ANTITUMOUR EFFECT OF PHYLLANTHIN AND HYPOPHYLLANTHIN FROM PHYLLANTHUS AMARUS AGAINST EHRLICH ASCITES CARCINOMA IN MICE.

Aminul Islam¹,²*, Thamil Selvan¹, U.K. Mazumder¹, M. Gupta¹, Shibnath Ghosal³

¹Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India.
²Natreon Inc, R&D Centre, CL -18A, Salt Lake City, Kolkata-700091.
³Research Adviser, R&D Centre, Indian Herbs Ltd. Saharanpur-24700(U.P).

*Corresponding Author’s contact id: aminulislamju@gmail.com

Summary

A mixture (1:1) of Phyllanthin and Hypophyllanthin isolated from Phyllanthus amarus (P. amarus) exhibited antitumor activities against Ehrlich Ascites Carcinoma in Swiss albino mice. Animals were pre-treated orally with the extract at a dose of 25mg/kg, 50 mg/kg, 100mg/kg body weight and then after 24hrs after EAC (at a dose of 2 x 10⁶ cells /mouse) administration and following an 18hr fasting; mice were sacrificed for studying of antitumor activity. The decrement of tumor volume, and packed cell volume and viable cell count were observed in lignans treated mice when compared only to EAC tumor bearing mice. Treatment with test compounds increased the survival time and normal peritoneal cell count. Hematological parameters, PCV which were altered by tumor volume inoculation, were restored considerably. Thus, this study was an attempt to evaluate the preventive and curative role of P. amarus lignans in tumor bearing mice.

Keywords: Ehrlich Ascites Carcinoma, Phyllanthus amarus, lignans, phyllanthin, hypophyllanthin

Introduction

Cancer is the leading cause of mortality worldwide and most of the chemotherapeutic agents have been reported to exhibit sever normal tissue toxicity, accompanied by undesirable side effects. Moreover these drugs are highly expensive mutagenic and carcinogenic . Therefore the substitute of the conventional chemotherapeutic agents to control the high mortality rate are needed which will be highly effective at non toxic doses and inexpensive and accessible to general people¹. This can be achieved by in-depth research and continuous screening of new molecules or natural agents which will provide the anti-tumor activity in Indian traditional system of medicine (Ayurvedic system) uses plant derived medicines in health care from ancient period of time.
These natural medicines have played a great role to treat various disorders in humans including cancer\textsuperscript{2,3}. Recent surveys indicate that plants, vegetables and herbs used as folk and traditional medicine have been accepted widely as a major resources of chemo preventive agents\textsuperscript{4}. \textit{Phyllanthus amarus} Schum Thonn. (Syn \textit{P.niruri}) is a small herb belong to the family Euphorbiaceae, popularly known as Bhuin Amla (Hindi, Bengali) or Chanca piedra (Spanish) means “stone breaker” or shatter stone. It grows throughout the world\textsuperscript{5} but typically is found in the drier climates in India, Brazil and even Florida, Texas. \textit{P.amarus} has long history in traditional system of medicine\textsuperscript{6,7} in every tropical country where it grows and well known for the biologically active compounds it possesses\textsuperscript{8}. Particularly it has anti-viral activities\textsuperscript{9}. The extract of \textit{P.amarus} has been used to inhibit DNA polymerase of hepatitis B virus and other related hepatitis virus\textsuperscript{10-17}. \textit{P.amarus} extract has been shown to minimize the transcription and translation process of hepatitis B virus mRNA and also suppressed cell line. It is also reported that the aqueous extract of \textit{P.amarus} has potent anticarcinogenic activity against 20-methylcholanthrene induced sarcoma development. \textit{P.amarus} also used for the treatment of diabetes\textsuperscript{18-21} and dropsy\textsuperscript{22}. Several biologically active compounds of \textit{P.amarus} have been isolated and mainly include lignans\textsuperscript{23,24}, flavonoids ,gallotanoids\textsuperscript{25} etc. Phyllanthin and hypophyllanthin are the major lignans in \textit{P.amarus}. The proper studies focusing on the antitumor activity only of Phyllanthin and hypophyllanthin \textit{in-vivo} are lacking. Therefore this present study was undertaken to evaluate the antitumor activity of Phyllanthin and hypophyllanthin lignans of \textit{P.amarus} against EAC in Swiss albino mice.

