 2, respectively. Overexpression of PCNA and cyclin D1 was noticed in the buccal mucosa of hamsters treated with DMBA alone (group II). While myrtenal downregulated the expression of the above said molecular markers in the hamsters treated with DMBA (group III), it also improved the immunoexpression pattern of the markers significantly towards tumor suppression in the hamsters that received topical application of DMBA alone for the first 10 weeks, followed by the oral administration of myrtenal for further eight weeks (group IV). A similar immunoexpression pattern was noticed between the group I (liquid paraffin alone) and group V (myrtenal alone) treated hamsters.

Abnormal or dysregulation of biomarkers of cell proliferation could result in tumorigenesis. Cyclin D1 is an important gene that has been reported to play a pivotal role in the regulation of cell cycle phases and cell signals.[35] The cyclin D1 overexpression has been shown in the G_1 phase of the cell cycle and abnormal or aberrant cell proliferation.[36, 37] As several cancerous tissues have shown an aberrant expression of cyclin D1, inhibition of cyclin D1 expression has recently been considered as a major target for therapeutic implications. Sawair *et al*.[38] reported that the study of cyclin D1 expression pattern in the early stage of oral squamous cell carcinoma could help to assess the local recurrence of the tumor.

Cyclin D1 expression in oral cancer has been linked to the geographical variation in the consumption of etiological factors. A wide variation in the range of 10-75% of cyclin D1 overexpression in the oral tumor tissues have been reported.[39] Zhao *et al*.[40] pointed out that abnormal expression of cyclin D1 in the oral tumor tissues from Asian populations is associated with poor survival and clinicopathological outcome. Abnormal cyclin D1 expression gradually progressed from precancerous to cancerous lesion of the oral cavity. Overexpression of cyclin D1 in oral tumor tissues has been linked to poor prognosis of oral cancer, including survival outcome and unresponsive to treatment strategy.[41]

Poorly differentiated squamous cell carcinoma with lymph node metastasis showed a higher Cyclin D1 expression than well differentiated one.[42] Ramos-Garcia *et al*.[43] have correlated the expression of cyclin D1 with the clinicopathological parameters in oral carcinogenesis. They reported cyclin D1 over expression in 28.7% of oral cancer patients. A positive correlation between cyclin D1 expression and stage III and IV tongue carcinogenesis has been reported.[44] The malignant tumors of various organs have shown abnormal expression of cyclin D1.[44]

The expression of PCNA, a 36KD protein, generally increases from the mid G_1 point to S phase and then starts to reduce from G_2/M to G_1 phase of the cell cycle.[45] PCNA expression was found to be higher in poorly differentiated squamous cell carcinoma than well differentiated squamous cell carcinoma of the oral cavity.[46] Poosarla *et al*.[47] observed an over expression of PCNA from dysplastic lesions to various histological grades of oral cancers. Gupta *et al*.[48] suggested that the expression pattern of PCNA could be used to determine the extent of neoplastic transformation as well as to assess the histological grades of oral malignancy. Abdulkadir *et al*.[49] reported that PCNA immunoexpression pattern could be used as a prognostic indicator in all the histological grades of oral malignancy. Lončarević*et al*.[50] correlated the PCNA expression with the degree of peritumoral tissue infiltration.

The immunoexpression pattern of angiogenic marker [vascular endothelial growth factor (VEGF)] and matrix metalloproteinases (MMP)- 2 and 9] is depicted in Figures 3, 4 and 5, respectively.

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Higher expression of VEGF, MMP-2, and 9 was noticed in the buccal mucosa of hamsters treated with DMBA alone (group II). The immunoexpression pattern of the above markers was significantly downregulated in DMBA + myrtenal treated hamsters (group III) and was considerably decreased in DMBA → myrtenal (group IV) treated hamsters. A similar immunoexpression pattern was noticed between the group I (liquid paraffin alone) and group V (myrtenal alone) treated hamsters.

