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Effect of esculetin on the expression pattern of apoptotic markers in experimental oral carcinogenesis

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ABSTRACT

Evasion of apoptosis has been recognized as one of the salient features of the tumour cell. The programmed cell death is regulated by a spectrum of apoptotic proteins, which include both pro-apoptotic and anti-apoptotic markers. The aim of the present study is to explore the apoptotic induction potential of esculetin by analysing the expression pattern of apoptotic markers in 7,12-dimethylbenz(a)anthracene (DMBA) - induced oral carcinogenesis. Tumours were developed in the hamster's buccal mocosa using a site-specific carcinogen, DMBA [topical application, 3 times a week for 14 weeks]. While the buccal mucosa from the hamster treated with DMBA alone (tumour bearing hamster) exhibited abnormal pattern of apoptotic markers (p^{53}, Bcl-2, Bax, caspase 3 and caspase 9), the expression pattern was found to be reverted to near normal pattern in DMBA+esculetin treated hamster's buccal mucosa. The present results thus reveal the esculetin ability to induce apoptosis in DMBA-induced hamster buccal pouch carcinogenesis.

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INTRODUCTION

Extensive studies highlighted the putative role of the natural products in the prevention of several illnesses including cancer. The major phytochemical constituents of natural products include flavanoids, terpenoids, polyphenols and isothiocyanate (Batra and Sharma, 2013). The mechanism of action of these bioactive principles in several types of cancer has been well documented. Accumulating research studies explored the anticancer effect of various medicinal plants and their active constituents via their apoptotic efficacies (Wang *et al.*, 2012). Many bioactive constituents that explored apoptotic induction potential were tested for their anticancer efficacy in pre-clinical and clinical trials as well (Singh *et al.,* 2016; Pan *et al.,* 2012). The major goal of the present study is to explore a non-toxic and less side effect phytoconstituent for the treatment of cancer.

In recent years, numerous studies documented the molecular pathogenesis of oral carcinogenesis by analysing the spectrum of biomarkers in the tumour tissues (Santosh *et al.,* 2016). Investigation of the expression pattern of molecular markers in carcinogenesis would provide an insight into the tumour behavior and its aggressiveness (Bhatt *et al.,* 2010). p^{53} has been well pointed out as a key signalling molecule in the programmed cell death and cell arrest in response to DNA damage (Pietsch *et al.,* 2008). p^{53} has also been regarded as a ''Molecular policeman'' and ''Guardian of the genome''

(Efeyan and Serrano *et al.,* 2007). p^{53} is one of the major tumour suppressor genes and plays a pivotal role in the regulation of cell devision. Due to its crucial role in the cell cycle regulation, it is regarded as a mainstay of intrinsic anticarcinogenic defence mechanism (Oren and Rotter *et al.,* 2010). The tumour suppressor gene p^{53} has been recognized as a target gene for gene therapy and has been pointed out as a first molecular indicator of carcinogenic process (Rivlin *et al.,* 2011). The tumour suppressor gene p^{53} has been recognized as a target gene for gene therapy and chemotherapy due to its over expression in cancerous tissues (Hientz *et al.,* 2017). Mutant p^{53} protein over expression has been pointed out as a first molecular indicator of carcinogenic process (Rivlin *et al*., 2011). Shin *et al* (1996) pointed out that p^{53} expression has a prognostic significance in identifying oral cancer recurrence and secondary primary tumours. p^{53} functional status has been considered as a valuable predictor for chemotherapy response.

The anti-apoptotic protein Bcl-2 is a 26 kDa protein found in the nuclear envelope, outer mitochondrial membrane and endoplasmic reticulum (Tzifi *et al*., 2012). Bcl-2, B - cell lymphoma -2, has been recognized as a primary regulator of programmed cell death. Bcl-2 serves as an anti-apoptotic protein, by inhibiting apoptosis and by promoting the survival of damaged cell (Montero and Letai *et al*., 2018). Bcl-2, a membrane associated protein, was found to be over expressed in several cancers including oral carcinoma (Manimaran et al., 2017). Bcl-2 family, consist of at least 15 proteins both apoptotic and anti-apoptotic members, are found in basal and proliferating cells (Arya *et al.,* 2016).

