

Anticarcinogenic and Chemopreventive effect of *Andrographis Paniculata* extract in Swiss albino mice

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Abstract- An *Andrographis paniculata* leaf and stem extract was studied against 7, 12 - dimethylbenz (a) anthracene (DMBA) induced papillomagenesis in Swiss albino mice and Hela cells by In Vitro methods. *Andrographis paniculata* methanolic leaves and stem extracts were analyzed for anticarcinogenic activity. It was evaluated using a two stage protocol consisting of initiation with a single topical application of a carcinogen (7, 12 - dimethylbenz (a) anthracene (DMBA) followed by a promoter (croton oil) twice a week. Tumor incidence, tumor burden and papillomas numbers reduction were observed, along with an increase in the average latent period for in mice treated topically with *Andrographis paniculata* extract compared to the control group treated with DMBA and croton oil alone. The prevention of bone marrow micronucleus formation by *Andrographis paniculata* leaves and stem extract was also observed. The glutathione level was increased in the animals which received the treatment of *Andrographis* extract along with DMBA + Croton Oil. The revealing information about the anticancer and antimutagenic of an *Andrographis paniculata* extract was observed.

Key words: Papilloma, DMBA, Micronucleus, Bone marrow, Glutathione, Hela cells

Introduction: Recently there has been a growing awareness that dietary non-nutrient compounds can have important Chemopreventive effects. Considerable work examining the cancer Chemopreventive effects of such compounds in animal models has been undertaken. A number of common medicinal plants have antioxidant properties and therefore may act as chemoprotector or radioprotectors. There is worldwide scientific interest in herbal based medicines to boost immune cells against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, herbal formulations can be designed to attack the cancerous cells without harming normal body cells.

Andrographispaniculata is known as “Kalmegh”. It has been used for centuries in Asia to treat gastrointestinal (GI) tract and upper respiratory infections, fever, herpes, sore throat, and a variety of other chronic and infectious diseases. It appears in the *Indian Pharmacopoeia* and is prominent in at least 26 Ayurvedic formulas. Studies have confirmed that *Andrographis*, properly administered, has a broad range of pharmacological effects, some of them are beneficial¹. The stem and leaves of the plant, used medicinally, contains a large number of chemical constituents, including lactones, diterpenoids, diterpene glycosides, flavonoids, and flavonoid glycosides. Controlled clinical trials report suggest its safe and effective for use in reducing symptoms of uncomplicated upper respiratory tract infections. A hydroalcoholic extract of *Andrographispaniculata* possesses antioxidant activity against oxidative alterations in the myocardium and confer significant cardio protection by facilitating normal cardiac function². Compounds were isolated from chloroform and methanolic extract of *Andrographispaniculata* possess cytotoxic activity against cancer cell lines Hep G2, HCT-116 with MTT assay³. Antimicrobial activity against eleven bacterial strains by ethanol extract of the aerial part of *Andrographispaniculata* have been reported⁴. A purified extract and andrographolide have been reported to decrease blood glucose, triglyceride, and LDL levels when compared to controls. No changes were observed in the serum cholesterol or body weight of rats. Metformin has also shown similar effects in these parameters⁵. *Andrographispaniculata* Antiulcer activity was reported in cyst amine induced

duodenal ulcer model in rats⁶. *Andrographispaniculata* antioxidant and hepatoprotective effect on acetaminophen (Paracetamol) induced hepatotoxicity in albino rats are also reported⁷. An andrographolide was reported to induce apoptosis in TD-47 human breast cancer cell line in a time and concentration-dependent manner by increase expression of p53, bax, and caspase-3 and decrease expression of bcl-2 determined by immune histochemical analysis⁸. *Andrographispaniculata*, dry leaf powder when fed orally to male albino rats, at a dose level of 20 mg per day for 60 days was reported to spermatogenesis cessation, cessation of degenerative changes in the seminiferous tubules, Leydig cells regression and regressive and/or degenerative changes in the epididymis, seminal vesicle, ventral prostate and coagulating gland⁹. An intraperitoneal injection of an ethanol extract of the aerial parts (25 g/kg body weight) in to mice poisoned with cobra venom delayed respiratory failure and death. These data suggest that extracts of the aerial parts do not modify the activities of the nicotinic receptors but produce significant muscarinic activity, which accounts for its antivenom effects¹⁰. Many of the conditions commonly treated with *Andrographispaniculata* in traditional medical systems are considered self-limiting, which requires purported benefit in cancer treatment.