The present study utilized the immunoexpression pattern of angiogenic (VEGF) and invasive (MMP-2 and 9) markers to evaluate the chemopreventive and chemotherapeutic of myrtenal in DMBA induced hamster buccal pouch carcinogenesis. VEGF, a powerful cytokine, plays a major role not only in tumor angiogenesis but also in the production of vasoactive molecules and mast cell chemotaxis.[51] Overexpression of VEGF has been reported in oral epithelial dysplasia.[52] VEGF has been reported to play a crucial role in the stimulation of motility of tumor cells, secretion of metalloproteinases and in favoring metastasis. Anti-VEGF therapies have been recently approached to treat various disorders, including inflammation, degenerative diseases and cancer.[53] The immunoexpression pattern of VEGF, a key regulator of tumor angiogenesis, showed lower expression in well differentiated squamous cell carcinoma to intense expression in poorly differentiated squamous cell carcinoma.[54] The expression of VEGF in tumor tissues is directly proportional to the invasion depth of carcinogenesis.[55]

Atla *et al.*[56] focused on MMP-9 as an important molecular and histopathological marker of carcinoma and thus could be used as a therapeutic target in oral cancer. A positive correlation between MMP-2 and 9 expression and survival outcome has been reported in several types of cancers.[57] It has been reported that MMP-9 could be used as a prognostic indicator in tumor staging as well as survival outcome.[58] Wiegand *et al.*[59] demonstrated that abnormal expression of MMP-2 and 9 in oral tumor tissues could lead to lymph node metastasis. Several studies documented overexpression of immunoexpression pattern of MMP-2 and 9 in oral carcinogenesis and suggested that abnormal expression of MMP-2 and 9 is associated with lymph node metastasis.[60] An association between MMP-2 expression status and various histological grades of oral malignancy has been studied.

A positive correlation has been demonstrated between MMP-2 expression and tumor metastasis.[61] MMP-2 expression pattern was found to be abnormal in poorly differentiated squamous cell carcinoma.[62]

Ren *et al.*[63] pointed out that MMP-9 over expression may serve as a valuable indicator of poorly differentiated oral squamous cell carcinoma. Ikebe *et al.*[64]pointed out that MMP-9 overexpression could lead to invasion but not metastasis in oral carcinogenesis. Atla *et al.*[56] reported over expression of MMP-9 in stage III oral tumors. Champatyray *et al.*[65] reported immunoexpression pattern of MMP-9 in different grades of oral squamous cell carcinoma. Oral squamous cell carcinoma patients showed higher levels of serum and salivary MMP-9 as compared to control subjects. MMP-9 mediates carcinogenesis through degradation of collagen, fibronectin and elastin and by promoting angiogenesis.[66] MMP-9 gene polymorphism has been associated with increased risk of oral cancer. MMP-9 expression pattern could be used as a valuable biomarker to predict early stage of oral carcinogenesis as well as to utilize for intervention therapy.[67] MMP-9 has been shown to promote angiogenic switch at an early stage of carcinogenesis itself. Studies have documented MMP-9 as an essential element requires for the progression of oral cancer.[68] MMP-9 overexpression was noticed in the cytoplasm of the keratinocytes of all the primary oral malignant tumors.[68]

In the present study, myrtenal effectively downregulated the expression of PCNA and Cyclin D1,VEGF, MMP-2 and MMP-9 in the buccal mucosa of tumor bearing hamsters (group III) and improved the expression pattern of these molecular markers towards tumor size reduction (tumor suppression) in the buccal mucosa of DMBA → myrtenal treated hamsters (Group IV). The results obtained from this study thus show the anti-cell proliferative, anti-invasive and anti-angiogenic potential of myrtenal in DMBA-induced oral cancer in golden Syrian hamsters, as evidenced by no tumor formation in DMBA + myrtenal treated hamsters (group III) and reduced tumor size (small tumors) in DMBA → myrtenal treated hamsters (group IV).

