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Chemopreventive potential of *Annona squamosa* leaves extract in Swiss albino mice

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ABSTRACT

The effect of *Annona squamosa* leaves extract against 7,12-dimethylbenz(a)anthracene (DMBA) induced papillomagenesis in Swiss albino mice was studied. The methanolic extract of *A. squamosa* was analyzed for chemopreventive activity. Chemopreventive activity was evaluated by employing two stage protocol, consisting of initiation with a single topical application of a carcinogen (DMBA) followed by a promoter (croton oil), twice a week. A significant reduction in tumor incidence, tumor burden and cumulative number of papillomas was observed, along with a significant increase in average latent period in mice treated topically with *A. squamosa* extract as compared to the control group treated with DMBA and croton oil alone. The anticarcinogenic activity of *A. squamosa* extract was also observed in Melanoma tumour model. The antibacterial and antimutagenic activities of *Annona* leaves extract were also observed. The different dose of *A. squamosa* leaves extract showed dose dependent protection of chromosomal aberrations and micronucleus formations in bone marrow of Swiss albino mice. The above studies revealed information about the prevention of cancer. Therefore, the study is immensely important in future drug development programs for cancer treatment.

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INTRODUCTION

Cancer is a major killer disease against which no unified treatment concept has emerged so far. Surgery, radiotherapy and chemotherapy often remain the methods of choice in the treatment of cancer. A major hurdle in treating cancer patients using chemotherapy is the severe side effects. Radiation produces reactive free radicals, which cause DNA damage leading to cell death and genomic damage in the stem cells. Antioxidants, which can scavenge the free radicals, are considered for chemoprotector. The failure of research efforts to obtain more effective and low cost chemoprotector drugs using the synthetic compounds has turned the focus of research towards the natural products in the past decade. Most cancer prevention research is based on the concept of multistage carcinogenesis initiation → promotion → progression. In contrast to both the initiation and progression stages, animal studies indicate that the promotion stage occurs over a long period of time and may be reversible, at least early on. Therefore, the inhibition of tumor promotion is expected to be an efficient approach to cancer control. Cancer chemoprevention is defined as the use of specific natural and synthetic chemical agents to reverse or suppress carcinogenesis and prevent the development of invasive cancers (Kelloff,1999; Lippman et al 1998). There has been a growing awareness in recent years that dietary non-nutrient compounds can have important effects as chemopreventive agents, and considerable work on the cancer chemopreventive effects of such compounds in animal models has been undertaken(Watenberg,1985). A number of common medicinal plants have good antioxidant properties and therefore may act as chemoprotector and radioprotector. Scientists all over the

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world are concentrating on herbal medicines to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body.

Annona squamoas is commonly known as 'custard apple' and is edible. In folk medicine, the different parts of the plant are used. The leaves are used as a vermicide and are applied to abscesses; insect bites and other skin complaints. Scrapings of root-bark are used for toothache. Powdered seeds are used to kill head-lice and fleas. They are known to possess insecticidal, anti-ovulatory, abortifacient and anti-implantation properties (Vohora et al., 1975; Rao et al., 1979; Damasceno et al., 2002). In one study, the anti-cancer properties of custard apple has been reported due to a class of compounds called acetogenins which are very long chain fatty acids, specific to Annonaceae species (McLaughlin, 2008). Anti-diabetic properties of *Annona* spp. appear to be related to stimulation of insulin production and enhanced uptake of glucose by muscles leading to stabilization of blood sugar concentrations (Gupta et al., 2005a). Leaf extracts are effective in lowering blood glucose levels and several reports indicates that *Annona squamosa* leaf extract can substitute effectively for externally administered insulin (Gupta et al., 2005b).

The fruit of *Annona* spp. have been shown to have anti-microbial activities (Wiart et al., 2005; Neethu Simon et al., 2016) which shows good anti-bacterial activity of the crude methanol extract of sugar apple fruit, and an isolated diterpene, against *Staphylococcus aureus* and *Streptococcus pneumoniae*. Custard apple was listed as one of the foods with strong anti-obese activity (Niwano et al., 2009).

Beppu et al. (2009) showed that oral administration of ethanol extracts of fresh custard apple fruit potently lowered plasma triglyceride concentrations. Studies report widely differing levels of anti-oxidants in *Annona* spp. Studies conducted in India (Kaur and Kapoor, 2005), Taiwan (Chen et al., 2006) and Hole et al. (2006) reported the protective effect of aqueous extract of the fruits on isoproterenol induced myocardial infarction (death of heart tissue) in rats.

