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ANTI-INFLAMMATORY EFFECT OF MYRTENAL IN DMBA INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

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Abstract

Chronic inflammation has been documented as one of the key pathological features of several illnesses including cancer. The present study examined the anti-inflammatory effect of myrtenal by analyzing the immunoexpression pattern of inflammatory markers in 7,12-dimethylbenz(a)anthracene (DMBA) induced oral carcinoma in golden syrian hamsters. Topical application of DMBA (0.5%), a potent site specific carcinogen, three times a week for 14 weeks on the buccal pouches of hamsters resulted in tumor development, which was confirmed histopathologically as well differentiated squamous cell carcinoma. The immunoexpression pattern of inflammatory markers (NFκB, Cox-2, iNOS, TNFα, IL-1, IL-6 and IL-10) in the buccal mucosa was found to be up regulated in the tumor bearing hamsters. Myrtenal administration (230 mg/kg bw p.o) to the hamsters treated with DMBA down regulated the immunoexpression pattern of the above said molecular markers in the buccal mucosa. The present study thus suggests that the anti-inflammatory efficacy of myrtenal might have played a possible role in the prevention of DMBA induced oral tumor formation in the buccal mucosa of golden Syrian hamsters.

Key words: Inflammation, oral cancer, DMBA, Myrtenal.

Introduction

Inflammation has been focused as one of the major events in carcinogenesis and almost all types of solid tumors showed abnormal levels of inflammatory mediators (Qu *et al*., 2018). Chronic inflammation due to physical, chemical or biological agents could be considered as one of the major factors in mediating carcinogenesis. The major inflammatory mediators include cytokines, cyclooxygenases, nuclear factor kappa B (NFκB) and prostaglandins. Aberrant expression of these inflammatory markers could therefore lead to neoplastic transformation. They mediate carcinogenesis through epigenetic alterations such as altered DNA methylation pattern and by causing posttranslational modifications (Morgillo *et al*., 2018). NFκB, an ubiquitous transcription factor, plays a significant role in inflammatory processes and its over expression has been documented in several carcinogenesis including oral carcinoma (Patel *et al*., 2016). NFκB facilitates tumor growth and its progression via induction of pro-inflammatory cytokines release. NFκB regulated the expression of diverse genes that has a pivotal role in the process of cell proliferation, apoptosis and angiogenesis (Park and Hong, 2016). NFκB has an important role in the regulation of more than 500 genes, which have a vital and crucial role in the process of inflammation, apoptosis, angiogenesis and tumorigenesis (Hayden and Ghosh, 2004). NFκB expression could also result in chemoresistance, tumor invasion and metastasis.

Cyclooxygenases (COX) play a pivotal role in the conversion of arachidonic acid to prostaglandins. COX-2 plays vital role in both normal physiological and pathological conditions. COX-2 upregulation promotes increased abnormal cell proliferation, stimulates angiogenesis and inhibits immune surveillance (Nasry *et al*., 2018). The expression of COX-2 in the body is stimulated by a wide variety of factors, including growth factors for, cytokines and carcinogens (Wang *et al*., 2014). Moazeni-Roodi *et al*., (2017) suggested that pro-inflammatory mediators such as tobacco smoking could

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lead to over expression of COX-2 in oral carcinogenesis. A large number of research findings explored over expression of COX-2 in oral tumor tissues (Manoharan *et al*., 2016).

Nitric oxide production in the body is regulated by the family of nitric acid synthases (Liao *et al*., 2019). Nitric oxide synthase catalyses the conversion of L-arginine to L-citruline and nitric oxide generated during this reaction has a pivotal role *in vivo* in regulating multiple cellular signaling pathways such as vasodialation, neurotransmission and inflammation (Rasheed *et al*., 2007). Sangle *et al*., (2018) demonstrated over expression of nitric oxide in the tumor tissues of oral cancer patients. Nitric oxide plays a dual role in the process of carcinogenesis. While it's abnormal production suppresses the tumor formation, low levels of nitric oxide stimulate tumorigenesis by inhibiting angiogenesis (Vahora *et al*., 2016). The inducible nitric acid synthase (iNOS) has been considered to have a vital role in carcinogenesis. NOS family mediates carcinogenesis through inhibition of apoptotic induction, stimulating the angiogenic mechanism and by inducing gene mutation (Zhang *et al*., 2019). The expression of iNOS was found to be higher in several tumor tissues such as colon, breast and oral carcinoma (Fahey *et al*., 2017; Gao *et al*., 2019).

