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## **Chemopreventive and Antilipidperoxidative Efficacy of *Piper longum* (Linn.) on 7,12-dimethylbenz (a) anthracene (DMBA) Induced Hamster Buccal Pouch Carcinogenesis**

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**Abstract:** Aim of the present study was to find out the chemopreventive efficacy of *Piper longum*, a plant having diverse medicinal properties, in 7,12-dimethyl benz (a) anthracene (DMBA) induced oral carcinogenesis. The mechanistic pathway for its chemopreventive potential was analysed by measuring lipid peroxidation and antioxidants status in DMBA induced oral cancer. DMBA painting in hamster buccal pouch three times per week for 14 weeks resulted in well developed, well differentiated squamous cell carcinoma. Elevated lipid peroxidation and decline in antioxidants were noticed in tumor bearing hamsters as compared to control animals. Oral administration of ethanolic extract of *Piper longum* dried fruits (PLEFet) on alternate days to DMBA painting significantly prevented the tumor incidence, volume and burden and restored the status of lipid peroxidation and antioxidants in DMBA painted hamsters. Our results indicate that the dried fruits of *P. longum* has suppressing effects on cell proliferation, which is probably due to its antilipid peroxidative and antioxidative potential during DMBA induced oral carcinogenesis.

**Key words:** DMBA, oral cancer, lipid peroxidation, antioxidants

### **INTRODUCTION**

Carcinogenesis is the process by which malignant tumor develops in a living organism. The prevalence of several cancers increases exponentially with age in a human population from fourth to eight decade of life (Perin, 2001). Oral cancer is defined as any malignant tissue growth located in the oral cavity which includes lips, buccal mucosa, anterior tongue, floor of the mouth, hard and soft plates, upper gingiva and lower gingiva. Oral squamous cell carcinoma is the fifth most common cancer in the world. While it accounts for 3-4% of all malignancies in the Western countries, India has recorded the highest rate of oral cancer incidence than any other countries worldwide, where it accounts for 40-50% of all cancers (Gupta and Nandakumar, 1999). The habit of betel quid chewing with or without tobacco, tobacco smoking and alcohol consumption are the principal risk factors of oral cancer in India (Franceschi *et al.*, 1992).

Oral squamous cell carcinoma results due to repeated exposure of carcinogens to the entire epithelial cells of the oral cavity. 7,12 dimethylbenz (a) anthracene (DMBA) is the most widely used chemical carcinogen to induce oral

carcinogenesis in the buccal pouches of golden Syrian hamsters. It is a potent organ specific carcinogen and the pre-cancerous and cancerous lesions induced by DMBA are morphologically and histologically similar to that of human oral carcinoma. Also, DMBA induced oral cancer expresses similar biochemical and molecular markers that are expressed in human oral carcinoma (Miyata *et al.*, 2001).

Free radicals, a highly reactive, electrically charged chemical species with unpaired electrons, are frequently generated in biological systems either by normal metabolic pathways or as a consequence of exposure to physical, chemical and biological agents. They can alter genetic stability by attacking and damaging DNA, proteins and lipids if they are overproduced in the system (McCord, 2000). The important reactive oxygen species in biological systems include superoxide radical ( $O_2^-$ ), hydrogen peroxides ( $H_2O_2$ ), hydroxyl radical (OH), hypochlorous acid (HOCl) and peroxy nitrite (ONOO) (Freig *et al.*, 1994). A wide number of chemical carcinogens including DMBA, tumor initiators and promoters act through free radical mediated oxidative damage to cells and tissues (Farber, 1981).

Free radicals attack biomembranes, the major target of reactive oxygen species and lead to oxidative destruction of polyunsaturated fatty acids by a chain reaction known as lipid peroxidation. Free radical induced lipid peroxidation has been implicated in the pathogenesis of several clinical and pathological disorders including cancer (Ray and Husain *et al.*, 2002). Human body has an array of enzymatic [Superoxide Dismutase (SOD), catalase (CAT), Glutathione Peroxidase (GPx)] and non enzymatic [vitamin E, vitamin C, reduced glutathione (GSH)] antioxidants defence mechanism to protect the cells and tissues from the deleterious effects of free radical mediated oxidative damage. These antioxidants exert their protective role either by suppressing the formation of free radicals or by scavenging them (Halliwell, 1994). Manoharan *et al.* (1996, 2005) have demonstrated altered lipid peroxidation and disturbed antioxidant status in human and experimental oral carcinogenesis.

