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## Modifying Effects of *Piper longum* on Cell Surface Abnormalities in 7, 12-dimethylbenz(A)Anthracene Induced Hamster Buccal Pouch Carcinogenesis

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**Abstract:** Present study has investigated the modifying effects of ethanolic extract of *Piper longum* dried fruits (PLDFEE) on cell surface abnormalities in DMBA induced hamster buccal pouch carcinogenesis. DMBA painting in hamster buccal pouch three times per week for 14 weeks resulted in well developed, well differentiated squamous cell carcinoma. An increase in glycoconjugates (protein bound hexose, total sialic acid and fucose) in plasma and buccal mucosa tissues and decrease in erythrocyte membrane glycoconjugates were observed in DMBA painted hamsters as compared to control animals. Oral administration of (PLDFEE) restored the status of glycoconjugates and lipids during DMBA induced oral carcinogenesis. Our results indicate that (PLDFEE) has protected the cell surface and maintained the structural integrity of the cell membranes during DMBA induced hamster buccal pouch carcinogenesis.

**Key words:** DMBA, oral cancer, glycoconjugates

### INTRODUCTION

Oral cancer, a disfiguring disease, assumes a major health problem in terms of patient's, morbidity and mortality and this form of cancer represents 40-50% of all cancers in India (Gupta and Nandakmar, 1999). Cancer of the oral cavity has multifactorial aetiologies and are frequently associated with chewing of betel quid containing tobacco, in addition to smoking and alcohol consumption (Wamakulasuriya and Ralhan, 2007; Petti and Scully, 2005). Oral squamous cell carcinoma is one among the few human cancers, with the vast potential for prevention.

Oral carcinogenesis is preceded by distinct premalignant lesions such as leukoplakia and erythroplakia. 7, 12-dimethyl benz(a)anthracene (DMBA) is the most widely used chemical carcinogen to induce oral carcinogenesis in the buccal pouch of golden Syrian hamsters. DMBA induced precancerous and cancerous lesions in the hamsters resemble human oral precancerous and cancerous lesions. Also, DMBA induced oral cancer expresses similar biochemical and molecular markers that are expressed in human oral carcinoma (Miyata *et al.*, 2001).

Glycoproteins, a family of complex proteins, play a vital role in cell differentiation, intercellular recognition, tumorigenesis and as receptors for many hormones and viruses (Patel *et al.*, 1990). Sialic acid and fucose exist as

terminal units of oligosaccharides and are present in various mucoproteins and as the carbohydrate component of cell membrane glycolipids. Tumor associated carbohydrate changes have been used in the diagnosis of human cancers (Manoharan *et al.*, 2004). Altered expression of cell surface glycoconjugates is involved in the process of metastasis (Nicolson, 1984; Escrevente *et al.*, 2006).

Altered pattern of cell surface glycoconjugates are well documented in both human and experimental carcinogenesis including oral cancer (Manoharan and Nagini, 1995; Jiang *et al.*, 2006; Suresh *et al.*, 2007).

*Piper longum*, a well-known Indian medicinal spice, is commonly known as Long pepper in English and Thippili in Tamil. It is widely used in Siddha, Ayurveda and Unani systems of medicine for the treatment of several diseases due to its diverse biological activities and pharmacological functions. *Piper longum* is considered beneficial for arthritis, peptic ulcers, viral hepatitis, fertility and diabetes (Joshi, 2000; Virinder *et al.*, 1997). Piperine, an active constituent of *Piper longum*, has many pharmacological actions such as antifungal, anti-inflammatory, antioxidant and anticancer effects (Atal *et al.*, 1985; Singh *et al.*, 1984). Experimental studies have also shown their immunomodulatory and anticancer activity (Sunila and Kuttan, 2004; Selvendiran *et al.*, 2003). Previous studies from our Laboratory have demonstrated the

chemopreventive and antilipidperoxidative potential of *Piper longum* in DMBA induced hamster buccal pouch carcinogenesis (Senthil *et al.*, 2007). The major aim of the present study was to demonstrate the modifying effects of *Piper longum* on cell surface abnormalities in DMBA induced hamster buccal pouch carcinogenesis.