The interleukin-1 (IL-1) family is comprised of both pro-inflammatory and anti-inflammatory ligands. IL-1 plays a significant role in several biological processes and has a key role in the regulation of the immune system, both adaptive and innate immune response (Dinarello, 2018). Aberrant expression of IL-1 resulted in several pathological illness, including inflammatory disorders. Mounting evidences have correlated the expression of IL-1 and pathogenesis of cancer as well (Baker *et al*., 2019). IL-1 and tumor necrosis factor (TNFα) have been recognized as a major inflammatory cytokine, IL-1, has an important role in the pathogenesis of cancer-related inflammation. IL-1 has been reported to be involved in the NFêB activation (Liu *et al*., 2017). Tumor necrosis factor α is one of the inflammatory markers associated not only with cell cycle activation but also in tumor progression and metastasis. IL-6 has been pointed out to have diverse roles in carcinogenesis, including regulation of inflammatory responses, tumor growth and development, invasion and in angiogenesis (Guo *et al*., 2012). IL-10 favours tumor progression via inhibiting the synthesis of TNFα (Allavena *et al*., 2008). Ali *et al.,* (2018) suggested that IL-10 expression could be considered as a potent biomarker of oral carcinoma. Investigation of potent cytokine inhibitors could thus result in promising anticancer candidature for various cancers.

Myrtenal, an organic bicyclic monoterpenoid, is found in the essential oil of wide variety of medicinal plants such as cumin, mint, pepper and eucalyptus. Myrtenal has been shown to possess diverse biochemical, molecular and pharmacological effects. Profound studies well documented its anti-inflammatory (Dragomanova *et al*., 2019), antioxidant (Lokeshkumar *et al*., 2015), antidiabetic (Rathinam *et al*., 2016), neuroprotective (Kaufmann *et al*., 2011) and anticancer potential (Hari Babu *et al*., 2012) in experimental animal models. Recent research from our laboratory has explored its anticancer (Buddhan *et al*., 2020) and apoptotic potential in experimental oral carcinogenesis (Buddhan *et al*., 2020). Myrtenal thus attracted the researchers and scientists to assess its molecular mechanisms in the prevention of carcinogenesis. The present study evaluated the modulating effect of myrtenal on the immunoexpression pattern of inflammatory markers in DMBA induced oral carcinogenesis.

Materials and Methods

Animals

Thirty male golden Syrian hamsters (7-8 weeks old; 80-120g body weight) were procured from the National Institute of Nutrition (NIN), Hyderabad, India. The experimental animals were equally categorized into five groups (6 animals in each) and were maintained in the Central Animal House of Annamalai University as per the suggestions and the ethical principles of Annamalai University Institutional Ethical committee.

Experimental design

The experimental practice designed for the present research work was as follows. Group I hamsters were categorized as vehicle treated control and were received topical application of liquid paraffin alone on their buccal pouches (3 times a week for 14 weeks). Group II hamsters were assigned for the development of oral tumor formation and were received topical application of 0.5% DMBA alone on their buccal pouches for 14 weeks (3 times a week). Group III hamsters were treated with DMBA (3 times a week for 14 weeks) and myrtenal (230mg/kg bw) on alternate days of topical application of DMBA (3 times a week for 14 weeks) to assess the effect of myrtenal on the expression pattern of inflammatory markers in the chemoprevention phase. Group IV hamsters were treated with topical application of DMBA alone for first 10 weeks (3 times a week), which was followed by oral administration of myrtenal for 8 weeks (11^{th} to 18^{th} week, 3 times a week). This group was categorized to assess the effect of myrtenal on the expression pattern of inflammatory markers in the chemotherapeutic phase. Group V hamsters received oral

administration of myrtenal alone throughout experimental period (3 times a week). The buccal mucosa tissues from control and experimental groups were excised, after routine procedure and subjected to analyse the immunoexpresssion pattern of inflammatory markers in DMBA induced oral cancer.

