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Myrtenal averts apoptotic evasion of cancer cells in 7,12-dimethylbenz(a)anthracene induced experimental oral carcinogenesis

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ABSTRACT

Apoptotic avoidance is one of the foremost characteristic features of tumour cells. Apoptotic induction in cancer cells could thus help to identify new anticancer agents from the natural products. The present study has taken an effort to investigate the apoptotic efficacy of myrtenal, a monoterpene, in 7,12-dimethylbenz(a)anthracene (DMBA) induced oral carcinoma in male golden Syrian hamsters. The present study used the potent carcinogen, 7,12-dimethylbenz(a)anthracene, to develop oral tumours in the buccal pouches of the golden Syrian hamsters. Topical application of the above said carcinogen on the buccal mucosa (3 times a week for 14 weeks) resulted in well differentiated oral squamous cell carcinoma. The apoptotic potential of myrtenal was investigated using a spectrum of apoptotic markers, including pro-apoptotic and anti-apoptotic markers. Oral tumours developed in the buccal mucosa of hamsters showed higher expression of mutant p^{53} protein, Bcl-2 and lowered expression of Bax, Bad, Bid, caspase 3 and caspase 9. Myrtenal treatment at a dose of 230mg/kg bw orally to the hamsters treated with DMBA modulated the above said apoptotic markers towards tumour suppression or inhibition. The present findings thus suggests that the tumour preventive effect of myrtenal could partly be ascribed to its apoptotic induction potential during DMBA induced oral carcinogenesis.

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INTRODUCTION

Human body is endowed with a well balanced homeostatic mechanism to remove the unwanted or damaged cells arised through various cellular processes (Lee *et al.*, 2018) . Any defect in mitosis or removal of damaged cells could result in various illness especially cancer (Iyama and Wilson, 2013). The apoptotic process is an important phenomenon of multicellular organisms to maintain the tissue homeostasis and to control the proliferation of cells (Chen *et al.*, 2018). Apoptosis, a cell suicide, is regulated by a series of regulatory genes and of which some genes play a role in the apoptotic initiation, while the others are involved in the apoptotic inhibition (Goldar *et al.*, 2015). The family of apoptotic proteins is comprised of pro-apoptotic and anti-apoptotic proteins. Any disturbances in the ratio of pro- and anti-apoptotic proteins in the cell could lead to abnormal proliferation of cells (Jan R and Chaudhry G E, 2019; Pistritto *et al.*, 2016).

The apoptotic proteins expression could also help to assess the prognosis of cancer (Hassan *et al.*, 2014). It has been clearly pointed out that any defect or imbalance in intrinsic or extrinsic pathway of apoptosis could lead to carcinogenesis (Belkacemi, 2018). Altered status of pro-apoptotic and anti-apoptotic proteins have been explored in oral carcinogenesis. As apoptotic dys-regulation has massive impact on cell proliferation and cell cycle regulation, researchers target apoptotic markers to study the anticancer efficacy of natural products and their bioactive entities (Pfeffer and Singh, 2018).

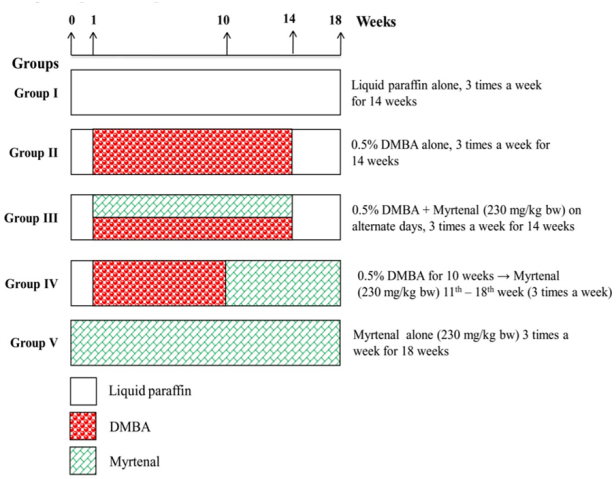

Figure 1: Experimental protocol

Myrtenal, in recent years, attracted the attention of several researchers due to its multiple biochemical and pharmacological efficacies such as anti-inflammatory (Dragomanova *et al.*, 2019; Rathinam *et al.*, 2019), neuroprotective (Kaufmann *et al.*, 2011), antioxidant (Lokeshkumar *et al.*, 2015; Dragomanova *et al.*, 2018), anti-diabetic (Pari and Rathinam, 2016), antihyperlipidemic (Pari and Rathinam, 2016) and anticancer (Lokeshkumar *et al.*, 2015) properties. Myrtenal has been shown to have protective effect against liver disorders (Lingaiah *et al.*, 2013), diabetes (Babu *et al.*, 2012; Rathinam *et al.*, 2019). A very few studies have also reported its cytotoxic potential against tumour cell lines (Suslov *et al.*, 2015; Martins *et al.*, 2019). The present study assesses the apoptotic induction potential of myrtenal in 7,12-dimethylbenz(a)anthracene induced oral carcinogenesis.

