

THE CHEMOADJUVANT POTENTIAL OF *PERGULARIA DAEMIA* (FORSK.) ON EXPRESSION PATTERN IN EXPERIMENTAL ORAL CARCINOGENESIS

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ABSTRACT

Pergularia daemia (Family: Asclepiadaceae), is a perennial herb growing widely along the road sides of India. Traditionally, the plant is utilized in many disease conditions. These findings provide valuable information that might help in the selection of possible biomarkers that can be used in early detection of the alarming problem of oral cancer in Southeast Asia. Topical application of DMBA for 14 weeks induced buccal pouch tumours that showed aberrant expression of cytokeratins, a marker for epithelial carcinomas. This was associated with increased cell proliferation and evasion of apoptosis as revealed by upregulation of proliferating cell nuclear antigen, NF- κ B, mutant p53, Bcl-2 and downregulation of Bax and caspase 3 protein expression. Our findings clearly suggest that PDME may play a role as a novel chemopreventive or therapeutic agent for oral carcinogenesis.

Keywords: *Pergularia daemia*, Chemoadjuvant, RT-PCR, Western Blotting,

INTRODUCTION

Oral cancer is a cancer that affects any part of the mouth, such as the jaw bone, oral muscles, the gums, lips, tongue, and/or the cheek. The most common type of oral cancer is squamous cell carcinoma and affects the sides and under surface of the tongue as well as the floor of the mouth (Tsantoulis *et al.*, 2007). In the world, annually more than 3,00,000 new cases are being diagnosed with oral squamous cell carcinoma (Jemal *et al.*, 2003). It is the first most common cancer in male and third most common in female in India (Fenley *et al.*, 2000). It constitutes around 12% of all cancer in male and 8% of all cancer among female. It accounts for 30-40 percent of all malignancies in India. Every year about 85,000 new cases of oral cancer are reported to be diagnosed. According to cancer incidence in five continents, one district in Madhya Pradesh, Bhopal has the highest Age Adjusted incidence Rate (AAR) for lung and mouth cancer in the world. The Ahmedabad urban registry has also a high AAR for tongue cancer (Parkin *et al.*, 2002).

Molecular biomarkers include altered or mutant genes, RNAs, proteins, lipids, carbohydrates and small metabolite molecules, and their altered expressions that are correlated with a biological behaviour or a clinical outcome. Identification of cancer biomarkers is one of the most promising approaches for the detection of early-stage malignant or even premalignant lesions. The role of biomarkers in cancer detection and progression is a major effort at various laboratories aimed at the development of novel and simple approaches for early detection of human cancer (Linkov *et al.*, 2007). Molecular profiling studies, the major contributors of cancer biomarker discoveries, are based on an association or correlation between a molecular signature and cancer behavior. One of the pioneering molecular profiling studies showed that gene expression patterns could classify tumors, yielding new insights into tumor pathology such as stage, grade, clinical course, and response to treatment (Golub *et al.*, 1999)

Chemical agents like poly aromatic hydrocarbon (PAH) appear to be the dominant etiological factor in oral cancer and use of carcinogen induced animal model is required to assess efficiency of new therapeutic approaches (Nagini 2009). Experimental cancer model using hamsters buccal pouch (HBP) and topical application of a chemical carcinogen-7,12-dimethylbenzanthracene [DMBA] a

member of PAH is an optimal model for the study of oral cancer.

Oral squamous cell carcinomas (OSCCs) induced by the application of 7, 12-dimethylbenz(a)anthracene (DMBA) to the cheek pouch of the Syrian hamsters are morphologically and histologically similar to human tumors (Gimenez-Conti IB and Slaga 1992). In addition, hamster tumors express many biochemical and molecular markers that are expressed in human oral cancer. This model has gained wide acceptance as the simplest and most effective system to analyse oral cancer development and investigate approaches to chemoprevention and chemointervention (Shkler 1998). A number of natural and synthetic compounds have been tested for their chemopreventive potential in the HBP model.