Immunohistochemical Staining

The immunoexpression pattern of the inflammatory markers in the buccal mucosa of the experimental hamsters was evaluated as described earlier (Buddhan *et al*., 2020). In brief, after the routine procedure and antigen retrieval from the tissue sections, they were treated with the primary monoclonal antibodies corresponding to the inflammatory markers (NFκB, COX-2, iNOS, TNFα, IL- 1, 6 and 10), after the retrieval of the antigens and routine procedure (Whiteside and Munglani, 1998). The slides were then incubated with the horseradish peroxidase labelled secondary antibodies. The slides were then treated with diaminobenzidine (DAB), the chromgenic substrate of the enzyme. The tissue sections were finally counterstained with hematoxylin and the inflammatory markers' immuno expression pattern was observed under the microscope.

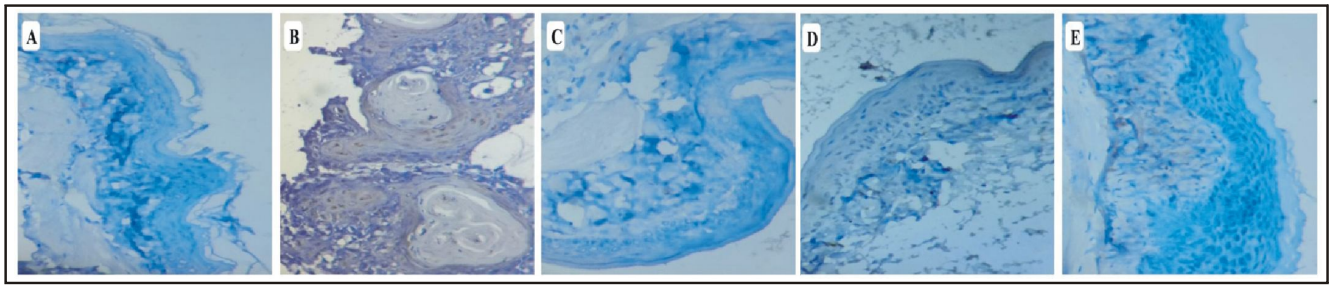

Fig. 1: NFκB expression in the buccal mucosa of experimental hamsters. (A) Tissues from control hamsters showed no NFκB expression; (B) Tissues from DMBA alone treated hamsters showed NFκB over expression in the epithelial islands; (C) Tissues from DMBA + myrtenal treated hamsters showed no NFκB expression in the epithelium; (D) Tissues from DMBA → myrtenal treated hamsters showed mild NFêB expression in the basal cells; (E) Tissues from myrtenal alone treated hamsters showed no NFκB expression in the basal cells.

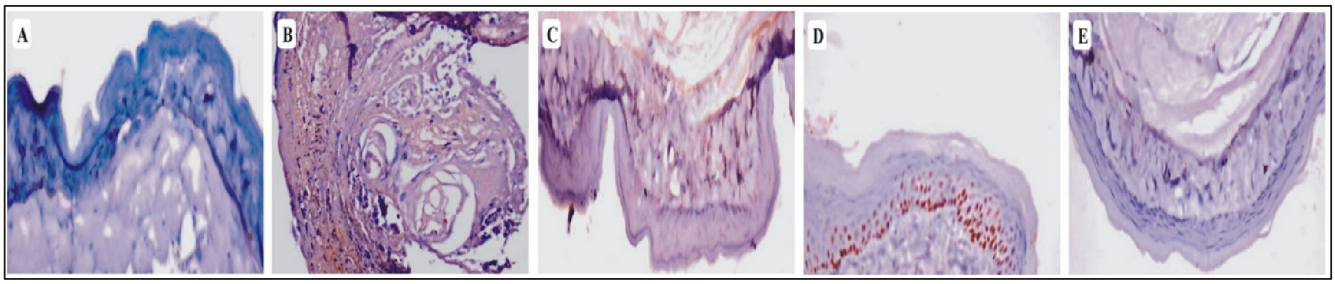

Fig. 2: COX-2 expression in the buccal mucosa of experimental hamsters. (A) Tissues from control hamsters showed no COX-2 expression in the basal cells; (B) Tissues from DMBA alone treated hamsters showed COX-2 over expression in few epithelial islands; (C) Tissues from DMBA + myrtenal treated hamsters showed no COX-2 expression in the epithelium; (D) Tissues from DMBA → myrtenal treated hamsters showed moderate COX-2 expression in the basal cells; (E) Tissues from myrtenal alone treated hamsters showed no COX-2 expression in the basal and parabasel cells.