MATERIALS AND METHODS

For the present study, thirty male golden Syrian hamsters (8-10 weeks old; 80—120g) were procured from National Institute of Nutrition, Hyderabad. The hamsters were then grouped into five categories and maintained in the well-sophisticated Central Animal House, Annamalai University, according to the ethics principles of Institutional ethics committees (Committee no.160/1999/CPCSEA, 25/11/1999, Proposal No.1175). All the animals received their diet and water ad libitum. The experimental protocol is represented in the Figure 1.

TUMOUR INDUCTION

The present study used the potent carcinogen, 7,12-dimethylbenz(a)anthracene, to develop oral tumours in the buccal pouches of the golden Syrian hamsters. Topical application of the above said carcinogen on the buccal mucosa (3 times a week for 14 weeks) resulted in well differentiated oral squamous cell carcinoma.

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Immuno-histochemical analysis was employed to analyse the expression pattern of apoptotic markers in the buccal mucosa of control and experimental hamsters. Paraffin embedded tissue sections were incubated with the corresponding primary monoclonal antibodies to the apoptotic markers (p^{53}, Bcl-2, Bax, Bid, Bad, caspase 3 and 9) after the retrieval of the antigens and routine procedure (Whiteside and Munglani, 1998). The slides were then treated with the horse radish peroxidase labelled secondary antibodies. The slides were subsequently incubated with the chromogen, Diaminobenzidine (DAB). The slides were than counterstained with hematoxylin. The immuno-expression pattern of the apoptotic markers were examined under microscope, after attaining the desirable colour intensity.

RESULTS AND DISCUSSION

The immunoexpression pattern of buccal mucosa apoptotic markers (pro-apoptotic and antiapoptotic) in control and experimental animals were represented in Figures 2, 3, 4, 5, 6, 7 and 8. The immunoexpression pattern of p^{53} (mutant) and Bcl-2 was found to be overexpressed in tumour bearing hamsters (group 2) as compared to control hamsters (group 1). On the other hand, the expression pattern of Bax, Bid, Bad, caspase 3 and 9 was found to be significantly decreased in hamsters treated with DMBA alone. Myrtenal administration downregulated the expression of mutant p^{53} and Bcl-2 and enhanced the expression of Bax, Bid, Bad, caspase 3 and 9 in the DMBA treated hamsters (chemopreventive phase) and significantly improved the expression pattern in the chemotherapeutic phase as well (DMBA→myrtenal treated hamsters; group 4). The immunoexpression pattern of the above said apoptotic markers in hamsters treated with myrtenal