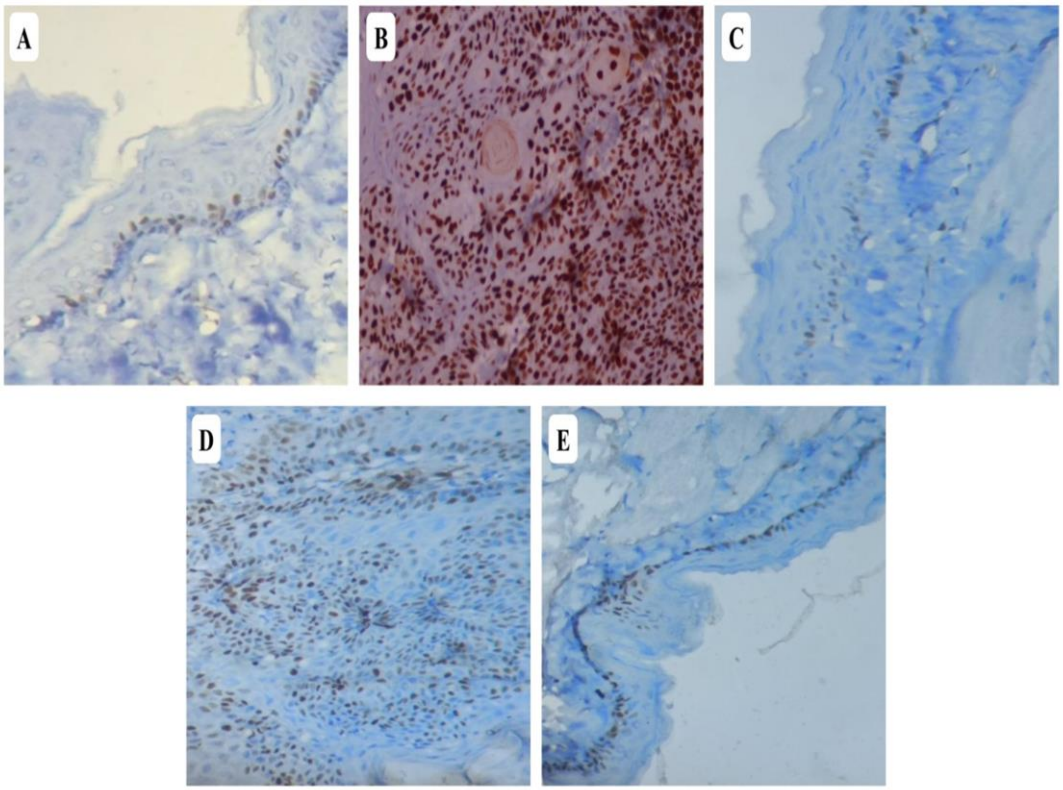

Figure 1: Cyclin D1 expression in the buccal mucosa of experimental hamsters. **(A)** Tissues from control hamsters showed mild cyclin D1 expression in the basal cells. **(B)** Tissues from DMBA alone treated hamsters showed severe cyclin D1 expression in epithelial island sand keratin pearls. **(C)** Tissues from DMBA + myrtenal treated hamsters showed mild cyclin D1 expression in basal and parabasal cells. **(D)** Tissues from DMBA → myrtenal treated hamsters showed moderate cyclin D1 expression throughout the cells. **(E)** Tissues from myrtenal alone treated hamsters showed mild cyclin D1 expression in basal and parabasal cells.