Chemoprevention, a novel approach in recent cancer research, deals with the prevention, inhibition, suppression and reversal of carcinogenic process by using natural plant products or their constituents and synthetic chemical agents (Aruna and Sivaramkrishnan, 1990). India is rich in medicinal plants biodiversity, where most of the prescribed modern medicine contains the active principles of medicinal plants or their derivatives. Previous studies from our laboratory have shown the chemopreventive potential *A. indica*, *Curcuma longa*, *Annona squamosa* and *Tephrosia purpurea* in experimental oral carcinogenesis (Suresh *et al.*, 2006; Kavitha and Manoharan, 2006).

Although a large number of medicinal plants and their bioactive constituents have been reported to have potent chemopreventive potential, several other medicinal plants and their active principles remain to be investigated.

*Piper longum*, one of the important piper species, is commonly known as long pepper in English and Thippilli in Tamil. It is widely distributed in the tropical and sub tropical regions of the world. This important spice is extensively used in Ayurvedic system of medicine for the treatment of several disorders including cancer (Joshi, 2000). *Piper longum* is widely used in China for tuberculosis, arthritis, cough, bronchitis, asthma, respiratory and inflammatory diseases (Virinder, 1997). Recent reports suggest that *Piper longum* and its constituents possess immunomodulatory and anticancer effects (Sunila and Kuttan, 2004). Piperine, an active constituent of *Piper longum*, has many pharmacological actions such as antimicrobial, antifungal, anti-inflammatory, antioxidant and anticancer effects (Atal *et al.*, 1985; Singh *et al.*, 1984).

No scientific studies were however published for its chemopreventive efficacy in experimental oral cancer. The

present study is therefore undertaken to evaluate the chemopreventive efficacy of *Piper longum* dried fruits in DMBA induced hamster buccal pouch carcinogenesis. The chemopreventive efficacy of *P. longum* was assayed by monitoring percentage of tumor bearing animals, number of tumors, tumor volume and burden and by estimating the status of lipid peroxidation and antioxidants 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

**Ethanolic extract preparation:** Five hundred grams 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 (Hossain *et al.*, 1992). 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.

**Experimental protocol:** The local institutional animal ethics committee, Annamalai University, Annamalai Nagar, India, approved the experimental design.

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 PLEFet (300 mg kg<sup>-1</sup> bw), starting 1 week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the sacrifice of the animals. Group IV animals received oral administration of PLEFet 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 blood of control and experimental animals in each group. For histopathological examination, buccal mucosal tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2-3 µm sections were cut in a rotary microtome and stained with haematoxylin and eosin.

**Biochemical analysis:** Plasma was separated from heparinized blood by centrifugation at 3000 rpm. After plasma separation, the erythrocyte membrane was prepared by the method of Dodge et al modified by Quist.

The activity of glutathione S transferase (GST) was assayed by the method of Habig *et al.* (1994). Glutathione reductase (GR) activity was assayed by the method of Carlberg and Mannervik (1985). Thiobarbituric acid reactive substances in plasma were assayed by the method of Yagi (1978) and in erythrocyte membranes by the method of Donnan (1950). Reduced glutathione (GSH) was determined by the method of Beutler and Kelley (1963). Vitamin C and E were measured according to the methods of Omaye *et al.* (1979) and Desai (1984), respectively. The activities of SOD, CAT and GPx in erythrocyte lysate were estimated according to the methods of Kakkar *et al.* (1984), Sinha (1972) and Rotruck *et al.* (1973), respectively.

**Statistical analysis:** Values are expressed as mean±SD. Statistical analysis was performed by One-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT). The values were considered statistically significant if the p-value was less than 0.05.

## RESULTS

Table 1 depicts the effect of PLEFet on tumor incidence; tumor volume and tumor burden in DMBA induced hamster buccal pouch carcinogenesis. We have observed 100% tumor formation with mean tumor volume

(395 mm<sup>3</sup>) and tumor burden (1777 mm<sup>3</sup>) in DMBA alone painted hamsters (Group II). Oral administration of PLEFet at a dose of 300 mg kg<sup>-1</sup> bw completely prevented the tumor incidence, tumor volume and tumor burden in DMBA painted hamsters (Group III). No tumors were observed in control animals (Group I) and PLEFet alone administered animals (Group IV).

