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Chemopreventive and Antilipidperoxidative Potential of Thespesia populnea (L.) on Experimental Buccal Pouch Carcinogenesis

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Abstract

The present study has investigated the chemopreventive potential and antilipidperoxidative effect of ethanolic extract of Thespesia populnea (L.)bark on 7,12-dimethylbenz[a] anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Oral squamous cell carcinoma had developed in Syrian golden hamsters by exposing the buccal mucosa to 0.5 % DMBA in liquid paraffin thrice a week for 14 weeks. The tumor incidence, tumor volume and tumor burden that occurred in the hamster buccal pouch were determined. Oral administration of ethanolic extract of *T.populnea* bark at a dose of 300 mg / kg b.wt to DMBA painted animals on alternate days of DMBA treated for 14 weeks significantly prevented the tumor incidence, tumor volume and tumor burden. Ethanolic extract of *T.populnea* bark showed potent antilipidperoxidative effect as well as enhanced the antioxidants status in DMBA painted animals. We thus conclude that ethanolic extract of *T.populnea* bark has potent chemopreventive efficacy and significant antilipidperoxidative effect in DMBA induced oral carcinogenesis. Further studies are needed to isolate and characterize the bioactive chemopreventive component from the bark of *T.populnea*.

Keywords: Oral cancer, *Thespesia populnea*, Chemoprevention, Lipidperoxidation

Introduction

Oral cancer is a major health problem in terms of patient morbidity and mortality and its incidence is rapidly increasing worldwide (1). India has recorded the highest incidence, accounting about 30 - 40% of all cancers (2). Epidemiological studies have shown that chewing of betel quid with tobacco is the major etiological factor of oral carcinogenesis in India. Several biochemical markers that were reported to be altered in oral carcinogenesis include oncofetal proteins, enzymes, hormones, lipid peroxidation byproducts, antioxidants, lipids, glycoproteins etc (3,4).

An imbalance in oxidant and antioxidant status has been implicated in the pathogenesis of several disorders including cancer. Over production of reactive oxygen species within tissues can damage DNA and possibly contribute to mutagenesis and carcinogenesis (5). Human body contains an array of sophisticated antioxidant defense mechanisms to fight against reactive oxygen species charges and antioxidants status play an important role in modulating lipid peroxidation.

Squamous cell carcinoma can be induced in animals by continued application of certain chemical carcinogens, such as 7,12-dimethylbenz[a]anthracene (DMBA). The hamster buccal pouch has been used as an experimental model for oral cancer for many years (6). The hamster oral cancer model has also histological, biochemical and molecular similarities to human oral cancer (7). Several medical plants and their constituents have been reported to prevent multi-stage carcinogenesis (8). Numerous medicinal plants and their formulations are used for cancer in ethno medical practices as well as traditional system of medicine in India (9).

Thespesia populnea Soland ex Correa (Family: Malvaceae) is a large avenue tree found in the tropical regions and coastal forests in India. A decoction of the bark is commonly used for the treatment of skin and liver diseases. Gossypol was found to be the major component of *T.populnea* (10) producing anti-inflammatory (11) and antifertility effects in rats (12,13) as well as in human beings (14). Four naturally occurring quinines, viz. thespone, mansonone-D, mansonone-H, thespone and thespesone, have also been extracted from heartwood of *T.populnea* (15). In the indigenous system of medicine, the paste of the fruits, leaves and roots of *T.populnea* are applied externally for various skin diseases. The leaves are applied locally for their anti-inflammatory effects in swollen joints (16). The fruits of the plant are used in Ayurveda for the control of diabetes (17). The barks and flowers possess astringent, hepatoprotective and antioxidant activity in rats (18-20). However, no reports are available on the antilipidperoxidative and antioxidant potential of *T.populnea* in DMBA induced oral carcinogenesis. The present study was thus designed to evaluate the anticarcinogenic potential of *T.populnea* in DMBA-induced oral carcinogenesis by investigating the role of *T.populnea* on the status of lipid peroxidation and antioxidants in experimental animals.

