

RESEARCH COMMUNICATION

Anti-tumor Activity of *Phyllanthus niruri* (a Medicinal Plant) on Chemical-induced Skin Carcinogenesis in Mice**Priyanka Sharma, Jyoti Parmar, Preeti Verma, Priyanka Sharma, PK Goyal*****Abstract**

Chemoprevention is an important strategy to control the process of carcinogenesis. The potential of using medicinal herbs as cancer chemopreventive nutraceuticals and functional food is promising. Thus, there is a need for exploring drugs/agents which act as chemopreventive agents. *Phyllanthus niruri* is a well known medicinal plant which has been used in Ayurvedic medicine as hepatoprotective, antiviral, antibacterial, analgesic, antispasmodic and antidiabetic. The present study was carried out to evaluate the anti-tumor activity of a hydro-alcoholic extract of the whole plant, in 7-9 week old male Swiss albino mice, on the two stage process of skin carcinogenesis induced by a single topical application of 7, 12-dimethylbenz (a)anthracene (100µg/100µl acetone) and two weeks later promoted by repeated application of croton oil (1% in acetone/three times a week) till the end of experiment (16 weeks). The oral administration of *P. niruri* at a dose of 1000 mg/kg/b.wt. at peri- (i.e. 7 days before & 7 days after DMBA application) and post- (i.e. starting from the croton oil application) initiational phase of papillomagenesis caused significant reduction in tumor incidence, tumor yield, tumor burden and cumulative number of papillomas as compared to carcinogen-treated controls. Furthermore, the average latent period was significantly increased in the PNE treated group. The results thus suggest that *P. niruri* extract exhibits significant anti-tumor activity, which supports the traditional medicinal utilization of this plant.

Key Words: *Phyllanthus niruri* - DMBA skin carcinogenesis - tumor incidence/burden - average latent period

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Introduction

Epithelial carcinogenesis is a multistep process in which an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma. Cancer chemoprevention, as first defined by Sporn in 1976, uses of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression (Sporn, 1976). Skin cancer accounts for approximately 40% of all new cancer diagnosed (Jemal et al., 2003). Most skin cancers (80%) result from basal cell carcinomas (BCC); another 16% are squamous cell carcinomas (SCC), and 4% are melanomas (Einspahr et al., 2002). Associated risk factors for skin cancer include childhood and chronic sun exposure, individual susceptibility with red or blond hair and fair skinned phenotype, older age, polycyclic aromatic hydrocarbon, immunocompromised status, or xeroderma pigmentosum.

Agents effective in inhibiting skin carcinogenesis are identified in a two-stage skin carcinogenesis protocol utilizing DMBA and TPA, which are applied topically to the back skin of SENCAR or CD-1 mice (McCormick and Moon, 1986; Warren and Slaga, 1993). Skin

papillomas appear as early as 6 weeks post-carcinogen treatment, eventually progressing to squamous cell carcinomas by 18 weeks (DiGiovanni, 1992). Test agents are generally administered in the diet, or in some experiments are topically applied according to several predefined treatment regimens.

The use of natural products as anticancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine. Several drugs currently used in chemotherapy were isolated from plant species or derived from a natural prototype. They include the Vinca alkaloids, vinblastine and vincristine, isolated from *Catharanthus roseus*, etoposide and teniposide, the semisynthetic derivatives of epipodophyllotoxin, isolated from species of the genus *Podophyllum*, the naturally derived taxanes isolated from species of the genus *Taxus*, the semisynthetic derivatives of camptothecin, irinotecan and topotecan, isolated from *Camptotheca acuminata*, and several others (Cragg et al., 1993; 1994; Wang, 1998). According to Cragg and Newman (2000), over 50 % of the drugs in clinical trials for anticancer activity were isolated from natural sources or are related to them. Human epidemiological and animal studies have indicated that cancer risk may be modified by changes in dietary habits

or dietary supplements. Experimental studies indicate that phytochemicals with anti-oxidative and anti-inflammatory properties can inhibit tumour initiation, promotion and progression. The scientific validation of traditional medicine is worthwhile for its possible use in the prevention and treatment of cancer.