Pergularia daemia has a vast application in different folk medicine even in the ayurveda and are believed to increase defense against various diseases. The leaves are useful in leprosy and haemorrhoids. The fresh, pulped leaves are applied as a poultice to relieve carbuncles. Leaf juice is used as an amenorrhea, catarrhal infections and dysmenorrhea, infantile diarrhea and also used to reduce body pain. In addition dried leaves are used as an antirheumatic, asthma, amenorrhea, dysmenorrhea, bronchitis, whooping cough, heals cuts and wounds and finally to facilitate parturition. The stem bark of this plant was a good remedy for cold, it is also used to treat malaria and the twig is used as an antipyretic and appetizer. Latex of the plant is used for boils and sores. Dried roots are used for cough, asthma and constipation, while the fresh roots are used as an abortifacient and used to treat gonorrhoea. The whole plant is used as an anthelmintic, antiseptic, antivenin and expectorant. Extract of this plant is taken orally for gastric ulcers, uterine and menstrual complaints (Karthishwaran K and Mirunalini 2010).

Moreover whole plant extract is also used for uterine and menstrual troubles and to facilitate parturition. However, very less work has been carried out on the aerial parts of plant and there is a wide scope for investigation. So in our present study, anticarcinogenic effect of aerial parts of *P.daemia* methanolic extract on certain biochemical aspects like, molecular aspects like NF- κ B, Bax, Bcl-2, p53, Caspase-3, Cox-2, CYP1B1 VEGF, PCNA expressions have been taken in account which is being explored in DMBA induced HBP cancer.

MATERIALS AND METHODS

Chemicals. DMBA was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Polyvinylidene difluoride membrane was purchased from Millipore, p53, COX-2, NFκB and caspase-3 antibodies were purchased from Santa-cruz Biotechnology, Inc, USA and goat anti-rabbit, anti-mouse and rabbit anti-goat secondary antibodies were purchased from Genei, Bangalore, India. Enhanced chemiluminescence (ECL)-kit was purchased from GenScript ECL kit, USA. All other chemicals used in this study were of analytical grade obtained from E. Merck and HIMEDIA, India.

Plant Material. The fully matured aerial parts of *Pergularia daemia* (Forsk.) were collected from Pudukkottai District, Tamil Nadu, India. The plant was identified by Dr. V. Venkatesalu, Professor, Department of Botany, Annamalai University. A voucher specimen (ACC: 196) was deposited in the herbarium of Department of Botany, Annamalai University.

Preparation of extracts. The flowering aerial parts of plant were dried in the shade and powdered so that all the material could pass through a mesh not larger than 0.5 mm. The powdered materials of each plant (1000 g) were soaked in 3L of methanol (Merck Co., Germany) for 1 day, and the steps were repeated twice, followed by Soxhlet apparatus by using methanol for 72 hrs. At the end of extraction, it was passed through Whatman filter paper No.1 (Whatman Ltd., England). The PDME (9%) was concentrated to dryness under vacuum on rotary evaporator at 40°C then reconstituted in minimum amount of DMSO and stored at 4°C for further use (Nongyao and Kitja 2005).

Experimental animals and diet. The whole experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Animal Ethical Committee of Annamalai University (Proposal no.647: Dated 25.09.2009). The study was conducted on 48 golden male Syrian hamsters (*Mesocricetus auratus*) weighing 90-120g, obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India. They were housed in polypropylene cages (47 x 34 x 20 cm), (six hamsters /cage) lined with husk, renewed every 24 h under a 12:12 h light dark cycle at around 22°C and had free access to tap water and food. The animals were fed on a standard pelleted diet (Pranav Agro Industries Limited, Maharashtra, India).

Experimental Design. The animals were divided into four groups of six animals each. Group I: Untreated control, animals were painted with paraffin alone using no.4 brush; Group II: Animals were given the methanolic extract of *P.daemia* at a concentration 200 mg/kg bw dissolved in 0.5% DMSO on alternative days for a period of fourteen weeks using intragastric tube; Groups III and IV: The left buccal pouches of animals in group 3 and 4 were painted with 0.5% DMBA three times a week. In addition the group 4 animals received the intragastric administration of *P.daemia* extract at a concentration of 200 mg/kg bw on days alternate to DMBA applications.

At the end of 15 weeks all the animal sacrificed. The buccal pouch tissues obtained were subdivided into four parts. The first portion of the tissue was immediately frozen in liquid nitrogen for subsequent RNA extraction, the second part was processed using lysis buffer for Western blot analysis. Third portion was used for the preparation of S9 fraction which was used for biochemical analysis. The remaining tissues were fixed in 10% formalin, which were used for histopathological and immunohistochemical analysis.

