RESEARCH COMMUNICATION

Anticancer and Antimutagenic Properties of *Acacia nilotica* (Linn.) on 7,12-Dimethylbenz(a)anthracene-induced Skin Papillomagenesis in Swiss Albino Mice

Punar Dutt Meena, Pallavi Kaushik, Shalini Shukla, Anil Kumar Soni, Manish Kumar, Ashok Kumar*

Abstract

We report the chemopreventive activity of *Acacia nilotica* (Linn.) gum, flower and leaf aqueous extracts, on 7,12–dimethylbenz(a)anthracene (DMBA) induced skin papillomagenesis in male Swiss albino mice. Animals were divided into following groups: Group I (Controls) given DMBA and croton oil, with no extract ; Group II (treatment) animals treated with *Acacia nilotica* gum (Group II-a) (800 mg/kg body weight), flowers (Group II-b) (800 mg/kg body weight), or leaves (Group II-c) (800 mg/kg body weight) during the peri- and post initiation periods of DMBA and croton oil application. A significant reduction in the values of tumor burden, tumor incidence and cumulative number of papillomas was observed in mice treated by oral gavage with the *Acacia nilotica* gum, flower and leaf extracts as compared with the control group. The latency period in treatment Group-II (b) and Group-II (c) was significantly increased as compared with the control group. A significant reduction in the frequency of micronuclei was also observed in mice treated by oral gavage with the aqueous extracts, along with significant decrease in total chromosomal aberrations in the form of chromatid breaks, chromosome breaks, centric rings, dicentrics, acentric fragments and exchange. Treatment with *Acacia nilotica* flower (Group II-B) and leaf (Group II-C) aqueous extracts by oral gavage for 15 days resulted in a highly significant decrease in the lipid peroxidation (LPO) level in the liver, but this was less evident with the gum (Group II-A) . Conversely, reduced glutathione (GSH) content was observed to be significantly elevated as compared with the control group with leaves (Group II-C) and flowers (Group II-B). The chemopreventive and antimutagenic activity of the leaf extract of *Acacia nilotica* was most significant followed by the flower extract and then by gum.

Key Words: DMBA - croton oil - chemoprevention - chromosomal aberrations - micronuclei - Acacia nilotica - papilloma - GSH - LPO

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Introduction

Laboratory studies and epidemiological evidence lead credence to chemoprevention strategy in attenuating the risk of developing cancer in human beings (Bertram et al., 1987; Boone eta l., 1990). Many nutrient and non-nutrient dietary constituents of plant origin have evidence of chemoprevention by inhibiting and/or suppressing or reversing cancer incidence trend evoked by multitude of factors including environmental chemical carcinogens.

Acacia nilotica (Linn.) belonging to the Leguminosae family and sub-family Mimosaceae has been subjected to long term clinical trials in folk medicine (EI-Tahir et al.,1999). The bark, root, gum, leaves and flowers have found use for skin diseases, diarrhoea, dysentery, cough, diabetes, eczema, wound healing, burning sensation and as an astringent, demulcent, anti-asthmatic.The tender twings are used as toothbrushes. It has been reported that various parts of the plant are rich in tannins (ellagic acid, gallic acid, tannic acid), stearic acid, vitamin-C (ascorbic acid), carotene, crude protein, crude fiber, arabin, calcium, magnesium and selenium (Basu et al., 1947; Swain,1965; Gupta and Bokadia, 1975; Singh and Pandey, 1980; Singletone,1981; New, 1984; Reed,1985; Srivastava et al.,1986; Chakraborti and Banerjee,1988; Siddique, 1989; Tybirk, 1989; Marohasy, 1995; Mohanty et al.,1996; Sotohy et al., 1997).

Tannins are plant polyphenolic compounds that are contained in large quantities in food and beverages (tea, red wine, nuts, etc.) consumed by humans daily. It has been shown that various tannins exert broad cancer

*Radiation & Cancer Biology Laboratory, Department of Zoology,University of Rajasthan, Jaipur-302 004, India *For Correspondence:*

chemoprotective activity in a number of animal models (Nepka et al.,1999). Therefore the present study was undertaken to evaluate the anticancer and antimutagenic activity of *Acacia nilotica* (Linn.) gum, flower and leaf aqueous extracts on DMBA and croton oil induced skin papillomagenesis in Swiss albino mice.

Materials and Methods

Animals

Random bred male Swiss albino mice (8-9 weeks old) were obtained from the animal facility (JNU, New Delhi). The animals were provided with standard mouse feed (Hindustan Lever Ltd. India) and tap water ad libitium.