Materials and Methods

Male golden Syrian hamsters (*Mesocricatus auratus*), 8-10 weeks old, weighing 80-120 g were used for the study. The animals were obtained from the National Institute of Nutrition, Hydrabad, India. The hamsters were housed in polypropylene cages and were maintained in controlled atmosphere (temperature of 22 ± 2°C and 55 ± 5 % humidity) with a 12 hr light:dark (LD) cycles in an experimental room. The local institutional animal ethics committee (Register Number 160/1999/CPCSEA), Annamalai University, Annamalai nagar, India, approved the experimental design (Proposal No. 291, date 29.08.2005).

Chemicals

The carcinogen, 7,12-dimethylben[a]anthracene (DMBA) were obtained from Sigma Aldrich Chemical Pvt Limited, Bangalore, India. All other chemicals used were of analytical grade.

Preparation of plant extracts

Thespesia populnea barks were collected in and around Thanjavur, Tamil Nadu, India and identified by the Botanist, Department of Botany, Annamalai University. A voucher specimen was deposited in the department of botany, Annamalai University, India. The ethanolic *T.populnea* bark extract (TpBet) was prepared according to the method of Hossain *et al* (21). 500 gm of fresh bark of *T.populnea* were dried, powdered and then soaked in 1500 ml of 95% ethanol overnight. After filtration, the residue obtained was again resuspended in equal volume of 95% ethanol for 48 hrs, and filtered again. The above two filtrates were mixed and the solvent was evaporated in a rota vapour at 40-50°C under reduced pressure. A 16% semi solid light yellow material of *T.populnea* bark obtained was stored at 0-4°C until used. A known volume of the 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 design

A total of 40 hamsters were randomized into four groups of ten animals each. The experimental protocol for the present study is given in figure 1. Group I animals served as control and were painted with liquid paraffin thrice a week for 14 weeks on their left buccal pouches. Group II and III animals were painted with 0.5% DMBA in liquid paraffin thrice a week for 14 weeks on their left buccal pouches. Group II animals received no other treatment. Group III animals were orally given TpBet at a dose of 300 mg/kg body weight, starting one week before 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 TpBet alone throughout the experimental period. The experiment was terminated at the end of 14 weeks and all animals after giving anesthesia were sacrificed by cervical dislocation between 7-9 AM, after overnight fasting. Biochemical studies were conducted on blood and buccal mucosa of control and experimental animals in each group. For histopathological studies, tumor tissues and 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 were stained with hematoxylin and eosin.
The plasma was separated by centrifugation at 3000 rpm for 15 minutes. After separation of the plasma, the buffy coat was removed and the packed cells were washed thrice with physiological saline. A known volume of erythrocytes was lysed with hypotonic buffer at pH 7.4. The haemolysate was separated by centrifugation at 10,000 rpm for 15 minutes at 20°C. The erythrocyte membrane was prepared by the method of Dodge *et al* (22) modified by Quist (23). Thiobarbituric acid reactive substances (TBARS) were assayed in plasma, erythrocyte and buccal mucosa according to the methods of Yagi (24), Donnan (25) and Okhawa *et al* (26), respectively. Reduced glutathione (GSH) was determined by the method of Beutler and Kelly (27). Vitamin C and E were measured according to the methods of Omaye *et al* (28) and Desai (29), respectively. The activities of enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were estimated by the methods of Kakkar *et al* (30), Sinha (31) and Rotruck *et al* (32), respectively.

The data are expressed as mean ± SD. Statistical comparisons were performed by one way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at p-values less than 0.05.