Phyllanthus niruri (Euphorbiaceae) originated in India and usually occurs as a winter weed throughout the hotter parts, contains over 600 species of shrubs, trees and annual or biennial herbs distributed throughout the tropical and subtropical areas. Whole plants have been used in traditional medicine for treatment of jaundice, asthma, hepatitis and malaria, and because of diuretic, antiviral, and hypoglycemic properties (Eisei, 1995; Mehrotra, 1990; Calixto et al., 1998). *P. niruri* extracts show potential therapeutic actions in the management of hepatitis B (Mehrotra et al., 1990; Calixto et al., 1998). Its antiviral activity extends to HIV-1 RT inhibition (Ogata et al., 1992; Naik and Juvekar, 2003). Its role in urolithiasis is related to the inhibition of calcium oxalate endocytosis by renal tubular cells (Campos et al, 1993; Freitas, et al., 2002).

According to the Ayurvedic system of medicine, it is considered acrid, alexipharmic and useful in thrust, bronchitis, leprosy, anemia, urinary discharge, anuria, biliousness, asthma, for hiccups and diuretic (Warrior et al, 1996). According to the Unani system of medicine this herb is stomachic and good for sores and chronic dysentery. In many parts of India, it is commonly used for the treatment of snake bite. The active compounds phyllanthin and hypophyllanthin have been isolated from leaves and they are major components of many popular liver tonics in India including Liv.-52. Due to the presence of various medicinal properties, the present study was an endeavour to examine the anticarcinogenic action, if any, of *Phyllanthus niruri* using a two-stage skin carcinogenesis model in mice.

Materials and Methods

Animal care & handling

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 study was conducted on random-breed male Swiss albino mice (7-9 weeks old) weighing 24 ± 2 gm. These animals were housed in polypropylene cages in the animal house under controlled conditions of temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and light (14 light :10 dark). The animals were fed a standard mouse feed procured from Aashirwad Industries, Chandigarh (India), and water ad libitum. Four animals were housed in one 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.

Chemicals

The initiator, 7, 12-dimethylbenz[a]anthracene (DMBA) and the promoter croton oil were procured from Sigma Chemicals Co., St Louis, USA. DMBA was

dissolved at a concentration of 100 $\mu\text{g}/100 \mu\text{l}$ in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

Preparation of Phyllanthus niruri Extract (PNE)

The whole plant *P. niruri* was collected after proper identification (Voucher No. RUBL 20247) by a competent botanist from the Herbarium, Department of Botany, University of Rajasthan, Jaipur. The whole plant was powdered in a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) in a round bottom flask for 36 hrs (3x12 hrs) at 60°C . The liquid extract was filtered, cooled and concentrated by evaporating its liquid contents in oven and collected. The powdered extract, termed Phyllanthus niruri extract (PNE), was redissolved in DDW prior for the oral administration in mice. The required dose for treatment was prepared by dissolving the extract in DDW at a dose level of 1,000 mg/kg body weight.

Experimental Design

The inhibition of tumor development by *P. niruri* extract (PNE) was evaluated on two-stage process of skin carcinogenesis, induced by a single application of 7,12-dimethylbenz(a)anthracene (initiator) and two weeks later, promoted by repeated application of croton oil (promoter) thrice weekly, using the following protocol for 16 weeks. The dorsal skin of the animals in the back area was shaven 3 days before the commencement of the experiment and only those animals in the resting phase of hair cycle were chosen for the study.

A total of 40 animals were assorted into the following groups (n=10 in each case): **Group-I:** Animals of this group (control) were applied topically a single dose of DMBA (100 $\mu\text{g}/100\mu\text{l}$ of acetone) over the shaven area of the skin. Two weeks later, croton oil (100 μl , 1% croton oil in acetone) was applied three times per week until the end of experiment (i.e.16 weeks). These animals were given orally double distilled water equivalent to the PNE (100 $\mu\text{l}/\text{mouse}$) throughout the experiment and served as carcinogen control group. **Group-II:** A single dose of DMBA (100 $\mu\text{g}/100\mu\text{l}$ of acetone) was applied topically over the shaven area of the skin of the mice and two weeks later, promoted by repeated application of croton oil (1% in 100 μl of acetone /thrice a week) till the end of experiment. These animals received PNE (1,000 mg/kg/b. wt./animal/day) by oral gavage starting from 7 days before and 7 days after DMBA application and served as PNE treated Experimental- 1 or peri-initiational group. **Group-III:** Animals in this group received the same treatment as for group I and also received PNE (1,000 mg/kg/b. wt./animal/day) by oral gavage, starting from the time of croton oil treatment and continued till the end of experiment (i.e.16 week) and served as PNE treated experimental-2 or post-initiational group. **Group-IV:** Such animals received PNE (1,000 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 to serve as the PNE treated group.