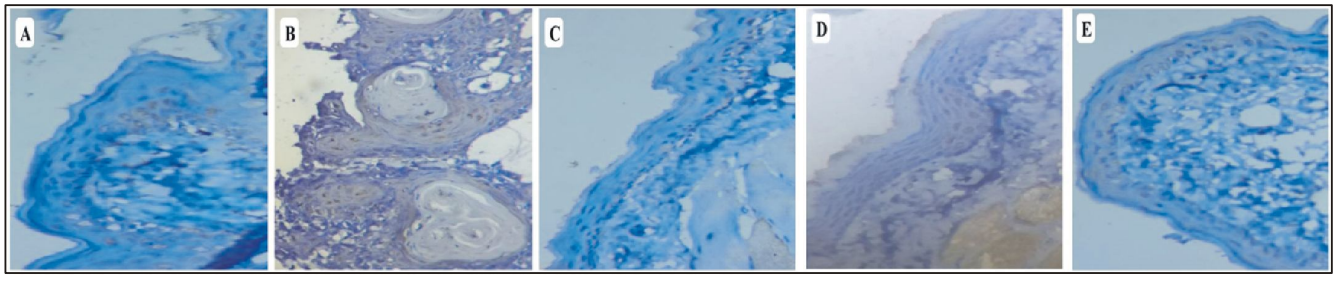

Fig. 3: iNOS expression in the buccal mucosa of experimental hamsters. (A) Tissues from control hamsters showed very mild iNOS expression in the basal cells; (B)Tissues from DMBA alone treated hamsters showed higher iNOS expression in few epithelial islands and keratin pearls; (C) Tissues from DMBA + myrtenal treated hamsters showed no iNOS expression in the basal and parabasal cells; (D) Tissues from DMBA → myrtenal treated hamsters showed moderate iNOS expression in the basal and parabasal cells; (E) Tissues from myrtenal alone treated hamsters showed very mild iNOS expression in the basal cells.

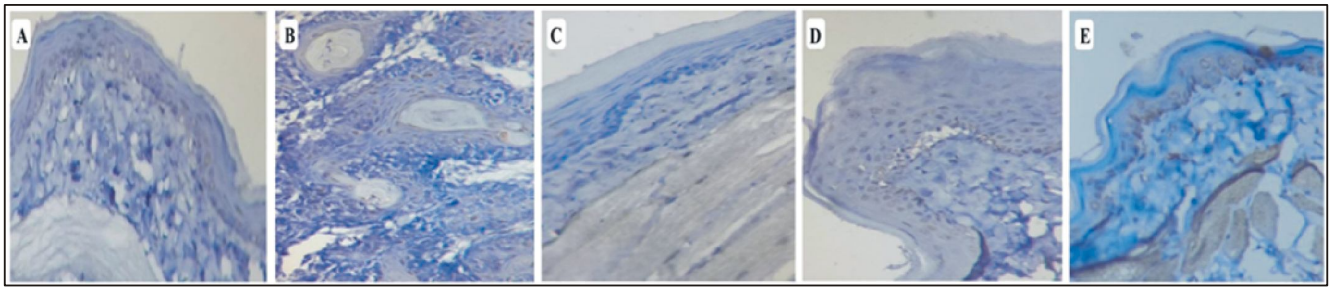

Fig. 4: TNFα expression in the buccal mucosa of experimental hamsters. (A) Tissues from control hamsters showed mild TNFα expression in the basal and parabasal cells; **(**B) Tissues from DMBA alone treated hamsters showed TNFα over expression in the hyperplstic epithelial islands; **(**C) Tissues from DMBA + myrtenal treated hamsters showed mild TNFα expression in the basal and parabasal cells; **(**D) Tissues from DMBA → myrtenal treated hamsters showed moderate TNFα expression in the basal cells; (E) Tissues from myrtenal alone treated hamsters showed mild TNFα expression in the basal cells.