MATERIALS AND METHODS

Chemicals

DMBA and croton oil were obtained from Sigma Chemicals Co. (St. Louis, MO. USA). The other chemicals were obtained from local firms and were of the highest purity. DMBA was dissolved in acetone at a concentration of 100 ug/100 ul and croton oil was diluted in acetone to give a 1% dilution.

Animals

Random bred male Swiss albino mice and C57 Bl (7-8 weeks old), weighing 24±2 mg were used for the experiments. These animals were housed in polypropylene cages in the animal house at temperatures of 24±3°C. The animals were provided with standard mice feed and tap water *ad libitum*. The experiment was approved by animal ethical committee of Priyamvada Birla Cancer Research Institute, Satna before the conduction of the experiments. The ethical approval No. 2/2014 was assigned to the experiment, dated 10.7.2014 of the Institutional Animal Ethic Committee of Priyamvada Birla Cancer Research Institute, Satna, India.

Preparation of *A. squamosa* extract

The dried leaves of *A. squamosa* were collected from herbal garden of Satna. Dry leaves were powdered with the help of grinder. Powdered material was weighed and soaked in petroleum ether for half an hour and then the extract was taken out and dried and then again, it was soaked in 50% methanol in a pear shaped separating funnel. The solvent was added to soaked powder up to a level such that a layer of solvent appears above the powder bed. The mixture was agitated at regular intervals for 24 h. The filtrate was taken out and fresh solvent was again added to the treated powder. The filtrate was concentrated in a water bath at 40°C. The concentrated extract was dried at 40°C in oven. The dried extract was powdered and then packed in an air tight container.

Experimental design for skin carcinogenesis

The dorsal skin on the back area of the animals was shaved a day before the commencement of the experiment and only those animals in the resting phase of the hair cycle were chosen for the study. For induction of tumors, a two stage protocol consisting of initiation with a single topical application of a carcinogen (DMBA) followed by a promoter (croton oil) twice a week were employed as adopted from previous modified method of Berenblum (1975) and Agrawal et al. (2009). The animals were randomly allocated into 7 groups comprising six mice each. The treatment was provided topically on shaven area.

Treatment groups

Group I (Vehicle control): 100 µl acetone 2 times/week for up to 16 weeks.

Group II (DMBA alone): 100 µg DMBA was dissolved in 100 µl acetone and single application was given.

Group III (croton oil alone): 1% Croton oil was applied on skin twice a week for up to 16 weeks.

Group IV custard apple alone (*A. squamosa* leaves extract alone): The extract was applied on skin twice a week for up to 16 weeks at the dose of 400 mg/kg b. wt.

Group V (DMBA + croton oil): 100 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards 1% croton oil was applied on skin twice a week for up to 16 weeks.

Group VI (DMBA + *A. squamosa* extract + croton oil): 100 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards the 100 µl dose of *A. squamosa* extract at the dose of 400 mg/kg b. wt. dose was given one hour before each application of 1% croton oil twice a week for up to 16 weeks.

The animals of all groups were kept under observation for gross and microscopic changes in the skin. During the period of 16 weeks of experimentation, the mice of all groups were weighed carefully, examined once a week for skin papillomas and these were recorded. The following parameters were taken into consideration.

Tumor study

Body weight: Change in mean body weight was measured weekly.

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

Cumulative number of papillomas: Total number of tumors bearing mice was recorded.

Tumor yield: The average number of papillomas per mouse was recorded.

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

Cytogenetic study

The cytogenetic damage in the bone marrow cells were studied by chromosomal aberrations and micronuclei induction.

Chromosomal aberrations analysis

For the chromosomal aberrations assay, the *A. squamosa* leaves extract at the volume of 0.2 ml was injected 24 h before the treatment of cyclophosphamide. The positive control group received single intraperitoneal injection of 50 mg/kg cyclophosphamide in 0.9% saline. Colchicines (4 mg/kg b. wt.) was administered intraperitoneally 2 h before the harvest of the cells. Animals were sacrificed by cervical dislocation and bone marrow cells were harvested. The slides were prepared essentially as adopted from the modified method of Preston et al. (1987) for chromosomal aberrations and Schmid (1975) and Agrawal et al. (1998, 1999), for micronucleus evaluations. The femur was excised and the bone marrow was extracted in 0.56% KCl. The harvested cells were incubated at 37°C for 20 min and then centrifuged for 10 min at 1000 rpm. Cells were fixed in Carney's fixative (Methanol: Acetic acid, 3:1) and burst opened on a clean slide to release the chromosomes. The slides were stained with 5% Giemsa solution for 15 min and then put in xylene and mounted with DPX. A total of 100 well spread metaphase plates were scored for chromosomal aberrations at a magnification of 1000× (100×10) for each group. Different types of chromosomal aberrations such as chromatid breaks, gaps, pulverization, polyploidy, centromeric association, etc. were scored and expressed as percentage chromosomal aberrations.

