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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (14): 2600-2605, 2006
ISSN 1028-8880

Chemopreventive and Antilipidperoxidative Efficacy of *Annona squamosa* Bark Extracts in Experimental Oral Carcinogenesis

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Abstract: The present study has investigated the chemopreventive and antilipidperoxidative potential of *Annona squamosa* bark extracts in DMBA induced hamster buccal pouch carcinogenesis. Oral squamous cell carcinoma was induced in hamster buccal pouches by painting with 0.5% 7,12-dimethylbenz (a) anthracene (DMBA) three times per week for 14 weeks. We observed 100% tumor formation in DMBA painted hamsters. Oral administration of aqueous and ethanolic bark extracts of *Annona squamosa* at a dose of 500 mg kg^{-1} body weight and 300 mg kg^{-1} body weight, respectively prevented the tumor formation as well as decreased the levels of lipid peroxidation byproducts and enhanced the antioxidants defense mechanism in DMBA painted hamsters. The effect of ethanolic bark extract is however more potent than aqueous extract of *Annona squamosa* barks. Our results suggest that *Annona squamosa* bark extracts exert their anticarcinogenic effect by modulating the status of lipid peroxidation and antioxidants in DMBA painted hamsters.

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

INTRODUCTION

Cancer of the oral cavity, the disfiguring disease of human populations, accounts for major morbidity and mortality worldwide. While oral squamous cell carcinoma accounts for 3-5% of all cancers in Western industrialized countries, it accounts for 40-50% of all malignancies in developing countries including India. India has recorded the highest incidence for oral cancer where the habits of excessive tobacco chewing with or without betel quid, smoking and alcohol consumption are attributed to the highest incidence of oral cancers (Notani, 2000; Gupta and Nandakumar, 1999). DMBA is known to induce multistep carcinogenesis preceded by a sequence of hyperplasia, dysplasia and carcinoma, which is quite similar to that of tumors that develop in oral cancer patients (Schwartz *et al*., 2000). DMBA induced hamster buccal pouch carcinogenesis is therefore used as an ideal model for studying chemoprevention of oral cancer.

Free radicals are chemical species that possess unpaired electrons, which are highly reactive. Free radicals induced oxidative stress results in the etiopathogenesis of several cancers including oral cancer. Excessive generation of reactive oxygen species has been demonstrated in betel quid chewers (Schwartz *et al*., 1993). An imbalance of pro oxidant and antioxidant has been linked with the mutagenicity and genotoxicity of biological organs that in turn results in cancer (Stitch and Anders, 1989). However, human body contains an array of defense mechanism including non-enzymatic [Vitamin E, C and reduced glutathione (GSH)] and enzymatic antioxidants [Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] to protect the deleterious effects of free radical induced oxidative damage. Previous studies from our laboratory have shown elevated lipid peroxidation and disturbed antioxidants defense mechanism in both experimental and human oral squamous cell carcinoma (Manoharan *et al*., 2005).

Annona squamosa, a small evergreen tree, is cultivated throughout India for its fruits. Different parts of *Annona squamosa* are used in folkloric medicine for the treatment of several disorders including cancer (Joshi, 2000). It is considered beneficial for cardiac diseases, diabetes, hyperthyroidism and cancer (Shirwaikar *et al*., 2004; Sunanda and Anand, 2003; Chopra, 1958). However, no scientific reports were available on the literature for its chemopreventive and antilipidperoxidative effects in DMBA induced hamster buccal pouch carcinogenesis. Thus, the present study is designed to fill up the lacunae in the literature for its chemopreventive and antilipidperoxidative efficacy in experimental oral carcinogenesis.

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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 in polypropylene cages 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: *Annona squamosa* barks were collected in and around Chidambaram, Tamil Nadu, India and authenticated by the Botanist, Dr. S. Sivakumar, Department of Botany, Annamalai University. A voucher specimen (AU04218) was also deposited.

