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Chemopreventive efficacy of *Phyllanthus emblica* L. (amla) fruit extract on 7,12-dimethylbenz(a)anthracene induced oral carcinogenesis – A dose–response study

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ABSTRACT

Phyllanthus emblica L. (Euphorbiaceae), a novel natural fruit has long been used as a home remedy by the medical practitioners. In this report, we investigated the chemopreventive effect of *P. emblica* fruit methanolic extract (PFMet) on oxidant–antioxidant status in hamster buccal pouch carcinogenesis. Buccal pouch carcinoma was induced in hamsters by painting with DMBA (0.5% in mineral oil) on the left buccal pouch three times a week for 14 weeks. By means of HPLC analysis, ascorbic acid (24.13%), gallic acid (10.45%), ellagic acid (1.74%) and quercetin (0.009%) were identified and quantified in the PFMet. The results showed that depleted activities of SOD, CAT and TBARS level and significant elevation were observed in the levels of GSH, vitamin E and activity of GPx in DMBA group of buccal pouch. The level of TBARS was significantly enhanced and the activities of enzymatic (SOD, CAT and GPx) and non-enzymatic (vitamin E, vitamin C and GSH) antioxidants were diminished significantly in plasma of tumor bearing animals. The effects were dose dependent and the above noted parameters were renovated to near normal after supplementation with different doses of PFMet (50, 100 and 200 mg/kg BW). The data obtained in this study clearly indicate that PFMet at a dose of 200 mg/kg BW possesses optimum chemopreventive effect against DMBA-induced buccal pouch carcinogenesis.

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1. Introduction

Squamous cell carcinoma (SCC) is the most common cancer of the oral cavity, accounting for more than 300,000 new cases annually worldwide. The disease is usually diagnosed in patients of advanced stage, which is based on currently available clinical assessments and treatment methods, and the 5-year survival rate remains low (Schliephake, 2003). The majority substantial exogenous factors implicated in carcinogenesis are tobacco and alcohol consumption (Hahn et al., 2002). Nevertheless, it is generally accepted fact that option to an animal model is a virtually inevitable prelude to under-standing how neoplasms develop and appraising the efficacy of innovative therapeutic approaches. Thus, animal models that accurately delineate the cellular and molecular

Abbreviations: PFMet, *Phyllanthus emblica* fruit methanolic extract; DMBA, 7,12-dimethylbenz(a)anthracene; BW, body weight; GAE, gallic acid equivalence; QE, quercetin equivalence; TAE, tannic acid equivalence; HPLC, high performance liquid chromatography; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; DMRT, duncan multiple range test.

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amends are of crucial, when it relates with the initiation and progression of human cancers. Based on the several steps of oral carcinogenesis characterized by initiation, promotion and progression phases, which are revealed by genetic alterations, have been suggested to undergo malignant transformation of normal epithelium (Braakhuis et al., 2004). The polycyclic aromatic hydrocarbon, dimethylbenz[a]anthracene is a significant pro-carcinogenic substance, which is metabolized by cytochrome P_{450} enzymes to yield an ultimate carcinogenic form 7,12-DMBA-3,4-dihydrodiol-1,2-epoxide (Kleiner et al., 2002) and forms DNA-adducts that lead to mutations (Moody et al., 2003). The hamster model of DMBA-induced buccal pouch carcinogenesis is considered as one of the most accepted and widely used experimental models to study the pathogenesis of human malignancies. Despite anatomical and histological differences between pouch mucosa and human buccal tissue, experimental carcinogenesis in this model induced premalignant changes and carcinomas that resemble those that occur during analogous carcinogenic progression in human buccal tissue (Chen and Lin, 2010).