The over expression of Bcl-2 was shown in oral dysplastic lesions as well. Bax, a pro-apoptotic gene, plays a vital role in the apoptotic cell death in cooperation with p^{53} and by inhibiting the effect of Bcl-2 (Hata *et al*., 2015). Manoharan *et al.,* (2011) reported decreased expression of Bax in experimental oral carcinogenesis. The caspases, cysteine rich proteases, are categorised into three groups such as initiator caspases, effector caspases and inflammatory caspases. The proteolytic cleavage of pro-caspases results in active caspases (Elliott *et al.,* 2009). The caspases 8, 9 and 3 have pivotal functions in the apoptotic pathway. Caspase 9 has been involved in cell disassembly in response to agents that triggers cytochrome c release from mitochondria (Reiners *et al.,* 2002). Caspase 3 stimulates caspases 8 and 9 initiator signals in to full-fledged commitment to disassembly. Abnormalities of in the status of caspase-3 and 9 have been documented in various cancers including oral carcinogenesis (Parrish *et al.,* 2013). The present study has thus investigated the modulating effect of esculetin on the expression pattern of apoptotic markers in DMBA induced oral carcinoma.

MATERIALS AND METHODS

The present experimental design categorized the 24 experimental hamsters, procured from National Institute of Nutrition, Hyderabad, into four groups. Each experimental group consists of six hamsters. The experimental design strictly followed the principles of institutional animal ethical committee (CPCSEA Reg. no: 160/1999), Annamalai University and the animals were housed and maintained in the Central Animal House, Annamali University. The experimental design is depicted in the figure 1.

Molecular markers analysis

The expression patterns of p53 in the buccal mucosa of the experimental hamsters were analyzed using Western blotting as described earlier (Manimaran *et al*., 2018). Briefly, proteins that are separated in the gel electrophoresis were transferred to nitrocellulose membrane. The membrane was then treated with corresponding primary antibodies (p^{53}, caspase 3 and 9), followed by horseradish peroxidase labaelled secondary antibodies. Then, the substrate, diaminobenzidine, was added and the band obtained were densitometerically analysed.. The immunoexpression pattern of Bax and Bcl-2 was examined as per the method described earlier (Rajasekaran et al., 2012). Briefly, the antigen retrieved slides were incubated with primary antibodies (Bax and Bcl-2) followed by incubation with horseradish peroxidase labelled secondary antibodies. The substrate, diaminobenzcidine, was then added and was viewed under microscope when acceptable colour intensity was attained.

STATISTICAL ANALYSIS

The statistical significance between the experimental groups was analyzed using One way analysis of variance followed by Duncan's Multiple Range Test. The differences between two groups are considered statistically significant if the p values were found to be less than 0.05.

RESULTS

The present study noticed an abnormal status of apoptotic proteins in the buccal mucosa of hamsters treated with DMBA alone. While Bcl-2 was over expressed, Bax protein expression pattern was decreased in the buccal mucosa of tumour bearing hamsters (Immunohistochemistry Figures 2 and 3). The expression pattern of p^{53}, caspase 3 and 9 were found to be downregulated (Western blotting; Figures 4 and 5) in hamsters treated with DMBA alone as compared to vehicle treated control hamsters. Esculetin administration (50 mg/kg