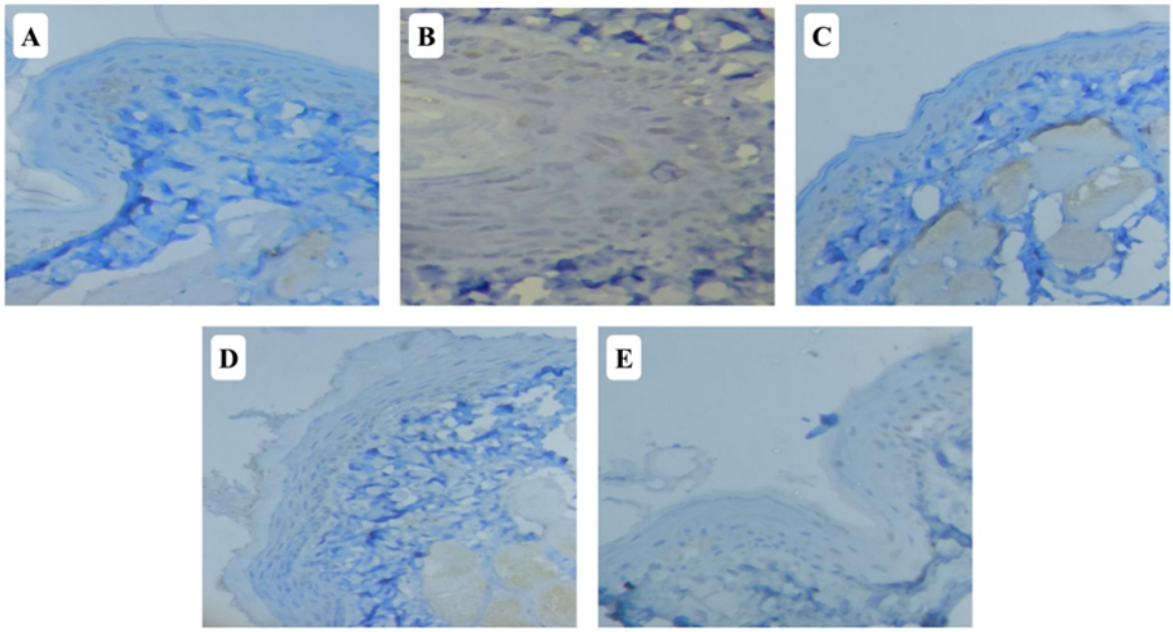

Figure 2: p^{53}expression in the buccal mucosa of experimental hamsters

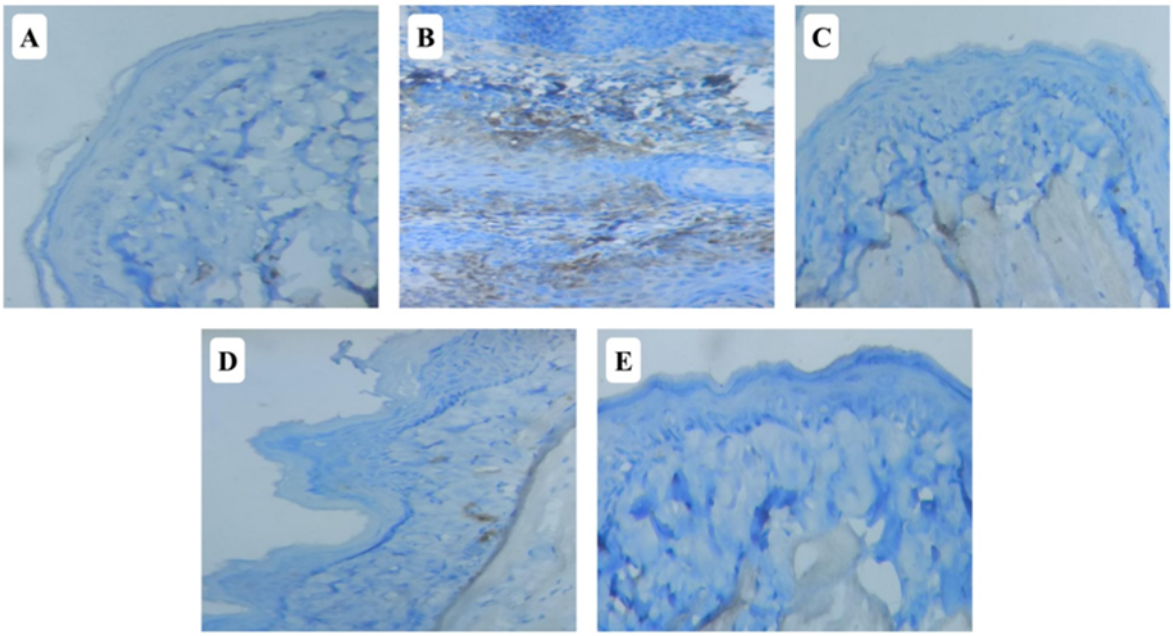

Figure 3: Bcl-2expression in the buccal mucosa of experimental hamsters

alone (group 5) was much comparable to that of control hamsters.

Figure 2 shows that

A- Tissues from control hamsters showed no p53 expression in the basal and parabasal cells.

B- Tissues from DMBA alone treated hamsters showed higher mutant p53 expression in the keratin pearls surrounded by dysplastic epithelial cells.

C- Tissues from DMBA + myrtenal treated hamsters showed very minimal p53 expression in the basal cells.

D- Tissues from DMBA → myrtenal treated hamsters showed minimal p53 expression in the basal cells.

E- Tissues from myrtenal alone treated hamsters showed no p53 expression in the basal and parabasal cells.

Figure 3 shows that

A- Tissues from control hamsters showed no Bcl-2 expression in the epithelium.

B- Tissues from DMBA alone treated hamsters dysplastic epithelium island are showed Bcl-2 overex-