During the 16 weeks of experimentation, mice of all

groups were observed daily and weighed weekly. The mice were carefully examined once per week for the presence of skin papillomas, and these were recorded.

Detection of papillomas

Papillomas appearing on the shaven area of the skin were examined and recorded at weekly intervals in all the above groups. Only those papillomas which persisted for two weeks or more, with a diameter greater than 2 mm, have been taken into consideration for final evaluation of the data. Skin papillomas which regressed after one week observation were not considered for counting.

The following parameters were taken into consideration: *i. Cumulative number of papillomas*: The total number of papillomas appeared till the termination of the experiment; *ii. Tumor incidence*: The number of mice carrying at least one tumor, expressed as a percentage incidence; *iii. Tumor yield*: The average number of tumors per mouse; *iv. Tumor burden*: The average number of tumors per tumor bearing mouse; *v. Size of tumor*: The diameter of each tumor was measured. *vi. Weight*: The weight of the tumors of each animal at the termination of each experiment was measured. *vii. Body weight*: The body weight of the mice was measured weekly. *viii. 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 = $\sum FX/N$ (where F is the number of tumors appearing each week, X is the numbers of weeks, and N is the total number of tumors). *ix. Inhibition of tumor multiplicity* = (Total no. of tumors in carcinogen control) – (total no tumors in PNE treated) X 100/Total no of tumors in carcinogen control

Results

The different treatment schedules of PNE did not appreciably alter the mortality rate and the average body weight gain during the experimental period (Table 1). The cumulative number of papillomas induced (Figure 1a), the percentage of mice with papillomas (Figure 1b), tumor burden (Figure 1c) and tumor yield (Figure 1d) during the observation period have been depicted to compare the PNE-induced alterations on the pattern and extent of DMBA/croton oil induced skin papillomagenesis. Oral administration of PNE during peri- (Group II) and post- (Group III) initiational stages of DMBA-induced papillomagenesis, significantly reduced the cumulative numbers of papillomas to 30 and 24 respectively (carcinogen control value 62) while the tumor burden were reduced to 5 and 4.8 respectively (carcinogen control value 6.2) and tumor yield were also reduced to 3.0 and 2.4 respectively (carcinogen control value 6.2). The mice assorted in Groups II & III and given two-stage protocol for tumor induction revealed 60% (6 mice out of 10; Group II) and 50% (5 mice out of 10; Group III) skin papillomas while the respective figure for carcinogen control (10 mice

Table 1. Effects of *Phyllanthus niruri* extract on Mouse Skin Tumorigenesis

	Modulator	Treatment	Body Initiator Promoter	Body weight (g)	Tumor Size*		Tumor weight (g)
					2-5	6-9	
I	Nil	DMBA	CO	30.8±2.3	44	18	1.672
II	PNE ¹	DMBA	CO	30.9±0.2	24	6	0.808
III	PNE ²	DMBA	CO	31.9±1.4	20	4	0.646
IV	PNE	Nil	Nil	33.2±1.0	0	0	0