Chemicals

7,12-dimethylbenz(a)anthracene (DMBA), croton oil, reduced glutathione (GSH), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), 1,1,3,3-tetramethoxy propane (TMP), Meta phosphoric acid (MPA),colchicine, giemsa stain, fetal calf serum (FCS), methanol, acetic acid, saline, may-grunwald's stain were obtained from Qualigens, Himedia Laboratories Ltd. India and Sigma Chemicals Co. (USA). The carcinogen, DMBA, was dissolved in acetone at a concentration of 100μg/50μl. Croton oil was diluted in acetone to give a 1 % dilution.

Preparation of Acacia nilotica (Linn.) extracts

Acacia nilotica Linn. [RUBL-19898] gum, flower, leaf were collected locally and identified at Herbarium, Dept. of Botany, University of Rajasthan-Jaipur. Gum, flowers and leaves were air-dried in shade without direct exposure to sun rays and powdered. Gum was dissolved in double distilled water (DDW) just before oral administration. Flower and leaf were distilled in a round bottom flask using double distilled water (DDW) at 60˚C.The procedure was repeated thrice for 12 hrs duration and the leftover residue after the third distillation was filtered and the remaining distilled water was allowed to evaporate. Thereafter, remaining material was dried in oven and collected. For a dose level of 800 mg/kg body weight, the aqueous extract was dissolved in the vehicle, double distilled water (DDW) and 0.1 ml of the gum, flower and leaf extracts was given orally to each mouse by oral gavage daily till the termination of the experiment.

Parameters for Biochemical Study

I. Reduced glutathione (GSH) assays: The hepatic level of reduced glutathione (GSH) was determined by the method as described by Moron etal. (1979). The absorbance was read at 412 nm using a UV-VIS Systronics Spectrophotometer. Glutathione was emplyed as a standard to calculate μmole GSH /mg tissue.

II. Lipid peroxidation (LPO) assays: The lipid peroxidation levels in liver specimens were measured using Thiobarbituric Acid Reactive Substances (TBARS) and the method of Ohkhawa et al (1979), with absorbance read at 532 nm using a UV-VIS Systronics Spectrophotometer.

Experimental design

(A) Tumor Induction: Experiments were designed to see the effect of Acacia nilotica extracts on DMBA induced skin papillomagenesis. A two stage skin carcinogenesis model was used as reported earlier by us (Qiblawi and Kumar, 1999). Group I (Control) – all the 9 animals of this group were treated with DMBA (100 μg/ 50 μl acetone / animal) on day 0 and then 2 weeks later, 0.1ml croton oil (1% in 100 μl acetone) was applied on the same area. This treatment was continued thrice weekly till the termination of the experiment (16 weeks). Group II (Treatment) – Animals of this group were sub-divided and given extracts of various parts of Acacia nilotica (9 animals in each group), Group II (a) gum, Group II (b) flower and Group II (c) leaf (800 mg/kg body weight / day) by oral gavage continuously at peri and post-initiational stages of papillomagenesis. The croton oil treatment was given thrice weekly 2 weeks after DMBA application for 16 weeks.

The dorsal skin on the back area of the animals were shaved 3 days before the commencement of the experiment and only those animals in the resting phase of the hair cycle were chosen for the study. During the 16 weeks of the experiments, mice were observed weekly and were weighed daily. The mice were carefully examined once a week for the presence of skin papillomas and the number of papillomas on each affected mouse was recorded.

Parameters studied were: 1. Tumor incidence, the number of mice carrying at least one tumor, expressed as percent incidence; 2. Diameter of each tumor; 3. Weight of tumors of each animal at the termination of the experiment; 4. Tumor burden, the average number of tumors per tumor bearing mouse; 5. Body weights of the mice measured daily; 6. Average latent period, the time lag between the application of the promoting agent and the appearance of 50% of tumors (Prashar etal. 1994), computed 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 the total number of tumors -

$$\text{Average latent period} = \sum FX / n$$

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

(B) GSH Content and LPO Levels: Experiments were designed to test the modulatory influence of *Acacia nilotica* extracts on mouse hepatic LPO (Lipid peroxidation) level and GSH (reduced glutathione) content.The effect of Acacia nilotica gum, flower and leaf aqueous extracts on GSH content and LPO level in the mouse liver in the following groups: Group I (Control), all the 9 animals of this group were placed on a normal diet and were given 100 μl double distilled water (vehicle) by orally for 15 consecutive days; Group II (Treatment), Animals of this group were sub-divided (9 animals in each) on normal diet and treated orally

with *Acacia nilotica* gum at 100µl (Group IIA), flowers (Group II B)) or leaves (Group II C) for 15 consecutive days. The body weights of mice were recorded daily.