Histopathological changes. After sacrifice, the buccal were macroscopically examined for the presence of tumors or other pathological lesions. Tissues with abnormal morphology were fixed in 10% buffered formalin and embedded in paraffin blocks. Histological sections stained with hematoxylin and eosin was used to confirm the presence and type of tumors by histopathological examination, which was performed by a pathologist unaware of the experimental codes.

Immunohistochemistry. Paraffin embedded tissue sections on glass slides were deparaffinised by heat at 60°C for 30 minutes,

followed by three washes in xylene. After gradual hydration through graded alcohol, the slides were incubated in citrate buffer (pH 6.0) for two cycles of 5 minutes in a microwave oven for antigen retrieval. The sections were allowed to cool for 20 minutes, rinsed with Tris-buffered saline (TBS) and treated with 3% H₂O₂ in distilled water for 15 minutes to inhibit endogenous peroxidase activity. Nonspecific antibody binding was reduced by incubating the sections with normal goat serum for 25 minutes. The sections were then incubated with PCNA, VEGF (Santa Cruz Biotechnology, CA, USA) mouse monoclonal antibody at 48C overnight. The slides were washed with TBS and then incubated with antimouse biotin-labeled secondary antibody followed by streptavidin-biotin-peroxidase for 30 min each at room temperature. The immunoprecipitate was visualized by treating with 3,3'-diaminobenzidine (DAKO) and counterstaining with haematoxylin. For negative controls, the primary antibody was replaced with TBS. Positive controls for each antibody were also processed simultaneously.

SDS-PAGE and Western blot analysis. SDS-Polyacrylamide gel electrophoresis was carried out by the method described by Laemmli (1970) using a discontinuous buffer system. The transfer of proteins resolved by SDS-PAGE to Polyvinylidene difluoride (PVDF) membrane was done according to Towbin *et al.* (1979) using a Wet Blotting transfer apparatus. The transfer buffer contained 23 mM Tris and 192 mM glycine. Polyvinylidene fluoride (PVDF) membrane was presoaked in methanol and equilibrated in transfer buffer. The gel was placed in contact with the PVDF membrane and sandwiched between two pieces of buffer soaked Whatman no. 1 filter paper on each side. This sandwich was placed between two graphite electrode with the membrane towards positive side. The efficiency of protein transfer was checked by staining the blot with Ponceau - S (0.5 % Ponceau S in 5 % v/v glacial acetic acid solution) for 5-10 minutes and washing with several changes of double distilled water.

The blot was treated for overnight with blocking solution containing 5% skimmed milk powder dissolved in tris buffered saline with Tween 20 (TBST) (pH 7.4) containing 150 mM NaCl, 50mM Tris and 500μL tween 20 at 4° C. The blot was rinsed once with TBST and incubated with 1:1000 dilution of anti p53 antibody for 2 hours. The blot was washed with TBST (0.1 % Tween 20) four times for 10 minutes each. p53 were detected by incubating corresponding horseradish peroxidase conjugated secondary antibodies (1:4000 diluted) to p53 for 2 hours at room temperature. After washing for four times in TBST (each wash for 10 minutes), the transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit according to the manufacturer's instruction. Chemiluminescence was captured by X-ray film and quantitated using Image J software. Percentage of expression was calculated by keeping expression of protein in control animals as 100%. Using specific expression of p53, caspase-3, Cox-2 and NF-κB were also analyzed using the same protocol.

Reverse transcriptase-Polymerase chain reaction (RT-PCR) analysis. Total RNA from the various tissue samples were isolated following the method of Chomczynski and Sacchi (1987). The tissue samples were minced and homogenized (100 mg/1 mL) in RNA isolation buffer. The homogenate was transferred to a 15 mL polypropylene tube and added in order: 2.5 mL of 2.5 M sodium acetate (pH 4.6), 0.5 mL of saturated phenol (80%) and 2.5 mL CHCl₃: Isoamyl alcohol (24:1). Following the addition of each reagent, the contents were mixed thoroughly by inversion. After incubation on ice for 15 min, the samples were centrifuged at 10,000 rpm for 15 min at 4°C. To the aqueous phase equal volume of ice-cold isopropanol was added and kept at -20 °C for 1 h. The RNA was precipitated at 12,000 rpm for 15 min at 4 °C, discard the supernatant and the pellet was washed with 80% ethanol. The resulting pellet was dried briefly in vacuum and dissolved in minimal volume of sterile DEPC treated MQ water. The amount of RNA was quantified spectrophotometrically at 260nm.

