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Research Article

Impact of Whole Plant Extract of *Pergularia daemia* on Glycoproteins in Dimethylbenz(A)Anthracene Induced Hamster Buccal Pouch Carcinogenesis

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Abstract

Background: Oral squamous cell carcinoma is a major component of a diverse group of neoplasms often referred to as 'head and neck cancer'. Frequent smoking and/or alcohol consumption are two major risk factors for oral cancer.

Objectives: The present study was aimed to investigate the protective role of *Pergularia daemia* ethyl acetate and methanolic extracts (PDEAE and PDME, respectively) on glycoproteins in dimethylbenz(a) anthracene (DMBA) induced hamster buccal pouch carcinogenesis.

Materials and Methods: Male golden Syrian hamsters were used and divided into six groups. Group 2 carried 0.5% 7,12-DMBA painting on left buccal pouch. Groups 3 and 4 were treated with DMBA and 300 mg/kg bwt of PDEAE and PDME by intragastric administration. Remaining groups served as untreated control. All the experiments were performed within 14 weeks.

Results: Body weight loss and 100% tumor incidence were observed treated hamsters with DMBA alone, whereas administration of PDEAE and PDME in animals with oral cancer caused significant alterations in body weight and tumor incidence. Further, plasma and buccal pouch tissue glycoprotein levels were increased and erythrocyte glycoprotein levels were depleted in DMBA treated hamsters. The levels were significantly reversed in hamsters treated with PDEAE and PDME at 300 mg/kg bwt.

Conclusion: PDEAE and PDME produce a significant protective effect against DMBA induced oral cancer by altering glycoproteins levels.

Keywords: *Pergularia daemia*, Hamsters, Dimethylbenz(a)anthracene, Glyco proteins, Histopathology

Background

Among the diseases threatening the world, oral cancer is the fifth leading one. In India, 9.8% of the nation has been reported to suffer from this disease. Studies have also shown that every year 75 000-80 000 people are affected by the disease (1). There are so many methods available to detect oral/precancerous lesions. The evaluation of glycoprotein levels is one of the methods to identify the severity of the disease. Glycoproteins are the major component of animal cells and the carbohydrate linked protein macromolecules found on the cell surface. Major glycoproteins are hexose, hexosamine, and sialic acid. Generally, glycoproteins are formed in the cytosol and play an important role in membrane transport, cell differentiation and recognition, the adhesion of macromolecules to the cell surface, and the secretion and absorption of macromolecules (2). Protein binds to carbohydrates through glycation process by cotranslational or post-translational modifications, by which free radicals are formed and cause the risk of developing carcinogenesis. Advanced glycation end-products (AGEs) modify galactose, fucose and sialic acid contents of specific cellular glycoproteins (3).

Plants and plant products have long been used as sources of medicine since in almost all non-industrialized societies. Therefore, various medicinal plants have been studied using modern scientific approaches. Medicinal properties of plants are due to their bioactive polyphenolic constituents that may be stored in roots, leaves, stem bark, fruits and seeds. These compounds can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (4).

In certain African countries, up to 90% of the people still rely on medicinal plants to satisfy their primary health care needs. *Pergularia daemia* (Forsk.) Chiov is an important medicinal plant that belongs to the family Asclepiadaceae. The whole plant, commercially known as *veliparuthi* in Tamil, has been traditionally used as an expectorant, anthelmintic, laxative, and antipyretic agent, and to treat infantile diarrhea (5). Similarly, studies reported the aerial parts of this plant to exert various pharmacological activities including contraceptive (6), antidiabetic (7), hepatoprotective (8), analgesic, antipyretic, anti-

inflammatory (9) and anticancer (10). In phytochemical investigations, the plant has been investigated for the presence of cardenolides, alkaloid, saponins and steroidal compounds (11). The curative properties of medicinal plants are due to the presence of various complex chemical substances of different compositions which occur as secondary metabolites.

Objectives

The present study was designed to investigate the effect of *P. daemia* ethyl acetate and methanolic extracts (PDEAE and PDME, respectively) on glycoproteins levels in dimethylbenz(a)anthracene (DMBA)induced hamster buccal pouch carcinogenesis.

Materials and Methods

Chemicals

All the chemicals used in this experiment were obtained from Sigma Chemical Company (St Louis, MO, USA), Hi Media (Mumbai, India), and SD-Fine Chemicals (Mumbai, India). All chemicals used were of analytical grade.

Plant Materials

Mature *P. daemia* was collected from the river bank (in and around) Pudukkottai district, Tamil Nadu, India. The plant was identified by Dr. V. Venkatesalu, the Professor at the Department of Botany, Annamalai University. A voucher specimen (ACC: 196) was deposited in the Herbarium of the Department.