## MATERIALS AND METHODS

**Chemicals:** The carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA) was obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore India. All other chemicals used were of analytical grade.

**Animals:** Male golden Syrian hamsters 8-10 weeks old, weighing 80-120 g, were purchased from National Institute of Nutrition, Hyderabad, India and maintained in Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed four or five in a polypropylene cage and provided standard pellet diet and water *ad libitum*. The animals were maintained under controlled conditions of temperature and humidity with a 12 h light/dark cycle.

**Plant material:** *Piper longum* dried fruits was purchased from traditional market in Chidambaram, Tamil Nadu and identified by the Botanist, Department of Botany, Annamalai University. A voucher specimen was deposited in the Department of Botany, Annamalai University.

### Preparation of plant extract:

**Ethanol extract preparation:** Five hundred gram of *Piper longum* dried fruits was powdered and then soaked in 1500 mL of 95% of ethanol overnight. After filtration, the residue obtained was again resuspended in equal volume of 95% ethanol for 48 h and filtered again. The above two filtrates were mixed and the solvent was evaporated in a rotavapor at 40-50°C under reduced pressure. A 12% semisolid dark brown material obtained was stored at 0-4°C until used. A known volume of the ethanolic residual extract is suspended in distilled water and was orally administered to the animals by gastric intubation using a force-feeding needle during the experimental period.

**Preparation of tissue homogenate:** Tissue samples from animals were washed with ice cold saline and dried between folds of filter paper, weighed and homogenized using appropriate buffer of concerned parameter in an all glass homogenizer with Teflon pestle. The homogenate was centrifuged at 1000 g for 5 min and the supernatant was then used for the biochemical estimations.

**Experimental protocol:** The local institutional animal ethics committee, Annamalai University, Annamalai Nagar, India, approved the experimental design. The present study was conducted during 2005-2006 in the Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar, Tamilnadu, India.

A total number of 40 hamsters were randomized into four groups of 10 animals in each. Group I animals were served as untreated control. Groups II and III animals were painted with 0.5% DMBA in liquid paraffin thrice a week for 14 weeks on the left buccal pouches. Group II animals received no other treatment. Group III animals were orally administered with PLDFEE (300 mg kg<sup>-1</sup> b.wt.), starting 1 week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the scarification of the animals. Group IV animals received oral administration of PLDFEE alone throughout the experimental period. The experiment was terminated at the end of 14 weeks and all animals were sacrificed by cervical dislocation. Biochemical studies were conducted on buccal mucosal tissues of control and experimental animals in each group.

**Biochemical analysis:** After plasma separation, the erythrocyte membrane was prepared by the method of Dodge *et al.* (1968) modified by Quist (1980). The defatted tissues obtained after treating buccal mucosa tissues with methanol and chloroform was used for the estimation of glycoproteins. To this dry defatted tissues, 0.1 N H<sub>2</sub>SO<sub>4</sub> was added and hydrolysed at 80°C for 1 h. It was cooled and the aliquot was used for sialic acid estimation. To the remaining solution, 0.1 N sodium hydroxide was added and kept in an ice bath for 1 h. From these aliquots, protein bound hexose and fucose were estimated. The protein bound hexose, hexosamine, total sialic acid and fucose in plasma, erythrocyte membrane and buccal mucosa tissues were estimated by the methods of Niebes *et al.* (1972), Wagner (1979), Warren (1959) and Dische and Shettles (1948), respectively. Plasma lipid bound sialic acid level was determined by the method of Katopodis and Stock (1980).

**Statistical analysis:** Values are expressed as mean±SD. Statistical analysis was performed by One-way analysis of variance (ANOVA) followed by Student's t-test. The values were considered statistically significant if the p-value was less than 0.05.

