PUBLICATIONS
Chemopreventive action of *Phyllanthus urinaria* Linn on DMBA-induced skin carcinogenesis in mice

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The inhibition of tumor incidence by hydro-alcoholic extract of the whole plant of *P. urinaria* was evaluated in 6-7 weeks old female albino mice on two-stage process of skin carcinogenesis induced by a single application of 7,12-dimethylbenz(a)anthracene (50 μg/50 μl of acetone), and 2 weeks later, promoted by repeated application of croton oil (1% in acetone/three times a week) till the end of the experiment (15 weeks). Topical application of the extract at a dose of 5mg/kg body weight/day for 15 weeks at the peri-initiational stage (i.e., 7 days before and 7 days after DMBA application), promotional stage (i.e., from the time of croton oil application) and both peri and post-initiational stages (i.e., 7 days prior to DMBA application and continued till the end of the experiment) on the shaved backs of the mice recorded a significant reduction in tumor incidence to 50.33, 33.3 and 16.7% respectively in comparison to the control (i.e., the mice treated with DMBA and croton oil only) where tumor incidence was found to be 81.8%. The average number of papillomas per mouse was also significantly reduced. The results suggest a possible chemopreventive property of *P. urinaria* against DMBA-induced skin papillomagenesis in mice.

Keywords: Carcinogenesis, Chemoprevention, *Phyllanthus urinaria*

The medicinal herb *Phyllanthus urinaria*, Linn (Fam: Euphorbiaceae) is common in central and southern India extending to Ceylon, distributed throughout the plains from Punjab to Assam. It is noted for its effectiveness in stimulating sluggish liver and is a therapy for regeneration of liver tissue in jaundice. The plant is also considered to be useful in dropsical gonorrhoea and other urinogenital troubles like menorrhagia. Leaf decoction is stomachic and employed in dispesia and dysentery. Unander et al. have reported absence of toxicity in *Phyllanthus* spp. and stated that LD₅₀ > 1 g/kg body weight in mice. *P. urinaria*, used widely as a healing agent and also against skin diseases was tried in the present study for its effectiveness as a chemopreventive agent against DMBA-induced skin carcinogenesis in mice.

Animals — Female Swiss albino mice of random breed, 6-7 weeks old were used. The animals were obtained from the animal house of Biotechnology Department, Gauhati University and housed under normal condition having natural photoperiod (12 hr light/dark cycle) at 25±1°C and 50-60% RH. They were provided with standard pellet diet and tap water *ad libitum*, under hygienic conditions. Two animals were kept per cage. Sixty animals were taken for investigation at the beginning of the experiment. Three days before the onset of the experiment, the hair on the interscapular region of the mice were clipped and the resting phase of the hair growth cycle observed. Only 48 mice showing no hair growth were considered for the experiment. Weekly body weight of the mice was recorded to keep a constant vigil on the health of the animals.

**Chemicals** — The carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), and croton oil were procured from Sigma Chemicals Co., St. Louis, USA. DMBA was dissolved at a concentration of 50 μg/50 μl in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

**Preparation of *P. urinaria* extract** — The plant, *P. urinaria* was collected locally from various parts of Guwahati after proper identification by a competent botanist. The whole plant, including roots was washed and dried in shade without direct exposure to sunlight. It was then ground and material of known weight (50g) was subjected to soxhlet extraction using 300ml of hydro-alcoholic solvent (80% ethanol: 20%, distilled water). The process was repeated three times with fresh material of the same amount. The alcohol was allowed to evaporate and the residue obtained...
was stored at 4°C. The yield was 14.98%. The required dose for treatment was prepared by diluting the residue in acetone at a dose level of 5 mg/kg body weight at par with the doses of initiator and promoter concentrations. The aliquot obtained was a fine homogenous suspension in acetone.

Experimental design — Mice were divided into the following 4 groups of 12 each:

Group I: A single dose of 50μg of DMBA in 50μl acetone was applied topically over the shaven area of the skin of mice. Two weeks later, croton oil (100μl of 1% croton oil in acetone) was applied three times per week until the end of the experiment (15 weeks).

Group II: Animals received a topical treatment (on shaven area of the skin of the mice) of an ethanolic extract of the whole plant of *P. urinaria* (5mg/kg body weight/day) in 100μl acetone for 14 days i.e., 7 days before and 7 days after the application of DMBA. Croton oil was given as in Group I. The experiment was carried out for 15 weeks.

Group III: Animals received a topical treatment of *P. urinaria* extract (5mg/kg body weight/day) in 100μl acetone, starting from the time of croton oil treatment and continued till the end of the experiment (15 weeks). DMBA was given as in Group I.

Group IV: Animals were treated topically with *P. urinaria* extract continuously for 15 days (i.e., 7 days prior to DMBA application and continued till the end of the experiment) and also at the promotional stage. Croton oil was given as in Group I. The experiment was carried out for 15 weeks.

Papillomas appearing on the shaven area of the skin were recorded at weekly intervals. Only those papillomas that persisted for two weeks or more were considered for final analysis of the data. All the animals were sacrificed by cervical dislocation at the end of the experiment. The differences in the incidence of tumors among different groups were found to be significant at 5% probability level when evaluated by Chi-square test.

Results are presented in Table 1.