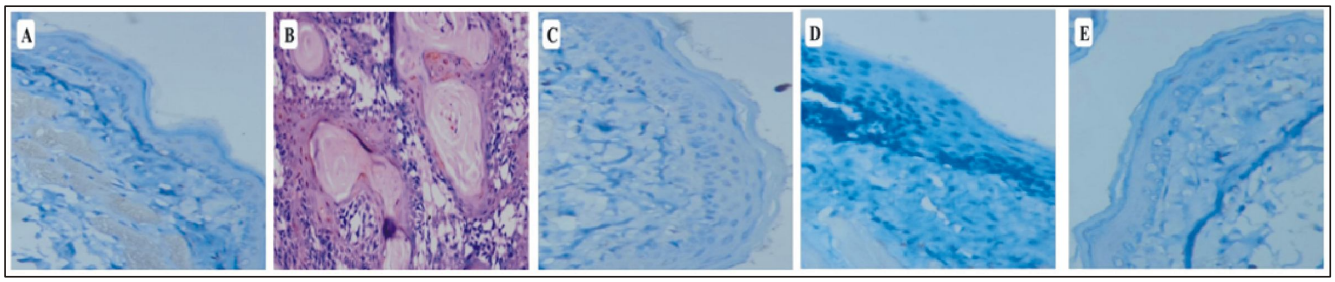

Fig. 5: IL-1 expression in the buccal mucosa of experimental hamsters. (A) Tissues from control hamsters showed no IL-1 expression in the basal cells; **(**B) Tissues from DMBA alone treated hamsters showed moderate IL-1 expression in the epithelial islands; (C) Tissues from DMBA + myrtenal treated hamsters showed no IL-1 expression in the epithelium; (D) Tissues from DMBA → myrtenal treated hamsters showed no IL-1 expression in the basal cells; (E) Tissues from myrtenal alone treated hamsters showed no IL-1 expression in the basal cells.

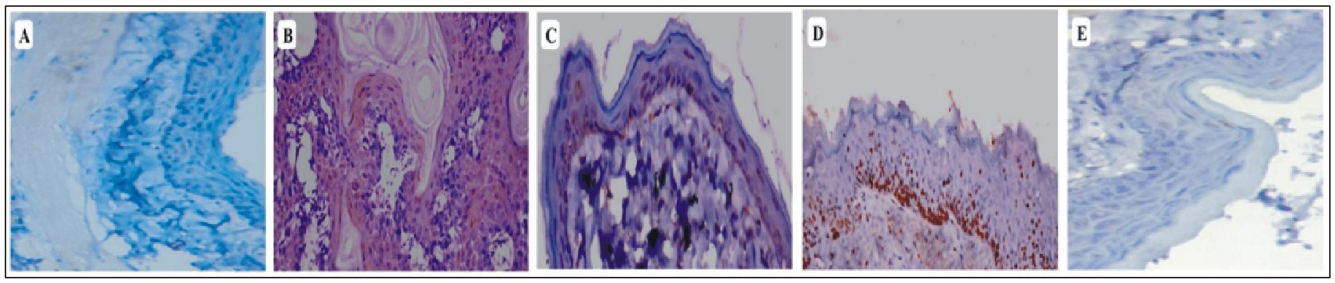

Fig. 6: IL-6 expression in the buccal mucosa of experimental hamsters. (A) Tissues from control hamsters showed no IL-6 expression in the basal cells; (B) Tissues from DMBA alone treated hamsters showed higher IL-6 expression in island of the epithelium; **(**C) Tissues from DMBA + myrtenal treated hamsters showed mild IL-6 expression in basal and parabasal cells; (D) Tissues from DMBA → myrtenal treated hamsters showed moderate IL-6 expression in basal and parabasal cells; (E) Tissues from myrtenal alone treated hamsters showed no IL-6 expression in basal cells.

