Modifying Effects of *Annona Squamosa* Linn on Glycoconjugates Levels in 7, 12-Dimethylbenz (A) Anthracene (DMBA) Induced Hamster Buccal Pouch Carcinogenesis

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**Abstract:** The present study was examined modifying effects of *Annona squamosa* on glycoconjugates levels in 7,12-dimethyl benz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Oral squamosa cell carcinomas were induced in buccal pouches of male golden syrian hamsters by painting with 0.5% DMBA in liquid paraffin three times per week for 14 weeks. Oral administration of aqueous and ethanolic extracts of *A. squamosa* bark at a dose of 500 mg kg-1 b.w and 300mg kg-1 b.w respectively. The total number of tumors, tumor burden, tumor volume as well as the incidence of tumor was significantly decreased on *A.squamosa* bark extracts treated hamsters and significantly normalized levels of glycoconjugates in tumor bearing animals. Our results suggest that *A. squamosa* bark extracts protect the cell surface glycoconjugates during DMBA induced hamster buccal pouch carcinogenesis.

**Key words:** Oral cancer, DMBA, *Annona squamosa*, Glycoconjugates.

**INTRODUCTION**

Oral squamous cell carcinoma (OSCC), is the fifth most common malignancy worldwide, a major cause of cancer morbidity and mortality in India, it represents approximately 40- 50% of all cancers in India[1]. The highest incidence rates have been observed in the Indian sub-continent[2]. It has been suggested that use of tobacco either smoked or chewed is associated with more than 70-80% of oral cancers[3]. The risk of oral cancer, however, increases if the persons both consume alcohol and uses of tobacco[4]. DMBA, a potent organ and site specific carcinogen is commonly used 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[5]. The buccal mucosa of the Syrian hamster has been used as an ideal model for oral cancer development and intervention by chemopreventive agents[6].

Glycoproteins are complex proteins in which both a protein and a carbohydrate joined together in covalent linkage. Many functional proteins are released by cells to the blood circulation as glycoprotein[7]. The predominant sugar moieties in oligosaccharides are glucose, galactose, fucose, mannose and derivatives of sialic acid and acetylated derivatives of hexosamines[8]. Glycoproteins are major constituents of cell membrane, plays an important role in cell differentiation, cell proliferation, cell-cell interaction, carcinogenesis and act as a receptor for many hormones and viruses[9,10]. Measurement of serum glycoconjugates in oral pre-cancerous and cancerous lesions may be useful in the diagnosis of cancer patients and experimental animals[11]. Sialic acid is widely distributed in animal cell and occurs as a terminal component at the non-reducing end of the carbohydrate side chains of glycoprotein and glycolipids[12]. It has been implicated that number of ways including metastatic spread, contact phenomenon, tumor antigenicity, transport processes and viral receptors[13].

Sialylated glycoconjugates also seem to be involved in tumor biology, since aberrant glycosylation patterns, for example, high polysialic acid bearing N-CAMs, an increased level of sialic acid in α2,6-linkage to galactose, and the expression of sialyl-Tn, are very common in human and animal neoplasia[14]. Fucose is an essential sugar for function of human body cell-cell communication in the body[15]. An altered fucose metabolism is associated with several malignant states, which affects the protein turnover. It has been reported that elevation of serum fucose levels in oral cancer patients and these changes appear to be related to the stage of tumors and their importance in prognosis and detection of early recurrence has been advocated[16]. Lipid bound sialic acid is regarded as a tumor marker of several cancerous as well as to follow up the effects of anticancerous treatment [17]. Previous studies from
our laboratory have demonstrated that significant correlation between glycoconjugates levels and tumors stages of hamster buccal pouch of carcinogenesis\[18\]. Several studies were suggested that the chemoprevention is a new kind of approach for the prevention of cancer\[19\]. Cancer chemoprevention is a recent good approach in cancer biology use natural, synthetic, or biological chemical agents to reverse, suppress, or prevent to the tumor formation\[20\]. Recently, considerable attention has been directed on identifying phytochemicals, particularly those included in our diet, which has the ability to interfere with carcinogenic or mutagenic processes. A wide variety of phenolic substances in our diet have been shown to possess anticarcinogenic and antimutagenic effects\[21\].

