RESEARCH COMMUNICATION

Chemopreventive Action of *Syzygium cumini* on DMBA - induced Skin Papillomagenesis in Mice

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Abstract

***Syzygium cumini* L. is widely used for the treatment of diabetes in various parts of India. The protective efficacy of *S. cumini* seed extract (SCE) against peroxidative damage contributing to skin carcinogenesis in Swiss albino mice was tested in the present study. A single topical application of 7,12-dimethyl benz(a)anthracene (100μg/100μl acetone), followed 2 weeks later by repeated application of croton oil (1% in acetone three times a week) and continued till the end of the experiment (i.e., 16 weeks) caused a 100% tumor incidence. In contrast, mice treated with the SCE (125 mg/ kg/ b.wt./ animal / day)in either the peri (i.e. 7 days before & 7 days after the application of DMBA) or post-initiational (i.e. from the day of start of croton oil treatment & continued till the end of the experiment) phases demonstrated significant reduction in cumulative numbers of papillomas and tumor incidence (75%). The average latency period in the SCE treated group was also significantly increased (Pre Group – 11.1 weeks; Post Group – 10.9 weeks) as compared with the carcinogen control group (7.9). Results from the present study indicate that the anticarcinogenic activity of SCE during DMBA-induced skin papillomagenesis is mediated through alteration of antioxidant status. Thus, SCE can be considered as a readily accessible, promising novel cancer chemopreventive agent.**

Key Words: Carcinogenesis - chemoprevention - papillomagenesis - *Syzygium cumini* - peroxidative damage

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Introduction

Carcinogenesis, a multistage process that involves molecular and cellular alterations, largely consists of three separate, but closely linked stages - tumor initiation, promotion and progression (Armitage and Doll, 1957; Moolgavkar, 1978; Surh, 2003). Initiation is a rapid and irreversible process that involves a chain of extracellular and intracellular events. These include the initial uptake of or exposure to a carcinogenic agent, its distribution and transport to organs and tissues where metabolic activation and detoxification can occur, and the covalent interaction of reactive species with target-cell DNA, leading to genotoxic damage. In contrast to initiation, tumor promotion is recognized as a relatively lengthy and reversible process in which actively proliferating preneoplastic cells accumulate. Progression, the final stage of neoplastic transformation, involves the growth of a tumor with invasive and metastatic potential.

Chemoprevention, refers to the use of relatively non-toxic chemical substances either of natural or synthetic origin to impede, arrest or reverse carcinogenesis in early stages (Sporn, 1991). Chemopreventive agents which come under various chemical classes have been shown to inhibit initiation, and to act as blocking and suppressing agents. Oxygen radicals are associated in the activation of carcinogen as well as in the promotion of an initiated cell (Cerutti, 1985). Scavengers of oxygen radicals have been shown to inhibit the cancer causation in animals and in human trials (Sun, 1990).

Normal levels of antioxidant defense mechanism is not sufficient for the eradication of free radical induced injury, therefore, administration of antioxidants from a natural origin have a promising role to play. Several antioxidants of plant material are experimentally proved and widely used as more effective agents against oxidative stress (Battacharya et al., 1997; Ilavarasan et al., 2001; Manonmani et al., 2002).

Cancer prevention could be achieved by avoidance of cancer causing substances, and by using chemopreventive agents that can inhibit the metabolism of carcinogen or causing its detoxification (Williams, 1971). Plant derived natural products such as flavanoids (Osawa et al., 1990), terpenes (Giulia et al., 1999), alkaloids (Keith et al., 1990) have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects (Roja and Heble, 1994). India is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine such as Ayurveda, Unani and Sidha but only few of them were scientifically explored. Therefore, scientific validation of such materials is needed in order to find out their possible use in cancer prevention.

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To find a natural product that can be used in chemoprevention of cancer, we tested *Syzygium cumini* seed. The Jamun tree is large commonly seen evergreen tree of Indian subcontinent but is also found in countries of Southeast Asia and Eastern Africa. Botanical name for this fruit is *Syzygium cumini* L. and it belongs to the myrtaceae family. The Jamun fruit is a small oval shaped purple coloured fruit. The fruit ripens during June-July months. These huge Jamun trees continue to give fruits for 60 – 70 years. The berry is oblong, ovoid, green when just appearing, pink when attaining near maturity and shining purple when fully ripe. *Syzygium cumini* is also known as Java plum and by various other colloquial names such as Portuguese plum (Crawford, 1937), Malabar plum (Steinmetz, 1960), black plum, Indian blackberry (Dastur, 1951), jaman, jambu, jambul, jambool and duhat. *E. jambolana* seeds were further reported to have hypoglycemic (Chopra et al., 1958), anti-inflammatory (Chaudhuri et al., 1990), neuropsychopharmacological (Chakrabarty et al., 1985), anti- bacterial (Bhuiyan et al., 1996), anti-HIV (Kusumoto et al, 1995), and anti-diarrhoeal (Indira and Mohan, 1993) effects.