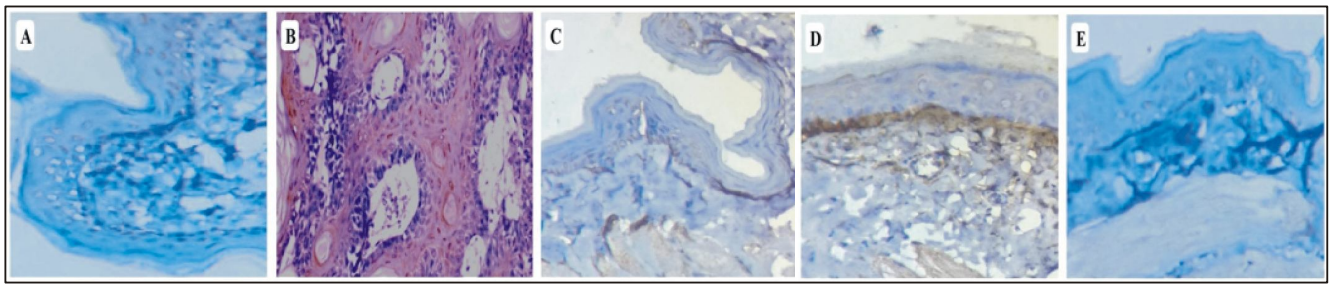

Fig. 7: IL-10 expression in the buccal mucosa of experimental hamsters. (A) Tissues from control hamsters showed very mild IL-10 expression in the basal cells; (B) Tissues from DMBA alone treated hamsters showed higher IL-10 expression in dysplastic epithelial island; (C) Tissues from DMBA + myrtenal treated hamsters showed mild IL-10expression in basal cells; (D) Tissues from DMBA → myrtenal treated hamsters showed moderate IL-10 expression in basal and parabasal cells; (E) Tissues from myrtenal alone treated hamsters showed no IL-10 expression in basal and parabasal cells.

Results

The immunoexpression pattern of the inflammatory markers (NFκB, COX-2, iNOS, TNFα, IL-1, IL-6, IL-10) in the control and experimental animals is shown in the figs. 1 to 7. The immunoexpression pattern of the inflammatory markers was significantly up regulated in the buccal mucosa of DMBA treated hamsters (group II). Myrtenal administration orally at a dose of 230mg/kg bw significantly down regulated the expression of the inflammatory markers in DMBA+myrtenal treated hamsters (group III) and considerably decreased the expression of the inflammatory markers in DMBA → myrtenal treated hamsters (group IV). A similar immunoexpression pattern of the above said inflammatory markers was noticed between liquid paraffin alone (group I) and myrtenal alone (group IV) treated hamsters.

Discussion

Tumor cells secrete a spectrum of biomarkers into the tissues or circulation, which could help to evaluate the prognosis of tumors (Hussein *et al*., 2018). Chronic inflammation induced by physical, chemical and biological agents has been associated with the pathogenesis of several illnesses, including cancer (Kawanishi *et al*., 2017). It is well known that the inflammatory diseases and autoimmune disorders could enhance the risk of cancer development. Immunosuppression has also been focused as a key factor in the pathogenesis and progression of cancer. Immunosuppression could result from changes in the expression of cytokines and imbalances in the populations of the immune cells (Jebreel *et al*., 2007).

Interleukins have a crucial role in the transmission of cellular signals and in the immune system. Johnson *et al*., (2014) suggested that NFκB inhibition could suppress the oral tumor formation in the experimental animals. Madankumar *et al*., (2017) have shown NFκB upregulation in 4-nitroquinoline induced tongue cancer. NFκB over expression has been shown at 52% of head and neck cancer (Yan *et al*., 2010). Jimi *et al*., (2016) pointed out that over expression of nuclear factor kappa β could contribute to the bone invasion in the oral squamous cell carcinoma. Gupta *et al*., (2018) have shown NFκB over expression in tongue cancer. NFκB expression in inflammatory conditions could in turn lead to abnormal expression of cox-2, iNOS and TNFα (Ramu *et al*., 2018).

A positive association has been shown between COX-2 over expression and poor prognosis, low survival outcome and tumor recurrence in oral cancer cases (Pannone *et al*., 2007). COX-2 upregulation has been shown to inhibit apoptosis and facilitates angiogenesis and invasion (Mendes *et al*., 2009). Pontes *et al*., (2013) demonstrated abnormal COX-2 expression of precancerous and cancerous lesions of the oral cavity. Silva *et al*., (2017) have demonstrated the role of COX-2 in the biological behaviour of the oral cancer. Soland *et al*., (2008) correlation the expression of COX-2 with various histological grades of oral malignancy.