Preparation of Plant Extracts

The shade dried plant materials (root, stem, leaves, flower and barks) of *P. daemia* of about 1000 g were subjected for size reduction to coarse powder. The powdered plant material was defatted by using petroleum ether (60-80°C) and then extracted with ethyl acetate and methanol using a Soxhlet apparatus for about 72 hours at 40°C. After the sediment was filtered with Whatman filter paper grade 1 (Whatman Ltd, England), both PDEAE and PDME were further concentrated under vacuum using a rotary vacuum evaporator (Buchi R-V120, Switzerland) at 40°C, and then reconstituted in a minimum amount of dimethyl sulfoxide (DMSO) and stored at 4°C for further use (12). The percentage yield of the extracts were found to be 4.5 % (w/w) and 8.1% (w/w), respectively.

Experimental Animals

Male golden Syrian hamsters weighing 120-150 g were obtained from National Institute of Nutrition, Hyderabad and maintained in the Central Animal House of the Department of Experimental Medicine, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India. The whole experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Animal Ethical Committee of Annamalai University (proposal no. 647: dated 25.09.2009). Animals were maintained in polypropylene cages (47 × 34 × 20 cm, 6 hamsters /cage) layered with husk, renewed every 24 hours, under a 12:12 h light/dark cycle at around 22°C. The animals were fed on a standard pelleted diet (Sai Enterprises, Chennai, India).

Experimental Design

The total number of 36 hamsters were randomized into 6 groups of 6 each. Group 1 were painted with liquid paraffin alone and served as untreated controls. Group 2 were painted with 0.5% of DMBA in liquid paraffin on the left buccal of the animals by number 4 brush three times a week for 14 weeks. Group 3 received 0.5% DMBA painting and PDEAE at the concentration of 300 mg/kg bwt by intragastric administration. Group 4 received 0.5% DMBA painting and PDME at the concentration of 300 mg/kg bwt by intragastric administration. Groups 5 and 6 received only PDEAE and PDME at the concentration of 300 mg/kg bwt.

All the experiment was conducted within 14 weeks and all the animals were sacrificed by cervical decapitation. Blood sample of each hamster was collected in two separate tubes. One with anticoagulant was collected for the separation of plasma and the other without anticoagulant for further biochemical analysis of the plasma, erythrocyte and buccal tissue.

Biochemical Assays

Extraction of Glycoproteins

To 0.1 mL of plasma, 5.0 mL of methanol was added, mixed well and centrifuged for 10 min at 3000 g. The supernatant was decanted and the precipitate was again washed with 5.0 mL of 95% ethanol, recentrifuged and the supernatant was decanted to obtain the precipitate of glycoproteins. This was used for the estimation of hexose and hexosamine. For extraction of glycoproteins from the tissues, a known weight of the tissue was homogenized in 7.0 mL of methanol. The contents were filtered and homogenized with 14.0 mL of chloroform. The homogenate was filtered and the residue was successively homogenized in chloroform-methanol (2:1 v/v) and each time the extract was filtered. The residue (defatted tissues) was obtained and the filtrate decanted. A weighed amount of defatted tissue was suspended in 3.0 ml of 2 NHCl and heated at 90°C for 4 hours. The sample was cooled and neutralized with 3.0 ml of 2 N NaOH. Aliquots from the sample were used for estimation of fucose, hexose, hexosamine and sialic acid (13).

Determination of Glycoproteins

Plasma and tissue hexose levels were determined by the method of Niebes (14), hexosamine levels were estimated by the method of Wagner (15), andsialic acid and fucose

levels were determined by the method of Warren (16) and Dische & Shettles (17), respectively.

Buccal Pouch Histopathological Evaluation

For histopathological examinations, sections of buccal, liver and kidney tissues of control, tumor and treated groups were fixed in 10% formalin and stained with haematoxylin and eosin (H & E) and examined under a microscope (40x magnification).

Statistical Analysis

The data on biochemical parameters were analyzed using analysis of variance (ANOVA) and the mean values in the groups were compared by Duncan's multiple range test using SPSS (Windows, version 13.0, Chicago, USA). Results were presented as mean ± SD. $P<0.05$ were considered significance level.

Results

Our previous study demonstrated the dose response effect of PDEAE and PDME on body weight changes, tumor incidence and oxidant/antioxidant status. The results of dose response study showed methanolic extracts had excellent activity compared to ethyl acetate extract at the concentration of 300 mg/kg bwt.