Materials and Methods

**Test Compounds:**
\textit{Phyllanthus amarus} was collected in and around Salt Lake, Kolkata in the month of Jun-July 2005 and authenticated Botanical Survey of India, Shibpur, Howrah West Bengal India. A voucher specimen [(No.CNH/I-I)(73)2005 Tech II./966] has been kept in our Laboratory for further reference.

The two major lignans, Phyllanthin and hypophyllanthin were isolated from \textit{Phyllanthus amarus} and used for the present investigation.

The total plant was pulverized after shade dry and powdered (particle size ~ 0.25 mm) using a laboratory mill. The powered material of the whole plant were extracted with methanol (yield 12\%) in a soxhlet apparatus. The solvent was evaporated under reduced pressure at 40\°C dried in vacum. The dried extract thus obtained was subjected to column chromatography for isolation of phyllanthin and hypophyllanthin, the two major lignan of \textit{Phyllanthus amarus}.

**Animals:**
Male Swiss albino mice of about 8 weeks of age with an average body weight of 20 ± 2 g were used for the study. The animals were obtained from Indian Institute of Chemical Biology (IICB), Kolkata, India. They were housed in standard environmental conditions of temperature, humidity and under clear and dark cycles of 12-h. The mice were fed standard laboratory diet (HLL, India) and were given sterilized water \textit{ad libitum}.

**Ethical clearance:** The Institutional Animals Ethical Committee approved the protocol used in this experiment.
Chemicals:
Analytical grade reagents were used for this experiment. Bovine serum albumin (BSA) was procured from Sigma Chemicals, Co. USA. Nitrozolium (NBT), Thiobarbuturic acid (TBA), Phenazonium metho sulphate and Nicotinamide adenine were purchased from Loba Chemie, Bombay, India. 5,5’-dithio bis 2-nitrobenzoic acid (DTNB); Folin-ciocalteau phenol and reduced Glutathione were procured from SRL, India.

Vehicle:
CMC was used for drug administration via oral route.

Doses:
Both of the lignanas Phyllanthin and hypophyllanthin were mixed in equal proportion and at the doses of 25 mg and 50 mg /kg body weight were administered for 14 days from day 1 to 14.

Tumor Cells:
The transplantable tumor cells namely Ehrlich Ascites Carcinoma (EAC) cells were used in the present study. The cells were obtained through the courtesy of Chittaranjan National Cancer Institute (CNCI), Kolkata (WB), India. The EAC cells were maintained in vivo in Swiss albino mice, by interperitoneal (ip) transplantation of 2 x 10^6 cells /mouse after every 10 days. EAC cells 9 days old were used for the experiment. These tumors in the ascites form are fast growing and kill the host animals within the period of three-four weeks approximately after tumor transplantation with 2x10^6 cells. The tumor uptake by the host was manifested by a very high initial growth rate followed by exponential growth up to a period of 10th day post transplantation (log phase) followed by gradual decline in this growth rate with progressive accumulation of ascites fluid (lag phase). By the 21st day post transplantation, the tumor volume reach its maximum. The tumor bearing mice survive 20-26 days after tumor transplantation.

Antitumor activity:
Male Swiss albino mice were divided into 5 groups (n=12). The EAC cells (200µl of 2x 10^6 cells /mouse) were injected to all the groups interperitoneally except the normal group. This was considered as zero day. On the first day normal saline (5ml/kg/day/mouse) was administered in group 1 (normal). Propylene glycol (5ml/kg/day/mouse) was administered in group 2 (EAC control). Lignans of two different doses (25 mg, 50mg/kg and 100mg/kg /day) and the standard drug 5-fluorouracil were administered in groups 3, 4 and 5 respectively for 14 day interperitoneally. After the application of last dose which was followed by 18h fasting 6 mice from each group were sacrificed for the study of antitumor activity, hematological parameters. The rest of the animal groups were kept to check the survival time of EAC tumor bearing hosts.