Micronuceus assay

The femur of mice was dissected out and the bone marrow was flushed out in Hank's balanced salt solution (HBBS) as described earlier (Agrawal and Kumar,1999). The smear was made in pre-cleaned slides, air dried and fixed in absolute methanol. The slides were stained with Maygrunwald and Giemsa stain. About 2000 cells were counted and numbers of micronucleated polychromatid erythrocytes cells were scored. Poly-chromatic erythrocytes to normochromatic erythrocytes (PCE/NCE) ratio was also calculated. The data are presented in MNPCE+SE. The statistical significance was evaluated using Student's *t* test.

Anti-bacterial activities

Antibacterial activities of hydro-methanolic extract from leaves of *A. squamosa* were investigated using the disk diffusion method given by Kerby-Bauer disk diffusion susceptibility test.

Bacterial strain

Following Gram negative and Gram positive bacterial strain, that is, *Escherichia coli*, *Klabsella*, *Staphylococcus* and *Pseudomonas* were used for the antibacterial activities which were received from stock culture of

Table 1. Cumulative number of papilloma in different group.

Group No.	Groups	Dose	Time of 1st appearance of papilloma	Cumulative No. of papilloma	Mean No. of papilloma	Tumour yield
I	Vehicle alone	100 µl/animal	_	_	_	_
II	DMBA alone	104 µg/animal	_	_	_	_
III	Croton Oil alone	1% per animal	_	_	_	_
IV	Custard apple Extract alone	mg/kg per animal	_	_	_	_
V	DMBA + CO (Control)	100 µg + 1% per animal	58th Day	47	47/6 (7.8)	6/6 (100%)
VI	DMBA + CO + *custard apple* Ext.	100 µg + 1% + 400 mg/animal	82th Day	18	18/5 (3.6)	3/5* (60%)

*Denotes statistical significant at P< 0.05 in students t test.

our laboratory.

Media

Nutrient agar broth media were used for the antibacterial activities. Nutrient broth was prepared thus: 1.3 g in 100 ml of double distilled water was poured in 6 different test-tubes and four bacterial strain were added into each test-tube. The nutrient agar media prepared was poured in Petri dishes and were allowed to solidify. After solidifying, the bacterial cultures on the plates were poured on them and were incubated at 37°C for 24 h.

Concentration

Four different concentrations of crude extract were prepared (100, 75, 50, and 25%). 100% = 1 g crude extract in 1 ml of freshly prepared double distilled water. Afterward, serial dilution was prepared: 75% = 75 mg in 1 ml, 50% = 50 mg in 1 ml, and 25% = 25 mg in 1 ml.

Study parameter

Measurement of zone of inhibition (in mm).

RESULTS

Effect of *A. squamosa* extract on DMBA induced skin papillomagenesis

The findings of the study are depicted in Table 1. Animals of group V (control) in which a single topical application of DMBA, followed by croton oil produced skin papillomas, which started appearing from the 8th week onwards. The incidence in DMBA/croton oil treated mice (carcinogen control) reached 100% by the termination of the experiment (that is, 16 weeks).

In the skin papilloma model, significant prevention of tumor incidences was observed in the *A. squamosa* extract treated experimental groups (which was 60% in group VI) as compared to carcinogen control (100%) group. The cumulative number of papillomas was also reduced in the *A. squamosa* extract treated experimental groups (18 in group VI) as compared to carcinogen control (47) group. The tumor burden and tumor yield were significantly decreased (3.6) as compared to DMBA treated control (7.8) group.