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

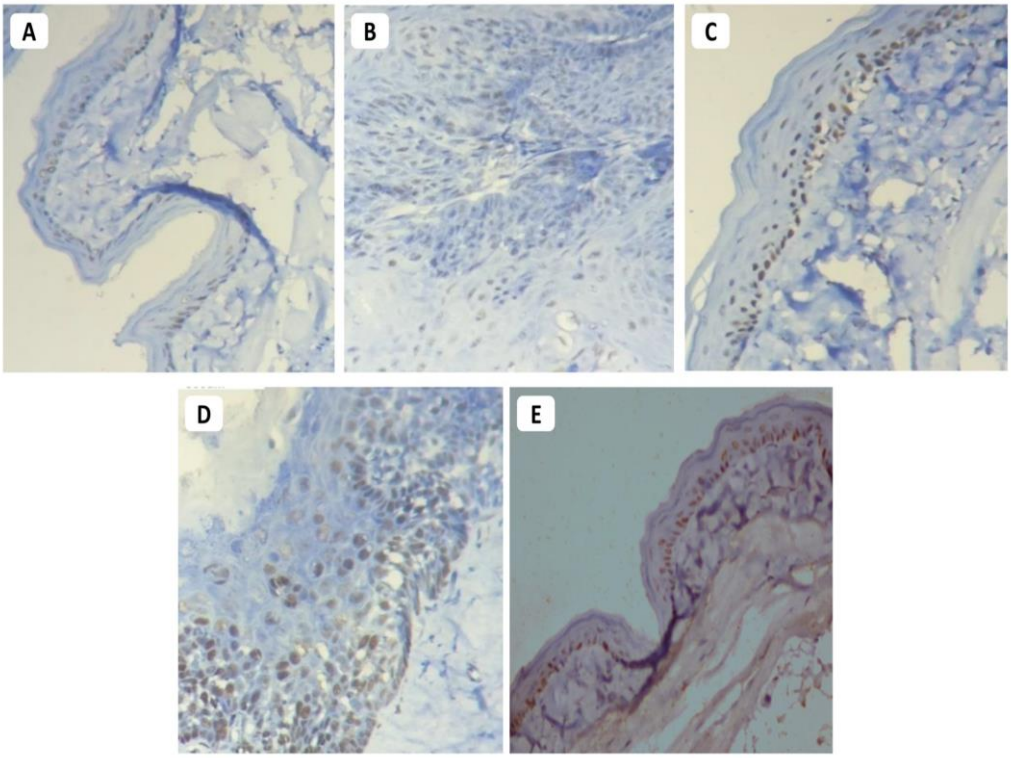

Figure 2: PCNA expression in the buccal mucosa of experimental hamsters. **(A)** Tissues from control hamsters showed mild PCNA expression in the basal and parabasal cells. **(B)** Tissues from DMBA alone treated hamsters showed over expression of PCNA in the hyperplastic epithelial islands. **(C)** Tissues from DMBA + myrtenal treated hamsters showed mild PCNA expression in basal and parabasal cells. **(D)** Tissues from DMBA→myrtenal treated hamsters showed moderate PCNA expression in basal cells. **(E)** Tissues from myrtenal alone treated hamsters showed mild PCNA expression in basal cells.