With the isolated RNA, Reverse transcriptase polymerase chain reaction was performed using a commercially available Kit (Genei, Bangalore) to study the expression of Bax, Bcl-2, CYP1B1, CDK2AP1 and β-actin was used as the internal standard. Primer sequence and band length of the amplicon are given below:

Gene	Primer	Sequences	Size
Bax	F	5'-GGCGCCACTGCTGGCACTTAT3'	375
	R	5'-GCCCTCAGTGTGGAGATCG-3'	
Bcl-2	F	5'-CGGAGGCTGGGATGCCTTTGTG-3'	600
	R	5'-GCCAGAAAGCCAGCTTCCCCAA-3'	
CYP1B1	F	5'-GAAAAGAAGGAGCCGAAAGGCC-3'	500
	R	5'-CGCCGTTTGCCACCGAAAAA-3'	
CDK2AP1	F	5'-CTGGTGGACAACATCGCTCTG-3'	600
	R	5'-GGTCTGCTGACCTCACTTGTG-3'	
β Actin	F	5'-CGGCCTCCAGCTCCTCCCTA-3'	341
	R	5'-GCCCGCATCCACACGGAG- 3'	

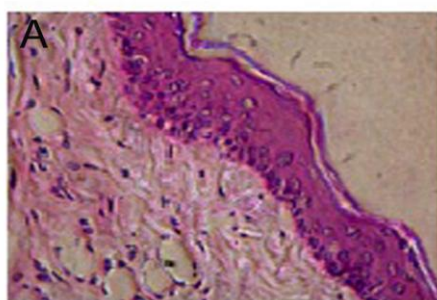
Statistical analysis. Values are expressed as means ± S.D. The data were statistically analysed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) using a statistical package program (SPSS 10.0 for Windows). p-values of less than 0.05 were considered as statistically significant.

RESULTS

A myriad of histopathological changes (severe hyperkeratosis, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma of the epithelium), were observed in hamsters painted with DMBA-alone induced group (Fig. 1). A mild to moderate preneoplastic lesion (hyperplasia, hyperkeratosis and dysplasia)

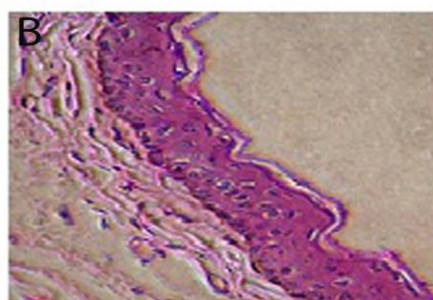
were noticed in DMBA + PDME treated hamsters. Hamsters administered with PDME alone showed well defined and intact epithelial layers similar to that of the control hamsters. The immunohistochemical expression of PCNA and VEGF proteins in control

and experimental animals was shown in fig. 2a, b. The levels of VEGF and PCNA showed high positive nuclei staining in DMBA treated hamsters groups when compared with control group. Oral administration of PDME (200 mg/kg bw) to DMBA painted groups significantly restored the expression of above mentioned or noted proteins to near normal when compared with DMBA group. However, control and PDME alone treated groups showed no significant changes of expression of PCNA and VEGF].