The current investigation also addressed the body weight changes of control and DMBA induced oral cancer animals. We observed the body weight changes in the animals cotreated with PDEAE and PDME. The results revealed that the severe body weight loss in hamsters treated with DMBA alone, while a significant body weight increment was noticed in PDEAE and PDME treated hamsters (Table 1).

Table 1. Effects of *Pergularia daemia* Ethyl Acetate and Methanolic Extracts (PDEAE and PDME, Respectively) on Body Weight Changes in Control and Experimental Animals

Groups	Initial Weight (g)	Final Weight (g)	Net Weight Gain (g)
Control	133± 7.19^{a}	167± 7.63^{a}	34
DMBA alone	130± 7.08^{a}	133± 5.31^{b}	3
DMBA+ PDEAE (300 mg/kg bwt)	129± 9.18^{a}	149± 7.95^{c}	20
DMBA+ PDME (300 mg/kg.bwt)	135± 7.85^{a}	162± 8.3^{a}	27
PDEAE (300 mg/kg.bwt)	130± 8.3^{a}	169± 8.19^{a}	39
PDME (300 mg/kg.bwt)	130± 6.08^{a}	172± 7.42^{a}	42

The values are expressed as mean ± SD of 6 experiments in each group (ANOVA followed DMRT). Values not sharing the common superscripts differ significantly at $P<0.05$.

The carcinogenic parameters like tumor incidence, tumor volume and tumor burden were analysed in control and experimental hamsters. The results showed 100% tumor incidence in hamsters treated with DMBA alone, while the carcinogenic features were significantly reduced in those cotreated with PDEAE and PDME (Table 2).

The changes in the levels of plasma and buccal pouch glycoprotein in control and experimental hamsters are illustrated in Figures 1 and 2. The levels of plasma and buccal pouch glycoprotein were significantly higher in animals treated with DMBA alone when compared with control animals. Oral supplementation of PDEAE and PDME at the concentration of 300 mg/kg bwt reduced the glycoprotein levels significantly when compared to animals treated with DMBA alone. Among the 2 extracts used in this study, PDME exhibited splendiferous activity when compared to PDEAE.

Figure 3 shows the levels of erythrocyte glycoprotein in control and experimental animals. Results revealed that the depleted glycoprotein levels were observed in the erythrocytes of hamsters treated with DMBA alone, while the levels were significantly increased in the animals cotreated with PDEAE and PDME at the concentration of 300 mg/kg bwt. A higher activity was observed in PDME treated animals when compared to PDEAE treated ones

Figure 4 shows the histopathological image of buccal tissue excised from control and experimental hamsters. Well differentiated squamous cell carcinoma was observed in animals treated with DMBA alone, while mild hyperplasia and dysplasia were observed in hamsters cotreated with PDEAE and PDME at the concentration of 300 mg/kg bwt. Normal tissue was observed in control group and the groups treated with PDEAE and PDME.

Discussion

Glycoproteins are the diagnostic tools that can be used for investigation of the treatment response of several cancers,

Table 2. Effects of *Pergularia daemia* Ethyl Acetate and Methanolic Extracts (PDEAE and PDME, Respectively) on Tumor Incidence, Volume and Burden in Control and Experimental Animals

Groups	Tumor Animals	Tumor Incidence	Tumor Volume (cm^3)	Tumor Burden (cm^3)
Control	0/6 (0)	-	-	-
DMBA alone	6/6 (30)	100%	24.31± 2.19^{a}	121.55± 9.33^{a}
DMBA + PDEAE (300 mg/kg bwt)	2/6 (8)	33%	5.28± 0.41^{b}	7.04± 0.39^{b}
DMBA + PDME (300 mg/kg bwt)	2/6 (5)	33%	2.65± 0.17^{c}	2.21± 0.27^{c}
PDEAE (300 mg/kg bwt)	0/6 (0)	-	-	-
PDME (300 mg/kg bwt)	0/6 (0)	-	-	-

Values are expressed as mean ± SD for 6 rats in each group; values not sharing a common superscript differ significantly at $P<0.05$ (DMRT). Tumor volume was measured using the formula $V = 4/3\pi$ (D1/2) (D2/2) (D3/2), where D1, D2, and D3 are the three diameters (cm) of the tumor. Tumor burden was calculated by tumor volume × total number of tumors/number of animals; ( ) indicates total number of tumors.