Preparation of the plant extracts: Five hundred grams of dried and finely powdered *Annona squamosa* barks were soaked in 1500 mL of 95% ethanol over night. The residue obtained after filtration was again resuspended in equal volume of 95% ethanol for 48 h and filtered again. The above two filtrates were mixed and the solvents were evaporated in a rotovapour at 40-50°C under reduced pressure. A dark semisolid material (9%) obtained was stored at 4°C until used.

Hundred grams of dried and finely powdered *Annona squamosa* barks were suspended in 250 mL of water for 2 h and then heated at 60-65°C for 30 min. The extract was preserved and the process was repeated for three times with the residual powder, each time collecting the extract. The collected extract was pooled and passed through the fine cotton cloth. The filtrate upon evaporation at 40°C yielded 16% semisolid extract. This was stored at 0-4°C until used.

A known volume of the residual extracts 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, has approved the experimental design. A total number of 60 golden Syrian hamsters were randomized into six groups of 10 animals in each. Group I animals were served as untreated control. Groups II -IV animals were painted with 0.5% DMBA in liquid paraffin three times per week for 14 weeks on the left buccal pouches. Group II animals received no other treatment. Groups III and IV were orally administered AsBAet (500 mg kg^{-1} bw) and AsBEet (300 mg kg^{-1}, bw) respectively starting 1 week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the scarification of the animals. Groups V and VI were received AsBAet (500 mg kg^{-1} bw) and AsBEet (300 mg kg^{-1} bw) alone respectively throughout the experimental period. The experiment was terminated at the end of 15th week and all animals were sacrificed by cervical dislocation. Biochemical studies were conducted on blood and buccal mucosa 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: After plasma separation, 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 hemolysate was separated by centrifugation at 10,000 rpm for 15 min at 20°C. The erythrocyte membrane was prepared by the method of Dodge *et al.* (1963) modified by Quist (1980). Thiobarbituric acid reactive substances were assayed in plasma, erythrocytes and buccal mucosa according to the methods of Yagi (1987), Donnan (1950) and Ohkawa *et al.* (1979) respectively. 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 enzymatic antioxidants, SOD, CAT and Gpx were estimated by 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 shows the effect of *Annona squamosa* bark extracts on tumor incidence, tumor volume, tumor burden and histopathological features in DMBA induced hamster buccal pouch carcinogenesis. We have noticed 100% tumor formation with mean tumor volume (362.28 mm^3) and

tumor burden (1267.98 mm^3) in DMBA alone painted hamsters (group II). Oral administration of AsBAet and AsBEet at a dose of 500 mg kg^{-1} body weight and 300 mg kg^{-1} body weight respectively significantly prevented the tumor incidence, tumor volume and tumor burden in DMBA painted hamsters (groups III and IV). No tumors were observed in control animals (group I) and AsBAet and AsBEet alone administered animals (groups V and VI).

We have observed severe keratosis, hyperplasia, dysplasia and squamous cell carcinoma in the buccal mucosal tissues of hamsters painted with DMBA alone (group II). A mild to moderate preneoplastic lesions (hyperplasia, keratosis and dysplasia) were noticed in groups III and IV animals.

Table 2 and 3 show the status of TBARS and antioxidants in plasma and erythrocytes respectively of control and experimental animals in each group. The concentration of TBARS was increased whereas the levels of nonenzymatic antioxidants (GSH, Vitamin-C and Vitamin-E) and activities of enzymatic antioxidants (SOD, CAT and GPx) were significantly decreased in DMBA alone painted hamsters (Group II) as compared to control animals (Group I). Oral administration of AsBAet and AsBEet significantly decreased the levels of TBARS and improved the antioxidants status in DMBA painted hamsters (Groups III and IV). Hamsters treated with AsBAet and AsBEet alone (Groups V and VI) showed no significant difference in TBARS and antioxidants status as compared to control animals (Group I).

