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Research Article

SINAPIC ACID ATTENUATES 7,12-DIMETHYLBENZ[A] ANTHRACENE-INDUCED ORAL CARCINOGENESIS BY IMPROVING THE APOPTOTIC ASSOCIATED GENE EXPRESSION IN HAMSTERS

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ABSTRACT

Objectives: The main objective of the present study is to examine the histological changes and apoptotic associated gene expression during 7,12-dimethylbenz[a] anthracene (DMBA)-induced buccal pouch carcinogenesis in male golden Syrian hamsters.

Methods: Squamous cell carcinoma was induced in the buccal pouch of male Syrian golden hamsters by painting with 0.5% solution of DMBA in liquid paraffin 3 times per week for 16 weeks to induce the development of oral tumors. Sinapic acid (50 mg/kg b.wt) were either applied topically to the oral tumor lesions or administrated orally at varying dosage to hamster animals with oral tumor for 14 weeks. The experiment was terminated at the end of 16th weeks. The development of oral carcinogenesis was confirmed by the histopathological analysis and expressions of apoptotic associated genes were analyzed by the immunohistochemical methods.

Results: We observed altered status of apoptotic associated gene expression (P53, B-cell lymphoma [Bcl-2], Bax, and caspase-3) was observed in the DMBA alone painted hamsters as compared to control hamsters. Oral administration of sinapic acid improved the histological changes and significantly stimulate the apoptotic associated genes expression, especially caspase-3 but decreasing Bcl-2 protein production.

Conclusion: It can be evident from the findings of this research work concluded that oral administration of sinapic acid are effective at inhibiting tumor cell proliferation and stimulating apoptosis in oral cancer suggesting that sinapic acid have chemopreventive effects on DMBA-induced experimental oral carcinogenesis.

Keywords: Apoptosis, 7,12-dimethylbenz[a] anthracene, Hamster, Histology, Sinapic acid.

INTRODUCTION

The field of cancer research has continued to rapidly evolve, and in recent years, there have been advancements not only in the knowledge regarding the molecular biology and epigenetics of oral cancer [1]. The molecular mechanisms implicated in the malignant transformation and progressions of oral cancer are still not well-understood. Neoplastic transformation of cancer is characterized by alterations of oncogenes and tumor suppressor genes [2]. The interaction between activated oncogenes and mutations that result in a loss of function of tumor suppressor genes appears to be the driving force directing normal cells to uncontrolled growth and invasion [3]. Apoptosis is a critical pathway that selectively allows cells with damaged DNA to undergo cell death. It is an active cell suicide mechanism consisting of an evolutionarily conserved cascade that includes characteristic features such as cell shrinkage, condensation of chromatin, and formation of specific oligonucleotide fragments [4]. Deficiencies in apoptosis also contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations conferring resistance to cytotoxic anticancer drugs and radiation [5].

Numerous studies have pointed to the importance of the p53 tumor suppressor gene in the process of carcinogenesis. In particular, a high incidence of p53 mutation has been found in carcinogen mediated cancers status [6]. Tumor suppressor genes are now thought of as either "gatekeepers or caretakers" for cells p53 is a phosphoprotein barely detectable in the nucleus of normal cells. On cellular stress, particularly that induced by DNA damage, p53 will arrest cell cycle progression, thus, permitting DNA to be repaired or it will cause cell death [7]. p53 mediated apoptosis involves the multiple mechanisms including the expression of B-cell lymphoma (Bcl-2), Bax and other Bcl-2 homology domain 3 (BH3) proteins, amplification of death signals, and activation of caspases [8]. Caspase-3, a key factor in apoptosis execution, is the active form of procaspase-3. Caspase 3 expression could be detected in several human malignancies such as non-small cell lung carcinoma, squamous cell carcinoma, esophageal squamous cell carcinoma, and gastric cancer. It has been identified as being a key mediator of apoptosis of mammalian cells [9,10].

Bax and Bcl-2 are membrane of bound pore forming proteins that interact through heterodimerization. Bax and Bcl-2 protein were collectively regulating the mitochondrial transmembrane passage of cytochrome c that successively activates the caspase passages it leads to apoptosis [11]. Bcl-2 functions as a suppressor of apoptosis leading to the survival of neoplastic cells, expression of other Bcl-2 family proteins, such as Bax, induces mitochondrial apoptosis with apoptosis related morphological changes, caspase activation, and subsequent substrate proteolysis [12]. Bax belongs to the Bcl-2 family of proteins and is a key player in apoptosis. The pro-apoptotic protein Bax, which belongs to the Bcl-2 family, the pro-apoptotic protein Bax binds to the anti-apoptotic protein Bcl-2, leading to inactivation of the anti-apoptotic function of Bcl-2 [13]. It inhibits the release of cytochrome C from the mitochondria through specific mitochondrial channels, and it serves as essential effectors of the mitochondrial apoptotic pathways and participates in executing p53-mediated apoptosis [14].