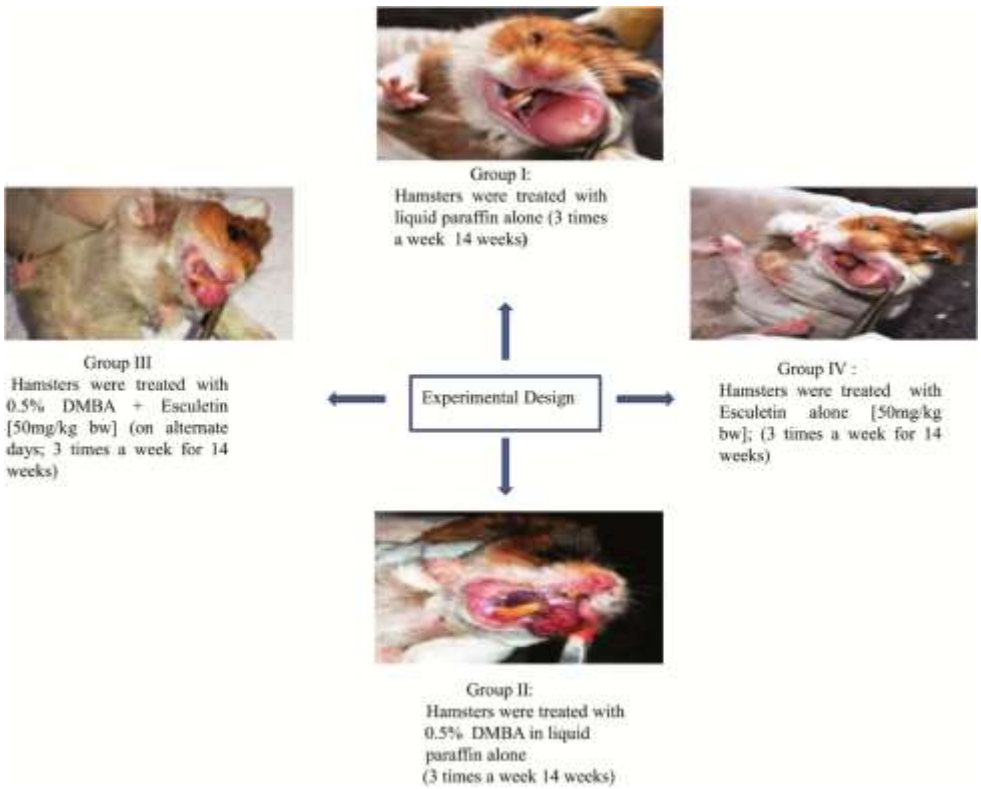

Figure 1: Experimental protocol

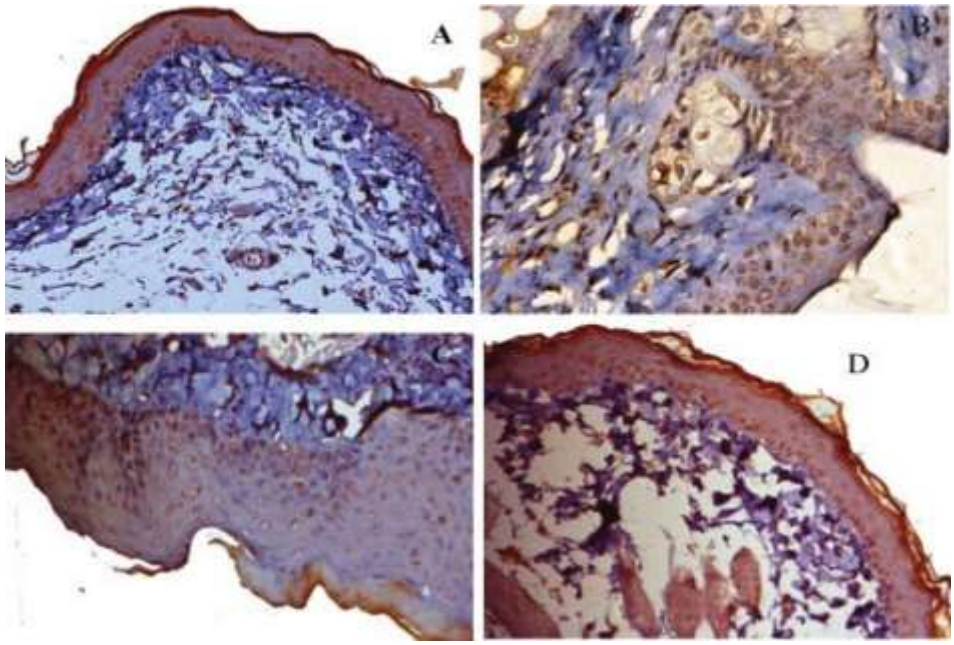

Figure 2: Immunoexpression pattern of Bax proteins observed in the buccal mucosa of control and experimental hamsters in each group. Bax: A and D - Control and esculetin alone (nuclear expression positive), B-DMBA alone (nuclear expression negative), C- DMBA+ esculetin treated (nuclear and cytoplasmic expression positive)

bw) orally to hamsters treated with DMBA restored the expression pattern of above said molecular markers to near normal pattern.