Annona squamosa (Annonaceae) commonly known as custard apple, is a native of West Indies and is now cultivated throughout India, mainly for its edible fruits\[22\]. It is a fruit tree, and the seeds are famous to contain many acetogenins, waxy substances, consisting long chain fatty acids, they showed antimalarial, immnosuppressive, antifeedent, antiparasitic, and pesticide, cell growth inhibitory and particularly remarkable cytotoxic activity\[23\]. Squamocin is another annonaceous acetogenin has been reported to exert antiproliferative effects on HL-60 cancer cells via activation of caspase-3. The preliminary pytochemical screening of this plant revealed a number of alkaloids, terpene derivatives and a normal diazepine, squamolone\[24\].

To our best of knowledge, there were no scientific studies on chemopreventive potential of A.squamosa bark extracts and its modifying effects on cell surface glycoconjugates in hamster buccal pouch carcinogenesis. Thus, the present study is examined to focus on modifying effects of A.squamosa bark 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 80-120g 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 poly propylene cages and provided standard pellet and water *ad libitum*. The animals were maintained under controlled conditions of temperature and humidity with a 12h light dark cycle.

The Annamalai university animal ethics committee has approved the experimental design. A total number of 60 golden syrian hamsters were randomized into 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 AsABet (500 mg kg b.w) and AsEBet (300 mg kg b.w) 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 AsABet (500 mg kg b.w) and AsEBet (300 mg kg b.w) 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 estimations were conducted on blood, erythrocytes and buccal mucosa of control and experimental animals in each group. For histopathological examination, buccal mucosa tissues were fixed in 10% formalin and routinely processed.

**Plant Material:** Annona squamosa bark was collected in and around Chidambaram, Tamil nadu, India and authenticated by the Botanist, Dr.S.Sivakumar, Department of Botany, Annamali University. A voucher specimen (AU04218) was also deposited. The ethanolic extracts of Annona squamosa bark were prepared according to the method of Hossain *et al*\[25\].

**Preparation of the Plant Extracts:** Five hundred grams of dried and finely powdered Annona squamosa barks were soaked in 1500 ml of 95% ethanol overnight. The residue obtained after filtration was again resuspended in equal volume of 95% ethanol for 48h and filtered again. The above two filtrates were mixed and solvent were evaporated in a rotavapour at 40-50.,a°C under reduced pressure. A dark semisolid material (9%) obtained was stored at 0- 40.,a°C until used.

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

A known volume of the residual extracts was suspended in distilled water and was orally administered to the animals by gastric intubations using a force-feeding needle during the experimental period.

**Experimental Protocol:** The Annamalai university animal ethics committee has approved the experimental design. A total number of 60 golden syrian hamsters were randomized into 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 AsABet (500 mg kg body weight) and AsEBet (300 mg kg, body weight) 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 AsABet (500 mg kg b.w) and AsEBet (300 mg kg b.w) 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 estimations were conducted on blood, erythrocytes and buccal mucosa of control and experimental animals in each group. For histopathological examination, buccal mucosa tissues were fixed in 10% formalin and routinely processed.
and embedded with paraffin, 2-3μm sections were cut in a rotary microtome, stained with haematoxylin and eosin.

**Biochemical Analysis:** After plasma separation, the erythrocyte membrane was prepared by the method of Dodge *et al.*[26] modified by Quist[27]. The protein bound hexose, hexosamine, total sialic acid and fucose in plasma, erythrocyte membrane and buccal mucosa were estimated by the methods of Niebes *et al.*[28], Wagner[29], Warren[30] and Dische and Shettles[31] respectively. Plasma lipid bound sialic acid level was determined by the method of Katopodis and Stock[32].