Table 1: Incidence of oral neoplasm and histopathological features in control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA+AsBAet	Group IV DMBA+AsBEet	Group V AsBAet alone	Group VI AsBEet alone
Tumor incidence (Squamous cell carcinoma)	0	100 (%)	0	0	0	0
Total number of tumors	0	35 (10)	4 (2)	0	0	0
Mean tumor volume (mm^3)	0^a	362.28 ± 28.5^b	76.12 ± 6.2^c	0^a	0^a	0^a
Mean tumor burden (mm^3)	0^a	1267.98 ± 92.34^b	158.24 ± 12.18^c	0^a	0^a	0^a
Keratosis	No change	Severe	Moderate	Mild	No Change	No change
Hyperplasia	No change	Severe	Moderate	Mild	No change	No change
Dysplasia	No change	Severe	Moderate	Mild	No change	No change
Squamous cell carcinoma	No change	Moderately differentiated	Well differentiated (2)	No change	No change	No change

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 D1, D_2, D_3 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. Values are expressed as mean±SD for 10 hamsters in each group values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT) AsBAet-Aqueous bark extract of *Annona squamosa.* AsBEet-Ethanolic bark extract of *Annona squamosa*

Table 2: Status of plasma TBARS and antioxidants in control and experimental animals in each group (n = 10)

Parameters	Group I Control	Group II DMBA	Group III DMBA+AsBAet	Group IV DMBA+AsBEet	Group V AsBA*et al*one	Group VI AsBE*et al*one
TBARS (nmoles mL^{-1})	2.74 ± 0.21^a	4.78 ± 0.40^b	3.72 ± 0.18^c	3.01 ± 0.21^a	2.70 ± 0.15^a	2.65 ± 0.17^a
GSH (mg dL^{-1})	28.32 ± 2.6^a	18.08 ± 1.52^b	21.56 ± 1.9^c	26.42 ± 2.1^a	28.35 ± 2.1^a	29.06 ± 1.9^a
Vitamin C (mg dL^{-1})	1.32 ± 0.07^a	0.72 ± 0.05^b	1.02 ± 0.07^c	1.22 ± 0.09^a	1.38 ± 0.09^a	1.42 ± 0.10^a
Vitamin E (mg dL^{-1})	1.15 ± 0.10^a	0.74 ± 0.05^b	0.87 ± 0.05^c	1.04 ± 0.06^a	1.20 ± 0.10^a	1.28 ± 0.12^a
SOD (U* mL^{-1})	2.57 ± 0.20^a	1.65 ± 0.12^b	2.02 ± 0.15^c	2.42 ± 0.18^a	2.60 ± 0.18^a	2.18 ± 0.21^a
CAT (U** mL^{-1})	0.48 ± 0.03^a	0.25 ± 0.02^b	0.37 ± 0.03^c	0.43 ± 0.04^a	0.50 ± 0.03^a	0.52 ± 0.04^a
GPx (U*** L^{-1})	121.7 ± 10.8^a	85.8 ± 7.2^b	98.2 ± 5.82^c	113.81 ± 8.31^a	122.31 ± 10.14^a	123.81 ± 9.32^a

Values are expressed as mean±SD for 10 hamsters in each group, Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT) *-The amount of enzyme required inhibiting 50% NBT reduction, **-Micromoles of H_2O_2 utilized sec, ***-Micromoles of glutathione utilized min, AsBAet -Aqueous bark extract of Annona squamosa, AsBEet-Ethanolic bark extract of Annona squamosa