## RESULTS

The biochemical parameters were significantly increased (p<0.001) in the plasma of tumor bearing hamsters (Group II) as compared to control animals (Group I) (Table 1). Oral administration of PLDFEE at a

Table1: Protein bound hexose, hexosamine, total sialic acid, lipid bound sialic acid and fucose in plasma of control and experimental animals in each group

Parameters	Protein bound hexose (mg dL <sup>-1</sup> )	Protein bound hexosamine (mg dL <sup>-1</sup> )	Total sialic acid (mg dL <sup>-1</sup> )	Lipid bound sialic acid (mg dL <sup>-1</sup> )	Fucose (mg dL <sup>-1</sup> )
Control	83.5±6.2	72.50±4.9	42.9±3.5	10.90±0.9	6.89±0.47
DMBA	128.4±10.6 <sup>a</sup>	110.60±9.8 <sup>a</sup>	80.6±8.7 <sup>a</sup>	24.50±2.2 <sup>a</sup>	15.82±1.3 <sup>a</sup>
DMBA+PLDFEE	91.2±7.5 <sup>a,b</sup>	79.51±7.2 <sup>a,b</sup>	47.5±4.5 <sup>a,b</sup>	12.40±1.7 <sup>a,b</sup>	7.61±0.82 <sup>a,b</sup>
PLDFEE alone	82.9±5.9 <sup>NS</sup>	71.90±5.2 <sup>NS</sup>	42.4±2.8 <sup>NS</sup>	10.69±1.1 <sup>NS</sup>	6.84±0.53 <sup>NS</sup>
F-value	45.10	47.67	76.41	116.67	204.38

Values are expressed as mean±SD, (n = 10); PLDFEE Piper longum dried fruit ethanolic extract; Significantly different from control animals, <sup>a</sup>p<0.001, <sup>b</sup>p<0.05; Significantly different from DMBA alone painted animals, <sup>b</sup>p<0.001; NS: Not significant from control animals

Table 2: Protein bound hexose, hexosamine and total sialic acid levels in erythrocyte membranes of control and experimental animals in each group (n = 10)

Parameters	Protein bound hexose (µg mg <sup>-1</sup> protein)	Protein bound hexosamine (µg mg <sup>-1</sup> protein)	Total sialic acid (µgmg <sup>-1</sup> protein)
Control	123.9±8.7	79.5±5.6	35.9±2.9
DMBA	76.9±8.2 <sup>a</sup>	60.3±5.8 <sup>a</sup>	21.4±2.3 <sup>a</sup>
DMBA+PLDFEE	114.9±8.9 <sup>a,b</sup>	72.3±6.9 <sup>a,b</sup>	32.5±3.32 <sup>a,b</sup>
PLDFEE alone	124.7±9.1 <sup>NS</sup>	78.4±6.4 <sup>NS</sup>	35.7±3.1 <sup>NS</sup>
F-value	41.76	14.59	36.56

Values are expressed as mean±SD, (n = 10); PLDFEE- Piper longum dried fruit ethanolic extract; Significantly different from control animals, <sup>a</sup>p<0.001, <sup>b</sup>p<0.05; significantly different from DMBA alone painted animals, <sup>b</sup>p<0.001; NS: Not significant from control animals

Table 3: Protein bound hexose, total sialic acid and fucose levels in buccal mucosa of control and experimental animals in each group

Parameters	Group I control	Group II DMBA	Group III DMBA+PLDFEE	Group IV PLDFEE alone	F-value
Protein bound hexose (mg g <sup>-1</sup> protein)	108.12±8.08	151.23±11.51 <sup>a</sup>	117.65±9.01 <sup>a,b</sup>	104.25±7.93 <sup>NS</sup>	32.28
Total sialic acid (mg g <sup>-1</sup> protein)	12.05±0.91	27.19±2.07 <sup>a</sup>	13.77±1.88 <sup>a,b</sup>	11.98±0.91 <sup>NS</sup>	153.23
Fucose (mg g <sup>-1</sup> protein)	19.39±0.98	27.91±2.12 <sup>a</sup>	20.80±1.58 <sup>a,b</sup>	12.80±0.97 <sup>NS</sup>	103.36

Values are expressed as mean±SD, (n = 10); PLDFEE- Piper longum dried fruit ethanolic extract; Significantly different from control animals, <sup>a</sup>p<0.001, <sup>b</sup>p<0.05; Significantly different from DMBA alone painted animals, <sup>b</sup>p<0.001; NS: Not significant from control animals

dose of 300 mg kg<sup>-1</sup> body weight to DMBA painted animals (Group III) restored the status of plasma protein bound hexose, hexosamine, total sialic acid, lipid bound sialic acid and fucose (p<0.001). Hamsters treated with PLDFEE alone (Group IV) showed no significant difference in glycoconjugates levels as compared to control animals.