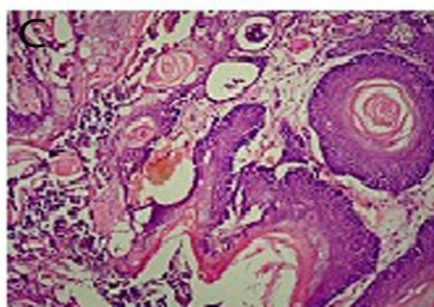
Control

Normal mucosa of a control hamster (H& E, 40 x)



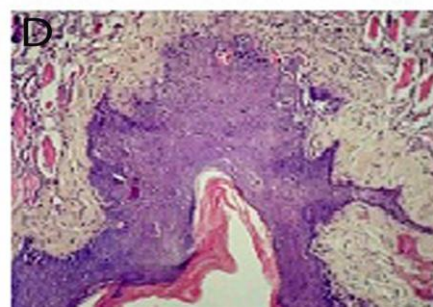
PDME Alone

Photomicrograph of PDME treated pouch mucosa of hamster exhibiting well defined and intact epithelial layers: ((H& E, 40 x)



DMBA

DMBA induced buccal mucosal tumors exhibiting hyperplasia, dysplasia and hyperkeratosis: (H& E, 40 x)

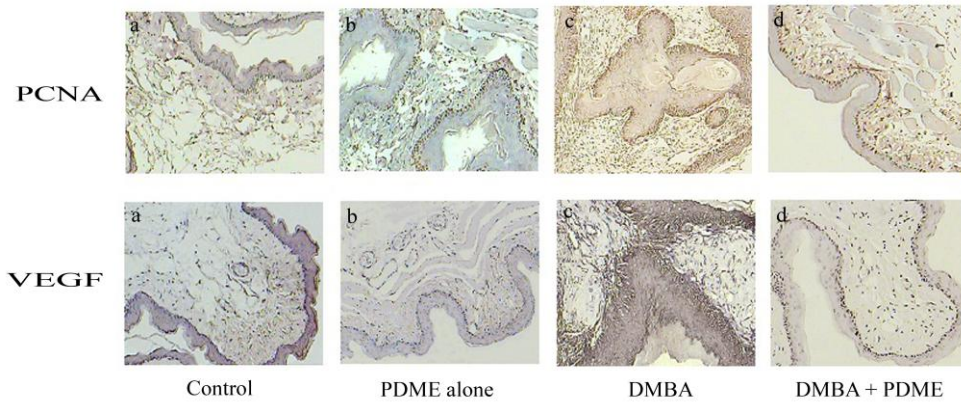


DMBA+ PDME

Photomicrograph of DMBA + PDME treated buccal pouch exhibiting moderate pre neoplastic lesions: (H& E, 40 x)

Using immunoblots analysis, the alteration in the expression of proteins p53 and caspase-3 in control and experimental animals were shown in fig 3. The expression levels of p53 and caspase-3 was significantly upregulated in DMBA alone treated animals. On supplementation with PDME to DMBA treated animals significantly

modulated the expression patterns of p53 and caspase-3 when compared with DMBA alone group. However, there were no significant difference in p53 and caspase-3 expressions between control and PDME alone treated groups.



Chronic DMBA-induced buccal pouch tissues section. (a, b) Shows lack of positive nuclei staining (c). The PCNA and VEGF expression were increased in DMBA group (d). The PCNA and VEGF expression were decreases in DMBA + PDME.

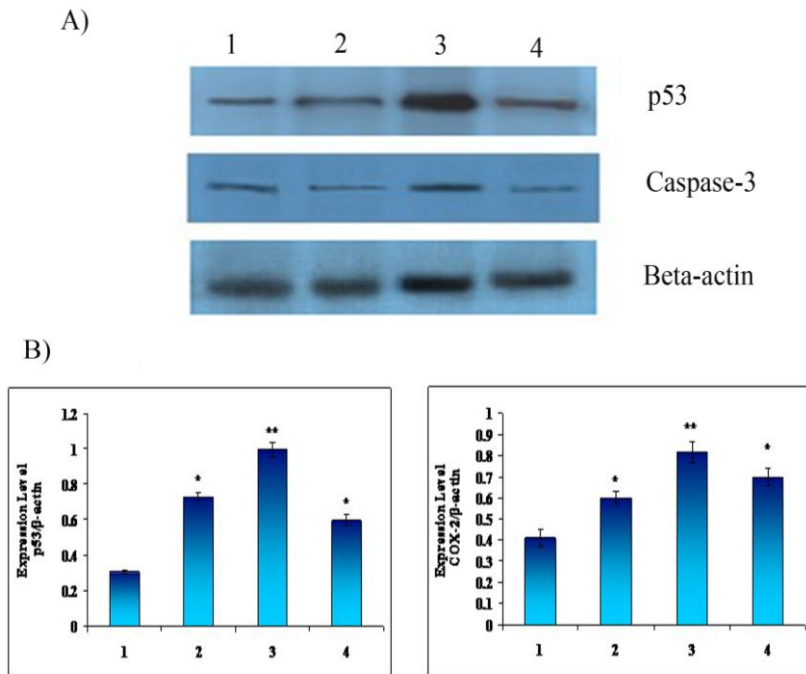
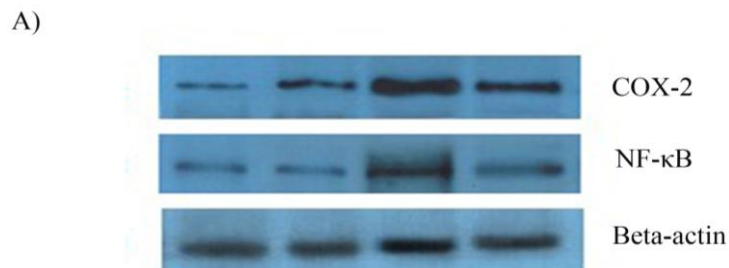


