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Modifying Effects of *Annona squamosa* on Glycoconjugates Levels in 7,12-dimethylbenz(a)Anthracene Induced Hamster Buccal Pouch Carcinogenesis

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Present aim was to study the modifying effects of *Annona squamosa* leaf extracts in 7,12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis. Oral squamous cell carcinomas were induced in buccal pouches of Syrian golden hamsters by painting with 0.5% 7,12-dimethylbenz(a)anthracene in liquid paraffin three times per week for 14 weeks. The incidence of total number of tumors, tumor burden and tumor volume were recorded in 7,12-dimethylbenz(a)anthracene painted hamsters. The status of glycoconjugates in plasma, erythrocyte membranes and tumor tissues were analysed in tumor bearing hamsters and *Annona squamosa* leaf extracts treated 7,12-dimethylbenz(a)anthracene painted hamsters. Oral administration of aqueous and ethanolic extracts of *Annona squamosa* leaf extracts at a dose of 500 mg kg^{-1} body weight and 300 mg kg^{-1} bodyweight respectively, reduced the tumor formation as well as protected the levels of glycoconjugates in 7,12-dimethylbenz(a)anthracene painted hamsters during carcinogenesis. Present study suggests that *A. squamosa* leaf extracts have potent chemopreventive efficacy and can modify the abnormalities in cell surface glycoconjugates during neoplastic transformation.

Key words: Oral cancer, *Annona squamosa*, glycoconjugates, DMBA

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INTRODUCTION

Cancer of the oral cavity assumes a major health problem in terms of patient's morbidity and mortality and it represents approximately 40-50% of all cancers in India. Tobacco either smoked or chewed is associated with more than 70-80% of oral cavity cancers. The risk of oral cancer, however, increases if the person both consumes alcohol and uses tobacco (Moore *et al*., 2000; Gupta and Nandakumar, 1999). 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis is an excellent model for the evaluation of potent cancer chemopreventive agents due to its marked similarities with human tumors. The evaluated results reported in hamster buccal carcinogenesis may assist the clinicians in the treatment of oral cancer patients (Schwartz *et al*., 2000).

Glycoproteins are complex proteins in which carbohydrates are linked covalently to aspargine or serine or threonine residues of polypeptides. The predominant sugar moieties in oligosaccharides are glucose, galactose, fucose, mannose and derivatives of sialic acid and acetylated derivatives of hexosamine. Neoplastic transformation is usually associated with molecular changes such as glycosylation of glycoproteins and glycolipids (Tarner *et al*., 1985). Glycoproteins play a vital role in cell differentiation, intercellular recognition, tumorigenesis and as receptors for many hormones and viruses (Patel *et al*., 1990).

The measurement of serum glycoconjugates in oral pre-cancerous and cancerous lesions may be useful in the diagnosis of patients with oral pre-cancer or cancer. Altered expression of cell surface glycoconjugates is involved in the process of metastasis (Nicolson, 1984). Sialic acid, the terminal sugar residue of oligosaccharides of cell surface glycoconjugates in animal cells and tissues, is involved in the regulation of cell surface phenomenon and is therefore altered during malignant transformation (Narayana, 1994).

Human body requires fucose as one of the essential sugar for optimal function of cell-cell communication. Fucose plays a significant role in many diseases including cancer and its spread. Fucose and mannose are the most effective of the essential sugars when it comes to slowing the growth of cancer cells (Rao *et al*., 1998). Lipid bound sialic acid is regarded as a tumor marker of several cancers as well as to follow up the effects of anticancerous treatment (Schutter *et al*., 1992). Previous studies from our laboratory have demonstrated significant correlation between glycoconjugates levels and tumor stages of oral carcinoma (Manoharan *et al*., 2004).