The tumor growth response of *P. amarus* lignans was measured in EAC animals with respect to the following parameters such as –

1. Tumor volume:
2. Tumor Cell Count:
3. Viable/non-viable tumor cell count:
4. Mean survival time and Percentage increase in life span (% ILS):
5. Body weight:
6. Hematological Parameters
Tumor Volume: The ascetic fluid was collected from the peritoneal cavity of the dissected mice. The fluid volume was calculated in a graduated centrifuge tube and the Packed Cell Volume (PCV) was measured after 5 min centrifugation at 1000 rpm.

Total Cell Count:
The collected ascetic fluid was diluted 100 times by taking in at the 64 small squares was counted by Neubeauer counting chamber.

Viable – Nonviable tumor cell count: The trypan blue dye (0.4% in Normal Saline) was used for identifying the viable and non-viable cells. Viable cells were negative to the dye but the non-viable cells were taken the stain. This behavior of the cells was recorded.

\[
\text{Cell count} = \frac{\text{(no. of cells x Dilution)}}{\text{(Area x Thickness of liquid film)}}
\]

Percentage of Increased Life Span:
The efficacy of the lignan on the tumor proliferation was observed by recording the mortality daily. This observation was conducted for a period of 6 weeks and percentage increase in life span \(^{27, 28}\) (% ILS) was measured

\[
\text{% ILS} = \frac{\text{(Mean survival of treated group) x 100}}{\text{(Mean survival of control group)}}
\]

\[
\text{Mean survival} = \frac{\text{(Day of 1st death + day of last death)}}{\text{2}}
\]

Body weights:
During the period of treatment the body weights of the experimental mice were recorded in all (treated and control) groups at the “0” day (beginning of the experiments) and sequentially on every 5th day

Hematological Parameters:
At the end of the experimental period, all the mice were sacrificed after 18 h of fasting by decapitation. Blood was collected from freely flowing tail vein and used for the estimation of Hemoglobin (Hb) content \(^{30}\), red blood cell (RBC) count \(^{31}\) and white blood cell (WBC) cell count. WBC differential count \(^{32}\) was carried out from Leishman stained blood smears.

Estimation of Hemoglobin:
0.1 ml of heparinized blood was taken in Sahli’s hemoglobinometer and diluted with 0.1 N HCl until the color matched with standard. The reading was then taken from the graduated cylinder and expressed as mg/100 ml of blood.

Estimation of Erythrocyte (RBC) count:
For the erythrocyte (RBC) counts, blood samples were diluted 1:200 with the Rees-Ecker diluting fluid containing Sodium citrate (3.8g), HCHO (0.2 ml) and Brilliant Cresyl Blue, volume made up with 100 ml of sterile distilled water.

Blood samples were drawn by the fine tips (capacity 20 µl attached to an auto adjustable micropipette transferred to the micro centrifuge tubes containing the dilution fluid. The tubes were mixed thoroughly and a drop of the resultant suspension was discharged under the cover glass of an improved Neubuer bright field hemocytometer and the corpuscles were allowed to settle. The number of erythrocytes in 80 small squares was counted.
under light microscope. The number of cells in 1µl of undiluted blood was calculated
following the standard formula.
Erythrocyte count = N x 1/0.02 x 200.
N x 10,000.Where N = no. of cells in 80 small squares i.e 0.02 µl.