DISCUSSION

Apoptotic disruption has a major impact on neoplastic phenotype and thus dysregulation in the apoptotic pathway or the expression pattern of apoptotic genes would result in tumour progression. Profound studies highlighted that the cytotoxic anticancer drugs mediate their therapeutic effects via apoptotic induction (Liu *et al* 2017). Previous studies have also documented the apoptotic induction efficiency of various natural products and synthetic constituents (Manoharan *et al*.,

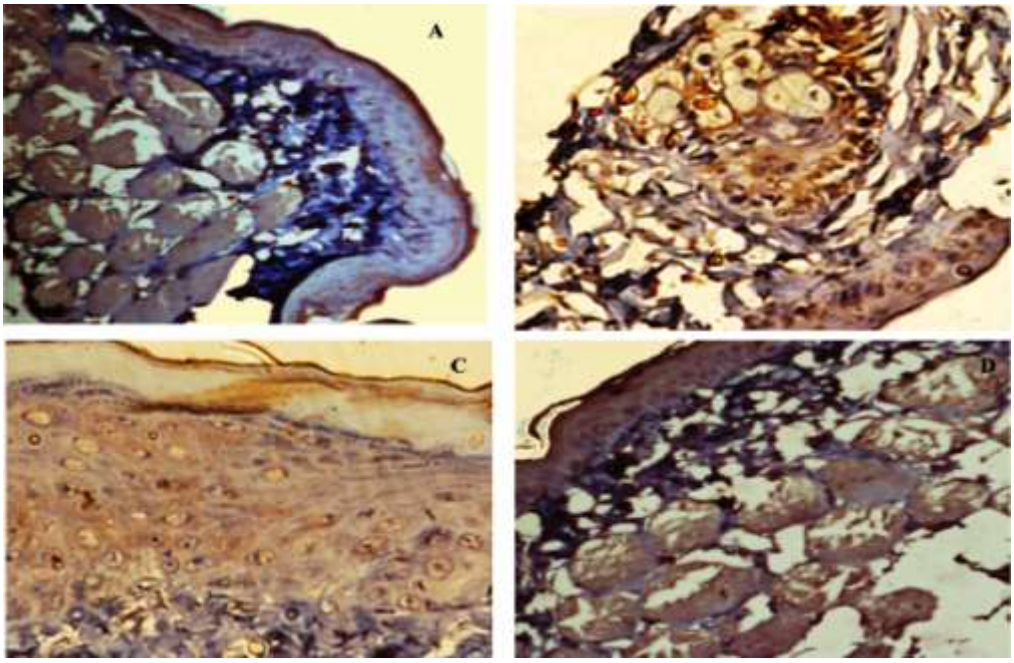

Figure 3: Immunoexpression pattern of Bcl-2 proteins observed in the buccal mucosa of control experimental hamsters in each group Bcl-2 A and D- Control and esculetin alone (expression not detectable); B- DMBA alone (over expression); C-DMBA + esculetin (down regulated)

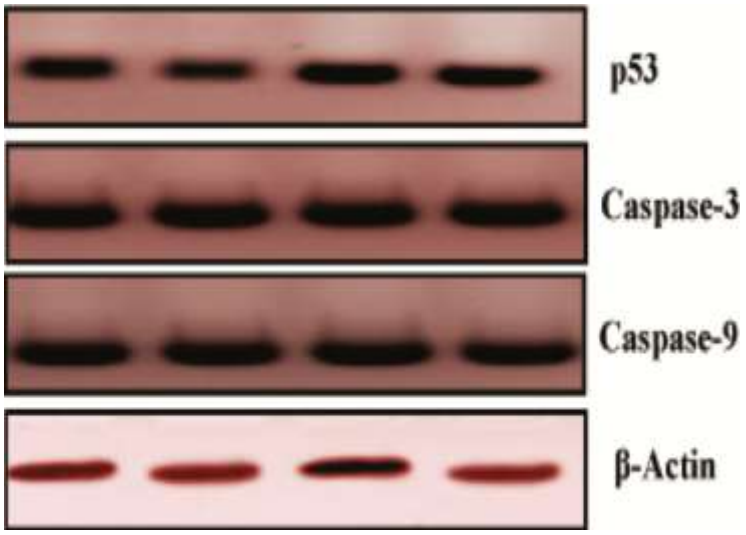

Figure 4: Expression pattern of p53, Caspase 3 and caspase 9 in the buccal pouch tissues of control and experimental animals. (a) Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA + esculetin, Lane 4: esculetin alone

2015). The present study focuses the apoptotic potential of esculetin in experimental oral carcinogenesis.