Annona squamosa, belonging to family Annonaceae, is cultivated in several parts of India. A. squamosa has been used in folkloric medicine to treat several types of diseases including cancer. The aqueous leaf extract has been used to ameliorate hyperthyroidism (Sunadha and Anad, 2003). The boiled extract of *A. squamsoa* leaves possesses hypoglycemic and antihyperglycemic effects (Joshi, 2000). A 50% of ethanolic extract of leaves and stem showed anticancer activity (Chopra, 1958). The preliminary phytochemical screening of this plant revealed a number of alkaloids, terpene derivatives and a normal diazepine, squamolone (Vohora *et al*., 1975). To our best knowledge, there were no scientific studies on chemopreventive potential of *A. squamosa* and its modifying effects on cell surface glycoconjugates in experimental oral carcinogenesis. Thus, the present study is designed to focus the above-mentioned effects of *A. squamosa* leaf extracts 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 in 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: *A. squamosa* leaves were collected in and around chidambaram, Tamil Nadu, India. The Botanist Dr. S. Sivakumar, Department of Botany, Annamalai University verified the identity of the plant and a voucher specimen (AU04218) was also deposited.

Preparation of plant extract: Five hundred gram of dried finely powdered *A. squamosa* leaves were soaked with 1500 mL of 95% ethanol overnight. 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 rotavapour at 40-50°C under reduced pressure. A dark semisolid material (9%) obtained was stored at -4°C until used.

Hundred gram of dried finely powdered *A. squamosa* leaves was 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 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 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 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 thrice a week for 14 weeks on the left buccal pouches. Group II animals received no other treatment. Groups III and IV animals were orally administered with *A. squamosa* aqueous leaf extract AsLAet (500 mg kg^{-1} body weight) and *A. squamosa* ethanolic leaf extract AsLEet (300 mg kg^{-1} body weight), respectively starting 1 week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the sacrification of the animals. Groups V and VI were received AsLAet (500 mg kg^{-1} body weight) and AsLEet (300 mg kg^{-1} body weight) alone 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 erythrocyte membrane was prepared by the method of Dodge *et al*. (1968) modified by Quist (1980). 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 (972), 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 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 tumor incidence, tumor volume, tumor burden and histopathological changes in DMBA induced hamster buccal pouch carcinogenesis. We have observed 100% tumor formation with mean tumor volume (318.15 mm^3) and tumor burden (890.82 mm^3) in group II animals. Oral administration of AsLAet (500 mg kg^{-1} body weight) and AsLEet (300 mg kg^{-1} body weight) 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 AsLAet (Group V) and AsLEet (Group VI) alone administered animals. Severe keratosis, hyperplasia, dysplasia and squamous cell carcinoma were observed in Group II animals. A mild to moderate preneoplastic lesions (hyperplasia, keratosis and dysplasia) were noticed in Groups III and IV animals.

Table 1: Incidence of oral neoplasms and histopathological changes observed in control and experimental animals in each group

		Tumor				Histopathological changes			
Groups	Treatments	incidence (%)	No. of tumors	Mean tumor volume (mm^3)	Mean tumor burden (mm^3)	Keratosis	Dysplasia	Hyperplasia	Squamous cell carcinoma
I	Control	0	0	0^a	0^a	No change	No change	No change	No change
II	DMBA	100	28/(10)	318.15 ± 20.05^b	890.82 ± 42.3^b	Severe	Severe	Severe	Moderately and well differentiated
III	DMBA + AsLAet	20	4/(2)	70.32 ± 3.82^c	140.64 ± 7.88^c	Moderate	Moderate	Mild	Well differentiated (2)
IV	DMBA + AsLAet	10	2/(1)	64.81 ± 2.46^c	129.62 ± 9.18^c	Moderate	Mild	Mild	Well differentiated (1)
V	AsLAet alone	0	0	0^a	0^a	No change	No change	No change	No change
VI	AsLEet alone	0	0	0^a	0^a	No change	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 D_1, 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. Parenthesis indicates total number of animals bearing tumors. Values are expressed as mean±SD for 10 hamsters in each group, AsLAet- Aqueous leaf extract of *A. squamosa*. AsLEet-Ethanolic leaf extract of *A. squamosa*

