RESEARCH COMMUNICATION

Chemomodulatory Effects of *Aegle Marmelos* Against DMBA-Induced Skin Tumorigenesis in Swiss Albino Mice

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Abstract

***Aegle marmelos* is widely used in Indian Ayurvedic medicine for the treatment of diabetes mellitus. In the present study, cancer chemopreventive properties were evaluated on 7, 12-dimethylbenz (a) anthracene (DMBA) induced skin papillomagenesis in Swiss albino mice. A single topical application of DMBA, followed 2 weeks later by repeated application of croton oil till the end of the experiment (i.e. 16 weeks) caused a 100% tumor incidence. In contrast, mice treated with the AME (50 mg/kg b. wt./animal/day) in the peri-initiational phase (i.e. 7 days before & 7 days after DMBA application; Group IV) and post-initiational phase (from the day of croton oil treatment till the end of the experiment; Group V), exhibited a significant reduction to 70 and 50% respectively. The cumulative number of papillomas after 16 weeks was 67 in the control group, but 26 and 23 in the animals treated with AME at peri-initiational and post-initiational stages, respectively. The tumor burden and tumor yield were significantly decreased (Group IV-3.7, 2.6; Group V- 4.6, 2.3) as compared to carcinogen treated control group (6.7, 6.7). The present study demonstrates the chemopreventive potential of *Aegle marmelos* fruit extract on DMBA induced skin tumorigenesis in mice.**

Keywords: Chemoprevention - DMBA - tumorigenesis - *aegle marmelos* - Swiss albino mice

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Introduction

Skin cancer is the most common type of cancer in the United States (NIH, 1993), with more than a million reported cases (ACS, report) and approximately 11,590 deaths (8,650 from melanoma and 2,940 from other nonepithelial skin cancers) were estimated in the year 2009 (ACS, 2009). Increasing incidence of skin cancer due to constant exposure of skin to environmental carcinogens, including both chemical agents and ultraviolet radiation, provides a strong basis for chemoprevention with both synthetic and natural, and internal and topical, remedies (Gupta et al., 2002). The two stage skin-tumorigenesis protocol (initiation & promotion) in the mouse model has provided an insight towards understanding the stages and mechanisms of carcinogenesis. It is useful for the study of various morphometric and biochemical studies that were reflected in the skin and to screen for new cancer protective, natural and synthetic agents, as during carcinogenesis it displays a pre-neoplastic condition in the form of papillomas that are visible and can be confirmed histopathologically.

DMBA, an ubiquitous environmental pollutant, is considered to be one of the etiological factors in human cancer as it is found in cigarette smoke and environmental mixtures. Tumor initiation by DMBA is an irreversible process that probably involves a somatic mutation (Slaga et al., 1995; 1996). Most tumor initiating agents either generate, or are metabolically converted to electrophilic reactants that bind covalently to cellular DNA (Di Giovanni, 1992).

Free radicals and the DNA bases modified by such radicals have also been strongly implicated in the process of carcinogenesis in general (Ames et al., 1993; Malins, 1993). TPA, the most active phorbol ester, present in croton oil acts a strong promoter through an oxygen - mediated mechanism; oxygen components are the critical components of the tumor promotion process (Huachen and Krystyna, 1991).

Chemoprevention refers to the administration of chemical agents to prevent the initiational and promotional events that occur during the process of neoplastic development. Chemoprevention aims to directly modulate specific steps in the carcinogenic process, i.e. block mutagenic carcinogens, prevent DNA damage by free radicals and suppress epithelial cell differentiation and apoptosis. Chemoprevention with food phytochemicals is currently regarded as one of the most important strategies for cancer control.

Aegle marmelos, commonly known as bael, is a spinous tree belonging to the family Rutaceae. It is widely found in India, Bangladesh, Burma and Sri Lanka. It is distributed mainly within the sub-himalayan forest, in dry hilly regions. It is called "Shivadume" the tree of lord Shiva. *Aegle marmelos* has an important place in indigenous system of medicine. Its edible leaf, root, bark, seed and fruit are valued highly in Ayurvedic medicine in India (Sharma, 1988). In fact as per Charaka (1500 BC) no

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drug has been longer or better known or appreciated by the inhabitants of India than the bael (CHEMEXCIL, 1992). The fruit is bitter, acrid , sour, astringent, aids digestion and stomach irritation, and is useful in treating diarrhea, dysentery and stomachalgia. Aqueous *aegle marmelos* fruit extract exhibits an anti-hyperlipidaemic (Miyazaki, 2007) and hypoglycaemic (Kamalakkannan et al., 2003) effect in streptozotocin-induced diabetic rats. The ripe fruit used in different formulation for treatment of chronic diarrhea (Citarasu, 2006).

Looking towards the medicinal properties of this plant, the present study is undertaken to obtain insight into the possible anti-cancer activity of *A. marmelos* against DMBA-induced skin tumorigenesis in mice.