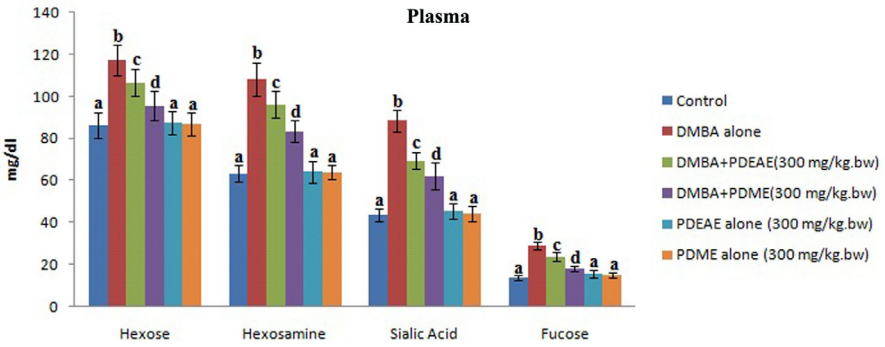

Figure 1. Effect of PDEAE & PDME on plasma glycoproteins of control and experimental hamsters. The values were given as mean ± SD of six experiments in each group (ANOVA followed DMRT). Values not sharing the common superscripts differ significantly at $P < 0.05$.

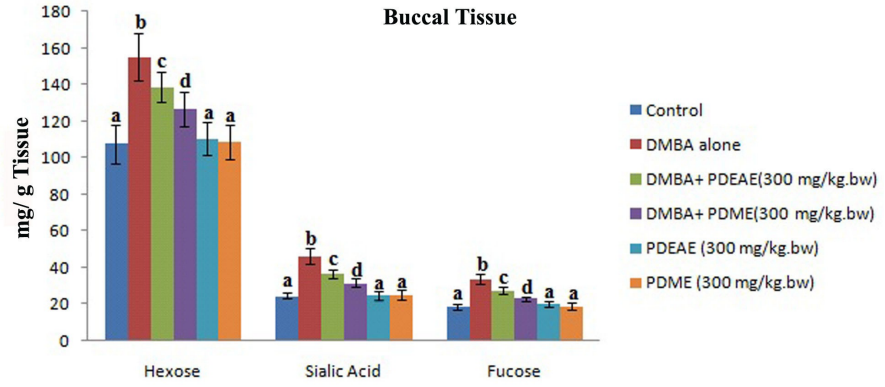

Figure 2. Effect of PDEAE & PDME on buccal tissue glycoproteins of control and experimental hamsters. The values were given as mean ± SD of six experiments in each group (ANOVA followed DMRT). Values not sharing the common superscripts differ significantly at $P < 0.05$.

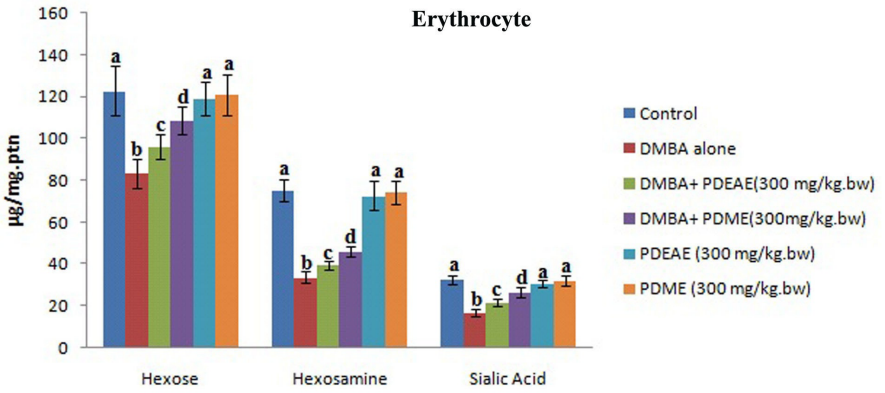

Figure 3. Effect of PDEAE & PDME on erythrocyte glycoproteins of control and experimental hamsters. The values were given as mean ± SD of six experiments in each group (ANOVA followed DMRT). Values not sharing the common superscripts differ significantly at $P < 0.05$.

including oral cancer. Thus, the simultaneous evaluation of hexose, hexosamine and sialic acid residues of glycoproteins can be helpful in monitoring the diagnosis and treatment of cancer patients (18). Atypical glycosylation of cell surface carbohydrates leads to neoplastic transformation of oral epithelium. Aberrant expression of glycoproteins could contribute to defects in cell-cell communication, invasiveness, and metastatic characteristics of cancer cells. Overexpression of glycoproteins, sialic acid, lipid-bound sialic acid, and fucose has been well documented in DMBA-treated animals and human cancers (19). Recent studies have reported that the concentrations of fucose and sialic acid are overexpressed in the cell surface during malignant transformation. Several studies reported that increase in existing specific sialylated sequence or tumor associated denovo synthesis of specific sialylated sequence could contribute to elevating the levels of sialic acid in tumor tissues. Thus, it could be argued that total sialic acid