(C) Cytogenetic Study: Cytogenetic damage in the bone marrow cells were studied by chromosomal aberrations and micronuclei induction.

(i) *Chromosomal aberration analysis*. Animals were administered (i.p.) with 0.25% colchicine to arrest the dividing cells at metaphase. The animals were sacrificed after 75 minutes by cervical dislocation and the bone marrow cells from femur were flushed with 0.56% KCl and incubated at 37°C for 20 min. Suspension was centrifuged and the pellet was kept in acetic acid-methanol (1:3) for 1 hr and centrifuged. Pellet was resuspended in acetic-methanol, incubated for 10 min and centrifuged. This cycle was repeated thrice. The final cell suspension was dropped on pre-chilled slides, flame dried and stained with 4% Giemsa (Sigma, USA). Well spread (100) metaphases were examined per animal to score the aberrations (Tjio and Whang, 1962). Chromosomal aberrations were scored under a binocular microscope. A total of 400 metaphase plates were scored/ animal. Different types of aberration like chromatid breaks, chromosome breaks, centric rings, dicentrics, acentric fragments and exchanges were scored. When breaks involved both the chromatids, it was termed "chromosome type aberration", while "chromatid type aberration" involved only one chromatid. If the deleted portion had no apparent relation to a specific chromosome having two centromeres, known as dicentric, chromosome whose ends have joined to from a circle with a centromere, it was termed centric ring type aberration. Chromatid interchange involving two or more chromosomes with either symmetrical or asymmetrical distortion of the usual chromatid pattern called a exchange (Savage, 1975; Bender et al., 1988).

(ii) *Micronuclei assay*: The femurs of mice were dissected out and the bone marrow was flushed out, vortexed and centrifuged. The pellet was resuspended in a few drops of fetal calf serum. Smear were made on a pre-cleaned,dry slides, air dried and fixed in absolute methanol. The slides were stained with May-Grunwald's and Giemsa stain (Sigma,USA). Micronuclei appeared in normochromatic erythrocyte (NCE) and polychromatic erythrocyte (PCE). At least 2000 erythrocytes were observed and counted. The micronuclei in them were recorded and micronuclei per 1000 cells were calculated (Schmid,1975).

Statistical analysis

The statistical significance of differences between control and experimental groups was determined by the Student's t-test or the chi-square test.

Results

In the control Group-I, with a single topical application of DMBA followed 2 weeks later, by repeated application of croton oil, skin papillomas appeared in all animals. The cumulative number of papillomas as induced during the observation period was 43±0.56. The average number of tumors per tumor bearing mouse (tumor burden) was 4.77±0.09 and the average latent period was 10.8 ±1.05 weeks.

Mice of the treatment group II (a), II (b) and II (c) given a continuous treatment of Acacia nilotica gum, flower and leaf extracts orally at peri and post-initiational phases, showed a significant reduction in the tumor incidence as compared with the control group (66.7±1.13, 66.7±1.06, 66.7±0.75 %, respectively) ($p<0.001$). The cumulative numbers of papillomas during the observation period (17±0.72, 18±0.86 and 14±1.03) were significantly less than the control group ($p<0.01$). The average weights of tumors were also significantly reduced to 137±5.17, $p<0.005$; 87.8±6.03, $p<0.001$; and 70±4.13, $p<0.001$ mg.

In the control group-I the first tumor appeared at week 6 whereas in the treatment group II (a), II (b) and II (c) the appearances of tumors were considerably delayed (weeks 7, 9, 11) respectively. All the treatment groups II (a), II (b) and II (c) animals showed a marked reduction in the size of the papillomas with respect to control.

The animals treated orally with *Acacia nilotica* gum(II-a), flower(II-b) and leaf(II-c) extract showed a significant reduction in the frequency of micronuclei (16.8±0.76; 11.1±0.83; and 7.13±0.67, all $p<0.005$) as compared to the control group (26.3±0.98). All the treatment groups (II(a), II(b), II(c) also showed a significant reduction in the total chromosomal aberrations (85.5±1.05; 63.6±1.14; 39.3±1.07 , all $p<0.005$) in the form of chromatid breaks, chromosome breaks, centric rings, dicentrics, acentric fragments and exchanges as compared to the control group-I (157.3±3.48).