For thousands of years, the natural products have played an important role throughout the world in the treatment and prevention of human diseases. Over 60% of currently used anticancer agents are derived in one way or another from natural sources [15]. Sinapic acid is a cinnamic acid derivative, which possesses 3,5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of cinnamic acid. Sinapic acid is found

in cereal bran with ferulic acid, though the latter is the most abundant sources [16]. Recent data support the sinapic acid act as preventive and/or therapeutic agents in several oxidative stress related diseases, such as atherosclerosis, inflammatory injury, and cancer [17-19]. Sinapic acid is a major free phenolic acid in rapseed meal, with the majority found in the form of sinapine. It has potent antioxidant capacity, and its efficiency is always higher than ferulic acid and sometimes of caffeic sources [20]. Our previous studies in 7,12-dimethylbenz[a] anthracene (DMBA)-induced experimental carcinogenesis in hamster have shown that [6] paradol facilitate the apoptosis as evidenced by the expression of p53 related gene expression sources [21]. However, no scientific reports were available on literature about p53, Bcl-2, Bax, and casp-3 gene regulation of sinapic acid on DMBA-induced carcinogenesis of male golden Syrian hamsters. Hence, the current study was designed to investigate the role of sinapic acid as an effective chemopreventive agent: Mechanism behind the induction of apoptosis was assessed by immunohistochemical techniques.

METHODS

Chemicals

DMBA, sinapic acids were purchased from Sigma Chemical Company, USA. All other chemicals used were of analytical grade, marketed by Himedia laboratories, Bengaluru, India.

Animals

Male golden Syrian hamsters (6-week-old) weighing 80-120 g were purchased from National Institute of Nutrition, Hyderabad. The animals were housed in polypropylene cages at room temperature (27±2°C) with relative humidity (55±5%) in an experimental room, the LD (light:dark) cycle is almost 12:12 hrs. All animals were given Commercial pelleted feed (M/s Kamdhenu Ltd., Bengaluru), and tap water ad libitum. The experimental design was approved by Institutional Animal Ethics Committee for the care, and use of laboratory animals were strictly followed throughout the study.

Experimental design

The hamsters were divided into four groups of 8 animals each. Group I hamsters served as control, the Group II, and Group III were painted with 0.5% DMBA in liquid paraffin three times a week for 16 weeks on the left buccal pouches using (No. 4 sable hair brush) to induce the buccal pouch carcinogenesis. The Group II received no other treatment. Group III hamsters were orally treated with sinapic acid (50 mg/kg b.wt: Dissolved in 0.5% DMSO) starting 1 week before the exposure to the carcinogen and continued on alternate days of the DMBA painting until within 1 week after completing the treatment, the hamsters were sacrificed by cervical dislocation after an overnight fast at the end of 16^{th} week, and the gross tumors were excised and analyzed for the presence of p53, Bax-2, and caspase 3. However, Group IV was orally administrated with sinapic acid to exclude any toxic effects.

Tumor study

After the experimental period, the hamsters were sacrificed by cervical decapitation. The number of visible tumors in the oral cavity was counted: The length, width, and height of each tumor were measured. The tumor weight was estimated according to the method of Geren *et al*., [22]. The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axis. The two short axes were measured with vernier calliper. The tumor volume was calculated by the formula $v = 4/3\ (\pi) \times (D_1/2) \times (D_2/2) \times (D_3/2)$, where D_1, D_2, and D_3 are the three diameters (mm) of the tumors.

Histopathological examination

Histological slides were prepared by according to the method of Klitgaard [23]. Tumors and cheek pouch tissue samples were harvested. Buccal tissues were fixed in 10% buffered formalin, embedded in paraffin using a conventional automated system. The blocks were cut to obtain 5 mm thick sections and stained with hematoxylin-eosin. Serial paraffin sections of each tissue image were captured by light microscopy.