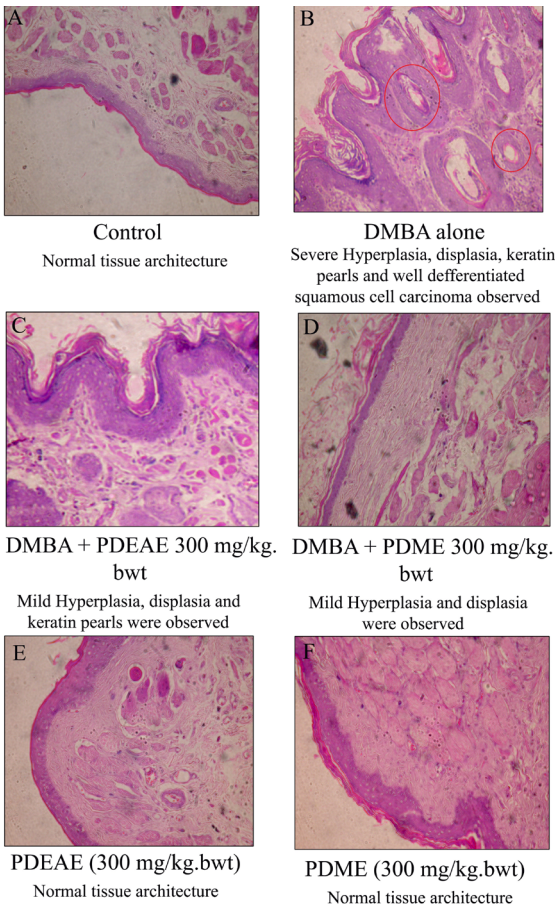

Figure 4. Histopathological Appearance of Buccal Tissue Excised From Control and Experimental Hamsters.

level in plasma or serum could be considered a supportive evidence of tumor marker for the diagnosis of cancer (20). Delphine et al also observed that sialic acid and fucose concentrations were increased compared to the adjacent counterparts. Based on that study, increased plasma sialic acid levels in cancer were due to the increased synthesis or secretion of sialoglycoproteins from tumor cells (21). Thirunavukarasu et al reported that malignant tumor in the body stimulates the synthesis of glycoproteins in the liver, which subsequently enter into the circulation. In that study, the amount of erythrocyte membrane glycoproteins was observed to decrease. Our results showed the increased levels of plasma and buccal tissue glycoproteins and decreased levels in erythrocyte membrane. This is due to the increased membrane degradation or increased shedding into circulation (22).

Hexose is a monosaccharide with six carbon atoms that plays a vital role in cellular energy release, signaling, and carbohydrate synthesis, and regulates gene expression. Cancer cells divide rapidly and need favorable energy production rates. In these cells, glucose is more produced as bioavailable and metabolizable through upregulation of glucose transporters and metabolic enzymes. Glycosaminoglycans are large complexes of negatively charged polysaccharide chains derived from amino-sugars or hexosamines. Glycosaminoglycans are the body's ground substances which support various protein structures and provide a stable aqueous environment in tissues. Glycosaminoglycan chains are derived from hexosamine repeating units. The elevated hexose and hexosamine is probably due to shedding or secretion from tumor cell surfaces or due to membrane degradation (13,23). Researchers have elaborately revealed that the glycoproteins are often elevated more markedly compared to normal levels in the sera of cancer patients (24). It has been reported that the chemical carcinogens like DMBA increase the expression of cell surface glycoconjugates during cell differentiation. This may be due to the elevation of sialyl and glycosyl transferase activity. Altered cell surface carbohydrate composition of the cell membrane and the changes in the surface of tumor cells lead to abnormal growth, metastasis, and changes in cell adhesion (25). In the current investigation, the protein bound hexose, hexosamine, sialic acid and fucose in plasma and buccal pouch of DMBA treated animals increased, whereas depletion in erythrocyte membrane was observed. Supplementation of PDEAE & PDME to experimental hamster models resulted in a significant reversal of all these changes to near normal levels. This could be due to the cytostabilising property of flavonoids, alkaloids and other bioactive components present in the PDEAE and PDME; further, it may significantly alter the expression of glycosyltransferases, thereby modulating glycoprotein synthesis and protecting the structural integrity of cell surface and membrane.

Conclusion

The present study revealed that PDEAE and PDME maintained the integrity of cell membrane during DMBA induced oral carcinogenesis. Thus, it could be concluded that the PDEAE and PDME can be used as chemopreventive agents for treating various types of malignancies.

Conflict of Interest Disclosures

The authors declare no potential conflicts of interest relevant to this article.

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