Materials and Methods:

1 Chemicals

The croton oil, 7, 12 - Dimethylbenz (a) anthracene (DMBA), purchased from Sigma Chemicals Co. (St. Louis, MO. USA). Other chemicals were obtained from local firms and were of the highest purity. DMBA was dissolved in acetone at a concentration of 100µg /100µl. Croton oil was diluted in acetone to give a 1% dilution.

2 Animals

Random bred male Swiss albino mice (7- 8 weeks old), weighing 24 ± 2gm were used. These animals were housed in polypropylene cages at temperatures of 24 ± 3°C. animals and animals were provided with standard mice feed and tap water ad libitum.

3 Preparation of *Andrographispaniculata* leaves and stem extract

Plant material (*Andrographispaniculata*) was collected and the specimen was authenticated by the botanist of Deendayal Research Institute, Chitrakoot (MP), India.. leaves and stem was washed, air dried, powdered and extracted separately using 50 % methanol in a separating funnel.Extract thus obtained were vacuum evaporated into powder. These extract was again dissolved in DDW immediately prior topical application.

2.4 Experimental design for Skin Carcinogenesis

The dorsal skin on the animal's back was shaved 1 day before the experiment commenced .Only animals in the hair cycle resting phase were chosen . Two stage protocol initiated by a single topical application of a carcinogen (7, 12 - dimethylbenz (a) anthracene (DMBA) and then followed by a promoter (croton oil) twice in a week were employed per Berenblum¹¹ as standardized by Agrawal et al,¹²) used to induce tumours. Animals were randomly allocated into 7 groups of comprising six mice each. The treatment was provided topically to the shaved area

2. 4.1 Treatment Groups

Group 1 (Untreated control): No treatment

Group 2 (Vehicle control): Twice a week administration of 100 µl acetone up to 16 weeks

Group 3 (DMBA Alone): - Single administration of 100 µg DMBA dissolved in 100 µl acetone.

Group 4 (Croton Oil Alone): - Twice a week application to skin of 1 % Croton oil up to 16 week.

Group 5 (Andrographispaniculataleaves extract Alone): - Twice a week application to skin at the dose of 500 mg/kg b. wt up to 16 week.

Group 6 (DMBA + Croton Oil): - Single application to skin of 104 µg DMBA in 100 µl acetone afterwards 1 % croton oil was applied on skin twice a week up to 16 week.

Group 7 (DMBA + Andrographispaniculataleaves extract + Croton Oil): -

Single application to skin of 100 µg DMBA in 100 µl acetone afterwards the 100 µl dose of *Andrographispaniculata* extract at the dose of 500 mg/kg b. wt. was given one hour before the each application of 1% croton oil twice a week up to 16 weeks.

Group 8 (Andrographispaniculata stem extract Alone): - Twice a week application to skin at the dose of 500 mg/kg b. wt up to 16 week.

Group 9 (DMBA + Andrographispaniculatastem extract + Croton Oil): -

Single application to skin of 104 µg DMBA in 100 µl acetone afterwards the 100 µl dose of *Andrographispaniculata* extract at the dose of 500 mg/kg b. wt. was given one hour before the each application of 1% croton oil twice a week up to 16 weeks.

All animals groups were observation for gross and microscopic skin changes weekly during the 16 weeks of experimentation period, All mice were weighed and examined for skin papillomas and results were recorded. The following parameters were considered

2. 4.2 Tumor study:

Body weight: Mean body weight changes were measured weekly.

Tumor incidence: The number of mice at least one tumor expressed as percent incidence.

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

Tumor yield: The average number of papillomas per mouse.

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

Average latent period: The lag between the application of the promoting agent and the appearance of 50% 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

$Average\ latent\ period = \sum fx/n$

where f is the number of tumors appearing in each weeks , x is the numbers of weeks and n is the total number of tumors.