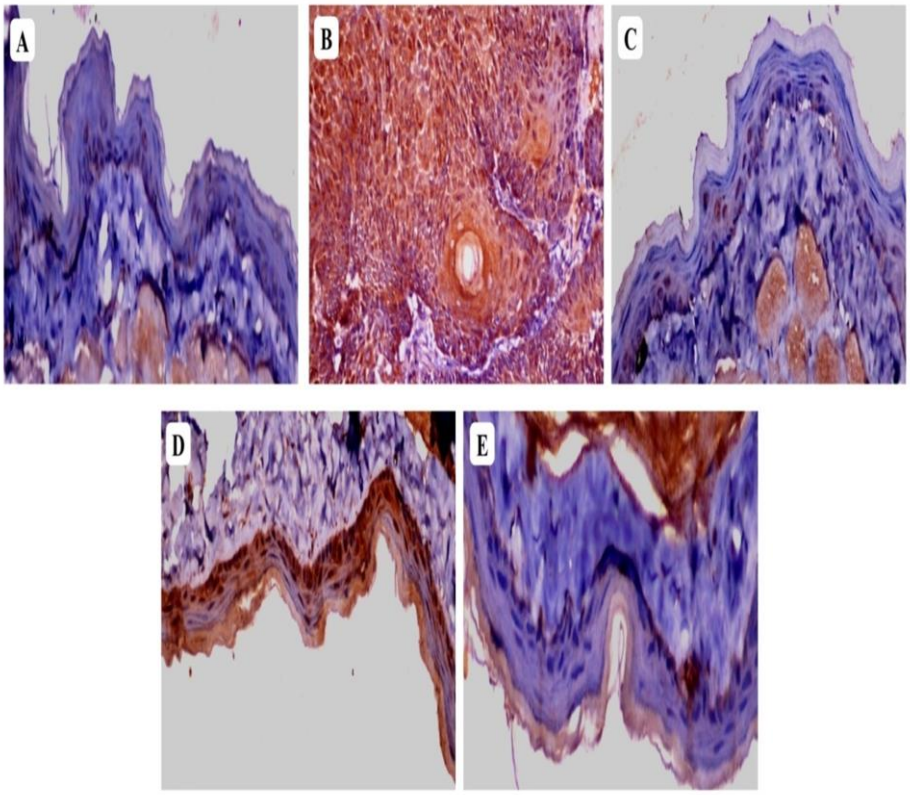

Figure 3: VEGF expression in the buccal mucosa of experimental hamsters. **(A)** Tissues from control hamsters showed few basal cells of VEGF expression in the epithelium. **(B)** Tissues from DMBA alone treated hamsters showed severe VEGF expression in dysplastic epithelium. **(C)** Tissues from DMBA + myrtenal treated hamsters showed VEGF expression in a few of the basal and parabasal cells. **(D)** Tissues from DMBA → myrtenal treated hamsters showed moderate VEGF expression throughout the basal and parabasal cells. **(E)** Tissues from myrtenal alone treated hamsters showed few basal cells of VEGF expression in the epithelium.

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

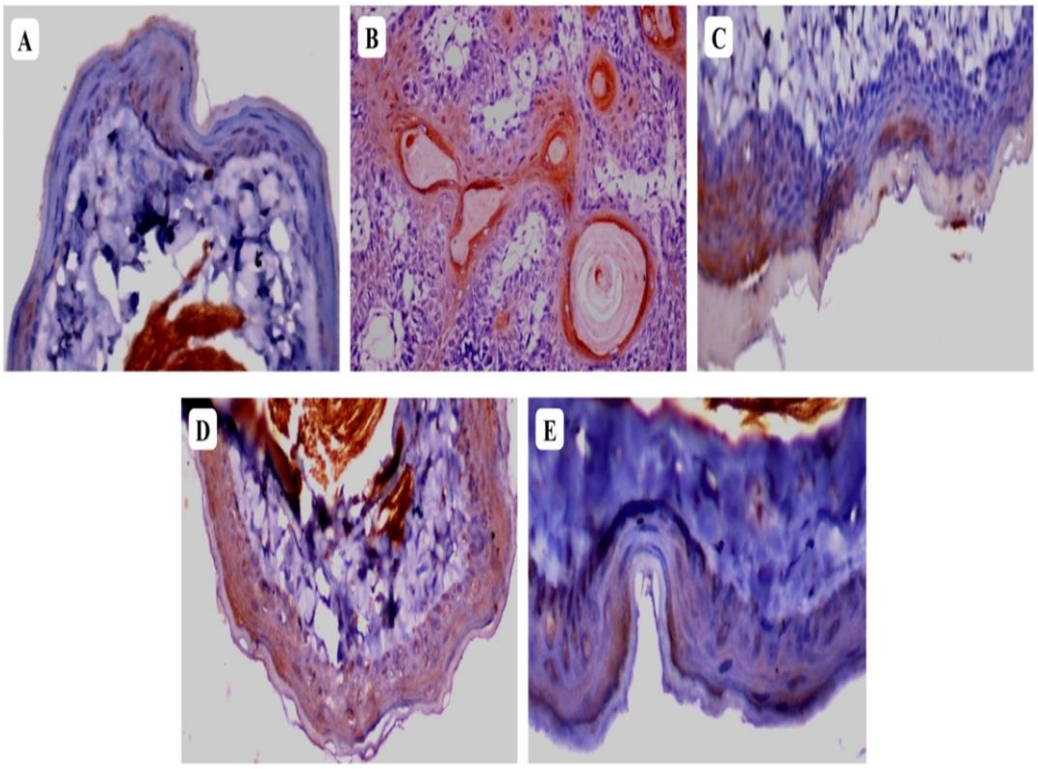

Figure 4: MMP-2 expression in the buccal mucosa of experimental hamsters. **(A)** Tissues from control hamsters showed no MMP-2 expression. **(B)** Tissues from DMBA alone treated hamsters showed moderate MMP-2 expression in the keratin pearls and its layer cells. **(C)** Tissues from DMBA + myrtenal treated hamsters showed no MMP-2 expression in the epithelium. **(D)** Tissues from DMBA → myrtenal treated hamsters showed MMP-2 expression throughout the epithelium. **(E)** Tissues from myrtenal alone treated hamsters showed no MMP-2 expression in the basal cells.