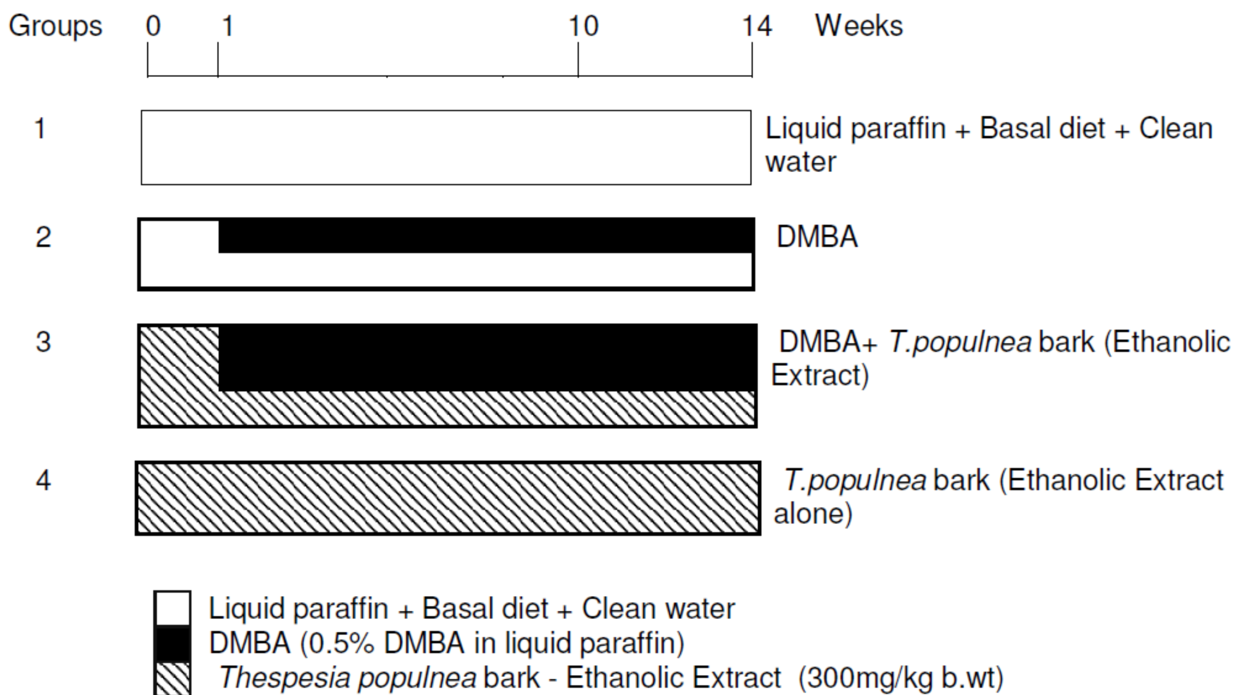

Figure 1. Experimental protocol

Results

Table 1 shows the tumor incidence, tumor volume, and tumor burden of control and experimental animals. We have observed 100% tumor formation with a tumor volume of 374.14 ± 31.30 mm^3 and tumor burden of 1122.42 ± 75.03 mm^3 in group II DMBA-painted animals. In TpBet treated group III animals (300 mg/kg b.wt) the tumor incidence (90%), tumor volume (75.92 ± 5.15 mm^3) and tumor burden (151.84 ± 9.19 mm^3) were all significantly decreased. No tumor was observed in group I control as well as group IV TpBet alone treated animals.
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 of the 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 + TpBet). The severity of pathological changes was assessed by Dr. CR. Ramachandran, Dean, Faculty of Dentistry, Rajah Muthaiah Medical College and Hospital, Annamalai University, India, when examining the histopathological slides under the microscope.

Table 3 and 4 shows the status of TBARS and antioxidants in plasma and erythrocytes of the control and experimental groups. The concentration of TBARS was increased, whereas the levels of non-enzymatic antioxidants (GSH, Vitamin C and E) and activities of enzymatic antioxidants (SOD, CAT and GPx) were significantly decreased in group II (DMBA alone), as compared to control animals. Oral administration of TpBet (group III) significantly decreased the levels of TBARS and improved the antioxidants status in DMBA-painted hamsters. TpBet alone treated hamsters showed no significant difference in TBARS and antioxidants status, as compared to control animals.

Table 5 indicates the concentration of TBARS and antioxidants status in the buccal mucosa of control and experimental animals in each group. Decrease in TBARS concentration and alteration in the antioxidants status (vitamin E, GSH and GPx were increased; SOD and CAT were decreased) were noticed in cancer animals (group II) as compared to control (group I) animals. However, oral administration of TpBet (300 mg/kg b.wt, group III) reverted the concentration of TBARS and antioxidants to near normal range. Hamsters treated with TpBet alone in group IV showed no significant difference in TBARS and antioxidants status as compared to control animals.

Table 1: Effects of TpBet on tumor incidence, volume and burden in DMBA golden Syrian hamsters.

Tumor volume was measured using the formula $v = \frac{4}{3}\pi\left(\frac{D_1}{2}\right)\left(\frac{D_2}{2}\right)\left(\frac{D_3}{2}\right)$ where D_1, D_2 and D_3 are the three diameters (mm) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors / animals.