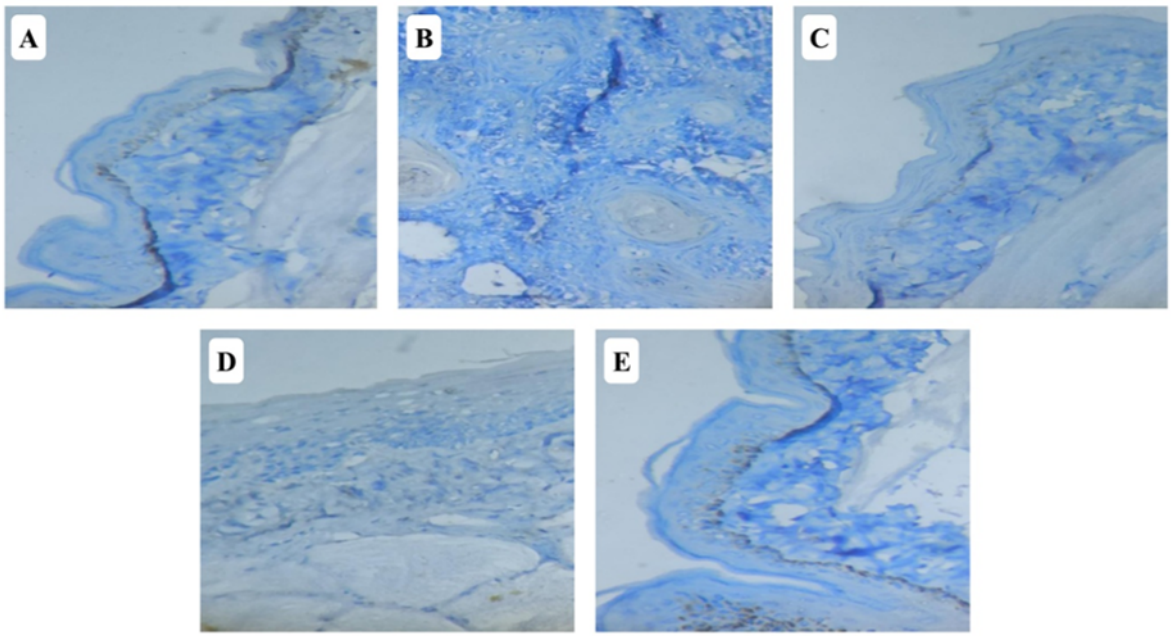

Figure 4: Baxexpression in the buccal mucosa of experimental hamsters

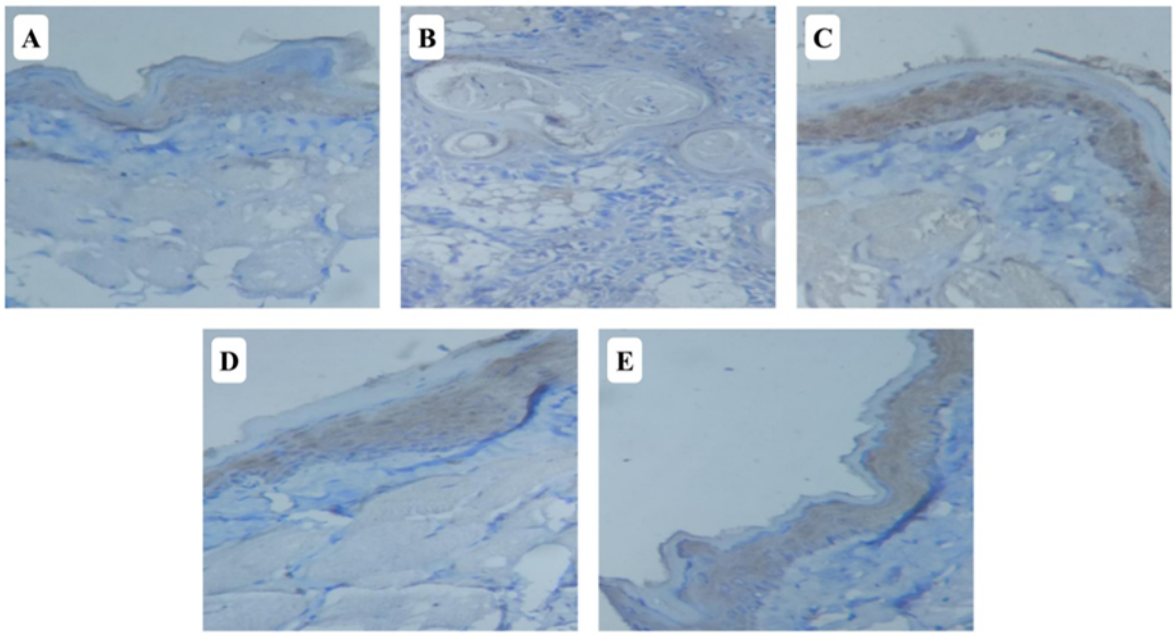

Figure 5: Bid expression in the buccal mucosa of experimental hamsters

pression.

C- Tissues from DMBA + myrtenal treated hamsters showed no Bcl-2 expression.

D- Tissues from DMBA → myrtenal treated hamsters showed minimal expression of Bcl-2 in both basal and parabasal cells.