Table 2 shows the histopathological features of control and experimental animals in each group. A myriad of histopathological changes (severe keratosis, hyperplasia, dysplasia and squamous cell carcinoma in malignant epithelium) were observed in hamsters painted with DMBA alone (Group II). A mild to moderate preneoplastic lesions [hyperplasia (++), keratosis (+) and dysplasia (+)] were noticed in Group III animals (DMBA + PLEFet).

Table 3 shows the activities of detoxification enzymes [glutathione S-transferase (GST) and glutathione reductase (GR)] in control and experimental animals in each group. The activities of detoxification enzymes were significantly decreased in DMBA painted hamsters (Group 2) as compared to control animals. Oral administrations of PLEFet to DMBA painted hamsters (Group 3) significantly revert back the activities of these enzymes to normal levels. No significant difference was observed between control animals (Group 1) and PLEFet alone administered animals (Group 4).

Table 1: Incidence of oral neoplasm in control and experimental animals in each group (n = 10)

Parameters	Group I control	Group II DMBA	Group III	
			DMBA+ PLEFet	Group IV PLEFet alone
Tumor incidence (squamous cell carcinoma)	0	100%	0	0
Total number of tumors/animals	0	45/(10)	0	0
Tumor volume (mm <sup>3</sup> )/animals	0	395/(10)	0	0
Tumor burden (mm <sup>3</sup> )/animals	0	1777/(10)	0	0

Tumor volume was measured using the formula  $V = \frac{4}{3}\pi \left(\frac{D1}{2}\right) \left(\frac{D2}{2}\right) \left(\frac{D3}{2}\right)$

where D<sub>1</sub>, D<sub>2</sub> and D<sub>3</sub> are the three diameters (mm) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal. ( ) indicates total number of animals bearing tumors

Table 2: Histopathological changes in the buccal pouch of hamsters in control and experimental animals in each group (n = 10)

Parameters	Group I Control	Group II DMBA	Group III	
			DMBA+ PLEFet	Group IV PLEFet alone
Keratosis	No change	Severe	Moderate	No change
Hyperplasia	No change	Severe	Moderate	No change
Dysplasia	No change	Severe	Mild	No change
Squamous cell carcinoma	No change	Well differentiated	---	No change

**Table 3: Activities of detoxification enzymes in control and experimental animals in each group**

Parameters	Group I control	Group II DMBA	Group III DMBA+PLEFet	Group IV PLEFet alone
GST (U <sup>A</sup> mg <sup>-1</sup> protein)	23.78±1.87	12.34±1.09	19.78±1.75	21.67±2.17
GR (U <sup>B</sup> mg <sup>-1</sup> protein)	18.59±1.13	8.82±0.91	14.13±1.20	16.18±1.44

Values are expressed as mean±SD for 10 animals in each group; Values not sharing a common superscript significantly differ at p<0.05 (DMRT) A- umoles of GSH-CDNB conjugate formed/min. B- B- μmoles of NADPH oxidized/hour

**Table 4: Status of plasma TBARS and antioxidants in control and experimental animals in each group**

Parameters	Group I control	Group II DMBA	Group III DMBA+PLEFet	Group IV PLEFet alone
TBARS (nmoles mL <sup>-1</sup> )	2.76±0.29 <sup>a</sup>	4.78±0.34 <sup>b</sup>	3.28±0.26 <sup>c</sup>	2.86±0.22 <sup>a</sup>
GSH (mg dL <sup>-1</sup> )	29.20±2.22 <sup>a</sup>	20.33±1.55 <sup>b</sup>	26.74±2.04 <sup>c</sup>	30.22±2.39 <sup>a</sup>
Vitamin C (mg dL <sup>-1</sup> )	1.42±0.11 <sup>a</sup>	0.81±0.06 <sup>b</sup>	1.21±0.09 <sup>c</sup>	1.49±0.12 <sup>a</sup>
Vitamin E (mg dL <sup>-1</sup> )	1.27±0.10 <sup>a</sup>	0.73±0.06 <sup>b</sup>	1.12±0.09 <sup>c</sup>	1.32±0.10 <sup>a</sup>
SOD (U L <sup>-1</sup> *)	2.91±0.02 <sup>a</sup>	1.72±0.13 <sup>b</sup>	2.59±0.20 <sup>c</sup>	2.98±0.23 <sup>a</sup>
CAT (U mL <sup>-1</sup> **)	0.45±0.04 <sup>a</sup>	0.26±0.02 <sup>b</sup>	0.37±0.03 <sup>c</sup>	0.48±0.04 <sup>a</sup>
GPx (U L <sup>-1</sup> ***)	135.22±10.30 <sup>a</sup>	96.82±7.37 <sup>b</sup>	128.26±9.82 <sup>c</sup>	139.37±10.90 <sup>a</sup>