Immunohistochemical staining

The expressions patterns of apoptotic associated molecular markers p53, Bcl-2, Bax, and caspase-3 were prepared immunohistochemical staining by according to the method of Cotran *et al*., [24]. Paraffin embedded tissue sections were incubated at 60°C for 1 hr in hot plate, allowed to cool, and then deparaffinised in xylene and brought to water rehydrated through descending grades of alcohol. Sections were washed in deionized water and the endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 minutes. The antigen retrieval was achieved by microwave oven in citrate buffer solution (2.1 g citric acid/L $D.H_2O$: 0.37g ethylenediaminetetraacetic acid/$D.H_2O$: 0.2g trypsin: pH 6.0) for 10 minutes. The sections were allowed to cool for 20 minutes and then rinsed with tris-buffered saline (8g NaCl: 0.605g Tris: pH 7.6). The tissue section was then incubated with power Block™ reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent, for 15 minutes at room temperature to block nonspecific reaction binding sites. The tissue sections were then incubated with the respective primary antibody of p53, Bcl-2, bax, and caspase-3 (Sigma-Aldrich, USA) overnight at 4°C. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA), for 30 minutes at room temperature. After rinsing with tris-buffered saline, the antigen-antibody complex was detected using 3,3'- diamminobenzidine, the substrate of horseradish peroxidase. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). The statistical analysis on the data for the tumor incidence, tumor volume, and tumor burden were performed by the one-way Analysis of Variance followed by Duncan's multiple range test. The percentage of positive cells in immunohistochemical was scored as follows: 0=negative, <1% of cell staining: 1 week staining, between 1% and 20% of cells were stained: 2=moderate staining, between 20% and 50% of cells were stained, and 3=strong staining, more than 50% of cells were stained: The statistical data were analyzed using the number of positively stained cells using the Chi-square (χ^2) test. The results were considered statistically significant if the p values were <0.05.

RESULTS

Status of neoplastic and histological changes in control and experimental animals

Table 1 and Fig. 1 display the tumor incidence, tumor volume, and tumor burden of hamsters in all experimental groups along with the histopathological features. In Group II animals (DMBA alone treated), we have observed 100% tumor formation with mean volume (270.37 ± 50.9^b) were noticed. The tumor sections of the DMBA alone treated hamster's specimen showing the severe hyperkeratosis, hyperplasia thickened epithelial layer and keratin pearl formation which is characteristic feature of well differentiated squamous cell carcinoma. Whereas, the histology of the sinapic acid + DMBA-treated hamster specimen showed thickening of epithelial layer. This change suggested that the sections were at dysplastic stage. Buccal pouch of control and sinapic acid alone animals resembles the same with no tumor formation and pre-neoplastic lesions.

Gene expression of p53 in control and experimental animals

Fig. 2a-d and Table 2 show the immunohistochemical gene expression of p53 in control and experimental animals of each group. In DMBA alone (Fig. 2b) treated hamsters, significant and sequential raise of the protein levels by p53 gene expression was observed with respect to control animals (Fig. 2a). However, Oral administration of sinapic acid at a dose of 50 mg/kg b.wt effectively restored the p53 gene expression levels in DMBA-treated hamsters as compared with control hamsters (Fig. 2a) and sinapic acid alone treated hamsters (Fig. 2d). Hamsters

Table 1: Incidence of oral neoplasm and histological changes in control and experimental animals in each group

Parameters	Control	DMBA	DMBA+sinapic acid	Sinapic acid alone
Tumor incidence (oral squamous cell carcinoma)	0	100%	20%	0
Total number of tumors/animals	0	32/(8)	6/(2)	0
Tumor volume (mm^3)	0[a]	270.37±50.9[b]	94.96±12.09[c]	0[a]
Keratosis	No change	Severe	Mild	No change
Hyperplasia	No change	Severe	Mild	No change
Dysplasia	No change	Severe	Mild	No change
Squamous cell carcinoma	-	Moderately differentiated	Well differentiated	-

Values are expressed as ±SD for 8 animals in each group. Tumor volume was measured using the formula $v=4/3\ \pi\ (D_1/2)\ (D_2/2)\ (D_3/2)$ where D_1, D_2, and D_3 are the three diameters (mm^3) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumor/animals. Parenthesis indicates total number of animals bearing tumor, SD: Standard deviation

Table 2: The intensity of staining for mutant p53, Bcl-2, Bax, and caspase-3 expression in the buccal pouch of control and experimental animals