E- Tissues from myrtenal alone treated the hamsters showed no expression of Bcl-2 in both basal and parabasal cells.

Figure 4 shows that

A- Tissues from control hamsters showed Bax expression in the basal cells throughout the epithelial layer.

B- Tissues from DMBA alone treated hamsters showed no Bax expression.

C- Tissues from DMBA + myrtenal treated hamsters showed minimal expression of Bax in the basal cells.

D- Tissues from DMBA → myrtenal treated hamsters showed very minimal expression of Bax in the basal cells.

E- Tissues from myrtenal alone treated hamsters showed Bax expression in the basal cells.

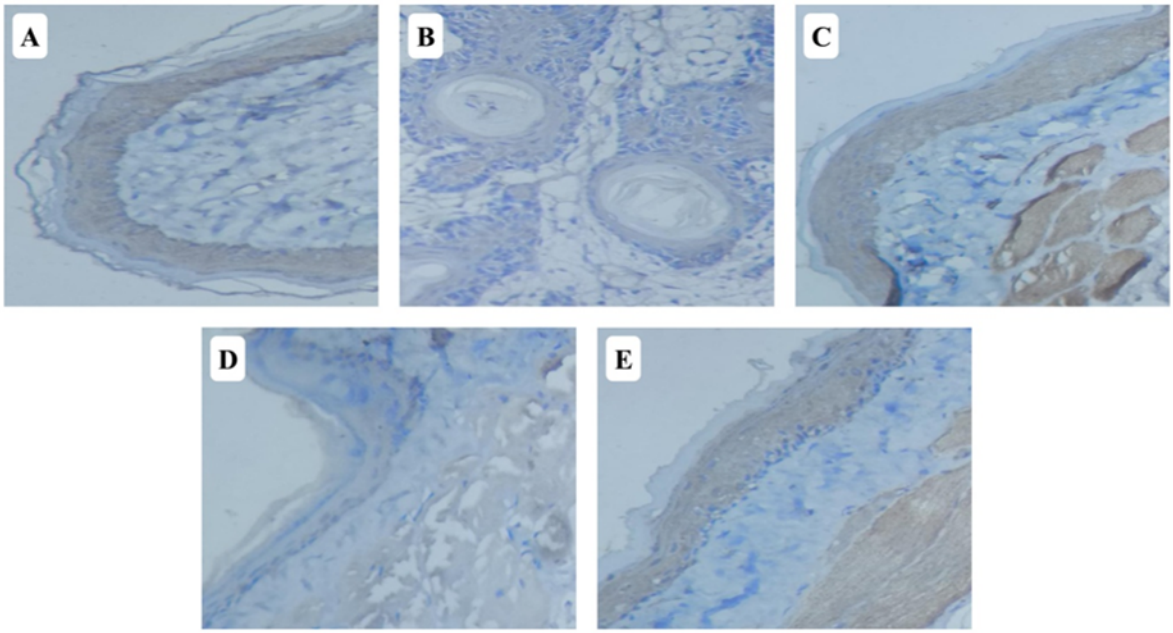

Figure 6: Bad expression in the buccal mucosa of experimental hamsters

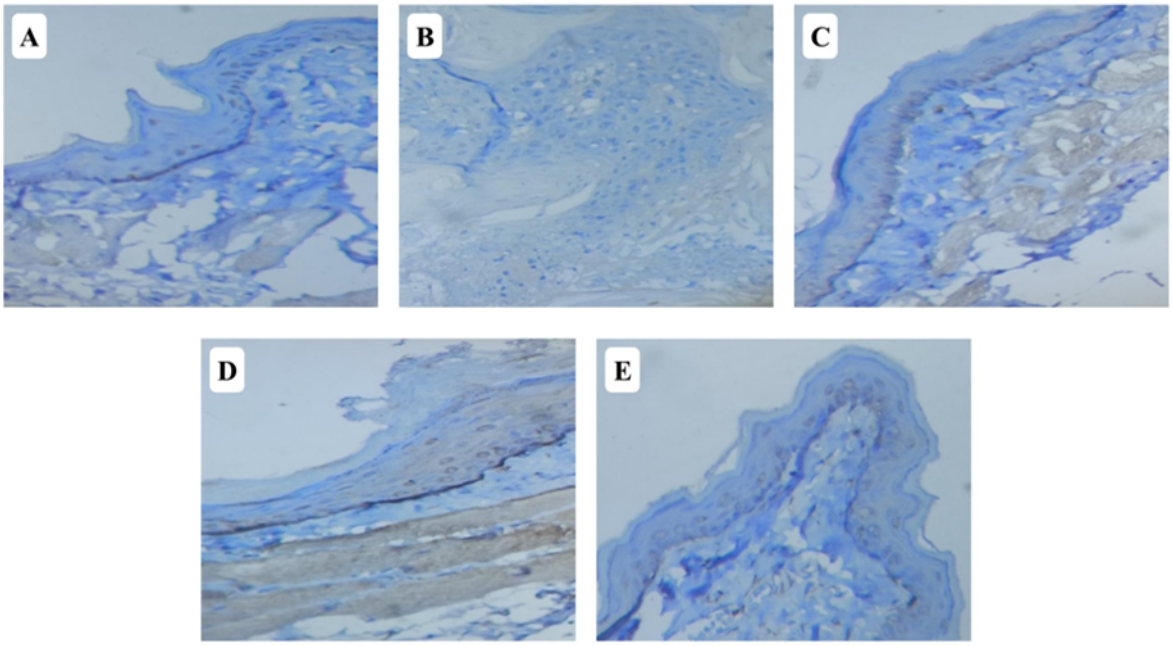

Figure 7: Caspase-3 expression in the buccal mucosa of experimental hamsters

Figure 5 shows that

A- Tissues from control hamsters showed expression of Bid throughout the epithelial layer.

B- Tissues from DMBA alone treated hamsters showed no Bid expression detectable.