The application of the plant extract did not affect the body weight of the animals during the experimental period. In Group I (control group), treated with a single dose of DMBA and two weeks later promoted by repeated application of croton oil, only 9 out of 11 affected mice developed skin tumors (81.8%). The average number of papillomas per mouse was 4.45 and the papillomas per papilloma-bearing mouse was 5.44. On the other hand, in animals of Group II, the percentage of tumor incidence, the average number of papillomas per mouse and papillomas per papilloma-bearing mouse were found to be 50, 1.25 and 2.5% respectively. In Group III, the percentage of tumor incidence, the average number of papillomas per mouse and papillomas per papilloma-bearing mouse were found to be 33.3, 0.5 and 1.75% respectively, when the mice received the application of the extract for 91 days (i.e., from the time of application of croton oil). When the mice of Group IV were treated with the *P. urinaria* extract continuously for 15 days (i.e., 7 days prior to DMBA application and continued till the end of the experiment) the percentage of tumor incidence, the average number of papillomas per mouse and papillomas per papilloma-bearing mouse were recorded to be 16.7, 0.25 and 1.5% respectively. The differences in the values of the results of groups II, III and IV were statistically analysed and found to be significant in comparison to the control group (P < 0.05).

Cumulative number of papillomas and percentage inhibition of tumor multiplicity in control and experimental groups during the observation period have been shown in Figs 1 and 2 respectively.

Chemoprevention is an important strategy to control the process of carcinogenesis. Thus, there is a need for exploring medicinal plants or other natural agents which act as chemopreventive agents. The present investigation demonstrates the chemopreventive

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of animals</th>
<th>Body weight(g) [Mean ± SD]</th>
<th>Papillomas per papilloma-bearing mouse</th>
<th>Mice with ( % )</th>
<th>Av. number of papillomas per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Effective</td>
<td>Initial</td>
<td>Final</td>
<td>5.4</td>
</tr>
<tr>
<td>I</td>
<td>12</td>
<td>11</td>
<td>13.27 ± 2.86</td>
<td>18.5 ± 4.1</td>
<td>2.5*</td>
</tr>
<tr>
<td>II</td>
<td>12</td>
<td>12</td>
<td>14.08 ± 2.24</td>
<td>16.7 ± 4.09</td>
<td>1.75*</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>12</td>
<td>14.3 ± 2.5</td>
<td>17.33 ± 2.49</td>
<td>1.5*</td>
</tr>
<tr>
<td>IV</td>
<td>12</td>
<td>12</td>
<td>14.8 ± 2.37</td>
<td>18.0 ± 1.5</td>
<td>1.5*</td>
</tr>
</tbody>
</table>

*P < 0.05
potential of *P. urinaria* extract on DMBA-induced skin tumorigenesis in female Swiss albino mice. Berenblum and Shubik suggested that one submininal dose of carcinogen initiates tumorigenesis and the treatment with croton oil promotes them to visible tumor stage. The present findings exhibited the same with 81.9% tumor incidence, an average number of 4.45 papillomas per mouse and 5.44 papillomas per papilloma-bearing mouse in group I (control group). This is perhaps due to the rapid accumulation of inflammatory cells such as neutrophils and macrophages, and increased formation of reactive oxygen species and other free radicals leading to skin tumor promotion. Moreover, skin tumor promoters, mediates the induction of epidermal ornithine decarboxylase, the rate limiting enzymes in the biosynthesis of polyamines which appear to be a pre-requisite for cell proliferation, differentiation and neoplastic transformation. On the contrary, the mice of Group II, III and IV receiving similar treatment of DMBA and croton oil, when subjected to a topical application of the hydro-alcoholic extract of *P. urinaria*, a significant reduction (p<0.05) in tumor incidence, average number of papillomas per mouse and papillomas per papilloma-bearing mouse were recorded (Table 1 and also Figs 1 and 2). The cumulative number of papillomas (Fig. 1) was found to be reduced in the *P. urinaria* extract-treated groups (II, III and IV) when compared to the control mice. The present study also showed an increase in the percentage inhibition of tumor multiplicity in the *P. urinaria* extract-treated groups (group II, III and IV) in comparison to the control mice (Fig. 2). The property of *P. urinaria* used as a healing agent and also against skin diseases leads to the supposition that the plant extract may have either acted as an anti-inflammatory agent inhibiting DMBA-induced skin papillomagenesis or inhibited the epidermal ornithine decarboxylase, thus reducing the percentage of tumor incidence, average number of papillomas per mouse and papillomas per papilloma-bearing mouse. Similar reduction of tumorigenesis due to the inhibition of epidermal ornithine decarboxylase, epidermal DNA synthesis and promotion of skin tumors by eucurmin have been reported by Mou et al.

Further, it is suggested that aryl hydrocarbon hydroxylase enzyme system leads to the formation of active chemical carcinogens that may characteristically interact with DNA to produce mutations or increase release of reactive oxygen species by the promoters that may induce strand breaks in DNA leading to mutagenic and carcinogenic effects. The present study is inadequate to claim the involvement of aryl hydrocarbon hydroxylase in the process of tumor formation in mouse skin. However, it is presumed that if there is any involvement of aryl hydrocarbon hydroxylase in the process of tumor formation in mouse skin, the inhibition of these pathways by *P. urinaria* may have caused reduced tumorigenesis in groups II, III and IV, forming innocuous products in nucleophilic chemical reactions. Similar inhibition of aryl hydrocarbon hydroxylase enzyme system in rats by the oral administration of garlic oil has been reported by Siddiqui and Pawar. This work demands additional study to evaluate the exact mechanism of chemoprevention of carcinogenesis by *P. urinaria*. Further studies on hepatic detoxifying and antioxidant enzymes by the extract of this medicinal plant in mice are in progress.

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