In the biochemical study, the group-I (control) value for liver lipid peroxidation (LPO) was 13.9±0.43 nmole/mg and reduced glutathione (GSH) content was 38.82±0.62 µmole /mg. In the treatment groups, animals treated orally with *Acacia nilotica* flower (Group II-B) and leaf (Group II-C) aqueous extract at the dose level of 800mg/kg body weight/ day for 15 days resulted in a highly significant decrease in the hepatic LPO levels 10.8±0.29 and 7.75±0.46,$p<0.005$, whereas non-significant decrease in the hepatic LPO level was observed in Acacia nilotica gum (Group II-A) treated animals 11.8±0.22, as compared to the control group. Highly significant increase in the GSH contents was observed with *Acacia nilotica* leaf treatment 62.5±1.07 ($p<0.005$), with a less prominent but significant increase with the flower extract 45.7±1.00 ($p<0.050$), whereas non-significant elevation was observed with the gum treatment, 43.2±0.85.

Discussion

Our studies show chemopreventive activity of aqueous extracts of *Acacia nilotica* (Linn.) gum, flower and leaf on DMBA and croton oil induced skin papillomagenesis in male Swiss albino mice. Thus a significant reduction in the tumor burden, tumor incidence, cumulative number of papillomas was noted, with a marked increase in the latency period as

compared to the animals treated with single topical application of DMBA alone and croton oil. Significant reduction in micronuclei number and chromosomal aberrations in the form of chromatid breaks, chromosome breaks, centric rings, dicentrics, acentric fragments and exchange was also apparent.

Several naturally occurring dietary or non-dietary constituents, as well as parts of several species of edible plants having pharmacological activity, may influence the hepatic biotransformation enzyme profiles that are involved in activation and detoxification of xenobiotic compounds, including chemical carcinogens (Wattenberg,1983; Bradifield and Bjeldanes,1984).

The antimutagenic and chemopreventive activities of *Acacia nilotica* and *Acacia auriculiformis* were ealrier evaluated based on the development of preneoplastic lesions in response to the chemical carcinogen (Kaur etal., 2002). Recent studies indicated that compounds with antioxidant or anti-inflammatory properties as well as certain phytochemicals can inhibit tumor initiation, promotion and progression in experimental animal models (Perchellet and Perchellet 1989; Chesson and Collins,1997). There is substantial evidence that reactive oxygen species (ROS) such as superoxide anion (O_2^{--}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen are found to be involved in both initiation and promotion of multistage carcinogenesis (Cerutti etal., 1987). These ROS can act as initiators and/or promoter, cause DNA damage active carcinogen, alter the cellular antioxidant defense system and phase-II metabolizing enzymes (Marnette,1987; Sun, 1990; Sultana etal.,1995). *Acacia nilotica* (Linn.) showed a marked anti-mutagent effect. Furthermore, it was more effective against indirect acting mutagens (Arora etal., 2003).

Acacia nilotica (Linn.) gum, flower and leaf extract may play an additional and synergistic antimutagenic role in the presence of tannins(ellagic acid, gallic acid and tannic acid), crude protein, crude fiber, arabin, calcium, magnesium, selenium, stearic acid with three phenoilic components (viz. Kaempferol-3-glucoside, isoquercetin, leucocyanidin) Vit. C (ascorbic acid), carotene, alkaloids, saponins.

The polyphenolic antioxidants, consumed as an integral part of vegetables, fruits and beverages are suggested as possessing anticarcinogenic properties (Khanduja et al., 1999). Ellagic acid is active in antimutagenesis assays and has been shown to inhibit chemically induced cancer in the lung, liver, skin and esophagus of rodents and TPA-induced tumor promotion in mouse skin (Stoner and Mukhtar, 1995).The role of dietary polyphenols was investigated in the promotional phase of carcinogenesis. Topical application of polyphenols simultaneously with phorbol-12-myristate-13-acetate (PMA) or mezerein resulted in significant protection against 7,12-dimethylbenz(a)anthracene induced skin tumors in mice (Kaul and Khanduja ,1998). The effects of ellagic acid on cell cycle events and apoptosis were studied in cervical carcinoma (CaSki) cells. Ellagic acid at a concentration of 10(-5) M induced G1 arrest within 48 hours, inhibited overall cell growth and induced apoptosis in CaSki cells after 72 hours of treatment. Activation of the cdk inhibitory protein p53/p21 by ellagic acid suggests a role for ellagic acid in cell cycle regulation of cancer cells (Narayanan etal.,1999). Epidemiological studies indicate that tannic acid, stearic acid, carotene and calcium may have chemopreventive activity for several type of cancers (Colditz etal.,1985; Habib etal.,1987; Wargovich and Ball 1989). Vitamin-C is necessary for the recycling of glutathione and is an effective quencher of reactive oxygen species. A number of laboratory studies has shown the pronounced effect of vitamin-C in decreasing the incidence and delaying the onset of malignant tumors (Bissett etal.,1990; Pauling, 1991).