Results

Short term toxicity studies: For the determination of short term toxicity, healthy Swiss
albino mice were treated interperitonealy. The single dose of the test compounds was
administered at doses of 25 and 50 mg/kg. The mice which were received 100mg/kg or
above showed toxic effect. These symptoms are inactiveness in general behavior slow
movement, loss of appetite, hypothermia, erected hairs etc.
Intraperitoneally administration of the drug daily at doses of 25, 50 and 100 mg/kg for 14
days consecutively did not affect the body weight of mice. The weights of the vital
organs (liver, kidney, brain, spleen) were also not altered by the treatment of this test
compounds.
Hematological parameters like Hb, RBC and WBC count (Fig.1), Serum glutamate
pyruvate transaminase (SGPT) and Serum glutamate oxlaoacetate transaminase (SGPT)
urea remain unaltered (Fig.2).
The antitumor activity of PAL was assessed by different hematological parameters. These
are tumor volume, total cell count (viable and non-viable), packed cell volume, mean
survival time and % Increased Life Span . The result is shown in table 1. The above
mentioned parameters like tumor volume, packed cell volume viable cell count were
found to be increased significantly (p<0.05) and non-viable cell count was significantly
(p<0.05) low in EAC control animals when compared with normal control animals.
Treatment with *P.amarus* lignans at the dose of 25 and 50 mg/kg significantly (p<0.05)
decreased the tumor volume viable cell count and packed cell volume. Non-viable cell
also significantly (p<0.05) higher in *P.amarus* lignans treated mice when compared with
EAC control animals.
Furthermore, the mean survival time was enhanced to 43.08 ±1.22 (% ILS =123.91) and
45.42 ±1.37 (% ILS =136.07) after administration of *P.amarus* lignans at 25mg and 50
mg /kg respectively (Table.1). Finally the change in body weight of the mice indicates the
tumor growth inhibiting action of *P.amarus* lignans (Table.2). Total bilirubin and total
protein also restored after treatment (Fig.3). All the above results clearly suggest that the
*P.amarus* lignans has remarkable effect to decrease the growth of solid tumor mass
induced by EAC cells in a dose-dependent manner in mice.

Hematological parameters:
Hematological parameters of EAC infected mice on day 14 were found to be remarkably
altered when compared to normal mice. The WBC count was increased but the Hb
percentage were found to be decreased with the modest change of the total number of
RBC. Treatment with phyllanthin and hypophyllanthin at the dose of 25 , 50 mg/kg and
100mg/kg changed these altered parameters more less normal values.
Fig 1. Effect of Phyllanthin and Hypophyllanthin lignans from *Phyllanthus amarus* on haematological parameters in Erlich’s ascites carcinoma bearing mice.

*\(p<0.05\) when compared to EAC control group where significance was performed by ANOVA followed by Dunnett’s test (n=6).

Fig 2. Effect of Phyllanthin and Hypophyllanthin lignans from *Phyllanthus amarus* on SGOT, SGPT and SALP level in Erlich’s ascites carcinoma bearing mice.

*\(p<0.05\) when compared to EAC control group where significance was performed by ANOVA followed by Dunnett’s test (n=6).
Fig 3. Effect of Phyllanthin and Hypophyllanthin lignans from *Phyllanthus amarus* on total bilirubin and total protein level in Erlich’s ascites carcinoma bearing mice.

Table 1. Effect of Phyllanthin and Hypophyllanthin lignans from *Phyllanthus amarus* on body weight in Erlich’s ascites carcinoma bearing mice. (Values are mean±SEM; six mice in each group)

<table>
<thead>
<tr>
<th>Groups</th>
<th>% change of body wt after Day 3</th>
<th>% change of body wt after Day 6</th>
<th>% change of body wt after Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (0.9% NaCl;10ml/kg)</td>
<td>0.87±1.25</td>
<td>5.47±2.32</td>
<td>7.70±2.49</td>
</tr>
<tr>
<td>EAC control (0.9%NaCl;10ml/kg)</td>
<td>5.86±0.92</td>
<td>11.69±1.09</td>
<td>20.79±1.56</td>
</tr>
<tr>
<td>EAC+Phyllanthin (50 mg/kg)</td>
<td>1.70±1.36</td>
<td>2.00±2.41</td>
<td>3.04±3.54</td>
</tr>
<tr>
<td>EAC+Phyllanthin (200 mg/kg)</td>
<td>1.55±1.43</td>
<td>-0.53±2.18</td>
<td>0.92±1.84</td>
</tr>
<tr>
<td>EAC+Hypophyllanthin lignans (500 mg/kg)</td>
<td>0.77±1.19</td>
<td>0.88±1.60</td>
<td>2.30±2.46</td>
</tr>
<tr>
<td>EAC+5-Fluorouracil (20 mg/kg)</td>
<td>-2.57±0.82</td>
<td>-4.45±1.13</td>
<td>-5.12±0.73</td>
</tr>
</tbody>
</table>