**Statistical Analysis:** Values are expressed as mean ± SD. The statistical analysis was performed by oneway analysis of variance (ANOVA). Followed by Duncan’s Multiple Range Test (DMRT). The Values were considered statistically compared if the p-value was less than 0.05.

**Results:** Table 1 shows the incidence of oral squamous cell carcinoma and histological changes in control and experimental animals in each group. We observed 100% tumour formation in hamsters painted with DMBA alone. The tumour volume and burden was also significantly increased in hamsters painted with DMBA alone as compared to control hamsters. Oral administration of Annona squamosa bark extracts to DMBA treated hamsters significantly prevented the formation of oral squamous cell carcinoma (75%) as well as reduced the incidence of pre-cancerous lesions such as keratosis, hyperplasia and dysplasia.

Table 2-4 show the status of glycoconjugates in plasma (protein bound hexose, protein bound hexosamine, total sialic acid, lipid bound sialic acid and fucose), erythrocyte membrane (Protein bound hexose, Protein bound hexosamine and total sialic acid) and buccal mucosa (protein bound hexose, total sialic acid, and fucose) in control and experimental animals in each group. The levels of glycoconjugates were significantly increased in plasma and buccal mucosa whereas decreased in erythrocyte membranes of tumour bearing hamsters as compared to control hamsters. Oral administration of Annona squamosa ethanolic and aqueous extracts of Annona squamosa alone showed no significant differences in glycoconjugates status as compared to control hamsters.

**Discussion:** Cell surface glycosal residues play an important role in regulating cell proliferation and epithelial growth[33]. Malignant transformation of oral epithelium is associated with atypical glycosylation of cell surface glycoconjugates[34]. A loss in epithelial cell surface carbohydrates during experimental oral carcinogenesis has been reported[35]. Neoplastic transformation is associated with altered cell surface carbohydrate composition of the cell membrane and changes in tumor cell surface due to their abnormal growth, metastasis, and changes in cell adhesion[36]. Dabelsteen et. al reported that measurement of glycoconjugates has been used for diagnosis, staging and monitoring of cancer in experimental carcinogenesis[37].

Glycosylation has been demonstrated to play a critical role during malignant transformation[38]. Several studies documented that various tumor markers such as glycoprotein and glycolipids were shed from the tumor cells[39]. The decreased erythrocyte membrane glycoconjugates may be due to increased membrane damage in the tumor tissue and released into the circulation. Several studies have reported that neoplasms often have increase concentration of sialic acid on tumor cell surface and sialoglycoproteins are shed or secreted by these cells increasing their concentration in blood[40].

Sialic acid residues on the surface of the erythrocyte membranes are responsible for net negative charge on the cell surface and confer rigidity to the cell membrane. The reduction in the erythrocyte membrane sialic acid may be due to decreased amount of deficiency of glycosyltransferase in oral cancer patients[41]. Several studies examined that 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[42]. Increased excretion of glycosidically bound sialic acid in urine of cancer patients reflects elevation of sialyl transferase activity in tumor tissues[43].

Oral administration of *A. squamosa* bark 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* bark 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 maintaining effects.
Table I: Incidence of oral neoplasm in control and experimental animals in each group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (Group I)</th>
<th>DMBA (Group II)</th>
<th>DMBA + AsABet (500mg/kg b.wt) (Group III)</th>
<th>AsABet Alone (500mg/kg b.wt) (Group IV)</th>
<th>AsEBet Alone (300mg/kg b.wt) (Group V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence (oral squamous cell carcinoma)</td>
<td>0</td>
<td>100(%)</td>
<td>20%</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Total number of tumors/animals</td>
<td>0</td>
<td>35(10)</td>
<td>4(2)</td>
<td>2(1)</td>
<td>0</td>
</tr>
<tr>
<td>Tumour volume (mm³)</td>
<td>0a</td>
<td>362.28±28.5b</td>
<td>76.12±6.2c</td>
<td>264.83±2.46a</td>
<td>0</td>
</tr>
<tr>
<td>Tumour burden (mm³)</td>
<td>0a</td>
<td>1267.98±92.34b</td>
<td>158.24±12.18c</td>
<td>129.62±9.18a</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are expressed as ± SD for 10 animals in each group. 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, D2 and D3 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 bearing tumors animals.