Table 2: Protein bound hexose, hexosamine, total sialic acid, lipid bound sialic acid and fucose in plasma of control and experimental animals in each group

Groups	Treatments	Protein bound hexose (mg dL^{-1})	Protein bound hexosamine (mg dL^{-1})	Total sialic acid (mg dL^{-1})	Lipid bound sialic acid (mg dL^{-1})	Fucose (mg dL^{-1})
I	Control	90.18±7.140[a]	76.12±5.08[a]	48.16±4.08[a]	12.28±1.02[a]	7.48±0.62[a]
II	DMBA	130.20±12.82[b]	108.92±8.14[b]	77.32±6.18[b]	30.14±2.62[b]	16.33±1.05[b]
III	DMBA + AsLAet	110.34 ±7.56[c]	90.36±7.15[c]	59.44±6.02[c]	26.76±2.30[c]	12.57±0.97[c]
IV	DMBA + AsLEet	102.44±8.650[c]	87.78±8.12[c]	53.75±5.18[c]	21.35±2.10[c]	10.68±0.82[c]
V	AsLAet alone	89.75±6.380[a]	74.14±5.12[a]	47.52±4.72[a]	11.92±0.84[a]	6.94±0.58[a]
VI	AsLEet alone	88.12±5.320[a]	72.63±4.78[a]	46.33±3.56[a]	10.54±0.92[a]	6.12±0.45[a]

Values are expressed as mean±SD for 10 hamsters in each group, AsLAet-Aqueous leaf extract of *A. squamosa*, AsLEet-Ethanolic leaf extract of *A. squamosa*
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Table 3: Protein bound hexose, hexosamine and total sialic acid levels in erythrocyte membranes of control and experimental animals in each group (n = 10)

Groups	Treatments	Protein bound hexose (μg mg^{-1} protein)	Protein bound hexosamine (μg mg^{-1} protein)	Total sialic acid (μg mg^{-1} protein)
I	Control	130.65±10.18[a]	85.32±6.33[a]	34.46±3.21[a]
II	DMBA	95.02±7.370[b]	68.17±5.84[b]	22.08±2.52[b]
III	DMBA + AsLAet	116.73±8.810[c]	76.43±6.85[c]	29.73±2.14[c]
IV	DMBA + AsLEet	123.48±9.340[c]	80.56±7.37[c]	31.57±3.12[c]
V	AsLAet alone	132.52±11.55[a]	86.12±5.88[a]	35.13±2.98[a]
VI	AsLEet alone	133.05±12.32[a]	87.42±6.23[a]	36.65±3.52[a]

Values are expressed as mean±SD for 10 hamsters in each group , AsLAet-Aqueous leaf extract of *A. squamosa*, AsLEet-Ethanolic leaf extract of *A. squamosa*
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Table 4: Protein bound hexose, sialic acid and fucose levels in buccal mucosa of control and experimental animals in each group (n = 10)

Groups	Treatments	Protein bound hexose (mg g^{-1} protein)	Total sialic acid (mg g^{-1} protein)	Fucose (mg g^{-1} protein)
I	Control	105.36±8.120[a]	15.58±1.32[a]	12.78±1.15[a]
II	DMBA	148.81±19.28[b]	30.85±2.86[b]	28.25±2.08[b]
III	DMBA + AsLAet	123.54±10.54[c]	22.73±2.08[c]	22.56±1.58[c]
IV	DMBA + AsLEet	115.72±11.18[c]	18.44±1.52[c]	17.02±0.92[c]
V	AsLAet alone	104.45±8.550[a]	16.37±1.32[a]	11.41±1.02[a]
VI	AsLEet alone	102.74±7.350[a]	18.02±1.18[a]	10.57±0.83[a]