7,12-dimethylbenz(a)anthracene, a potent carcinogenic agent, is commonly used to develop tumours at various organs including oral cavity, skin and mammary gland (Hassan *et al.,* 2014). DMBA induced apoptotic abnormality has been well documented. In the present study, dysregulation in the expression pattern of p^{53}, Bcl-2, Bax, caspase 3 and 9 was noticed in the buccal mucosa of the hamsters treated with the DMBA alone.

A spectrum of check points are involved in monitoring the cell growth by regulating the cell cycle arrest or its progression during the cell cycle (Barnum and O'Connell., 2014). Dysregulation of p^{53} expression would lead to various pathological disorders including cancer. More than 50% of human carcinoma showed p^{53} mutations. p^{53} mutation would lead to genomic instability and abnormal cell division (Yasutis and Kozminski, 2013). Bai *et al* (2015) reported that the abnormal proliferation of squamous cell carcinoma cells could be due to defective p^{53} dependant pathway. Li *et al*., (2015) demonstrated over expression of p^{53} in the cells adjacent to oral cancer cells. In most of the cancer,

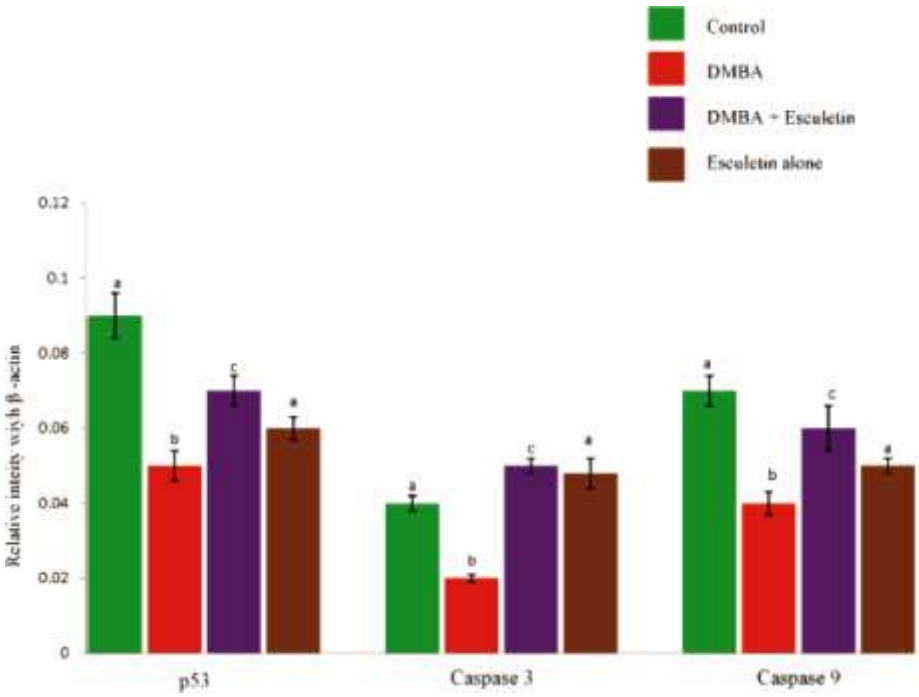

Figure 5: Densitometric analysis of p53, caspase 3 and caspase 9. Values are normalized with β-actin. Data are presented mean ± SD (n = 6). Common superscripts between two groups - not significant. Different superscripts between two groups – significant (p <0.05)

p^{53} mutation or its inactivation has been regarded as the key event. p^{53} triggers cytochrome c release via binding with Bcl-2 to induce apoptosis. Weissmueller *et al* (2014) reported that missense mutation in p^{53} gene promotes the metastasis pancreatic cancer. Wild type p^{53} has been shown to inhibit the proliferation and survival of precancerous cells (Chen *et a*l., 2015). Singh *et al* (2016) reported a spectrum of p^{53} mutation in oral cancer patients from Gujarat, India. They suggested that the assessment of p^{53} mutation could serve as a valuable indicator of early stage loco-regional recurrence among patients with oral carcinoma. Yang et al., (2013) have shown the p^{53} mediated apoptosis in oral cancer OC2 cells.