To reduce the prevalence of oral cancer, development of effective, nontoxic chemopreventive agents, offers a plausible approach. Chemoprevention refers to the administration of natural or synthetic agents to prevent initiation and promotion events linked with carcinogenesis, and is being increasingly appreciated as an effective approach for the management of neoplasms (Russo, 2007). Among various natural products with chemopreventive potential, our research focused on *Phyllanthus emblica* L. (Euphorbiaceae) commonly known as amla, one of the most versatile medicinal plants that have attracted the focus of attention owing to its medicinal properties. Epidemiological studies have highlighted that cancer reduction is closely associated with dietary supplementation of fruits and vegetables. It is extensively distributed in subtropical and tropical areas of China, India, Indonesia, Malaysia and well accepted by consumers for its special taste. The treatment modality using traditional Indian and Chinese medicines have been used for centuries to treat Jaundice (Scartezzini and Speroni, 2000) and diarrhea (Rani and Khullar, 2004). Our recent review on amla have been ascribed to possess antioxidant, anti-cancer, hyperlipidemic properties (Krishnaveni and Mirunalini, 2010). It acts as a chemopreventive agent by inhibiting two-stage carcinogenesis in mice (Sancheti et al., 2005). Previous investigations in our laboratory have demonstrated the chemopreventive potential of garlic oil on hamster buccal pouch (HBP) carcinogenesis model (Mirunalini et al., 2004).

In the present study we have designed a DMBA-induced hamster oral carcinogenesis model to ascertain the chemopreventive efficacy of amla on oxidant-antioxidant biochemical alterations.

2. Materials and methods

2.1. Chemicals

7,12-Dimethylbenz(a)anthracene (DMBA) and mineral oil were obtained from Sigma–Aldrich Chemical Co (USA). All other reagents and chemicals were of analytical grade.

2.2. Plant material and preparation of extract

Fresh fruits of *P. emblica* were collected from in and around areas of Chidambaram, Tamil Nadu, India. The plant was taxonomically identified and authenticated by Dr. V. Venkatesalu, Associate Professor, and the Department of Botany. A voucher specimen (No. 207) has been preserved at the herbarium of Botany, Annamalai University. *P. emblica* fruits were dried in shade condition and ground using blender. 500 g of dried fruit powder were soaked in 2 L methanol for 3 days. After 3 days, the extracts were filtered using muslin cloth and Whatman No. 1 filter paper and the procedure was repeated thrice. Then, the filtrates were collected and evaporated on a rotary evaporator (Buchi Rotavapour, Switzerland) under reduced pressure. The yield of the extracts was 9% (w/w) and stored at 4 °C.

2.3. HPLC analysis

For HPLC analysis, the PFMet and ascorbic acid were dissolved in HPLC grade (28.9 mg/8 ml and 1.0 mg/5 ml) and subjected to HPLC for the qualitative and quantitative analysis. The analytical HPLC was performed by a SHIMADZU SPD – 20A chromatograph linked with UV–vis detector using a phenamonex – 5 μm, C-18 column (4.6 mm × 250 mm). Acetonitrile in water was used as mobile phase. Orthophosphoric acid in 1 mM potassium dihydrogen phosphate (pH 2.5) was used as eluent A and 50% acetonitrile was used as eluent B. The flow rate of the mobile phase was 0.5 ml/min. The injection volume was 10 μl. Ascorbic acid, gallic acid, ellagic acid, and quercetin were used as authentic markers.

2.4. Determination of total polyphenol, total flavonoid and total tannin content

Total phenol contents in PFMet were determined with the Folin–Ciocalteau reagent by the method of Chew et al. (2008) using gallic acid as a standard. The total phenolic contents were then expressed as gallic acid equivalence (GAE) in mg/g extract. Total flavonoid content was determined by a colorimetric method described by Jia et al. (1999) with minor modifications. Results were expressed as quercetin equivalence (QE) in mg/g of extract. Tannin content in PFMet was determined by the previous method with modification (Polshettiwar et al., 2007). Results were expressed as mg of Tannic Acid Equivalence (TAE) per 100 mg of extract.

2.5. In vivo studies

The experiment was carried out in male Syrian hamsters of 8–10 weeks old (weighed 90–140 g), obtained from National Institute of Nutrition (NIN), Hyderabad. The animals were housed, six per polypropylene cage, with controlled temperature (22 ± 2 °C) and humidity (55 ± 10%) with 12 h light:dark cycle and provided with standard food pellet and water *ad libitium* during the experimental period. The standard pellet diet comprised of crude oil (4.12%), crude fibre (3.18%), crude protein (22.12%), ash (5.17%), and sand silica (1.13%) with energy value of 3625 Kcal/kg. Appropriate animal care and experimental protocols were performed according to the guidelines established by the Indian National law on animal

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