Group	p53			Bcl-2			Bax			Caspase-3		
	+	++	+++	+	++	+++	+	++	+++	+	++	+++
Control	2	3	0	5	1	0	7	2	0	7	4	0
DMBA	7	12	9	5	7	11	0	3	4	0	3	8
DMBA+sinapic acid	3	5	2	1	4	2	2	6	4	2	6	3
Sinapic acid alone	1	3	0	5	1	0	8	1	0	6	1	0

Bcl: B-cell lymphoma

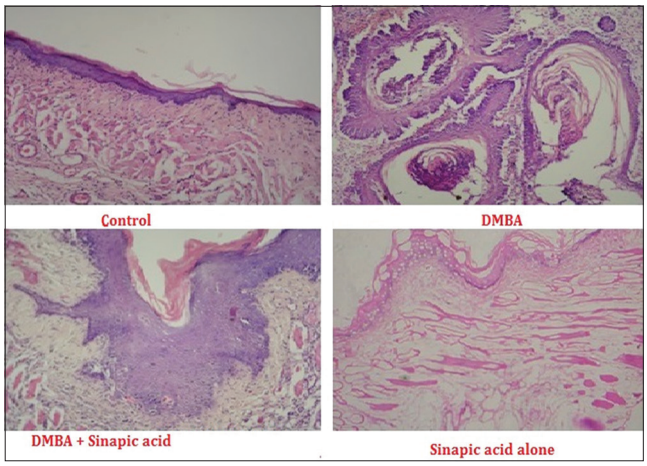

Fig. 1: Histopathological changes observed in the control and experimental animals in each group. Microphotograph of untreated control animals (Group I), picturising normal epithelium in buccal mucosa. Microphotograph of DMBA alone treated animals (Group II), picturising well-defined squamous cell carcinoma with hyper chromatic nuclei containing epithelial and keratin pearls. Microphotograph of DMBA + Sinapic acid treated animals (Group III), picturising mild to moderate dysplasia and hyperplasia. Microphotograph of sinapic acid alone treated animals (Group IV), picturising normal epithelium in buccal mucosa (H and E ×10)

treated with sinapic acid alone revealed no p53 staining compared to control hamsters.

Gene expression of Bcl-2 in control and experimental animals

Fig. 2e-h and Table 2 confirm the immunohistochemical expression of Bcl-2 in control and experimental hamsters. Buccal pouch of DMBA-treated animals (Fig. 2f) showed a significant and sequential up regulation of the Bcl-2 protein levels when compared with the respective time matched controls. The significant decreases in the expression of Bcl-2 were observed in sinapic acid treated hamsters (Fig. 2g). Control (Fig. 2e) and sinapic acid alone (Fig. 2h) treated groups show a negligible Bcl-2 expression, when compared to DMBA-treated animals.

Gene expression of Bax in control and experimental animals

Immunohistochemical expression of Bax in control and experimental animals buccal pouches were presented in Fig. 2i-l and Table 2. We observed a down regulation of Bax gene expression were observed in DMBA alone (Fig. 2j) treated hamsters as compared with control hamsters. The expression levels of Bax were significantly improved in DMBA + sinapic acid treated hamsters (Fig. 2k). Control and sinapic acid alone (Fig. 2i and l) treated hamster show a negligible Bax expression.

Gene expression of caspase-3 in control and experimental animals

Fig. 2m-p and Table 2 show the immunohistochemical expression of caspase-3 in control and experimental hamsters. Immunohistochemical analysis showed that the down regulation of caspase-3 in DMBA alone treated hamsters (Fig. 2n). However, there was a significant up regulation in the protein expression of caspase-3 were observed in sinapic acid treated animals (Fig. 2o). While, the control and sinapic acid alone treated animals (Fig. 2m and p) show a negligible caspase-3 expression, when compared to DMBA-treated hamsters.

DISCUSSION

This study designed to focusing the gene expression of p53, Bcl-2, Bax, and caspase 3 in DMBA-induced experimental oral carcinogenesis. The oral administration of sinapic acid treatment led to a significant increased expression of p53, pro-apoptotic proteins Bax, and caspase 3, whereas decreased the levels of anti-apoptotic Bcl-2 that correlated well with the induction of apoptosis. Incidentally, this is the first *in vivo* report which shows that sinapic acid directly or indirectly is associated with induction of apoptosis in DMBA-induced buccal pouch carcinogenesis.