Epidemiological studies and human intervention trials indicate that Selenium may have chemopreventive activity in human beings. In laboratory animal assays, chemopreventive effects of inorganic and organic selenium compounds have been observed in mammary gland, colon, lung, pancreas and skin at all stages of initiation, promotion and progression (Sugie etal., 2000).

Glutathione (GSH) function as an important antioxidant in the destruction of hydrogen peroxide and lipid peroxides by providing substrate for the glutathione peroxidases (GPX). Glutathione also function as an antioxidant by promoting formation of the reduced forms of other antioxidants such as ascorbic acid and detoxification of xenobiotics, carcinogens and free radicals and maintenance of immune functions (Orrneius and Moldeus,1994).

Animal treated orally with Acacia nilotica gum, flower and leaf aqueous extract for 15 days. These extracts were found to enhance the specific levels of GSH.The decrease level of LPO supported this possibility. Similar observations have been made by Khanduja et al(1999). They also reported decrease in lipid peroxidation (LPO) and increase in reduced glutathione (GSH) as one mechanism underlying anticarcinogenic properties of ellagic acid.

One possible explanation for the close relationship between chromosome breakage and carcinogenesis may be as follows. Most of breaks and gaps result from the inter change of DNA between chromosome or sister chromatids (Kurita et al., 1965; Brgoger and Johansen, 1972). Nichols has suggested a possible relationship between chromatid breakage and carcinogenesis with various chemical, viruses and radiations (Nichols,1969). This view is consistent with Sugiyama's observation that 7,12-dimethyl-benz(a)-anthracene induced chromosomal aberrations and leukemogenesis were equally enhanced in the erythropoietin rich conditions (Sugiyama,1972). Sugiyama also demonstrated that various benz(a)anthracene derivatives damage chromosome of bone marrow cells and this is linked with sarcoma incidence (Sugiyama,1973).Chromosome instability at a numerical or structural levels is a hallmark of malignant tumors and is particularly common in cancers of epithelial origin (Klausner, 2002).

The micronucleus technique is a viable alternative to chromosome aberration analysis for evaluating the cytogenetic damage in cells induced by chemical and physical agents. Micronuclei are formed from acentric

chromosome fragments or whole chromosome, lagging behind during the anaphase separation and hence excluded from the daughter nuclei (Heddle and Carrano,1977; Yamamoto and Kikuchi,1980). The bone marrow micronuclei assay using mice has proven to be a useful method for predicting the carcinogenicity of chemical substances. In our present investigation DMBA (PAHs) increased frequency of micronuclei because Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds which require metabolic activation by microsomal monooxygenase enzymes in order to become mutagenic and/or carcinogenic (Kodama and Bock,1970). Micronuclei as well the extent of lipid peroxidation and antioxidants of the glutathione redox cycle are useful surrogate markers for the study of initial steps in carcinogenesis and the effect of chemoprevention (Ketterer, 1988). b-Carotene and Vitamin-A reduce the occurrence of pathological micronuclei even on continuous exposure to mutagens (Stich etal.,1998). Our findings are also in line with the reports of other workers on the genotoxic effect of DMBA (Ito et al., 1986).

Antimutagens and anticarcinogens are natural or synthetic substances able to inhibit or to reduced spontaneous or induced DNA alteration.They react directly with mutagens or on the process of their activation (El Hamss and Idaomar, 2002). Dietary antimutagens may provide means of slowing progression towards cancer and be more acceptable to the population. Antioxidants have been suggested to scavenge free radicals and prevent their interaction with cellular DNA. Small molecule of dietary antioxidants include ascorbic acid (vit. C), vit. E, glutathione, various polyphenols, carotenids and selenium (Ferguson et al., 2004).

In conclusion, our results provide evidence that the *Acacia nilotica* gum, flower and leaf extracts have a modulatory influence on the two-stage skin carcinogenesis and exhibit chemopreventive and antimutagenic activity, probably through possession of antioxidative properties.

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