2.4.3 MTT Assay

The extract of whole plant and leaf of *Andrographispaniculata* on Hela cell line was determined by MTT assay as described earlier (Mosmann, 1983)¹⁶. For MTT assay, Hela cells were cultured and treated with different concentrations of extract (0-1000 µg/ml, 5 µl/100 µg of cell suspension)

for 24 hours. 2 hours prior to the termination of experiment, MTT was added to cell culture at 0.25 mg/ml (5 µl of 5 mg/ml in 100 µl of cell suspension) concentration. At the end of the experiment, culture supernatant was removed and cell layer was dissolved in DMSO and further read in a plate reader at 550 nm and 660 nm.

2.4.4 Micronucleus Assay:

For the micronucleus assay, the extract at the volume of 0.2 ml at different doses level such as 500, 1000 and 1500 mg/kg body weight was injected 24 hours before the treatment of Cyclophosphamide, to three animals. The positive control group received single ip.injection of 50 mg/kg Cyclophosphamide in 0.9% saline the animals were sacrificed by cervical dislocation and bone marrow cells were harvested. The slides were prepared essentially as described by Schmid (1975)¹³ and preston et al¹⁴ and standardised by Agrawal et al¹⁵ After staining with May-Gruenwald and Giemsa, a total 1000 cells were scored at the magnification of x1000 (100 x 10x) for each group. The data are expressed as the average number of micro nucleated cells/thousand polychromatied erythrocytes cells (PCE) cells/animals (±SE) for a group of six animals. The results were compared with the vehicle control group using Student's 't' test with significance determined at p<0.05.

3. RESULTS:

The study's findings depicted in Tables I. Animals of Group- V (control) in which a single topical application of DMBA, followed by croton oil produced skin papillomas, which began appearing in

5th week . Papilloma incidence in DMBA/croton oil treated mice (carcinogen control) reached 100% by the end of the experiment (16 weeks).

The significant tumour prevention was observed in the *Andrographispaniculata* extract treated experimental groups (25 % and 0 % in group VI & VII) compared to the carcinogen control (100 %) group. Papillomas cumulative number was also reduced in the *Andrographispaniculata* leaves and stem extract treated with DMBA + Croton Oil groups (5 and 0 in group VI & VII) compared to carcinogen control group. Tumor burdens and tumor yields were also decreased (0.5) compared to DMBA + Croton Oil treated l (3.5) group.

The induction of glutathione was also measured in the groups which was studied in papillioma models. Table 2 shows the induction of Glutathione levels in DMBA + Croton oil + AP extract leaves and stem extract treated groups as compared to DMBA + Croton Oil treated groups. The induction of glutathione level was significant in liver The anticarcinogenicity/ cytotoxicity of the plant was also studied using Hela Cell lines in Vitro models. Graph 1 &2 shows that whole plant extract of *Andrographispaniculata* had cytotoxic activity from lower concentration 10 µg/ml after 24 hour. However, leaf extract of *Andrographispaniculata* had cytotoxic activity from lower concentration 250 µg/ml after 24 hour

In the micronucleus assay CP has been used as a clastogen and anticlastogenic effect of *A.panniculata*(AP)has been observed in mice bone marrow cells (Table 3 & 4). A significant reduced number of micronuclei were found in AP along with CP as compared the CP alone. The result of Micronucleus assay showed that single application of *A.panniculata*Hydromethanolic leaves extract (i.p.) at the dose of 500 mg/kg, 1000 mg/kg and 1500 mg/kg body weight, 24 hours prior to the single i.p. administration of Cyclophosphamide (50 mg/kg) showed the reduction of micronucleus formation in PCE cell of bone marrow. The significant reduction of micronucleus formations was observed in all the dose of in micronucleus formation in AP leaves extract treated group in bone marrow cells of mice when compared to Cyclophosphamide whereas the protection was observed in AP stem extract treated group but these were not significant as compared to positive control group. The PCE/NCE ratio was comparable in all treated group as compared to CP which showed no toxicity of the extract in bone marrow cells of mice. While CP dose of 50mg/kg body wt. caused bone marrow toxicity as evidenced by a decrease in the proportion of PCE/NCE ratio.