The findings of the study on melanoma tumor model showed that the mice which received the treatment of *A. squamosa* leaves extract at the dose of 300 mg/kg showed decreased in the percentage tumour volume of 31.65 mm as compared to untreated group, that is, 135.4 mm. Whereas, the survival of the *A. squamosa* leaves groups of animals were 26 days as compared to 13 days for control animals. The histological sections of Melanoma tumor showed that maximum number of viable cells and focal areas of necrosis were observed in untreated group as compared to more necrosis and less viable cells in treated group. It seems that more necrosis in the tumor cells represent the anticarcinogenic nature of *Annona* leaves extract (Figure 1 and Table 2).

Histopathological studies of papilloma tumors

Histological sections in the *A. squamosa* treated groups showed marked papillomatosis, hypergranulosis and hyperkeratosis with papillary projections but in the DMBA+CO treated animals, it showed papilloma that consisted of fibrovascular core with lump of epithelia showing mild dysplasia, the signs of malignancy were prominent showing Keratinous pearl and full thick

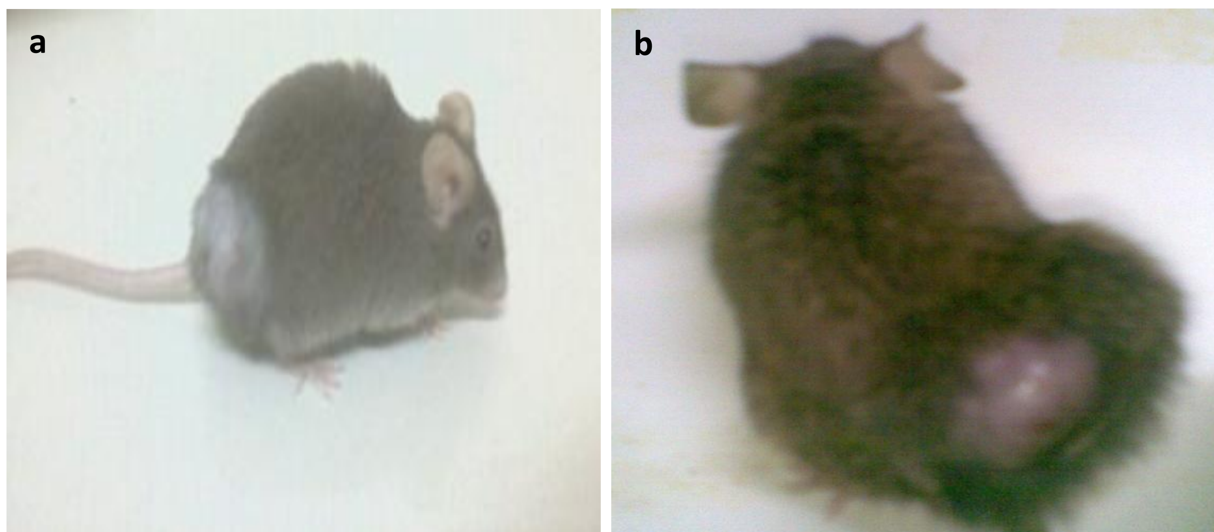

Figure 1. a) Mice treated with *Annona* extract; **b)** Untreated mice.

Table 2. Effect of *A. squamosa* leaves extract on melanoma tumor bearing mice.

S/N	Groups	Treatment	Tumor volume (mm)	Inhibition rate (%)	Survival (days)
2.	I	Untreated	135.4	-	13
3.	II	*A. squamosa* leaves extract (300 mg/kg)	31.6 5	75 %	26

dysplasia containing fibrovascular core with lining epithelia. Whereas, the animals which received the treatment of *A. squamosa* extract shows finger like papillary projections but none of malignancy was observed.

Effect of *A. squamosa* leaves extract on micronucleus formation in mouse bone marrow cells

The dose of 300, 600 and 900 mg/kg b. wt. have significantly prevented the micronucleus formation and chromosomal aberrations in bone marrow cells of mice in dose dependent manner. The PCE/NCE ratio was not affected by *A. squamosa* leaves extract treatment which showed no toxicity of the *A. squamosa* leaves extract (Figures 2 and 3 and Tables 3 and 4).

Antibacterial activity

50% methanolic extract of leaves of *A. squamosa* at the different concentration (25, 50, 75, and 100%) exhibited antibacterial activities against *Klebesella, E. coli, Staphlococcus and Psuedomonas* (Figure 4 and Table 5).