C- Tissues from DMBA + myrtenal hamsters showed Bid expression throughout basal and parabasal cells.

D- Tissues from DMBA → myrtenal treated hamsters showed moderate Bid expression.

E- Tissues from myrtenal alone treated hamsters showed Bid expression throughout the epithelial layer.

Figure 6 shows that

A- Tissues from control hamsters showed Bad expression in throughout the epithelial layer.

B- Tissues from DMBA alone treated hamsters showed no Bad expression.

C- Tissues from DMBA + myrtenal treated hamsters showed expression of Bad throughout the epithelial layer.

D- Tissues from DMBA → myrtenal treated hamsters showed minimal expression of Bad in the basal

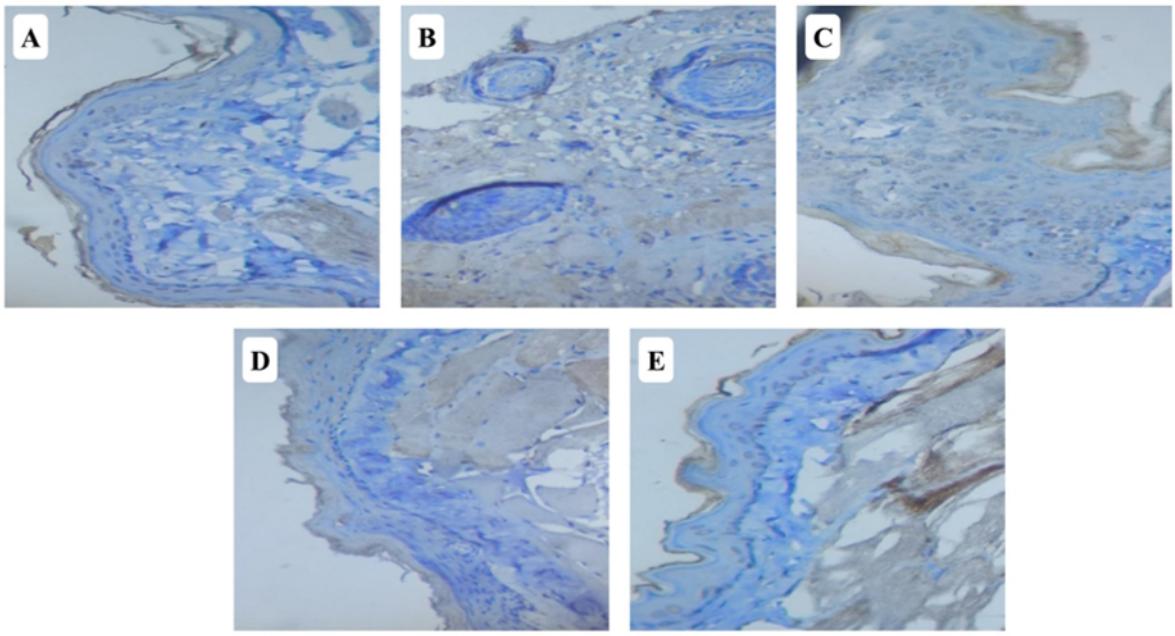

Figure 8: Caspase-9 expression in the buccal mucosa of experimental hamsters

and parabasal cells.

E- Tissues from myrtenal alone treated hamsters showed Bad expression in throughout the epithelial layer.

Figure 7 shows that

A- Tissues from control hamsters showed expression of caspase-3 in the basal cells.

B- Tissues from DMBA alone treated hamsters showed no caspase-3 expression.