DISCUSSION

Chemoprevention is currently an important strategy for controlling of cancer induction. There is a need to explore medicinal plants or other natural agents that may be work as chemopreventive agents. The current study demonstrates anticarcinogenic potential for andrographispanniculata extract for DMBA-induced skin tumorigenesis in male Swiss albino mice and Hela cells in Vitro models. The antimutagenic activity of AP extract was also observed in bone marrow micronucleus assay in Swiss mice. Skin carcinogenesis model in experimental animals has been found to be a useful when for investigating the chemopreventors influences both mechanistically and operationally [17]. The present study demonstrates that a topical application of andrographispanniculata leaves and stem extract (500 mg/kg body weight) at the pre promotion phase shows a significant reduction in tumor incidence, tumor burden, tumor weight, tumor size, and cumulative number of papillomas in andrographis treated groups relative to the

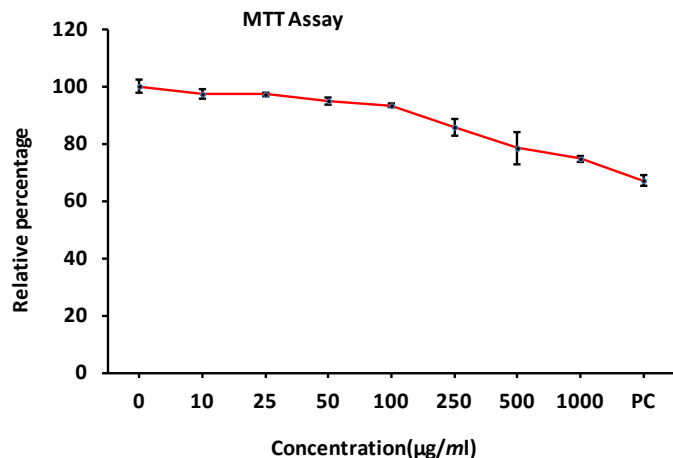
carcinogen treated control. In our previous study the anticarcinogenic activity of andrographispanniculata leaves has been reported¹⁸. The methanolic extract of leaves of AP was more effective than stem extract in both Papilloma and micronucleus assay. Evidence has accumulated suggesting that this may be due to a reactive oxygen species which play an important role in tumor initiation/promotion by enhancing or facilitating the metabolic activation and/or initiating effects of carcinogens^[22]. The plant extract may have inhibited the DMBA metabolism to its active form, delayed the carcinogenesis promotion phase or down regulated reactive oxygen species formation.^[19-21]. There are few reports on the cytotoxic and antiproliferative effects of *Andrographis.paniculata* up on in vitro cell lines [8, 13, 14]. It also increased expression of p53, bax and caspase-3 and decreased bcl-2 expression as shown by immunohistochemical analysis was observed [8]. The present study suggests the anticarcinogenic and antimutagenic activity of *Andrographis . panniculata* which is an important drug in traditional medicine and may be used to treat cancer..

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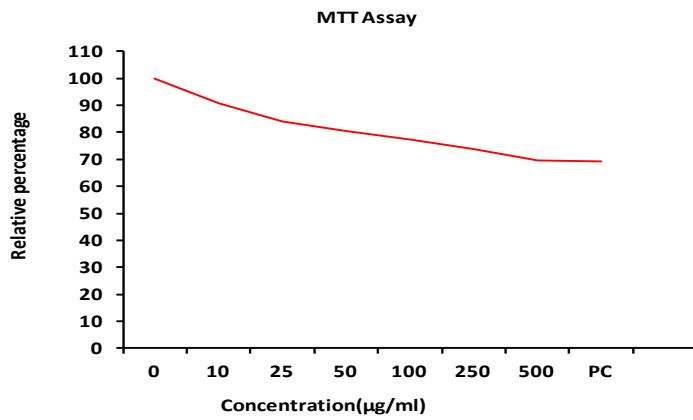
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Graph 1 : Effect of leaf extract of *Andrographispaniculata* on HeLa cell line for 24h by MTT Assay



Graph 2: Effect of whole plant extract of *Andrographis paniculata* on *HeLa* cell line for 24h by MTT Assay

			(µg/ml)	(µg/ml)
1	I.	Normal mice	4.58±0.65	53.8±8.34
2	II.	Carcinogen control (DMBA+CO)	2.7±0.06	14.95±9.75
3	III	DMBA+A.pan +CO	2.12±0.42	46.33±28.36
4	IV	(Leaves) DMBA+A.pan +CO (Stem)	3.07±2.07	30.54±14.62

Table 2 . Showing the level of Glutathione (GSH) in Blood and Liver of Papilloma bearing Swiss albino mice receiving treatment of *A. paniculata* extract.