DISCUSSION

Chemoprevention is currently an important strategy for controlling the process of cancer induction. Therefore, there is a need to explore medicinal plants or other natural agents that can work as chemopreventive agents. The study demonstrates an anticarcinogenic, anti-mutagenic and antibacterial potential of *A. squamosa* leaves (AP) extract. It also demonstrates that a topical application of *A. squamosa* leaves extract (400 mg/kg body weight) at the pre-promotion phase can cause significant reduction in tumor incidence, tumor burden, tumor weight, tumor size and cumulative number of papillomas in *A. squamosa* treated groups relative to the carcinogen treated control. Significant effects were achieved; implying that the plant extract may have either inhibited the metabolism of DMBA to it active form, delayed the promotion phase of carcinogenesis or down regulated reactive oxygen species formation. Few novel chemical constituent isolated from the *A. squamosa* showed anti-cancer, anti-HIV and anti-diabetic (type 2 diabetic) properties. Evidence has accumulated suggesting that anticarcinogenicity may be due to a reactive oxygen species. It is proposed that the inhibition of tumorigenesis by the plant extract might have been

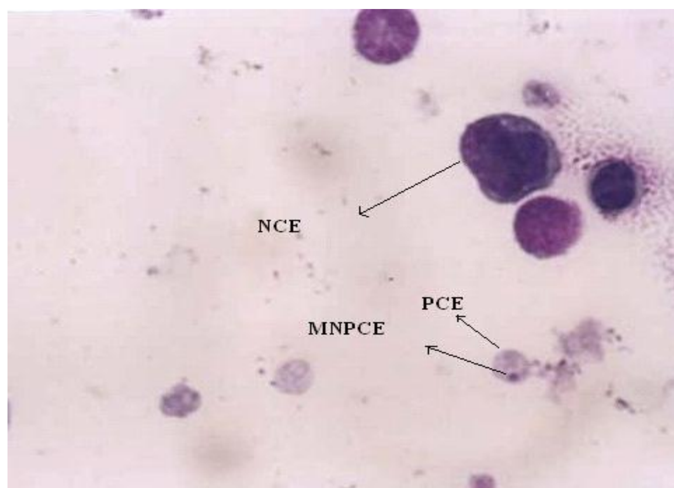

Figure 2. Showing MNPCE (micronucleated PCE) cells.

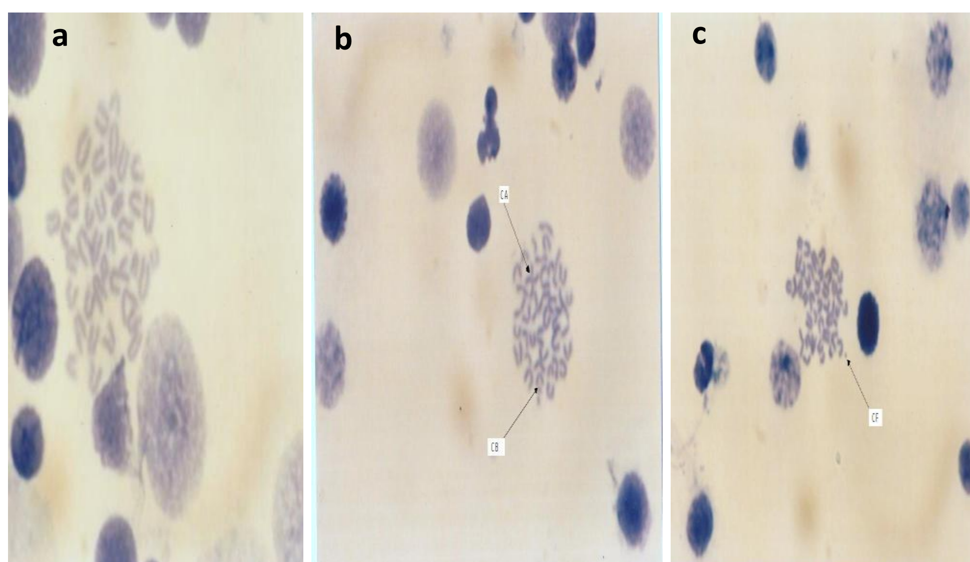

Figure 3. a) Showing normal chromosomes P; **b)** abnormal chromosome [showing chromatid break (CB) and centromeric association (CA)]; **c)** abnormal chromosome [showing chromatid fragment (CF)].

Table 3. Effect of *A. squamosa* leaves extract on micronucleus formation in mouse bone marrow cells.