Table 2 shows that biochemical parameters were significantly decreased (p<0.001) in the erythrocyte membrane of tumor bearing hamsters (Group II) as compared to control animals (Group I). Oral administration of PLDFEE at a dose of 300 mg kg<sup>-1</sup> body weight to DMBA painted animals (Group III) restored the status of erythrocyte membrane protein bound hexose, hexosamine and total sialic acid (p<0.001). Hamsters treated with PLDFEE alone (Group IV) showed no significant difference in glycoconjugates levels as compared to control animals.

The levels of biochemical parameters were significantly increased (p<0.001) in the buccal mucosa of tumor bearing hamsters (Group II) as compared to control animals (Group I). Oral administration of PLDFEE at a dose of 300 mg kg<sup>-1</sup> body weight to DMBA painted animals (Group III) restored the status of buccal mucosa protein bound hexose, total sialic acid and fucose (p<0.001).

Hamsters treated with PLDFEE alone (Group IV) showed no significant difference in glycoconjugates levels as compared to control animals (Table 3).

## DISCUSSION

Cell surface glycoconjugates play an important function in the regulation of epithelial tissue growth. Malignant transformation is associated with changes in composition of cell surface carbohydrates and cell membrane lipids and these changes could play a crucial role in the regulation of cell proliferation (Singhal and Hakomori, 1990; Zhao *et al.*, 2006). Atypical glycosylation and loss of epithelial cell surface carbohydrates have been reported in oral carcinogenesis (Dabelsteen, 1996; Dabelsteen *et al.*, 1992). Increased turnover of glycoprotein has been reported in cancer patients (Aranganathan *et al.*, 2005). Chemical carcinogens increase the expression of cell surface glycoconjugates during cell differentiation (Farber, 1981). Elevated sialyl and glycosyl transferase activity could be responsible for over expression of cell surface glycoconjugates in malignant tumor (Muller *et al.*, 2004).

The reduction in erythrocyte membrane glycoprotein in cancer bearing hamsters is probably due to increased membrane degradation or as a result of increased

shedding into circulation. An increase in serum glycoprotein due to increased turnover, secretion and or shedding from turnover cells in carcinogenesis has been well documented (Macbeth and Bekesi, 1964).

Sialic acid, the acetylated derivatives of neuraminic acid, is involved in the regulation of cell surface phenomenon and is therefore altered during malignant transformation (Yamamoto, 1995). Measurement of sialic acid is valuable in detecting the tumor growth and secondary metastasis or in monitoring tumor therapy (Sebzda *et al.*, 2006). Analysis of different kinds of cancer tissues revealed that malignant area contained almost twice the concentrations of sialic acid, as compared to normal areas of the same tissues (Narayana, 1994; Verazin *et al.*, 1990). Raval *et al.* (2004) recently reported that glycosidically bound sialic acids are excreted in larger amounts in urine of cancer animals due to elevated sialyl transferase activity in tumor tissues. Fucose has profound role in cell-cell communication and its altered pattern in the cell surface may lead to neoplastic transformation and metastasis (Escrevente *et al.*, 2006). Increase in sialic acid and fucose content in plasma and tumor tissues is probably due to increased turnover of malignant cells with subsequent shedding into plasma.

In the present study, oral administration of *Piper longum* dried fruits significantly restored the status of glycoconjugates in plasma, erythrocyte membrane and buccal mucosa tissues of DMBA painted hamsters. Present results thus demonstrate that *Piper longum* dried fruits have maintained the structural integrity of the cell surface and cell membrane, probably by interfering with the activities of enzymes involved in the glycosylation process, during DMBA induced hamster buccal pouch carcinogenesis.

Oral administration of PLDFEE restored the status of lipids in buccal mucosa tissues of DMBA painted hamsters. This suggests that PLDFEE has maintained the structural integrity of the cell membrane during DMBA induced oral carcinogenesis. The present study thus demonstrated the modifying effects of *Piper longum* dried fruits on cell surface abnormalities in DMBA induced chemical carcinogenesis.

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