Fig 3: (a) Western blot analysis of p53, caspase-3 and β-actin expression; (b) Band intensities were scanned by densitometer. Histograms of densitometric analysis represent the ratio of p53, caspase-3 and β-actin expression. Values that do not share a common superscript in the same column differ significantly at P < 0.05 (DMRT).

The effect of PDME on the expression pattern of COX-2 and NF-κB proteins in DMBA induced buccal pouch mucosa of experimental animals (Fig. 4). The immunoblots revealed that DMBA induced hamsters showed an increased expression of COX-2 and NF-κB when

compared with control group. Oral administration of PDME (200 mg/kg b.wt) to DMBA induced group showed a significant decrease in the expression level of COX-2 and NF-κB proteins



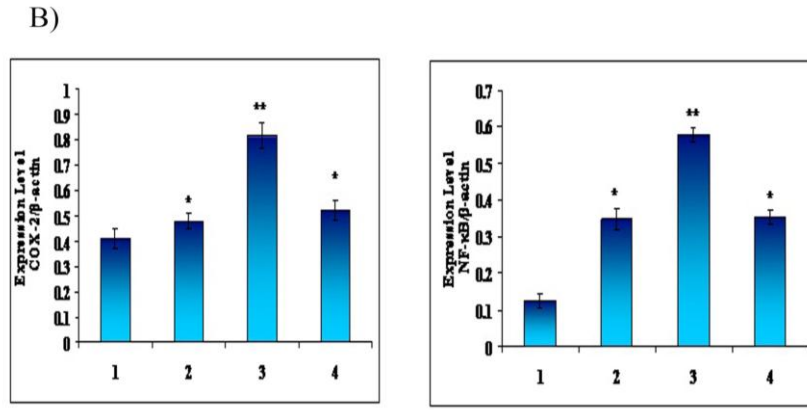


Fig 4: (a) Western blot analysis of COX-2, NF-κB and β-actin expression; (b) Band intensities were scanned by densitometer. Histograms of densitometric analysis represent the ratio of COX-2, NF-κB and β-actin expression. Values that do not share a common superscript in the same column differ significantly at P < 0.05 (DMRT).

The mRNA expression levels of Bax, CDK2AP1, CYP1B1 and Bcl-2 were examined using RT-PCR analysis on control and DMBA induced hamsters groups. DMBA induced animals showed decreased expression of Bax and CDK2AP1. In contrast, the mRNA levels of Bcl-2 and CYP1B1 were significantly increased in DMBA groups, when compared with control animals. Oral treatment with PDME (200

mg/kg bw) to DMBA induced group significantly decreased the Bcl-2 and CYP1B1 level and elevate the expression of Bax and CDK2AP1 in comparison with DMBA group. There was no significant alteration of above mentioned proteins between control and PDME alone treated groups (Fig. 5).