CO, Croton oil; ¹Peri-initiational; ²Post-initiational; *mm

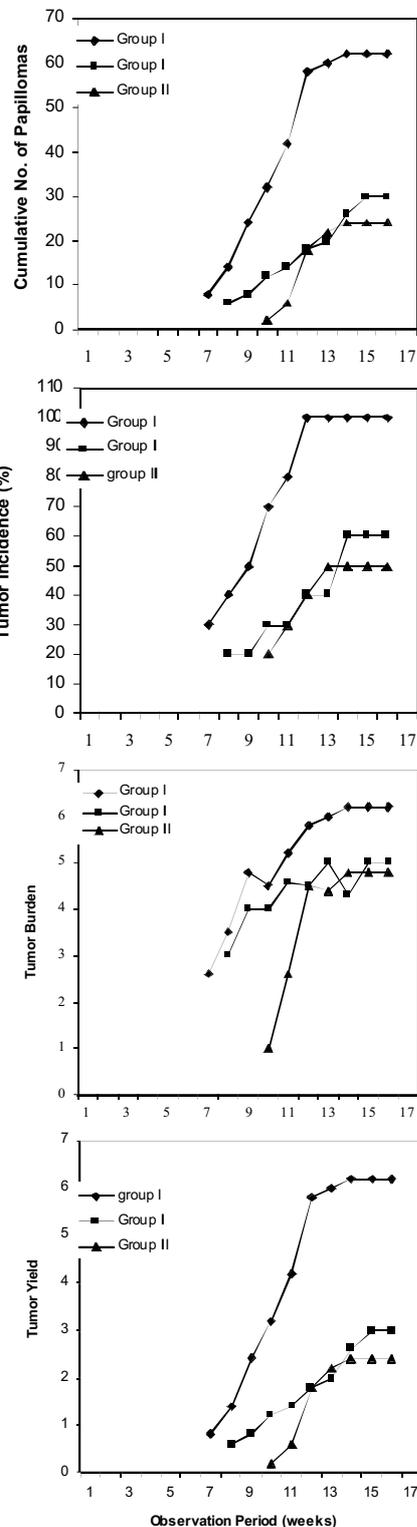


Figure 1 Tumor Data over Time. a) Cumulative number of papillomas; b) Incidence; c) Burden; d) Yield; Groups I-III as in the Materials and Methods

out of 10; Group I) was 100%.

In the carcinogen control group (Group I), the first tumor appeared at week 7 whereas in the PNE treated experimental Group II and III, the appearances of tumors were considerably delayed to weeks 8 & 10 respectively. The average latent period in the PNE treated experimental groups (Group II & III, 9.53 & 10 weeks respectively) was significantly higher than the carcinogen control group (Group I, 7.93 weeks).

The maximum inhibition of multiplicity of papillomas was evident in Group III (61%) in which the animals received PNE by oral gavage, starting from the time of croton oil treatment and continued till the end of experiment (i.e. 16 week) followed by Group II (48%) where the animals received PNE by oral gavage starting from 7 days before and 7 days after DMBA application. The weight of tumors was also significantly reduced to 0.80 gm. (Group II) and 0.64 gm. (Group III) as compared to weight 1.67 gm of Carcinogen control (Group I) (Table 1). Morphologically, tumors of the animals of the carcinogen control group were larger in size, darker in colour and more in number than in the PNE administered mice (Group II & III). No skin papillomas appeared in the animals orally treated only with PNE (Group IV) during the entire observation period.

Discussion

Substantial epidemiological data on population indicate an association between many human cancers and lifestyle or diet. Moreover, detailed studies of mutational events in human cancers have provided evidence for a direct action of environmental carcinogens in the development of certain cancers (Migliore and Coppede, 2002). This loss of genetic integrity may result due to several cumulative factors including exposure to harmful UV-radiation, potent mutagens and carcinogens, like genotoxic polyaromatic hydrocarbons (PAH), in humans and in experimental animal models (Hopkins et al., 2004; Das et al., 2005). Therefore, a new science of chemoprevention has appeared as an attractive alternative to control malignancy (Kapadia et al., 2000). This is a pharmacological approach to intervention in order to arrest or reverse the process of carcinogenesis (Azuine, 1998; Sporn and Suh, 2000).

A large number of agents including natural and synthetic compounds have been identified as having some potential cancer chemopreventive value (Kelloff, 2000), inhibiting mutagenesis, hyperproliferation or induce apoptosis or differentiation, which are critical characteristics of chemoprevention. Similarly, the results obtained in the present study clearly indicate that oral administration of *P. niruri* during initiational as well as tumor promotional stage of papillomagenesis significantly reduce the occurrence of skin papillomas induced by DMBA-croton oil in mouse skin after 16 weeks without causing any toxic effects. Extract of *P. niruri* appreciably decreased tumor burden by 19-22%, the cumulative number of papillomas by 51-61% and the incidence of skin papillomas by 40-50% in the PNE treated experimental groups (Groups II & III) as compared to the

carcinogen treated control (Group I).

Earlier studies have shown that a variety of plants exhibit chemoprotective properties by disrupting the different stages of multi step skin carcinogenesis, especially tumor promotion (Javed et al., 1998; Zhao et al., 1999). Therefore, it can be presumed that *P. niruri* may act in the similar way as it increased the average latency period of tumor occurrence by 16-20% thereby prolonging the promotional stage by delaying tumor formation and reducing the number of tumors in mouse skin as compared to the carcinogen treated control.