Materials and Methods

Animal care and handling were 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 *Aegle marmelos* pulp extract was evaluated on 2-stage skin carcinogenesis, induced by a single application of DMBA (as initiator), and 2 weeks later, promoted by repeated application of croton oil (as promoter) thrice per week, following the protocol for 16 weeks.

Animals

The study was conducted on random-bred, 6-7 weeks old and 24 ± 2 gm body weight bearing, male Swiss albino mice. Animals were maintained under controlled conditions of temperature (25°C ± 2°C) and light (14hrs. light: 10hrs. dark).The animals were fed a standard mouse fed procured from Aashirwad Industries, Chandigarh (India), and water *ad libitum*. These animals were housed in polypropylene cages containing saw dust (procured locally) as bedding material. As a precaution against infection tetracycline hydrochloride water was given to these animals once each fortnight. The Departmental Animal Ethical Committee approved this study. Three days before the commencement of the experiment, hair on the interscapular region of the mice were clipped. Only those animals in the resting phase of the hair cycle, showing no hair growth, were used for the study.

Chemicals

The initiator 7, 12-Dimethyl Benz (a) anthracene (DMBA) and croton oil were 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 *Aegle marmelos* L. were collected locally after their proper identification by a competent botanist (Voucher Specimen no: RUBL-20438) from the herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan (India). The pulp was removed from the fruit and shade dried, after that pulp 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 *Aegle marmelos* extract (AME) was stored at low temperature until further use. Such extract was redissloved in DDW prior to the oral administration in mice.

Experimental Design

Mice selected from the above mentioned random-bred colony, were assorted in to control and experimental groups; each group (I- V) comprised of 10 animals. The hair on the interscapular region of the mice was shaved 3 days prior to the experiment. Only the mice showing no hair growth were considered for the study. The body weight of the animals was recorded weekly. The inhibition of the tumor incidence of the pulp extract *A. marmelos* was evaluated on two- stage process of skin tumorigenesis using the following protocol.

Group-I: Vehicle treated control

Animals belonging to this group received topical application of acetone (100 µl/ mouse) on the shaven dorsal skin, and double distilled water(DDW), equivalent to AME (100 µl/ mouse), by oral gavage for 16 weeks.

Group-II: AME alone treated

Animals of this group were administered AME orally at a dose of 50 mg/kg b. wt, dissolved in 100 µl of DDW as vehicle to each mouse, once in a day for 16 weeks.

Group-III: Carcinogen treated (Positive Control)

These animals were applied topically a single dose of DMBA (100 µg/ 100 µl of acetone) over the shaven area of the skin of the mice. 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 AME (100 µl/ mouse).

Group-IV: AME treated (Experimental -1)

These mice were given the same treatment as in Group-III and also received AME at a dose of 50 mg/ kg b. wt. / animal/ day, orally for 7 days before and after DMBA.

Group-V: AME treated (Experimental- 2)

Animals in this group received the same treatment as for Group-III and were administered AME (50 mg/kg b. wt. / animal/ day) by oral gavage, starting from the time of croton oil treatment until the end of the experiment .

Detection of Papillomas

Papillomas appearing on the shaven area of the skin were recorded at weekly intervals. Only those papillomas that persist for 2 weeks or more (diameter<2mm) were taken into consideration for evaluation of the data. Papillomas that regressed after one observation were not considered for the counting.

Morphological Parameters

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

Table 1. Chemopreventive Effect of *A. marmelos* Extract (AME) Against DMBA-Induced Skin Tumorigenesis*

Groups	Body weight (gm) (mean ± S.E.)		Cumulative number of papillomas	Tumor Incidence (%)	Tumor burden	Tumor Yield	Tumor multiplicity	Average latent period (in weeks)
	Initial	Final						
Group III	26.58 ±1.49	31.38±1.99	67	100	6.7	6.7	-	7.88
Group IV	26.32±2.21	32.26±1.12	26	70	3.7	2.6	61.19	9.26
Group V	26.56±1.21	31.23.±1.84	23	50	4.6	2.3	65.67	10.34

*Treatment schedule of the groups is specified in materials and methods

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 weight of the mice was measured weekly throughout the experimental period. 7. Average latent period: The 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.

$$\text{Average Latent Period} = \frac{\sum FX}{n}$$

Where F is the number of tumors appearing each week, X is the numbers of weeks, and n is the total number of tumors.

8. Inhibition of tumor multiplicity = (Total no. of papillomas in carcinogen control) - (Total no. papillomas in treated) X100/ Total no of papillomas in carcinogen control

Results

The findings of the present study are depicted in Tables 1 and 2 and Figure 1. The gain in the body weight in mice was not affected either by carcinogen or by *Aegle marmelos* extract treatment. A gradual increase in the body weight was noted in all the animal Groups which was found near to normal weight of the control animals. The vehicle treated control animals of the Group I as well as the AME alone administered Group II did not show any tumor incidence.