Values are expressed as mean±SD for 10 hamsters in each group, AsLAet-Aqueous leaf extract of *A. squamosa*, AsLEet-Ethonolic leaf extract of *A. squamosa*
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Table 2-4 show the levels of protein bound hexose, hexosamine, total sialic acid, lipid bound sialic acid and fucose in plasma, erythrocyte membrane and buccal mucosa tissues respectively of control and experimental animals in each group. The levels of glycoconjugates were significantly increased in plasma and buccal mucosa tumor tissues whereas decreased in erythrocyte membranes of tumor bearing hamsters as compared to control animals. Oral administration of AsLAet and AsLEet at a dose of 500 and 300 mg kg^{-1} body weight to DMBA painted animals respectively protected the elevated levels of glycoconjugates. Hamsters treated with AsLAet and AsLEet alone showed no significant differences in glycoconjugates levels as compared to control animals.

DISCUSSION

Cell surface glycosyl residues play an important role in regulating cell proliferation and epithelial growth. Malignant transformation of oral epithelium is associated with atypical glycosylation of cell surface glycoconjugates. A loss in epithelial cell surface carbohydrates during experimental oral carcinogenesis has been reported (Dabelsteen *et al*., 1996; Dabelsteen, 1996). Plucinsky *et al*. (1986) reported that administration of carcinogen changes cellular carbohydrates during cell differentiation and behavior of cells with subsequent increase in the expression of glycoproteins. Measurement of glycoconjugates has been used for diagnosis, staging and monitoring of cancer in patients with malignant neoplasm (Manoharan *et al*., 2004). Aberrant glycosylation of cell surface glycoprotein has been observed for tumor cells and its involvement in the metastatic processes (Nicolson, 1984). Cell surface glycoproteins and glycolipids are released into the serum in carcinogenesis as a result of increased turnover, secretion and or shedding from tumor cells. Malignant tumor in the body stimulates the synthesis of glycoproteins in the liver, which subsequently enter into the circulation (Macbeth and Bekesi, 1964). The depletion of erythrocyte membrane glycoprotein may be due to increased membrane degradation or as a result of increased shedding into circulation. Elevated plasma glycoproteins in tumor bearing animals can therefore be related to an increased synthesis in liver or tumor tissue itself with subsequent shedding into plasma.

Several studies have documented that malignant cells have more sialic acid in their cell membranes than in normal cells (Verazin *et al*., 1990; Marth *et al*., 1988).

Marked elevation of total sialic acid and lipid bound sialic acid in serum were found to reflect tumor burden and correlated well with stages of cancer (Manoharan *et al*., 2004). Total sialic acid and lipid bound sialic acid elevation in serum and tumor tissues are probably related to increased turnover of malignant cells (Rao *et al*., 1998). Increased excretion of glycosidically bound sialic acid in urine of cancer patients reflects elevation of sialyl transferase activity in tumor tissues (Raval *et al*., 2004). Increased sialyl transferase activity may be responsible for increased expression of cell surface glycoconjugates during neoplastic transformation (Yamamoto *et al*., 1995). Present results corroborate these observations.

In the present study, oral administration of *A. squamosa* leaf extracts significantly prevented the tumor formation, tumor volume and burden in DMBA painted hamsters, which indicates their potent chemopreventive efficacy in experimental oral carcinogenesis. *A. squamosa* leaf extracts not only prevented the cancer formation but also inhibited the abnormalities seen in cell surface glycoconjugates in the tumor tissues and circulation which indicates their membrane stabilizing effects during neoplastic transformation. The protective effect of *Annona squamosa* leaf extracts on cell surface glycoconjugates is probably due to their inhibitory role on glycoprotein synthesis or on the activity of the glycosyl transferases. Although both aqueous and ethanolic extracts of *Annona squamosa* leaves exert chemopreventive efficacy in experimental oral carcinogenesis, the ethanolic extract was found to be more effective than that of aqueous leaf extract. Our results thus demonstrate the chemopreventive efficacy; of *Annona squamosa* leaf extracts and their modifying effects on cell surface glycoconjugates in DMBA induced hamster buccal pouch carcinogenesis. Further studies are warranted to identify and isolate bioactive anticarcinogenic principles from the leaves of *A. squamosa.*

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