C- Tissues from DMBA + myrtenal treated hamsters showed expression of caspase-3 in the basal cells.

D- Tissues from DMBA → myrtenal treated hamsters showed minimal expression of caspase-3 in the basal and parabasal cells.

E- Tissues from myrtenal alone treated hamsters showed expression of caspase-3 in both basal and parabasal cells of epithelium.

Figure 8 shows that

A- Tissues from control hamsters showed caspase-9 expression in the basal cells.

B- Tissues from DMBA alone hamsters showed no caspase-9 expression.

C- Tissues from DMBA + Myrtenal treated hamsters showed expression of caspase-9 in the basal cells of the epithelial layer.

D- Tissues from DMBA → Myrtenal treated hamsters showed very minimal expression of caspase-9 in the basal and parabasal cells of epithelium.

E- Tissues from Myrtenal alone treated hamsters showed expression of caspase-9 in the basal cells.

Apoptotic machinery is a complex cascade, which contains several signaling pathways to maintain the integrity of the genome (Hassan *et al.*, 2014). Apoptotic process causes diverse morphological alterations in the cell structure accompanied by a series of biochemical events, which together in turn clear the apoptotic cells from the body. The major morphological alterations that occur in the apoptotic cells include nuclear condensation, DNA fragmentation, blebbing of cell membrane and shrinkage of cells (D'arcy, 2019). The growth of cancer cells is mainly due to their ability to evade from the apoptotic cascade (Wong, 2011). Evasion of apoptosis could thus result in transcriptional and translational defects, which in turn lead to abnormal cell proliferation. p^{53}, a well known tumour suppressor gene, plays a vital and critical role in the regulation of cell cycle, DNA repair and in programmed cell death (Ozaki and Nakagawara, 2011). p^{53} is a nuclear phosphoprotein and is activated in the cell by both exogenous and endogenous stress stimuli. While normal wild type p^{53} has a very short life span, the mutant p^{53} has extended half- life due to its stability (Ozaki and Nakagawara, 2011).

p^{53} has been documented as the major target of several carcinogens to induce genetic alterations to cause abnormal cell proliferation. Immunohistochemical analysis of p^{53} expression always explored the higher expression of mutant p^{53} protein, as the wild type p^{53} protein cannot be measured due to its short half life. p^{53} expression should be enhanced in the tumour cells in order to provide effective therapy to eradicate the tumour cells (Parrales and

Iwakuma, 2015). (Dave *et al.*, 2016) reported that the immunoexpression pattern of p^{53} corresponds to its histologic grade of oral malignancy. (Mohite *et al.*, 2018) have shown p^{53} overexpression in epithelial dysplastic conditions.

(Patil *et al.*, 2016) have shown around 61% of positive p^{53} expression in oral squamous cell carcinoma. Mutant p^{53} expression has been documented in both human and experimental carcinogenesis (Manoharan *et al.*, 2015; Parrales and Iwakuma, 2015). More than 50% of the tumours excised from the oral cancer patients have shown mutation in the p^{53} gene (Blandino and Agostino, 2018). (Khanna *et al.*, 2012) pointed out that mutant p^{53} expression was found to be increased from premalignant lesions to malignant tumours. The status of p^{53} expression in tumour tissues could be used as a prognostic as well as treatment indicator of the cancer patients.

Bcl-2 family proteins serve as signal for apoptotic switch to decide the fate of the cell survival or death. Bcl-2 family proteins is comprised of both pro-apoptotic and anti-apoptotic proteins. While anti-apoptotic proteins suppress apoptosis by inhibiting the release of mitochondrial cytochrome C, the pro-apoptotic proteins promote the release of cytochrome C to the initiate apoptotic cell death (Wang and Youle, 2009). Bcl-2 family proteins play prominent role in the cell cycle and apoptotic pathway. Bcl-2, is involved in the process of apoptotic inhibition by means of inactivating caspases and by preventing translocation of cytochrome C. Overexpression of Bcl-2 has been shown in oral pre-neoplastic conditions such as dysplasia (Wong, 2011). (Arumugam *et al.*, 2017) reported that Bcl-2 overexpression occurs in parallel with tumour progression. (Pavithra *et al.*, 2017) have shown 33% of oral cancer cases showed positive Bcl-2 expression. It has been reported that the status of Bcl-2 family protein can be used as prognostic indicator of oral carcinoma. A positive correlation between tumour recurrence and Bcl-2 expression pattern has been documented well in various cancers including breast and oral carcinoma (Alam *et al.*, 2018). Bcl-2 overexpression support the abnormal proliferation of tumour cells by suppressing their death rather than inhibiting apoptosis (Selvasundaram *et al.*, 2018). Bax mutation has been shown in several cancers including colorectal and oral carcinoma (Ríos *et al.*, 2015; Selvasundaram *et al.*, 2018). Profound experimental and human carcinogenesis have explored an inverse relationship between the immunoexpression of Bax and Bcl-2 in tumour cells (Frenzel *et al.*, 2009). (Thomas and Sethupathy, 2015) have shown overexpression of Bcl-2 and suppressed Bax expression in human oral squamous cell carcinoma.