( ) indicates total number of animals bearing tumors.

Values are expressed as mean ± SD for 10 animals in each group.

Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Parameters	Group I (Control)	Group II (DMBA)	Group III (DMBA + TpBet)	Group IV (TpBet alone)
Tumor incidence (Squamous cell carcinoma)	0	100%	10%	0
Total number of tumors / animals	0	30/(10)	2/(1)	0
Tumor volume (mm^3)#	0^a	374.14 ± 31.30^b	75.92 ± 5.15^c	0^a
Tumor burden (mm^3)#	0^a	1122.42 ± 75.03^b	151.84 ± 9.19^c	0^a

Discussion

Oral cancer, a disfiguring disease, has multifactorial etiologies and occurs predominantly during the sixth to eighth decades of life. Worldwide, the prevalence of oral cancer is increased with increasing age (33). At the cellular level, the membrane is the primary target of carcinogens. DMBA, a potent organ specific carcinogen has been widely used to induce several malignancies including oral cancer in experimental models. Cancer induced by DMBA closely resembles human cancer, both histologically and morphologically (7). Neoplastic transformation induces profound alterations in the structure and function of the cell membrane (34). The positive association between free radical over production and neoplastic transformation has been reported (35). By-products of lipid peroxidation forming in various biochemical reactions are scavenged

by endogenous antioxidants defense mechanisms (36). Vitamin E, vitamin C and GSH can protect cells and tissues by eliminating or quenching excessively generated Reactive Oxygen Species (ROS) in the body (37).

Table 2: Histopathological changes in oral cheek mucosa of TpBet treated and DMBA painted golden Syrian hamsters (n=10)

Numbers within parentheses indicate the total number of animals bearing tumors.

Parameters	Group I (Control)	Group II (DMBA)	Group III (DMBA + TpBet)	Group IV (TpBet alone)
Keratosis	Absent	Severe	Moderate (++)	Absent
Hyperplasia	Absent	Severe	Mild (+)	Absent
Dysplasia	Absent	Severe	Mild (+)	Absent
Squamous cell carcinoma	Absent	Moderately differentiated (10)	Well differentiated (1)	Absent

Table 3: The status of TBARS and antioxidants of plasma in control and experimental animals.

Values are expressed as mean ± SD for 10 animals in each group.
* The amount of enzyme required to inhibit 50 % nitroblue tetrazolium (NBT) reduction.
** m moles of H_2O_2 utilized / sec; *** m moles of glutathione utilized / min.
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Parameters	Group I (Control)	Group II (DMBA)	Group III (DMBA + TpBet)	Group IV (TpBet alone)
TBARS (n moles / mL)	3.16 ± 0.24[a]	4.93 ± 0.49[b]	3.45 ± 0.24[a]	3.02 ± 0.32[a]
GSH (mg / dL)	24.33 ± 1.72[ab]	16.49 ± 1.76[c]	21.83 ± 1.81[b]	24.31 ± 1.94[a]
Vitamin C (mg / dL)	1.39 ± 0.11[a]	0.74 ± 0.09[b]	1.24 ± 0.21[c]	1.42 ± 0.12[a]
Vitamin E (mg / dL)	1.32 ± 0.10[a]	0.82 ± 0.06[b]	1.22 ± 0.08[c]	1.41 ± 0.10[a]
SOD (U* / mL)	2.59 ± 0.17[a]	1.47 ± 0.19[b]	2.32 ± 0.19[c]	2.63 ± 0.17[a]
CAT (U / mL)**	0.54 ± 0.04[a]	0.32 ± 0.02[b]	0.48 ± 0.03[b]	0.55 ± 0.04[a]
GPx (U* / L)**	123.89 ± 8.48[ab]	85.55 ± 9.29[c]	114.60 ± 9.45[b]	125.45 ± 9.06[a]

Table 4: TBARS and antioxidants status in erythrocytes in DMBA painted and TpBet treated animals.