Group	MNPCE + SERATIO	PCE/NCERATIO
Cyclophosphamide (50 kg/b wt.)	2.0 ± 0.816	0.693 ± 0.106
A. squamosa leaves ext. + CP (300 + 50 mg/kg b. wt.)	1.0 ± 0.09	1.73 ± 0.48
A. squamosa leaves + CP (600 + 50 mg/kg b. wt.)	0.6 ± 0.81*	1.40 ± 0.14*
A. squamosa leaves ext. + CP (900 + 50 mg/kg b. wt.)	0.33 ± 0.50*	1.50 ± 0.17*
A. squamosa leaves ext. alone (300 mg/kg b. wt.)	0.16 ± 0.4	3.21 ± 1.04
Solvent (water)	0.45 ± 0.03	0.549± 0.08

*Denotes statistical significance in *t* test as compared to cyclophosphamide treated group (4 animals were taken per group).

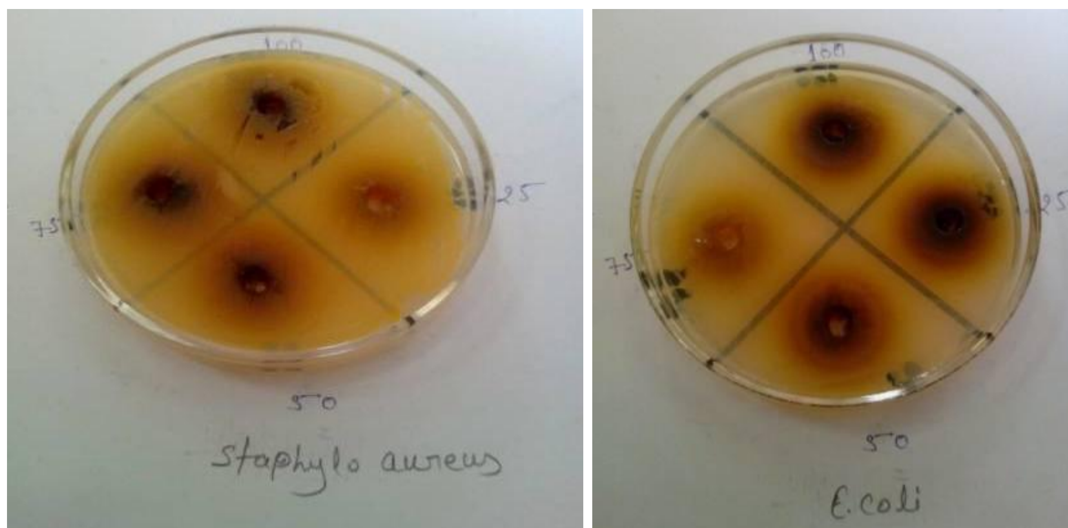

Figure 4. Zone of inhibition of *A. squamosa* extract against different strains of bacteria.

Table 4. Protection by *A. squamosa* leaves extract against cyclophosphosphamide induced chromosomal aberrations.

Treatment	Percentage chromatid	Percentage protection	Aberrations	Break	Fragmentation	Gap association	Ring
Cyclophosphamide (50 mg/kg)	18.6	3	-	5.08	11	47.8±3.16	-
A. squamosa leaves + cyclo 55.387 ± 320 (300 mg/kg + 50)	12	3	8	1	2	26±0.102	45%
A. squamosa + cyclo (600 mg/kg + 50)	4	3	9	2	3	21 ± 0.07	56%
A. squamosa + cyclo (900 mg/kg + 50)	4.8	3.8	5.8	1.9	1.9	19.4 ± 0.06	59%
A. squamosa alone 300 mg/kg	4	1	4	-	-	-	-
Solvent	5	6	-	-	-	10.00 ± 2.9	-

*Denotes statistical significance in *t* test as compared to cyclophosphamide treated group (4 animals were taken per group).

Table 5. Antibacterial activity of *A. squamosa* against bacterial strains.

Name of microorganisms	Percentage concentration of extract [zone of inhibition (mm)]			
	25	50	75	100
Klebsialla	-	-	16	18
E. coli	16	18	20	22
Staphylococcus	14	19	22	23
Psuedomonas	15	16	18	20

executed either by preventing the formation of active carcinogens from their precursors or by augmenting detoxification process, preventing promotional events in the mouse skin through free radical scavenging mechanism. The study suggests the anticarcinogenic and antimutagenic activity of *A. squamosa* which is an important drug in traditional medicine and may be useful in treatment of cancer patients. Further evaluation need to be carried out on *A. squamosa* in order to explore concealed areas and their practical clinical application,

which can be used for the welfare of the mankind.

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