Materials and Methods

The animal care and handling was approved by our institution and was done according to guidelines set by the World Health Organization, Geneva, Switzerland, and the Indian National Science Academy, New Delhi, India. The inhibition of tumor incidence by Syzygium cumini seed extract was evaluated on 2-stage skin carcinogenesis, induced by a single application of DMBA (initiator) and, 2 weeks later, promoted by repeated application of croton oil thrice per week, following the protocol for 16 weeks.

Animals

The study was conducted on random-breed male Swiss albino mice (7-8 weeks old), weighing 24 ± 2 g. These animals were housed in polypropylene cages in the animal house under controlled conditions of temperature (25°C ± 2°C) and light (14 light:10 dark). The animals were fed a standard mouse feed procured from Aashirwad Industries, Chandigarh (India), and water ad libitum. Eight animals were housed in 1 polypropylene plastic cage containing saw dust (procured locally) as bedding material. As a precaution against infections, tetracycline hydrochloride water was given to these animals once each fortnight.Three days before the commencement of the experiment, hair on the interscapular region of the mice was clipped. Only those animals in the resting phase of the hair cycle, showing no hair growth, were used.

Chemicals

7, 12-Dimethyl benz (a) anthracene (DMBA) and croton oil was procured from Sigma Chemical Co., USA. DMBA was dissolved at a concentration of 100 µg/ 100µl in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

Plant material & Extract Preparation

Fruits of *Syzygium cumini* L. were collected locally after proper identification. The identification of the plant Syzygium cumini L. (Family: Myrtaceae) was done by a botanist (Voucher Specimen No: RUBL- 20425) from Herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan (India). The pulp was removed from the fruit and the seed washed properly and shade dried, after that fruit was powdered in a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and Alcohol (3:1) for 36 (12 x 3) hrs at 40ºC. The liquid extract was cooled and concentrated by evaporating its liquid contents. The prepared Syzygium cumini extract (SCE) was stored at low temperature until further use. Such extract was redissolved in DDW prior for the oral administration in mice.

Experimental Design

A total of 32 animals were randomly divided into the following 4 groups to evaluate the anti-carcinogenic potential of SCE against DMBA-induced skin papillomagenesis in mice. Three days before the commencement of the experiment, hair on the inter-scapular region of the mice were clipped. Only the mice in the resting phase of the hair cycle were considered for the study. Body weights of the animals were recorded weekly until the term of experiment.

Group-I: Drug (SCE) treated Control: Animals received SCE (125 mg/kg/b. wt./animal/day) by oral gavage alone during the experimental period. The animals were not treated with DMBA and croton oil protocol for tumor induction.

Group-II: Carcinogen treated (Positive Control): Mice of this group were applied topically a single dose of DMBA (100 µg/ 100 µl of acetone) over the shaven area of the skin. Two weeks later, croton oil (1% v/v in acetone) was applied three times per week until the end of experiment. This group received double distilled water (DDW) equivalent to SCE (100 µl/ mouse) by oral gavage for 16 weeks.

Group –III: SCE treated (Experimental 1): These experimental animals received the same treatment as in Group-II and also received SCE at a dose of 125 mg/ kg body wt. / animal/ day, orally for 7 days before and 7 days after DMBA application.

Group –IV: SCE treated (Experimental 2): Animals of this group received the same treatment as in Group-II and were administered SCE (125 mg/kg b. wt. / animal/ day) by oral gavage, starting from the time of croton oil treatment till the end of experiment (i.e., 16 weeks).

The following morphological parameters were studied in Groups I - III:

1. Tumor incidence: The number of mice carrying at least one tumor expressed as a percentage incidence.

2. Tumor yield: The average number of papillomas per mouse.

3. Tumor burden: The average number of tumors per tumor bearing mouse.

4. Diameter: The diameter of each tumor was measured.

5. Weight: The weight of the each tumor appeared in animals at the termination of each experiment was

measured.