Syrian golden hamster buccal pouch carcinogenesis model closely mimics events in the development of precancerous lesions and epidermoid carcinomas of the oral cavity in humans using topically and chronically applied DMBA. This model produces 100% squamous cell carcinoma of the buccal pouch mucosa in 12-16 weeks [25]. It is an ideally accepted organ specific experimental carcinogen used in the present study has been reported to produce toxic and highly diffusible reactive oxygen species, capable of producing deleterious effects at the sites far from the tumor [26]. In DMBA painted animal cells exhibited severe dysplasia, hyperkeratosis and hyper chromatic nucleic with epithelial and keratin pearl formation. Oral administration of sinapic acid at a dose of 50 mg/kg b.wt for 16 weeks significantly prevented the tumor incidence and formation of oral squamous cell carcinoma; however, we observed premalignant lesions such as hyperplasia and dysplasia.

The importance of p53 gene can be drawn from the fact that this gene is reported to be mutated in 80% of the all human malignancies. Because of its role in regulation of cell cycle, alterations in p53 are critical events in carcinogenesis. Alteration of the p53 gene and high frequency of p53 expression were detected in a number of human solid

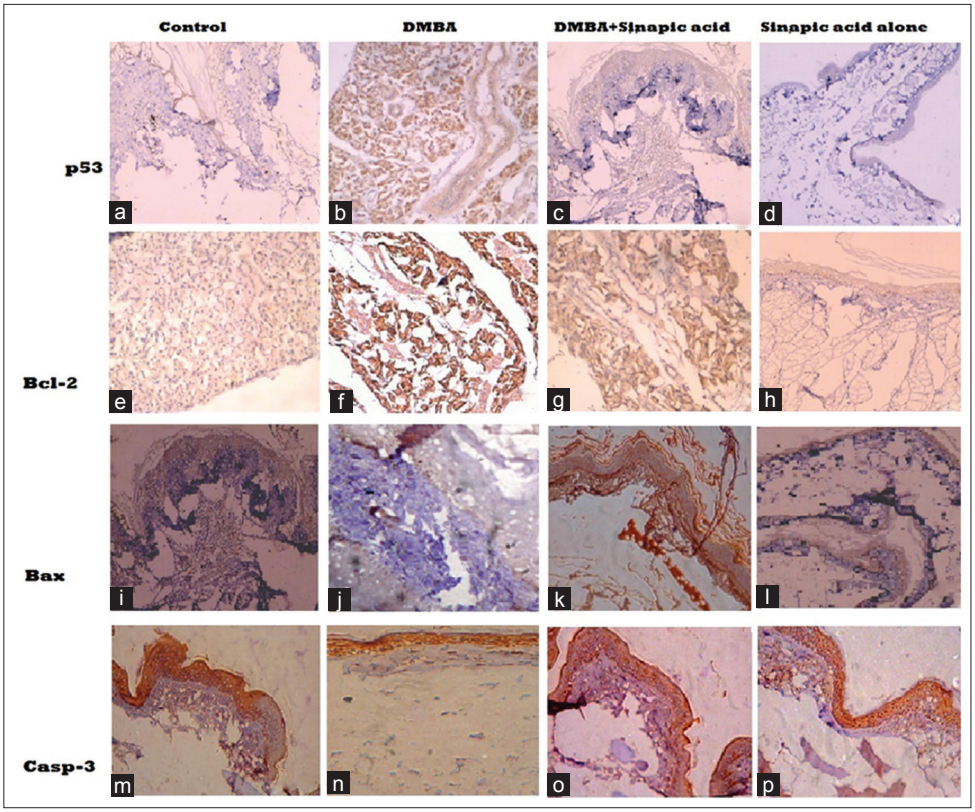

Fig. 2: Immunohistochemical expression of p53, Bcl-2, Bax, and caspase-3 in control and experimental animals of each group. (a,e,i,m) show the normal expression of mutant p53, Bcl-2, Bax, and caspase-3 genes in buccal tissues of control hamsters. (b,f,j,n) show the well-defined oral squamous cell carcinoma with significant up regulation of mutant p53, Bcl-2 and down regulation of Bax, and caspase 3 in buccal tissues of DMBA alone treated hamsters. (c,g) show the mild expression of mutant p53, bcl2 gene expression and up regulation of Bax and caspase 3 (k,o) in DMBA + sinapic acid treated hamsters. (d,h,l,p) show the Well-diminished expression of mutant p53, Bcl-2, Bax, and caspase-3 in buccal tissues of sinapic acid alone treated hamsters. +++: <50% of the cells are stained; ++: 10–50% of the cells are stained; +: <10% of the cells are stained; 0: Absence of staining