Values are expressed as mean ± SD for 10 animals in each group.
* The amount of enzyme required to inhibit 50 % nitroblue tetrazolium (NBT) reduction.
** µ moles of H_2O_2 utilized / sec; *** µ moles of glutathione utilized / min.
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Parameters	Group I (Control)	Group II (DMBA)	Group III (DMBA + TpBet)	Group IV (TpBet alone)
Erythrocytes TBARS (p moles / mg Hb)	2.16 ± 0.16[a]	3.03 ± 0.27[b]	2.45 ± 0.19[a]	2.15 ± 0.18[a]
RBC membrane:TBARS (n moles / mg protein)	0.37 ± 0.02[a]	0.98 ± 0.08[b]	0.46 ± 0.03[c]	0.31 ± 0.03[a]
Vitamin E (mg / mg protein)	1.98 ± 0.15[a]	1.33 ± 0.15[b]	1.88 ± 0.11[a]	2.15 ± 0.16[a]
Erythrocytes GSH (mg / dL)	54.42 ± 3.83[a]	36.31 ± 2.76[b]	47.68 ± 5.35[c]	54.32 ± 4.21[a]
Erythrocytes lysate: SOD (U* / mg Hb)	1.98 ± 0.15[a]	1.28 ± 0.17[a]	1.87 ± 0.21[c]	2.11 ± 0.15[a]
CAT (U / mg Hb)**	1.38 ± 0.11[a]	0.76 ± 0.08[b]	1.34 ± 0.18[a]	1.41 ± 0.09[a]
GPx (U* / g Hb)**	16.43 ± 0.79[ab]	9.55 ± 0.66[c]	15.48 ± 1.47[b]	17.34 ± 1.05[a]

Table 5: Buccal mucosa TBARS and antioxidants status in TpBet treated and DMBA painted golden Syrian hamsters.

Values are expressed as mean ± SD for 10 animals in each group.
* The amount of enzyme required to inhibit 50 % nitroblue tetrazolium (NBT) reduction.
** µ moles of H_2O_2 utilized / sec; *** µ moles of glutathione utilized / min.
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Parameters	Group I (Control)	Group II (DMBA)	Group III (DMBA + TpBet)	Group IV (TpBet alone)
TBARS (n moles / 100 mg protein)	68.91 ± 5.25[a]	46.28 ± 4.89[c]	61.58 ± 4.35[b]	67.34 ± 4.88[ab]
GSH (mg / 100 mg tissues)	7.30 ± 0.49[a]	11.50 ± 1.37[b]	7.9 ± 0.58[a]	7.43 ± 0.67[a]
Vitamin E (mg / 100 mg tissues)	1.69 ± 0.11[a]	2.51 ± 0.29[b]	1.69 ± 0.74[a]	1.94 ± 0.64[a]
SOD (U*/mg protein)	5.73 ± 0.44[a]	3.96 ± 0.30[b]	5.54 ± 0.43[a]	5.84 ± 0.45[a]
CAT (U/mg protein)**	40.11 ± 3.05[a]	23.88 ± 2.56[b]	38.61 ± 3.43[a]	40.5 ± 3.07[a]
GPx (U*/g protein)**	5.91 ± 0.35[a]	8.80 ± 0.79[b]	5.63 ± 0.51[a]	5.74 ± 0.34[a]

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Cancer chemoprevention deals with the prevention, inhibition and delay or reversal of carcinogenic process by using natural and synthetic agents (38). Medicinal plants may exert the carcinogenic potential by modulating carcinogen detoxification, inhibiting lipid peroxidation, or by improving *in vivo* antioxidants defense mechanism (8). Although some medicinal plants have been reported to have potent anticancer properties, many remain to be scientifically established. Also the chemopreventive potential of TpBet in DMBA induced hamster buccal pouch carcinogenesis has not been carried out worldwide. Thus the present study was designed to evaluate the chemopreventive potential of TpBet in DMBA induced hamsters was monitored by observing the status of tumor burden, percentage of tumor bearing animals and by determining the status of lipid peroxidation and antioxidants in blood and buccal mucosal tissues. We have observed hyperplasia, dysplasia and severe keratosis at the 8th to 10th week of carcinogen treatment and well differentiated squamous cell carcinoma at 14th week in DMBA-painted animals. Although mild precancerous lesions were observed in all animals, tumor formation was seen only in one DMBA-painted animal treated with TpBet (group III).