Over expression of iNOS has been associated with tumor progression and worst prognosis of cancer (Liao *et al*., 2019). A positive correlation has been demonstrated between VEGF and iNOS expression in tumor tissues (Chen *et al*., 2006). iNOS over expression stimulated mutant p53 accumulation in mammary cancer cell lines (Yang *et al*., 2015). Silva Servato *et al*., (2019) focused iNOS expression as a prognostic marker of oral dysplasia and oral carcinoma. Previous studies reported higher activities of iNOS in plasma, saliva and tumor tissues of oral carcinoma samples. Ramu *et al*., (2018) have shown iNOS, COX-2, TNFα and IL-6 aberrant expression in experimental oral carcinogenesis. iNOS expression pattern was found to be increased in the carcinogens and sarcoma of the oral cavity (Augustine *et al*., 2015). Yang *et al*., (2015) reported that iNOS knock down resulted in p53 over expression in oral carcinoma. iNOS expression was found to be higher in tobacco abusers than the subjects not habituated to tobacco use.

Cytokines play crucial role in the process of intercellular communication and their secretion in the tumor microenvironment facilitates tumor invasion and metastasis (da Cunha *et al*., 2019). Dantas *et al*., (2019) reported that the TNFα expression was found to be more significant in oral cancer cases with lymphnode metastasis. Abnormal expression of iNOS and TNFα in oral premalignant conditions such as oral submucous fibrosis has been associated with the pathophysiology of oral carcinogenesis (Gupta *et al*., 2017). Chronic inflammation due to abnormal levels of IL-6 and TNFα promotes angiogenesis at the site of inflammation (Lee *et al*., 2018). A positive correlation between TNFα and NFêB expression has been demonstrated. It has been well documented that aberrant TNFα expression could able to promote oral tumorigenesis (Goertzen *et al*., 2018). Zhang *et al*., (2019) reported that TNFα promotes angiogenesis in head and neck cancer.

Voiculescu *et al*., (2016) suggested that malignant cells secrete IL-1B, IL-6 and TNFα and these

inflammatory cytokines are responsible for the motility of keratinocytes and lymphnode metastasis. IL-1 and IL-6 are enormously generated in the squamous cell carcinoma in order to facilitate tumor progression (Almeida Vinícius *et al*., 2019). IL-6 and IL-10 over expression has been reported to be associated with poor prognosis of oral squamous cell carcinoma (Shinriki *et al*., 2011). Higher expression of IL-10 has been reported in tumor tissues and in lymphnode tumor metastasis (Arantes *et al*., 2016). Shinagawa *et al*., (2017) suggested that over expression of IL-6 in oral tumor tissues could be responsible for tumor recurrence and lymphangiogenesis. It has been reported that IL-6 over expression could cause vascular invasion in oral carcinogenesis. IL-6 expression was more prominent in the tumor cell membranes. Serum IL-6 levels have been utilized to assess the prognosis of several types of tumors, including oral carcinoma (Kim *et al*., 2017).

Goncalves *et al*., (2017) showed an over expression of IL-10 in oral leukoplakia and in oral squamous cell carcinoma. Abnormal IL-10 levels were reported in the saliva of the cancer patients. Higher IL-10 expression was noticed in 80 to 90% of the oral tumor damp (Fujieda *et al*., 1999). The salivary concentration of IL-6 could be utilized as an auxiliary diagnostic indicator in oral carcinogenesis (Zhang *et al*., 2017). Sahibzada *et al*., (2017) focuses salivary IL-6 and TNFα as oral cancer diagnostic biomarkers. Studies have also revealed, contrary correlations between IL-10 expression and various histological tumor grade (clinical tumor staging) (Ali *et al*., 2018). NFκB activation in the cell could also result in abnormal production of IL-6 in oral carcinogenesis (Woods *et al*., 1998).

In the present study, we investigated the modulating efficacy of myrtenal on the immunoexpression pattern of inflammatory markers (NFκB, COX-2, iNOS, TNFα, IL-1, IL-6, IL-10,) in DMBA induced experimental oral carcinogenesis. Abnormal expression of these inflammatory markers and pro-inflammatory cytokines were noticed in the buccal mucosa of tumor bearing hamsters. This clearly indicates the role of these inflammatory markers in the tumor cell proliferation and progression of oral cancer. Oral administration of myrtenal at a dose of 230mg/kg bw to hamsters treated with DMBA downregulated the expression of inflammatory markers to near normal pattern and significantly improved the immunoexpression pattern of these markers towards tumor suppression in the DMBA → myrtenal treated animals. The results observed in the present study thus clearly indicate the anti-inflammatory efficacy of myrtenal in experimental oral carcinogenesis.

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