Cabrera Ortega *et al.,* (2016) showed over-expression of Bcl-2 in 4-nitroquinoline-l-oxide induced oral carcinogenesis. A large number of studies pointed out Bcl-2 as a prognostic marker in several carcinogenesis including oral carcinoma (Arumugam *et al.,* 2017). Arya *et al*., (2016) demonstrated the correlation between Bcl-2 expression and prognostic significance in oral carcinogenesis and cancerous conditions. Bcl-2 abnormal expression was shown in oral, skin, colon and mammary cancer. Ramezani *et al*., (2017) suggested that analysing Bcl-2 expression could be helpful to distinguish skin sqaumous cell carcinoma and basal cell carcinoma. Gulati *et al*., (2017) has shown a gradual increase in the Bcl-2 expression pattern from stage I to stage IV of oral cancer patients. Bcl-2 overexpression facilitates the conversion of damaged mutated cells to malignant phenotype by favouring their cell survival (Kirkin *et al*., 2004).

Numerous studies clearly pointed out that the survival or death of the cells are determined by Bcl-2/ Bax ratio. Bcl-2 up-regulation and Bax down-regulation were reported in the tumour tissues of several cancers including oral cancer (Placzek *et al.,* 2010). It is well documented that caspases have significant contribution in the biochemical and morphological alterations during programmed cell death. Caspase 3 is one of the major executioner caspases involved in the DNA fragmentation and disassembly of the cell by targeting the structural substrates (Fuchs and Steller, 2011). The activated caspases are involved in the induction of apoptosis of several structural and functional proteins. The absence of caspase 3 was shown in the tumours tissues of the breast, liver and prostate (McIlwain *et al.,* 2015). It has been demonstrated that several anticancer drugs induced apoptosis via caspase 3and 9 activation (Jäger and Zwacka, 2010).

Oral administration of esculetin at a dose of 50mg/kg bw corrected the abnormalities of the above markers expression pattern during DMBA induced oral carcinogenesis (Witaicenisi *et al.,* 2013). A large number of in vitro studies demonstrated the pro-apoptotic potential of esculetin in cancer cell lines (Karandikar and Thangarajan, 2017). Wang *et al*., (2015) suggested that the anti-cell proliferative efficacy of esculetin is due to its ability to initiate mitochondrial mediated, caspase-

dependant apoptotic pathway in cancer. Kim *et al*., (2015a; and 2015b) reported that esculetin stimulated the process of apoptosis in human colon cancer cells through induction of endoplasmic reticulum stress and via ROS - mediated mitochondrial apoptotic pathway. It has been reported that esculetin might have induced apoptosis via down regulating Sp1 protein in human malignant melanoma cells (Jeon *et al.,* 2015). Bcl-2 over expression blocking and activation of JNK and ERK has been considered as the major mechanism for the esculetin's apoptotic efficacy in human leukemia cells (Park *et al.,* 2008). Chu *et al.,* (2001) proposed that the apoptotic efficacy of esculetin is probably due to its ability to enhance the cytosolic translocation of cytochrome c. Yang *et al*., (2010) pointed out ROS-mediated mitochondrial dysfunction pathway as a major mechanism for the apoptotic potential of esculetin.

CONCLUSION

The present study demonstrated the apoptotic efficacy of esculetin in vivo using DMBA-induced hamster buccal pouch carcinogenesis. Esculetin modulated the expression of apoptotic markers in favour of tumour suppression in DMBA treated hamsters. Esculetin up- regulated the expression of pro-apoptotic proteins p^{53}, Bax, Caspase 3 and 9 and down-regulated Bcl-2 expression in hamsters treated with DMBA. The observed findings thus suggest that the apoptotic potential of esculetin is attributed to its ability to maintain the Bcl-2/Bax ratio and by inducing the expression of p^{53}, caspases 3 and 9.

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