Values are expressed as mean±SD for 10 rats in each group,\*The amount of enzyme required to inhibit 50% NBT reduction, \*\*Micromoles of H2O2 utilized/sec, \*\*\*Micromoles of glutathione utilized/min, Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT)

**Table 5: Status of plasma TBARS and antioxidants in control and experimental animals in each group**

Parameters	Group I control	Group II DMBA	Group III DMBA+PLEFet	Group IV PLEFet alone
Erythrocyte TBARS (pmoles/mg Hb)	1.73±0.12 <sup>a</sup>	2.75±0.20 <sup>b</sup>	2.35±0.21 <sup>c</sup>	1.80±0.14 <sup>a</sup>
Erythrocyte membrane: TBARS (nmoles/mg protein)	0.32±0.03 <sup>a</sup>	1.25±0.11 <sup>b</sup>	1.02±0.10 <sup>c</sup>	0.30±0.03 <sup>a</sup>
Vitamin E (μg mg <sup>-1</sup> protein)	2.27±0.11 <sup>a</sup>	1.51±0.08 <sup>b</sup>	1.98±0.17 <sup>c</sup>	2.20±0.21 <sup>a</sup>
Erythrocytes GSH (mg dL <sup>-1</sup> )	38.15±2.7 <sup>a</sup>	20.32±2.3 <sup>b</sup>	28.14±2.1 <sup>c</sup>	39.72±2.5 <sup>a</sup>
Erythrocyte lysate: SOD (U*/mg Hb)	2.09±0.17 <sup>a</sup>	1.38±0.10 <sup>b</sup>	1.72±0.13 <sup>c</sup>	2.11±0.20 <sup>a</sup>
CAT (U**/mg Hb)	1.22±0.08 <sup>a</sup>	0.74±0.06 <sup>b</sup>	0.87±0.07 <sup>c</sup>	1.19±0.08 <sup>a</sup>
GPx (U***g Hb)	13.72±1.15 <sup>a</sup>	7.81±0.68 <sup>b</sup>	10.35±1.15 <sup>c</sup>	14.05±1.38 <sup>a</sup>

Values are expressed as mean±SD for 10 rats in each group; \*-The amount of enzyme required to inhibit 50% NBT reduction; \*\*-Micromoles of H2O2 utilized/ sec; \*\*\*-Micromoles of glutathione utilized/min; Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT)

Table 4 and 5 show the status of TBARS and antioxidants (plasma and erythrocytes, respectively) in control and experimental animals in each group. The concentration of TBARS was increased whereas the levels of nonenzymatic antioxidants in plasma and erythrocytes (GSH, Vitamin C and Vitamin E) and activities of enzymatic antioxidants (SOD, CAT and GPx) were completely decreased in group II animals (DMBA alone painted hamsters) as compared to control animals. Oral administration of PLEFet significantly decreased the levels of TBARS and improved the antioxidants status in DMBA painted hamsters. Hamsters treated with PLEFet alone showed no significant difference in TBARS and antioxidants status as compared to control animals.

## DISCUSSION

In recent years, research scientists have focused their attention on cancer chemoprevention using medicinal plants, due to the fact that the natural products are non toxic, having less side effects and affordable at low cost. Medicinal plants and their bioactive constituents exert their chemopreventive efficacy by preventing metabolic activation of carcinogens, increasing detoxification of the carcinogen, blocking the interaction of ultimate carcinogen with cellular macromolecules and by

suppressing the clonal expression of the neoplastic cells (Leena and Jaindra, 2003). Medicinal plants those possess intrinsic antioxidants, radical trapping and anti-inflammatory properties, can act as potent chemopreventive agents (Vaidyaratnam, 1994).

DMBA induced experimental oral carcinogenesis is an excellent model to test the chemopreventive efficacy of medicinal plants and their constituents, since this model closely resembles human oral tumor, both histologically and morphologically. Control animals showed well defined and intact epithelial layer whereas DMBA alone treated animals revealed hyperkeratosis, hyperplasia, dysplasia and well differentiated squamous cell carcinoma. A thick, rough and reddish oral mucosa was seen in DMBA alone treated hamsters after 4 weeks. Dysplasia with small tumor growth and well developed oral squamous cell carcinoma was seen in the mucosa of DMBA alone treated animals after 10 and 16 weeks, respectively. The tumor cells have pleomorphic hyperchromatic nuclei with epithelial pearl formation. DMBA painted animals treated with PLEFet showed severe hyperkeratosis, hyperplasia and dysplasia. The chemopreventive efficacy of PLEFet is probably due to its suppressing effect on cell proliferation. The chemopreventive potential of PLEFet is also due to the presence of several bioactive principles such as piperine and their synergistic effects.