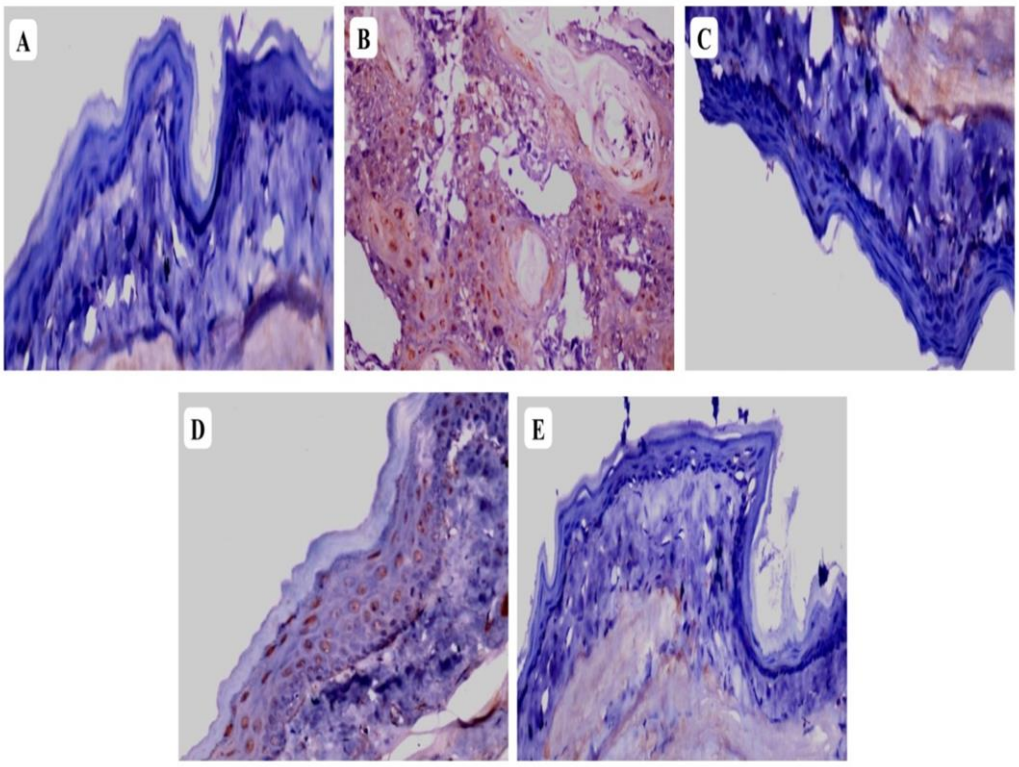

Figure 5: MMP-9 expression in the buccal mucosa of experimental hamsters. **(A)** Tissues from control hamsters showed no MMP-9 expression in the epithelium. **(B)** Tissues from DMBA alone treated hamsters showed moderate MMP-9 expression in the malignant cells. **(C)** Tissues from DMBA + myrtenal treated hamsters showed no MMP-9 expression in the basal and parabasal cells. **(D)** Tissues from DMBA → myrtenal treated hamsters showed MMP-9 expression throughout the basal and parabasal cells. **(E)** Tissues from myrtenal alone treated hamsters showed no MMP-9 expression in the basal cells.

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Conclusion

The present study concludes that the anti-cell proliferative, anti-invasive and anti-angiogenic effects of myrtenal could be partly responsible for its chemopreventive and chemotherapeutic potential in DMBA-induced oral carcinogenesis.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors gratefully acknowledge the financial assistance from ICMR, New Delhi and express their sincere appreciation to UGC-SAP and DST-FIST for providing infrastructural facilities to carry out this research work.

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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