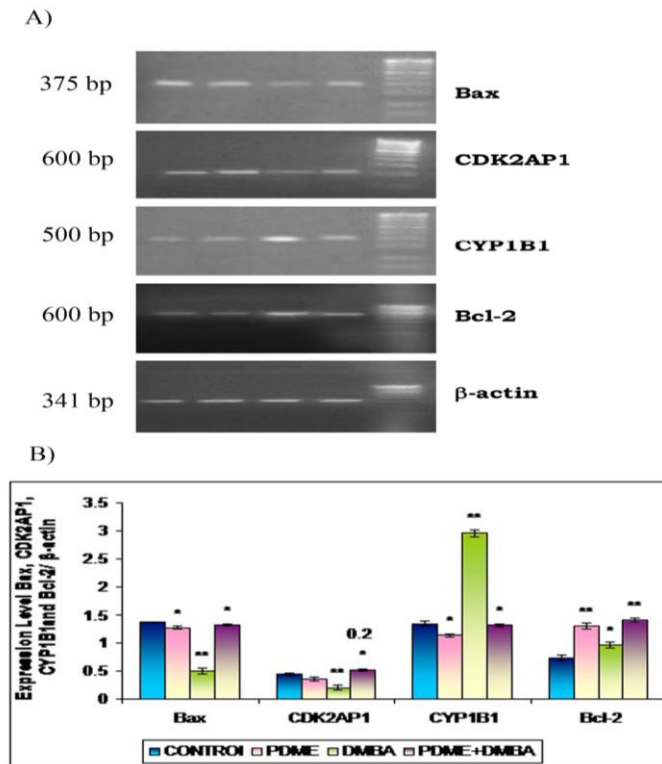


Fig 5: Values not sharing a common marking (*, **) differ significantly at P<0.05 (DMRT).

DISCUSSION

The Syrian hamster model is advantageous over other models examined because it is the most permissive for human Ad replication both in vitro and in vivo. This is reflected in the antitumor efficacy and i.t. replication observed in the hamster model. One of the best characterized animal models for OSCC is the hamster cheek pouch system which closely correlates with sequential common events involved in the development of remalignant and malignant human oral cancers (Miyata et al., 2001).

We noticed 100% tumor formation, which was histopathologically confirmed as well differentiated squamous cell carcinoma in the hamsters treated with DMBA alone. A myriad of histopathological changes (severe hyperkeratosis, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma of epithelium), were

observed in hamsters painted with DMBA-alone. A mild to moderate preneoplastic lesion (hyperplasia, hyperkeratosis and dysplasia) were noticed in DMBA + PDME treated hamsters. Hamsters administered with PDME alone showed well defined and intact

epithelial layers similar to that of the control hamsters. The present results suggest that oral administration of PDME at a dose of 200 mg/kg bw significantly prevented the tumor incidence and burden in DMBA treated hamsters, probably by inhibiting abnormal cell proliferation, occurring during DMBA-induced oral carcinogenesis.

On IHC analysis, over expression of VEGF markers was noticed in experimental animals treated with DMBA alone. Oral administration of PDME at a dose of 200 mg/kg bw to hamsters treated with DMBA significantly restored the expression of VEGF marker. Treatment with PDME alone revealed expression similar to that of control groups. It has been reported that, hypoxia inducing factor (HIF), nitric oxide, PDGF and mutant p53 can upregulate the expression of VEGF (Young *et al.*, 2001, Chen *et al.*, 2002). All these factors would have led to the elevation of VEGF. IHC analysis showed a low level of VEGF expression in the buccal pouch of PDME treated animals. In our study we have identified that PDME had significantly down regulated the expression of COX-2 and NF- κ B and maintained the functional status of p53. So the observed down regulation in VEGF expression by PDME could possess chemopreventive effect against DMBA-induced apoptotic signaling in buccal pouch tissues.

On the other hand, proliferating cell nuclear antigen (PCNA) is the most representative proliferating marker and plays important roles in DNA replication, chromatin remodeling, DNA repair, sister-chromatid cohesion, and cell-cycle control (Stoimenov and Helleday 2009). Functional alterations in PCNA activity are common genetic events in various types of cancers and have been found to be effective markers of cell proliferation, which can be used as an independent predictor of histological grade, recurrence rate, and prognosis (Staibano *et al.*, 1998). PCNA is a factor in DNA replication, and PCNA functions are regulated by an interaction with one of the cell-cycle inhibitors, p21 CIP1/ WAF1 induced by p53 (Qin *et al.*, 1998). However, the present study, oral administration of PDME at a dose of 200 mg/kg bw to hamsters treated with DMBA significantly correlated with cell differentiation, mode of invasion, recurrence and also restored the expression of PCNA marker in buccal pouch tissues.