tumors including oral cancer [27]. The topical application of DMBA in buccal pouches of the hamster can up regulated the p53 levels, whereas the oral administration of sinapic acid prevented the up regulation of p53 levels after DMBA treatment hence protected the cell death. The up regulation of p53 by chemopreventive agents is most likely responsible for the transcriptional induction of p21/waf1 by directly interacting with its regulatory elements [28]. In the same line of attack, oral administrations of sinapic acid improve the p53 gene expression. These observations in accordance with Kampa *et al*., who illustrated the selected phenolics acid including caffeic acid, syringic acid, sinapic acid, protocatechuic acid, ferulic acid, and 3,4-dihydroxy-phenylacetic acid were exert a direct anti-proliferative action through the p53 mediated apoptotic process in cancer cell lines [29]. The results of the present study are also in agreement with those suggested in previous reports about the role of p53 as a marker to confirm tumor regression by use of various chemopreventive agents against DMBA-induced oral carcinogenesis [30,31].

Apoptosis occurs through induction of the ratio of Bax: Bcl-2 protein expression and the cleavage of procaspase-3 to active caspase-3, which is a key step of apoptosis. The gene expressions profiles of Bax and Bcl-2 have been reported to play a crucial role in apoptotic response mediated by many agents [32]. The expression of the anti-apoptotic protein Bcl-2 is up regulated by the transcription factor NFkB and down regulated by p53. In contrast, the level of pro-apoptotic Bax is transcriptionally up regulated by p53 [33]. Therefore, we determined the effect of sinapic acid on expression of Bax and Bcl-2 in DMBA-induced buccal pouch carcinogenesis. Over expression of Bcl-2 gene has been reported in malignancies and several solid tumors including oral cancer. Bcl-2 has been documented to prolong cell survival and may inhibit apoptosis by inhibiting the release of cytochrome C from mitochondria and may promote tumor development [34]. We observed induction of apoptosis following administration of sinapic acid to animals painted with DMBA as revealed by the down regulation of Bcl-2 protein expression. Our results revealed that oral administration of sinapic acid significantly inhibited Bcl-2 expression in DMBA-treated animals. Therefore, the inhibitory effect of sinapic acid on DMBA-treated animals is possibly mediated via suppressing Bcl-2 expression and stimulating the products of genes of pro-apoptotic proteins.

Recently, Puangpraphant *et al*., reported that hydroxycinnamic acids and derivates, significantly improved the ratio of Bax: Bcl-2 protein expression and it facilitate the apoptosis in colon cancer cell lines [35]. In conjunction with sinapic acid mediated apoptosis, the study showed down regulation of Bax and up regulation of Bcl-2 in DMBA-induced buccal pouch carcinogenesis as reported in human and animal tumors [36]. Several reports were documented that the chemotherapeutic agent, induced apoptosis in human cancer cell line and hamsters by down regulation of Bcl-2 and up regulation of Bax [37,38]. Our results are concordant with a recent study that shows that the structural related phenolics compound of carnosic acid has significantly improve the expression of p53 and Bcl-2 during DMBA-induced oral carcinogenesis [39].

A large number of pro and anti-apoptotic proteins have been documented in various cell types and could be involved in the regulation of apoptosis in oral cancer. Apart from the action of pro and anti-apoptotic process: The caspase plays a vital role in the regulation of apoptosis. Caspase activation has been documented in several types of tumor cells when they have been successfully induced

to undergo apoptosis by various chemotherapeutic drugs [40]. In oral carcinogenesis, an alteration in the immunohistochemical expression of caspase-3 was also reported in the human and experimental studies, and this expression became stronger with tumor progression [41]. Burton *et al.* (2000) reported that activated caspase-3 over expression was associated with higher rates of disease invasiveness. There is an inverse relationship between Bcl-2 and caspase-3 immunoreactive cells in both control and DMBA-induced oral carcinogenesis animals [42]. Munshi *et al.,* (2001) indicated that the increase of Bcl-2 expression caused the decrease of caspase-3 activity. In the current study, we found that capsase-3 expressions in DMBA administrated animals were significantly less than its expression in control animals [43]. Our results corroborate these observations.

CONCLUSION

Findings of the present study conclude that DMBA causes carcinogenesis via up regulation of p53, caspase-3, Bax and down regulation of Bcl-2 levels and inducing mitochondrial mediated apoptosis mechanism. Further sinapic acid at a dose of 50 mg/kg b.wt confers protection of DMBA-induced carcinogenesis by decreasing proliferation and augmenting apoptosis of initiated cells. Thus, these observations hold promise for further molecular target oriented studies.

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