BH_3- interacting domain death antagonist (Bid) serves as a pro-apoptotic Bcl-2 family member and play a vital role in programmed cell death. Bid expression has been shown to sensitize the tumour cells for the chemotherapeutic effect of the anticancer agents (Ye *et al.*, 2019). Bad a pro-apoptotic protein stimulates apoptosis by inhibiting or blocking the activation of bcl-2. It has been reported that Bid activated the Bax in mediating apoptosis by binding with Bcl-2 family proteins. Bid has a crucial role in repairing the DNA damaged cells via programmed cell death. Impairment in Bad (Bcl-2 antagonist of cell death) expression play a crucial role in the carcinogenic process. Bad binds to anti-apoptotic proteins of Bcl-2 family and regulates the process of apoptosis.

Caspases are the most important apoptotic signalling molecules, which are categorized as initiator caspases and executioner caspases depending on their mechanism of action. While caspase 8 and 9 are involved in apoptotic initiation, caspases 3,6 and 7 serves as executioner caspases to complete the programmed cell death (Parrish *et al.*, 2013). Activation of caspases and externalization of phosphatidyl serine are the frequent mechanisms found during the apoptotic process (Zhang *et al.*, 2018). As caspases have vital role in the inhibition of apoptosis, caspase activation has been utilized as a target to search for new anticancer agents. (Huang *et al.*, 2017) suggested that caspase 3 expression in the tumour tissues can be used as a prognostic indicator for assessing the particular stage of the oral squamous cell carcinoma. Using immunohistochemistry, abnormal caspase 3 expression has been reported in oral tumour tissues as compared to their adjacent normal tissues. Almost all types of malignancy showed abnormal expression of caspase 3 (Hu *et al.*, 2014). Profound studies showed a positive association between higher caspase 3 expression and tumour size, tumour staging and lymphnode metastasis (Huang *et al.*, 2017). An association between caspase 3 expression and oral cancer prognosis has also been reported (Liu *et al.*, 2017). Overexpression of caspase 3 in oral carcinoma might be due to enhance or promote the apoptotic cascade. (Veeravarmal *et al.*, 2016) reported low or absence of caspase 3 expression in oral squamous cell carcinoma. They suggested that caspase 3 expression tends to be decreased as the tumour progress. Low expression of caspase 3 in oral tumour tissues was also reported by (Li *et al.*, 2017). An impairment in the status of caspase 3 and 9 has been reported frequently in cancer and its progression (Bernard *et al.*, 2019). (Fong, 2006) have shown the down regula-

tion of caspases in tumourigenesis.

Myrtenal, a monoterpene, plays a vital role in the inhibition or prevention of several disorders. (Babu *et al.*, 2012) pointed out that myrtenal suppressed the liver tumour formation in Wistar rats via down-regulating the antiapoptotic (Bcl-2) protein and upregulating pro-apoptotic (Bax) proteins. It has also been reported that myrtenal has the potent ability to up-regulate the expression of p^{53}, a pro-apoptotic and a tumour suppressive gene, in experimental hepatocarcinogenesis (Lingaiah *et al.*, 2013). In the present study, defect in pro-apoptotic and anti-apoptotic markers expression pattern was demonstrated in oral carcinogenesis using immunohistochemical studies. Administration of myrtenal to DMBA treated animals in the chemopreventive and chemotherapeutic phase modulated the immunoexpression pattern of both pro-apoptotic and anti-apoptotic proteins towards the inhibition of tumour formation (chemopreventive phase; group 3) and suppressed or reduced the size and burden of the tumours in the chemotherapeutic phase (group IV). The observed findings thus suggest that myrtenal might have exerted anticancer property via its pro-apoptotic potential during DMBA induced oral carcinogenesis.

CONCLUSIONS

The present study, for the first time, scientifically validated and explored the chemopreventive and chemotherapeutic potential of myrtenal in DMBA induced oral carcinogenesis by utilizing the expression pattern of apoptotic markers as a molecular end point during DMBA induced oral carcinogenesis. The results of the present study revealed a potent pro-apoptotic efficacy of myrtenal in DMBA induced oral carcinogenesis. Thus, the present study concludes that myrtenal could be considered as a promising candidature for the prevention and treatment of oral cancer.

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Conflict of Interest

None.

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