Expression of angiogenic factor VEGF were found to be down regulated in the group PDME treated animals, which could indicate the lower rate of angiogenic process when compared to DMBA alone treated animals. The reason for reduced antiproliferative, elevated apoptotic and down regulated angiogenic property observed in the PDME treated animals might be either due to the interference of phytochemicals present in the PDME on the progressive phase of carcinogenesis or due to the withdrawal of administration of DMBA before the occurrence of permanent changes in the animals that cause carcinogenesis.

The expression pattern of p53 in cell lysates was up-regulated in DMBA treated groups. Oral administration of PDME at a dose of 200 mg/kg bw to hamsters treated with DMBA significantly restored the expression of p53. Phytochemicals present in the fraction might have detoxified the genetic insult causing agents formed during the metabolism of DMBA. Thus the fractions might have prevented the DMBA induced p53 mutation. Along with p53, the pathway of programmed cell death (Apoptosis) is regulated by factors such as members of Bcl-2 protein family and caspases enzymes. It has been well established p53-associated OSCC cell lines to clearly demonstrate effective inhibition of growth and invasion following grape seed procyanidin (GSP) treatment in a dose-dependent manner.

The expression (or) activation of NF- κ B was upregulated in DMBA group. ROS and highly electrophilic metabolically activated compound produced from DMBA might have cause the activation of NF- κ B. It has been reported that natural antioxidant which has the chemopreventive property, due to its free radical scavenging capacity can block the NF- κ B activation process (Singh and Aggarwal 2005). Level of activated NF- κ B was found to be significantly low in PDME treated animals. This might be due to the influence of antioxidant phytochemicals present in the fraction on ROS mediated activation process. Moreover, PDME inhibited DMBA-induced activation of COX-2 inflammatory signaling in a dose dependent manner.

The Bcl-2 gene encodes a membrane protein localized to the nuclear membrane, the inner surface of the mitochondria, and the endoplasmic reticulum (Akao *et al.*, 1994), and which has been shown to function as a suppressor of apoptosis (Vaux *et al.*, 1988). Bax forms both homodimers and heterodimers with Bcl-2. Pervious study has investigated the expression profile of CYP1B1 in a panel of 51 OSCC cases and an epithelial dysplasia case. CYP1B1 was found to be downregulated at the mRNA level as determined by PCR in tumor tissues in comparison to their corresponding normal tissues and an epithelial dysplasia case (Shalmali *et al.*, 2011). Furthermore, the CYP1B1 down regulation in oral tumor tissues as compared to their matched normal tissues contradicts many studies which state that CYP1B1 is overexpressed in tumors (Sissung *et al.*, 2006). This down regulation is consistent over a range of tumor stages (TNM) lesion and etiology of the carcinogenesis. On the basis of our observations, we suggest that a level of caution should be observed for treatments based on CYP1B1 overexpression in tumors.

Cyclin-Dependent Kinase 2-Associated Protein (CDK2AP1) is serine/threonine protein kinases that play a critical role in cell cycle regulation (Hunter and Pines 1994). The present work investigated the mRNA expression pattern of Bax, CDK2AP1 and CYP1B1; they were significantly increased in DMBA- induced groups. Conversely, the mRNA levels of Bcl-2 were significantly decreased in DMBA-induced on buccal pouch mucosa of experimental animals. Oral administration of PDME (200 mg/kg b.wt) in DMBA-induced group has showed a significant decrease the expression levels and enhanced expression of Bcl-2 when compared to control (non-DMBA induced) groups. Thus, based on the present findings along with the previous reports it can be conclude that PDME possesses chemopreventive effect against DMBA-induced apoptotic signaling in buccal pouch tissues.

Taken together, the results of proapoptotic, apoptotic and anti-apoptotic markers on *in vivo* studies demonstrated that PDME had a significant chemotherapeutic and chemopreventive effect against oral cancer. However, it is worth emphasizing that the protective role of PDME can be considered as an anticancer agent to combat against oral cancer. Further studies are highly warranted to isolate the active compounds from *P.daemia* responsible for this potent activity against oral cancer.

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