Table 3: TBARS and antioxidant status in erythrocytes of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA+AsBAet	Group IV DMBA+AsBEet	Group V AsBAet alone	Group VI AsBEet alone
Erythrocyte TBARS (P moles mg Hb)	1.73 ± 0.12^a	2.75 ± 0.20^b	2.28 ± 0.21^c	1.82 ± 0.19^a	1.80 ± 0.14^a	1.75 ± 0.15^a
Erythrocyte membrane						
TBARS(nmoles mg protein)	0.32 ± 0.03^a	1.25 ± 0.11^b	1.02 ± 0.10^c	0.36 ± 0.04^a	0.30 ± 0.03^a	0.28 ± 0.03^a
Vitamin E (µg mg protein)	2.27 ± 0.11^a	1.51 ± 0.08^b	1.98 ± 0.17^c	2.16 ± 0.22^a	2.20 ± 0.21^a	2.25 ± 0.18^a
Erythrocytes GSH (mg dL^{-1})	38.15 ± 2.7^a	20.32 ± 2.3^b	28.14 ± 2.1^c	35.45 ± 1.9^a	39.72 ± 2.5^a	40.08 ± 3.2^a
Erythrocyte lysate SOD (U* mg Hb)	2.09 ± 0.17^a	1.38 ± 0.10^b	1.72 ± 0.13^c	1.95 ± 0.15^a	2.11 ± 0.20^a	2.18 ± 0.18^a
CAT (U** mgHb)	1.22 ± 0.08^a	0.74 ± 0.06^b	0.87 ± 0.07^c	1.13 ± 0.09^a	1.19 ± 0.08^a	1.27 ± 0.10^a
GPx (U*** gHb)	13.72 ± 1.15^a	7.81 ± 0.68^b	10.35 ± 1.15^c	12.53 ± 1.02^a	14.05 ± 1.38^a	14.82 ± 1.21^a

Values are expressed as mean±SD for 10 hamsters in each group, *-The amount of enzyme required to inhibit 50% NBT reduction, **-Micromoles of H_2O_2 utilized sec, ***-Micromoles of glutathione utilized min, Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT), AsBAet -Aqueous bark extract of Annona squamosa, AsBEet-Ethanolic bark extract of Annona squamosa

Table 4: TBARS and antioxidant status in buccal mucosa of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA+AsBAet	Group IV DMBA+AsBEet	Group V AsBA et alone	Group VI AsBEet alone
TBARS (nmole 100 mg protein)	72.8±5.32^a	48.17±3.52^b	60.13±4.34^c	68.2±5.18^a	73.66±6.8^a	75.14±7.1^a
GSH (mg 100 g tissue)	8.14±0.65^a	13.16±0.86^b	10.42±1.05^c	8.56±0.64^a	8.10±0.72^a	8.02±0.62^a
Vitamin E (mg 100 g tissue)	1.76±0.11^a	2.92±0.19^b	2.53±0.18^c	1.96±0.20^a	1.70±0.14^a	1.64±0.12^a
SOD U* mg protein)	4.63±0.32^a	3.18±0.24^b	3.92±0.39^c	4.35±0.32^a	4.68±0.30^a	4.71±0 .27^a
CAT (U** mg protein)	33.4±2.5^a	20.12±1.8^b	28.17±2.1^c	31.51±2.8^a	34.52±2.4^a	35.12±3.1^a
GPx (U*** g protein)	6.18±0.42^a	10.37±0.85^b	8.16±0.74^c	6.62±0.62^a	6.12±0.47^a	6.08±0.54^a

Values are expressed as mean±SD for 10 hamsters in each group, *-The amount of enzyme required to inhibit 50% NBT reduction, **-Micromoles of H_2O_2 utilized sec^{-1}, ***-Micromoles of glutathione utilized min^{-1}. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT), AsBAet -Aqueous bark extract of Annona squamosa, AsBEet-Ethanolic bark extract of Annona squamosa

Table 4 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 disturbances in antioxidant status [Vitamin E, GSH and GPx were increased; SOD and CAT were decreased] were observed in cancer animals (Group II) as compared to control animals (Group I). However, oral administration of AsBAet and AsBEet (Groups III and IV) prevented the alterations of buccal mucosa TBARS and antioxidants significantly in DMBA painted animals. Hamsters treated with AsBAet and AsBEet alone (Groups V and VI) showed no significant difference in TBARS and antioxidants status as compared to control animals (Group I).