S.No	Groups	Treatment Group	Glutathione level	
			Blood	Liver

Table 3 Effect of *A. paniculata* leaves extract on Micronucleus (MN) formation induced by Cyclophosphamide (CP) in bone marrow cells of *Swiss albino* mice.

S No.	Groups	Treatment Doses (mg/kg body wt)	MNPCE ± SEM	PCE/NCE Ratio ±SEM	% reduction in the frequency of CP induced DNA damage
1.	I	Cyclophosphamide Alone (50mg/kg b.wt)	3.5±1.1	0.99±0.08	-
2.	III	<i>A.paniculata</i> (500mg/kg b.wt)	2.2±1.3	0.61±0.14	37.2%

		+ CP (50mg/kg b.wt)			
3.	IV	<i>A.paniculata</i> (1000mg/kg b.wt) + CP (50mg/kg b.wt)	1.6±0.8	1.03±0.12*	55.3%
4.	V	<i>A.paniculata</i> (1500mg/kg b.wt) + CP (50mg/kg b.wt)	0.5±0.3	1.03±0.12*	85.8 %
5.	II	<i>A.paniculata</i> alone (1500mg/kg b.wt)	0.4 ±0.3	0.03 ±0.12	-
6.	VI	Vehicle alone (DDW)	0.16.±0.16	0.44±0.08	-

Table 4 Effect of *A. paniculata* stem extract on Micronucleus (MN) formation induced by Cyclophosphamide (CP) in bone marrow cells of *Swiss albino* mice.

S No.	Groups	Treatment Doses (mg/kg body wt)	MNPCE ± SEM	PCE/NCE Ratio ±SEM	% reduction in the frequency of CP induced DNA damage
1.	I	Cyclophosphamide Alone (50mg/kg b.wt)	3.5±1.1	0.99±0.08	-
2.	III	<i>A.paniculata</i> (500mg/kg b.wt) + CP (50mg/kg b.wt)	2.5±1.22	0.62±0.20	28 %
3.	IV	<i>A.paniculata</i> (1000mg/kg b.wt) + CP (50mg/kg b.wt)	2.6±0.89	0.587±0.12	25 %
4.	V	<i>A.paniculata</i> (1500mg/kg b.wt) + CP (50mg/kg b.wt)	2.6±1.14	0.68±0.12	25 %
5.	II	<i>A.paniculata</i> alone (1500mg/kg b.wt)	0.82 ±0.66	0.53 ±0.054	-
6.	VI	Vehicle alone (DDW)	0.16.±0.16	0.44±0.08	-

PCE – Polychromatic erythrocytes

NCE – Normochromatic erythrocytes

MNPCE – Micronucleated Polychromatic erythrocytes

Table 1. Showing Cumulative No. of Papilloma in the animals treated with *Andrographispaniculata* extract

Group	Treatment	Dose	Time of 1st appearance of Papilloma	Cumulative No. of Papilloma	Tumour yield	Tumour incidence
I	Vehicle alone	100µl/animal	–	–	–	–
II	DMBA alone	100µg/animal	–	–	–	–
III	Croton Oil alone	1% per animal	–	–	–	–
IV	Andrographis Extract alone	mg/kg per animal	–	–	–	–
V	DMBA + CO (Control)	100µg + 1% per animal	58 th Day	21	21/6 (3.5)	6/6 (100%)
VI	DMBA+ Andrographis (Leaves) Extract.+ CO	100µg + 1% + 500mg/animal	-	0	0/3	0/6
VII	DMBA+ Andrographis (Stem)Extract.+ CO	100µg + 1% + 500mg/animal	61 th Day	5	5/4 (1/4)	1/4 (25 %)