6. Body weight: The weights of the mice were measured weekly.

7. Average latent period: The time lag between the application of the promoting agent and the appearance of 50% of tumors was determined. The average latent period was calculated by multiplying the number of tumors appearing each week by the time in weeks after the application of the promoting agent and dividing the sum by total number of tumors.

8. Inhibition of tumor multiplicity =(Total no. of papillomas in carcinogen control) – (total no. papillomas in treated) X 100/Total no of papillomas in carcinogen control.

Results

A gradual increase in body weight of mice was noted in both the experimental groups (Group III & Group IV), while the similar increase was not evident in carcinogen treated control animals (Group II) (Table 1). The gain in the body weight in mice might be induced due to the administration of Syzygium cumini extract (SCE). Animals of both the groups survived throughout the experimental period.

Oral administration of SCE during peri- (Group III) and post- (Group IV) initiational stages of DMBA-induced skin papillomagenesis, significantly reduced the tumor burden to 4.00 and 4.66 in both the experimental groups (positive control value 8.5), while the cumulative numbers of papillomas were reduced to 24 and 28 respectively (positive control value 68). Furthermore, the size of papillomas in both the positive control (Group II) and experimental mice (Groups III & IV) also varied significantly (Table 2). The mice assorted in Groups II-IV and given two stage protocol for tumor induction revealed 75% (Groups III & IV) skin papillomas while the respective figure for positive control (Group II) was 100%. The onset of tumors (size-2 mm) commenced at 7 weeks and evident in the all carcinogen treated mice by 16 weeks, whereas in both the experimental Groups (III & IV) the onset was delayed until 9 weeks (i.e. .2 mm). At the end of the experiment (i.e., 16 weeks), the tumor weight in SCE treated animals was much lower than the tumor weight of carcinogen treated animals. The average papilloma weight of the control was 112 mg, whereas it was only 51.3 and 61.0 mg for both the SCE treated group at 16 weeks. The maximum inhibition of multiplicity of papillomas was occurred in SCE treated groups. No tumor development was recorded throughout the experiment in the animals treated orally with *Syzygium cumini* seed extract.

Table 1. Inhibitory Potential of *S cumini* extract (SCE) against DMBA-induced Skin Papillomagenesis

Group	Body Weight (g) (mean ± S.E.)		Number of	Tumor			Average
				Inc	Yield		Latent
	Initial	Final	Papillomas	Burden		Mult	Period
III	25.5±1.10	31.6±0.87	68	100	8.5	8.5	-	7.88
IV	23.5±1.19	31.8±1.61	24	75	4.0	3.0	64.7	11.1
V	28.0±1.53	36.0±1.38	28	75	4.7	3.5	58.8	10.9

Inc. Incidence (%); Mult, Multiplicity

Table 2. Tumor Size in DMBA-initiated and Croton Oil Promoted Skin Papillomagenesis in Mice with or without *S cumini* Extract

Treatment Group		Tumor size	
		2-5 mm	6-9 mm
III	Carcinogen control	30	38
IV	ROE experimental 1	11	13
IV	ROE experimental 2	13	15

Discussion

Carcinogenesis is a multi-step process exemplified by initiation, promotion, and progression steps in which genetic and epigenetic events determine the neoplastic conversion of normal cells (Zoumpourlis et al., 2003). The polycyclic aromatic hydrocarbon 7, 12-dimethylbenz (a)-anthracene (DMBA) can act as a complete carcinogen or an initiator of mouse skin carcinogenesis (Slaga et al., 1982; Brown and Balmain, 1995). It is well established that promotion with TPA produces oxidants and oxidatively damaged macromolecules (Bowden et al., 1993; 1995). On the other hand, the activity of xanthine oxidase, an enzyme capable of generating superoxide radicals, was noted to be increased in mice treated with TPA (Pence and Reiners, 1987; Reiners et al., 1987).

Previous reports suggest that 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes an enhanced release of reactive oxygen species (ROS), induction of epidermal ornithine decarboxylase (ODC), and overexpression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) proteins (Murakami et al., 2000; Seo et al., 2002).

Cancer chemoprevention is a new approach in cancer prevention, in which chemical agents are used to prevent cancer in normal and/or high-risk populations. Chemoprevention aims to halt or reverse the development and progression of precancerous cells through the use of noncytotoxic nutrients and: or pharmacological agents during the time period between tumor initiation and malignancy (Alberts and Garcia, 1995).