Estimation of the activities of detoxification enzymes in liver, the major metabolic organ, can be used as reliable biochemical markers to assess the chemopreventive potential of test compound. Recent studies have suggested that any medicinal plant that induces the activity of Glutathione-S-Transferase (GST) has potent chemopreventive activity (Schwartz and Shklar, 1996). It has been suggested that many chemopreventive substances convert DNA damaging entities into excretable metabolites through the induction of GST (McLellan and Wolf, 1999). Glutathione S-transferase detoxifies carcinogens either by destroying their reactive centers or facilitating their excretion by conjugation process. Glutathione Reductase (GR), an important enzyme required for reduced glutathione maintenance, catalyses NADPH dependent reduction of glutathione disulfide to reduced glutathione. The activities of detoxification enzymes (GST and GR) were decreased in tumor bearing animals, as compared to control animals. Oral administration of PLEFet significantly restored the activities of detoxification enzymes during pre-initiation phase, which indicates its crucial role in the detoxification process of the chemical carcinogens.

Over production of reactive oxygen species has been well documented in carcinogenesis. Profound studies have proposed that DMBA on metabolic activation into diol epoxide (ultimate carcinogen) generates toxic and highly diffusible reactive oxygen species, which cause severe cell damage or adducts on the biomolecules, contributing to carcinogenesis (Frei, 1994). The major target of reactive oxygen species is biomembrane polyunsaturated fatty acids and susceptibility of erythrocyte membrane to peroxide stress has been reported in several disorders including carcinogenesis. Measurement of lipid peroxidation by-products in plasma helps to assess the severity of tissue damage. We have previously demonstrated elevated lipid peroxidation and decline in antioxidant status in both experimental and human oral carcinogenesis. The elevated levels of plasma TBARS is probably due to a consequence of over production of Reactive Oxygen Species (ROS) during DMBA exposure or from elevated membrane lipid peroxidation with subsequent leakage into plasma. Oral administration of PLEFet significantly suppressed the formation of plasma and erythrocyte membranes TBARS in DMBA painted animals. Our results therefore indicate that PLEFet has potent antilipid peroxidative potential during oral carcinogenesis.

Antioxidants play an important role against free radical mediated oxidative tissue injury. Vitamin E, C and

reduced glutathione, the potent quenchers of reactive oxygen species, were reported to have inhibitory role in the process of carcinogenesis (Packer, 1997, Edge and Truscott, 1997; Trickler *et al.*, 1993). Reduced glutathione, a co-substrate of glutathione peroxidase, has an important role in maintaining the integrity of cell membrane and cellular homeostasis. Neoplastic cells can sequester nutrients from the circulation in order to meet the demands of growing tumor (Carpenter, 1991). The depletion of these nonenzymatic antioxidants observed in tumor bearing hamsters are therefore due to their utilization by malignant tumors or to combat the deleterious effects of lipid peroxidation by-products in circulation. Enzymatic antioxidants form the first line of *in vivo* antioxidant defense mechanism against free radical induced lipid peroxidation. Lowered activities of enzymatic antioxidants have been well documented in several pathological disorders including oral cancer (Kolanjiappan and Manoharan, 2005; Kavitha and Manoharan, 2006). Present results are in agreement with these observations. Oral administration of PLEFet at a dose of 300 mg/kg bw to DMBA painted animals restored the levels of non enzymatic antioxidants and activities of enzymatic antioxidants. Our results therefore indicate that PLEFet has potent free radical scavenging activity during

oral carcinogenesis, which is probably due to the presence of one or more antioxidant principles and their synergistic effects.

The present study thus demonstrated the potent chemopreventive and antilipid peroxidative efficacy of ethanolic extract of *Piper longum* dried fruits extract in DMBA induced hamster buccal pouch carcinogenesis. Although the exact mechanism of chemopreventive efficacy of PLEFet is unknown, its antilipidperoxidative and antioxidant potential could play a possible role in the prevention of DMBA induced hamster buccal pouch carcinogenesis.

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