AsABet – A. squamosa aqueous bark extract. AsEBet – A. squamosa ethanolic bark extract

Table II: Histopathological changes in oral cheek mucosa of A. squamosa bark extracts treated on DMBA painted golden syrian hamsters

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameters</th>
<th>Keratosis</th>
<th>Hyperplasia</th>
<th>Dysplasia</th>
<th>Squamous cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>II.</td>
<td>DMBA + AsABet (500mg/kg b.wt)</td>
<td>Severe</td>
<td>Severe</td>
<td>Severe</td>
<td>Moderately differentiated</td>
</tr>
<tr>
<td>III.</td>
<td>DMBA + AsEBet (300mg/kg b.wt)</td>
<td>Moderate</td>
<td>Mild</td>
<td>Mild</td>
<td>Well differentiated (3)</td>
</tr>
<tr>
<td>IV.</td>
<td>AsABet (500mg/kg b.wt) alone</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>V.</td>
<td>AsEBet (300mg/kg b.wt) alone</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

AsABet – A. squamosa aqueous bark extract. AsEBet – A. squamosa ethanolic bark extract

Table III: Plasma glycoconjugates in control and experimental animals in each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Protein bound hexose (mg/dl)</th>
<th>Protein bound hexosamine (mg/dl)</th>
<th>Total sialic acid (mg/dl)</th>
<th>Lipid-bound sialic acid (mg/dl)</th>
<th>Fucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control</td>
<td>83.5±6.5a</td>
<td>79.5±6.2a</td>
<td>44.9±3.7a</td>
<td>13.1±1.2a</td>
<td>7.27±0.5a</td>
</tr>
<tr>
<td>II.</td>
<td>DMBA</td>
<td>136.3±15.2b</td>
<td>120.7±13.9b</td>
<td>78.6±9.2b</td>
<td>30.8±4.2b</td>
<td>15.8±1.3b</td>
</tr>
<tr>
<td>III.</td>
<td>DMBA + AsABet(500mg/kg b.w)</td>
<td>112.4±10.9c</td>
<td>96.7±10.5c</td>
<td>60.7±8.1c</td>
<td>20.4±2.6c</td>
<td>11.3±1.1c</td>
</tr>
<tr>
<td>IV.</td>
<td>DMBA + AsEBet (300mg/kg b.w)</td>
<td>86.7±11.5a</td>
<td>83.4±8.7a</td>
<td>48.9±6.3a</td>
<td>14.3±1.7a</td>
<td>7.93±1.2a</td>
</tr>
<tr>
<td>V.</td>
<td>AsABet (500mg/kg b.w)</td>
<td>84.1±10.1a</td>
<td>80.1±7.5a</td>
<td>45.2±3.2a</td>
<td>13.3±1.5a</td>
<td>7.39±0.5a</td>
</tr>
<tr>
<td>VI.</td>
<td>AsEBet (300mg/kg b.w)</td>
<td>84.3±9.6a</td>
<td>79.7±6.9a</td>
<td>44.8±3.5a</td>
<td>13.4±1.1a</td>
<td>7.31±0.6a</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD, (n=10). Values not sharing a common superscript significantly differ at P < 0.05. (DMRT)

AsABet – A. squamosa aqueous bark extract. AsEBet – A. squamosa ethanolic bark extract

during neoplastic transformation and their inhibitory role on glycoprotein synthesis or on the activity of the glycosyl transferase. Although both aqueous and ethanolic extracts of A. squamosa bark exert chemopreventive efficacy in experimental oral carcinogenesis, the ethanolic extract was found to be more potent than that of aqueous bark extract.
Thus, the present study demonstrated that chemopreventive efficacy of *A. squamosa* bark 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 antitumour principles from the barks of *A. squamosa*.

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**REFERENCES**