Free radical induced lipid peroxidation has been implicated in the pathogenesis of over 100 different diseases including oral cancer. In the present study, TBARS was increased in circulation whereas decreased in oral tumor tissues of tumor bearing animals as compared to control animals. However the antioxidants were decreased in the circulation whereas disrupted in tumor tissues (Vitamin E, GSH and GPx increased whereas SOD and CAT decreased) in cancer bearing animals as compared to control animals. Oral administration of *T.populnea* bark extracts significantly normalized the status of lipid peroxidation and antioxidants.

Red blood cells are more prone to oxidative stress due to continue challenges with high oxygen tension. The red blood cell membrane has high content of polyunsaturated fatty acids (PUFA), which are more susceptible to peroxidative stress (37). Susceptibility of erythrocytes to oxidative stress has been implicated in several diseases (39,40). Profound studies suggested that insufficient antioxidant potential was responsible for increased lipid peroxidation in erythrocytes (41). Serum or plasma lipid peroxides are used increasingly to assess the extent of tissue damage. It has been suggested that lipid peroxides formed at primary sites could pass through the circulation to other sites and cause damage by propagating lipid peroxidation. Elevated plasma TBARS level observed in oral cancer rats (group II) can therefore be related to excessive generation of lipid peroxidation byproducts and diffusion from the damaged erythrocytes and erythrocytes membrane with subsequent leakage into plasma (42).

Insufficient antioxidant potential is indicative of oxidative cell damage that persists in the cell. Vitamin C has beneficial effects on the conversion of neoplastic cells into normal ones and is implicated in the free radical scavenging mechanisms (43,44). Vitamin E has been suggested as a universal stabilizer of biological membranes in normal oxygen metabolism and peroxidation as well as in disorders of normal metabolism (45). Vitamin E also has a crucial role in preventing several malignancies including oral carcinoma. Reduced glutathione (GSH) serves many biological functions including the prevention of oxidative damage, removal of hydroperoxides and stabilization of biological membranes (46). A decline in plasma and erythrocyte Vitamin E, Vitamin C and glutathione has been reported in both human and experimental oral carcinogenesis. It has been reported that tumor tissues sequester antioxidant nutrients from circulation. The decreases in non-enzymatic antioxidants are therefore due to elevated lipid peroxidation in circulation or utilization by cancer cells (47).

Oral tumor cells showed decreased susceptibility to lipid peroxidation as compared to normal tissues. Reactive oxygen species are involved in the cell growth differentiation, progression and death. The positive association between lipid peroxidation process and the rate of cell proliferation has been well documented (48). Low availability of PUFA has been suggested to be a responsible factor for the decreased levels of TBARS in tumor tissues.

Altered activity of an enzymatic antioxidant and non-enzymatic antioxidants were reported during carcinogenesis or after tumor formation (41). Glutathione and GPx have regulatory effects on cell proliferation. An increase in glutathione level and GPx activity has been shown in several types of tumor tissues including oral carcinoma (48). Cancer cells take up essential antioxidants and nutrients from circulation to supply the demands of the growing tumor (47). Vitamin E protects cells from carcinogenic chemicals by inhibiting lipid peroxidation and damaging free radical mediated consequences (36,49). Glutathione and GPx are over expressed in various malignant tumors. It has been postulated that diminished lipid peroxidation combined with elevated vitamin E, glutathione and GPx status in oral tumors facilitates cell proliferation offering selective growth advantage in tumor cells over their surrounding normal cells (40,50). Decreased SOD and CAT activities

were reported in several cancers including oral cancer. Our results are in line with these observations. Thus decrease in TBARS levels and functional compromise of antioxidant defense mechanisms observed in the tumor tissues of oral cancer bearing animals reflects a decreased susceptibility of oral tumor tissues to lipid peroxidation.

Oral administration of TpBet significantly improved the status of lipid peroxidation and antioxidants of DMBA-painted animals, which suggests their potent antilipidperoxidative and antioxidant functions in DMBA-induced oral carcinogenesis. Profound studies have proposed that the chemopreventive potential of medicinal plants are either due to antilipidperoxidative action or by improving the antioxidant defense system. The chemopreventive effects of TpBet are probably due to their antilipidperoxidative and antioxidants properties. Further studies are needed to isolate and characterize the principle bioactive chemopreventive component from the bark of *T.populnea*.

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