In the AME treated experimental Group, in which AME was given by oral gavage at the dose of 50 mg/ kg body wt. /animal, mice showed a significant decrease in the tumor number, diameter and weight as compared with that of the carcinogen treated control Group - III. Topical application of DMBA followed by croton oil produced skin papillomas which started appearing from week seventh onwards. The incidence of such papillomas in DMBA-croton oil treated mice (Group III) reached 100% by the end of the experiment (i.e. 16 weeks). The cumulative number of papillomas in these anima ls was recorded as 67. The average number of papillomas per mouse (tumor yield) as well as papillomas per tumor bearing mice (tumor burden) was found to be 6.7.

When AME was orally administered to animals of Group IV, in addition to the initiator (DMBA) and promoter (croton oil) for 16 days (i.e. 7 days before & 7 days after DMBA application), the tumor incidence was reduced to 70%. At the end of the observation period, the

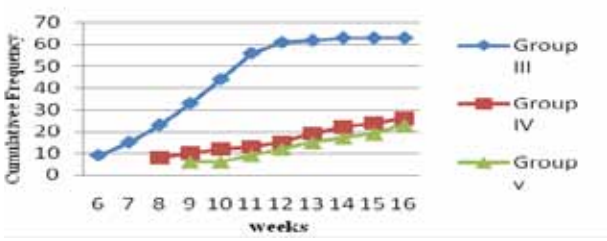

Figure 1. Variation in the Cumulative Frequency of Papillomas

Table 2. Tumor Size and Weight in DMBA-Initiated and Croton Oil Promoted Skin Tumorigenesis*

Treatment Group	Tumor size		Tumor weight (mg)
	2-5 mm	6-9 mm	
Group III	30	37	135.1
Group IV	12	14	50.0
Group IV	10	13	48.2

*Treatment schedule specified in materials and methods

value of cumulative number of papillomas, tumor yield and tumor burden was 26, 2.6, 3.7 respectively. Animals of Group V, administered AME for 14 weeks from the time of croton oil application, showed 50% tumor incidence. The cumulative number of papillomas, tumor yield and tumor burden were 23, 2.3, 4.6 respectively, which were significantly lowered than that noted in the DMBA-croton oil treated group.

The results also indicate that *A. marmelos* could prolong the average latency period (i.e. time lag between the application of the promoter and the appearance of 50% of tumors) of tumor occurrence. The latency period was found to be 7.88 weeks in the carcinogen control group, whereas it was significantly higher in the AME administered experimental Group IV (9.3) and V (10.3), respectively. Inhibition of tumor multiplicity was greater in Group V (65.7) than the Group IV (61.2).

Discussion

A great strength of chemoprevention is that a large number of compounds can prevent the occurrence of cancer and a variety of mechanisms exist for producing such protection (Belinsky, 1993). Numerous experimental and epidemiological studies show that chemoprevention has the potential of providing an important means for cancer prevention, for both the general population and even more importantly for individuals at high risk (Hakama,1998; Sporn, 2000). Oxidative stress arising due to the overproduction of reactive oxygen species (ROS) coupled with deficiency of antioxidant defence mechanism has been implicated in the pathogenesis of cancer (Das, 2002), inducing lipid peroxidation, DNA stand breaks

by modulating different biochemical pathways and gene expression (Halliwell, 1999). Topical application of TPA (active constituent of croton oil) has been reported to increase the production of free radicals (Huachen and Krystyna, 1991). Evidence has accumulated to suggest that this is perhaps due to reactive oxygen species, (Ather, 2002). Here, when AME was administered starting from the time of croton oil treatment until the end of the experiment, maximum inhibition of papillomas was recorded in the Group V. This fall may be due to the factors such as inhibition of DMBA metabolism to its active form or a delay in the promotion phase of tumorigenesis via down regulation in the production of ROS (Kausar et al., 2003). AME treatment was found to reduce the incidence of tumor appearance more during the promotion phase (Group V) than in the initiation phase (Group IV). Such depletion of tumorigenesis owing to similar factors and in various plants has been investigated by others (Sancheti et al., 2005; Kumar et al., 2006). Experimental studies have demonstrated that vitamin C (ascorbic acid) can inhibit the formation of nitroso compound both in vivo or in vitro (Marvish, 1981).Vitamin E and C are natural antioxidants present in *Aegle marmelos* fruit. Ascorbic acid is most powerful antioxidant under physiological condition, which can directly scavenge superoxides, hydroxyl radicals and single oxygen. Such Vitamin reduces H_2O_2 to water via ascorbate peroxidase reaction (Noctor and Foyer, 1998).

Aegle marmelos (Bael) fruit exhibits antidiabetic, anti-hyperlipidaemic and antioxidant properties. The exact mechanism of action of AME is not known. AME contains imperatorin that has been reported to show anti-proliferative effect on several cancer cell lines (Kawaii et al., 2001). The imperatorin present in AME has been reported to induce cytohrome c-dependent apoptosis in human promyelocytic leukemia, HL-60 cells (Pae et al., 2002). Therefore, induction of apoptosis by AME may have killed the tumor cells. Traditional use of natural plant part as food ingredients may confer some protection from cancer. The present work demands additional study to explore the exact mechanism and clinical applicability of *Aegle marmelos* as a chemopreventor.

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