Over millions of years, plants have developed the capacity to synthesize a diverse array of chemicals. There are many families of phytochemicals and they help the human body in a variety of ways. Phytochemicals may protect human from a host of diseases. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases. There are many phytochemicals in fruits and herbs and each works differently. The different combinations and polymers of the aforementioned form the large, diverse group of compounds known as polyphenols, which show potent antioxidant capacity and possible protective effects on human health (Santos-Buelga and Scalbert, 2000).

Foods and beverages rich in flavonoids and other polyphenols have been associated with decreased risk of

age related diseases in several but not all epidemiological studies (Hertog et al, 1993; Yochum, 1999; Hirvonen, 2001; Geleijnse et al., 2002; Mukamal et al., 2002; Huxley and Neil, 2003; Lin et al, 2007).

The fruits of *Syzygium cumini* (L.) skeels are edible and reported to contain gallic acid, tannins, anthocyanins and other components (Benherlal and Arumughan, 2007). Extract of seed, which is traditionally used in diabetes, has a hypoglycaemic action and antioxidant property in alloxan diabetic rats (Prince et al., 1998) possibly due to tannins (Bhatia et al., 1971). Flavanoid diglycosides (Yang et al., 2000) (Quercetin & Myricetin), hydrolysable tannins (1-0- galloyl castalagin and casuarinin (Slowing et al., 1994) and a triterpene, oleanolic acid (Rajasekaran et al., 1998) were isolated from seeds of *Eugenia jambolana*. In the present study, all these constituents of *Syzygium cumini* might be responsible to reduce DMBA induced skin papilloma formation both in terms of incidence of tumour as well as mean number of papillomas/ animal.

In a structure-dependent manner, flavonoids and phenolic acids are capable of scavenging ROS, RNS and chelating transition metal ions such as iron and copper, which play vital roles in the initiation of free radical reactions (Anafas'ev, 1989; Bourne and Rice-Evans, 1998; Van Acker, 1998; Rice-Evans et al., 2000). Several studies have shown the flavonoids to act as scavengers of superoxide anions, singlet oxygen, hydroxyl radicals, and lipid peroxyl radicals (Torel and Cillard, 1986; Robak and Gryglewski, 1988). There are also reports of flavonoids inhibiting the activities of many enzymes, including lipoxygenase, cyclooxygenase, monooxygenase, xanthinoxidase, mitochondrial succinate dehydrogenase and NADH-oxidase, phospholipase A2, protein kinases, and nuclear transcription factor (NF-κB) (Cao et al., 1996; Morton et al., 2000). The chemopreventive activity of flavonoids is dependent on their structural features. A study has indicated that the number, position and substitution of hydroxyl groups of the A and B rings of flavonoid, and unsaturation of the C2-C3 bond are important factors, affecting the action of these flavones as assessed in terms of FPTase inhibition activity in a cell line. Flavonoids have also been investigated with respect to their interaction with enzymes associated with DNA topology. Some of the flavones act through topoisomerase II activity modulation. DNA topoisomerase II is an enzyme that catalyzes the doublestrand breakage and rejoining of DNA; it is pivotal for several cell functions (Holt et al., 2001). The natural product quercetin, a flavonoid, found in many fruits and vegetables have shown antitumor, antiinflammatory, antiallergic, and antiviral activities.

It was found that quercetin significantly inhibited the expression of specific oncogenes and genes, controlling G(1), S, G(2), and M phases of the cell cycle. Moreover, it has been shown to reciprocally up-regulate the expression of several tumor suppressor genes. Activation of these tumor suppressor genes, inhibition of expression of oncogenes and modulation of topoisomerase II activity inhibits the cell cycle thereby reduce the risk of cancer incidence, tumor burden, tumor yield and cumulative number of papillomas. Thus, result from this study shows the anti-tumor and chemopreventive property of hydroalcoholic extract of *Syzygium cumini* seed due to the presence of flavanoids, quercetin tannins etc. They are working as blocking as well as suppressing agent. Mainly flavanoids block the initiation event and therefore Group IV shows the greater decrease in tumor burden, tumor yield, tumor incidence and cumulative number of papillomas as compared to Group V. Because of the presence of quercetin in SCE, cell cycle arrest at the point where damage occurs thereby inhibits initiational events. The results obtained from the present study demonstrate that extract derived from *Syzygium cumini* seed may be useful for chemoprevention owing to have high content of flavanoids and other anti-oxidants.

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