The chemopreventive effects of *P. niruri* might be attributed to the presence of two potentially strong chemopreventive agents - quercetin and rutin. Such components have demonstrated chemopreventive activity in a variety of laboratory animal models, including azoxymethane (AOM) induced colonic tumorigenesis in mice and rats (Deschner, 1991; Tanaka, et al., 1999), dimethylbenz-(a)-anthracene (DMBA) and N-nitrosomethyl treated mammary glands of rats (Verma, et al., 1988) and DMBA treated skin (Nishino et al., 1984). Since quercetin was shown to be an effective protein kinase inhibitor (Nagasaki and Nakamura, 1998; Noonberge and Benz, 2000), an alternative possibility is that the death inducing action of this compound resides, at least partly, in promoting dephosphorylation of key protein involved in the control of apoptotic events. Generally, the growth rates of pre-neoplastic or neoplastic cells outpace that of normal cells because of malfunctioning or dysregulation of their cell-growth and cell-death machineries (Jacks and Weinberg, 2002). Therefore, induction of apoptosis or cell cycle arrest by dietary chemopreventive compounds can be an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically damaged, pre-initiated or neoplastic cells from the body. Many dietary chemopreventive agents, including retinoic acid, phenylethyl isothiocyanate (PEITC), sulforaphane, curcumin, EGCG, apigenin, quercetin, chrysin, silibinin, silymarin and resveratrol elicit their inhibitory effect on carcinogenesis through the induction of apoptosis (Hu and Kong, 2004).

Plant extracts that contain several pluriphar-macological compounds have been reported to act on multiple molecular and cellular targets, and such approach is gaining support to fight cancer. Among them *Phyllanthus niruri* is a plant having a wide and complex spectrum of phytochemicals. The results from previous study have revealed that the many active bioconstituents of *P. niruri* constitute potential qualities in its curative action (Calixton et al., 1998). Most common active compounds are lignans like phyllanthin and hypophyllanthin (Somanabandhu et al., 1993), flavonoids like quercetin, astragalins (Nara et al., 1977), ellagitannins like amaric acid (Foo, 1995) as well as amarins (Foo, 1993) and phyllanthin D (Foo and Wong, 1992).

It has been hypothesized that the antioxidant properties of flavonoids and other phytochemicals (Kandaswami and Middleton, 1994) may protect tissues against oxygen derived free radicals and lipid peroxidation which might be involved in several pathological conditions such as

atherosclerosis, cancer and chronic inflammation (Halliwell, 1994). Since *Phyllanthus niruri* has potent free radical scavenging activity and could scavenge superoxides and hydroxyl radicals and can inhibit lipid peroxides (Joy and Kuttan, 1995). Similarly, results of present study showed significant increase in GSH, catalase level and significant reduction in lipidperoxidation in liver and skin of PNE administered groups (unpublished data). Therefore, it may be possible that flavonoids and other phytochemicals present in *P.niruri* as antioxidants might contribute to the prevention of free radical generated diseases like cancer.

Tannins are also plant polyphenols, comprising a heterogeneous group of compounds as active constituents, are responsible for many pharmacological activities (Haslam, 1996). An increasing body of experimental evidence supports the hypothesis that tannins exert antioxidant and anticarcinogenic activity in chemically induced cancers in animal models (Chung, et al., 1998). Hydrolysable tannins purified from *Phyllanthus niruri* were found to be potent inhibitors of rat liver cyclic AMP-dependent protein kinases. It also showed anti-genotoxic properties (Gowrishankar and Vivekanandan, 1994). Gallic acid and ellagic acid found in *P. niruri* have also been reported to have antioxidant activity and cancer chemopreventive properties (Singh et al., 1999).

A study documented that *P.niruri* increased the life span of mice with liver cancer from 33 weeks to 52 weeks (Rajeshkumar et al., 2000). Another research group tried to induce liver cancer in mice that had been pretreated with a water extract of *P. niruri*. Their results indicated the *P. niruri* extract dose dependently lowered tumor incidence, levels of carcinogen-metabolizing enzymes, levels of liver cancer markers, and liver injury markers. (Jeena et al., 1999). Both studies indicate that the plant has more of a protective and anti-proliferative effect against cancer than a direct anti-tumor effect or selective ability to kill a cancer cell.

Although the insights made in the present study provide a very small aspect of the modulation of the carcinogenesis process yet it can be concluded that *P. niruri* acts as a modulator of two stage skin carcinogenesis in Swiss albino mice since it prolongs the formation of tumors in skin, decrease the tumor multiplicity and yield, elicited by DMBA/croton oil. The anti-carcinogenic function of *P. niruri* might be attributed to a combination of its cytoprotective effect on normal cells and cytotoxic effect on pre-neoplastic and/or neoplastic cells. Since *P. niruri* is a complicated phytochemically rich plant, it can be suggested that the cumulative/synergistic effects of phytochemicals present in this plant including antioxidants, flavonoids and tannins etc. may be the underlying principle behind the protective potential of *P.niruri*. The present work demands further investigation for its possible use as chemopreventive agent against other types of tumors also.

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