DISCUSSION

Cancer chemoprevention is a novel approach to reverse, suppress or prevent the incidence of cancer. Continued search for novel chemoprotective agents offers a promising new strategy for improving current cancer treatment. *Annona squamosa* bark extracts significantly prevented the formation of oral squamous cell carcinoma in the hamster buccal pouches, which indicates its potent chemopreventive role in DMBA induced oral carcinogenesis. Although the exact mechanism of chemopreventive potential of *Annona squamosa* is not clear, the possible mechanisms include induction of phase 2 detoxification enzymes and increased enzymatic degradation of DMBA by liver and or enhance the antioxidant defense mechanism to neutralize the toxic effects of Reactive Oxygen Species (ROS) generated by DMBA.

An association between lipid peroxidation and rate of cell division has been suggested (Loo, 2003). Low levels of lipid peroxidation byproducts were reported in highly proliferating malignant tumors including oral cancers (Cohen and Ellwein, 1990). Low PUFA content in oral tumor tissues is responsible for decreased levels of lipid peroxides in oral carcinoma (Nagini and Saroja, 2001). Antioxidants have been shown to inhibit both initiation and promotion in carcinogenesis and counteract cell immortalisation and transformation. Lowered activities of SOD and CAT enzymes were reported in patients with malignant tumors as well as carcinogen induced experimental carcinogenesis (Kolanjiappan *et al*., 2003; Balasenthil *et al*., 2000; Nagini *et al*., 1998). The greater accumulation of H_2O_2 in tumors due to insufficient activity of catalase was shown (Eaton, 1991). Increased levels of glutathione and enhanced activity of glutathione peroxidase in oral tumors due to their regulatory effects on cell proliferation has been reported (Wong *et al*., 1994). Our results corroborate these observations.

A close relationship between free radical induced lipid peroxidation and cancer has been proposed (Stitch and Anders, 1989). DMBA produces excessive ROS during its metabolic conversion to become an ultimate carcinogen (Dix and Marnett, 1983). Excessively generated ROS reacts with membrane lipids and cause serious damage to cell membranes by inducing membrane lipid peroxidation. The pathological consequences of membrane lipidperoxidation include increased membrane fragility, decreased red cell fluidity and altered cell function and structural integrity (Van Ginkel and Sevanian, 1994). Enormous production of free radicals in the system has been reported in several cancers (Guyton and Kensler, 1993). Elevated level of TBARS in plasma indicates the extent of tissue damage (Gutteridge, 1995).

Enzymatic and non-enzymatic antioxidants form the first and second line of defense mechanism respectively against the deleterious effects of oxidative stress induced cell damage (Martins *et al*., 1991). Vitamin E and C have the ability to scavenge a wide variety of oxygen free radicals and thereby interfering with the process of lipid peroxidation during carcinogenesis (Van Ginkel and Sevanian, 1994). Glutathione, a substrate for several enzymes, plays a crucial role in scavenging toxic oxygen free radicals and keeps up the cellular levels of vitamin C and E in an active form (Exner *et al*., 2000). Lowered levels of antioxidants cause overproduction of free radicals and lipid peroxides, which in turn induce damage to cell membranes and cellular biomolecules and thereby leading to neoplasia (Kong and Lillehe, 1998). The observed increase in plasma TBARS can therefore be correlated to

insufficient antioxidants potential or enhanced production of lipid peroxides in damaged tissues and erythrocyte membranes with subsequent leakage into plasma.

Oral administration of *Annona squamosa* bark extracts to DMBA painted hamsters significantly protected the status of antioxidant and lipid peroxidation byproducts, which indicates their potent antilipidperoxidative potential during neoplastic transformation. The antilipidperoxidative property of the plant extracts suggests the presence of one or more potent antioxidant principles in *Annona squamosa* barks. Thus, the present study demonstrates the chemopreventive and antilipidperioxidative potential of *Annona squamosa* bark extracts in DMBA induced hamster buccal pouch carcinogenesis. Further studies are needed to isolate and characterize the bioactive chemopreventive principles from the barks of *Annona squamosa*.

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