Percentage Change of body weight of animals in different intervals (n=6) calculated by compared the day 0 of the experiment; + indicates increase percentage change; - indicates decrease percentage change.
Table 2. Effect of Phyllanthin and Hypophyllanthin lignans from *Phyllanthus amarus* on mean survival time, ILS, tumor volume, packed cell volume, percentage of viable and nonviable tumor cell count in Erlich’s ascites carcinoma bearing mice. (Values are mean SEM, 6 mice in each group)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean survival time (days)</th>
<th>% Increase of life span (ILS)</th>
<th>Tumor volume (ml)</th>
<th>Packed cell volume (ml)</th>
<th>Percentage of Viable cells (%)</th>
<th>Percentage of Non-viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAC control (0.9%NaCl;10ml/kg)</td>
<td>19.24±0.86</td>
<td>10.68±0.26</td>
<td>2.523±0.05</td>
<td>91.87±0.49</td>
<td>8.13±0.49</td>
<td></td>
</tr>
<tr>
<td>EAC+Phyllanthin (50 mg/kg)</td>
<td>45.42±1.37**</td>
<td>136.07</td>
<td>3.85±1.44**</td>
<td>0.64±0.07**</td>
<td>70.14±4.42*</td>
<td>29.86±4.42*</td>
</tr>
<tr>
<td>EAC+Phyllanthin (200 mg/kg)</td>
<td>43.08±1.22**</td>
<td>123.91</td>
<td>3.13±0.23**</td>
<td>0.75±0.03**</td>
<td>72.78±1.75*</td>
<td>27.22±1.75*</td>
</tr>
<tr>
<td>EAC+Hypophyllanthin lignans (500 mg/kg)</td>
<td>32.52±3.21*</td>
<td>69.02</td>
<td>5.62±0.56</td>
<td>1.36±0.11</td>
<td>83.55±2.15</td>
<td>16.45±2.15</td>
</tr>
<tr>
<td>EAC+5-Fluorouracil (20 mg/kg)</td>
<td>51.38±4.05**</td>
<td>167.05</td>
<td>1.93±0.79**</td>
<td>0.52±0.06**</td>
<td>56.43±5.76**</td>
<td>43.57±5.76**</td>
</tr>
</tbody>
</table>
Discussions

The present experiment was evaluated to investigate the antitumor activity of lignans of *Phyllanthus amarus* in EAC tumor infected mice. The lignans treatment of mice at the dose of 50, 100 mg/kg pronouncedly inhibited the tumor volume, packed cell volume, total cell count and also regained the hematological parameters near or near to normal conditions. The best parameters for gauging the effectiveness of any anticancer agents are the prolongation of life span of experimental animals. The reduction of tumor volume viable total cell count finally reduce the tumor burden and amplified the life span of EAC bearing mice. A regular rapid increase in ascetic fluid tumor volume was observed in EAC tumor bearing mice. The tumor cells directly draw the nutrition from ascetic fluid; it does mean the ascetic fluid continuously supply the nutritional requirement to tumor cells. Treatment with lignans suppressed the tumor volume, viable cell count and enhanced the life span of the tumor bearing mice. It can be concluded that PAL by reducing the nutritional fluid volume and inhibiting the tumor growth increases the life span of EAC –bearing mice. The major problems in the cancer chemotherapy are myelosuppression and anemia. Mainly the reduction of RBC and Hb % in tumor bearing mice lead to the formation of anemic condition and this may occur either due to iron deficiency or due hemolytic or myelopathic conditions. The treatment with *P. amarus* lignans restored the hemoglobin (Hb) count, RBC and WBC count more or less to normal levels. This findings clearly indicates that PAL posses effective protective properties on the hemopoietic system.

Acknowledgement

We thank Natreon Inc, 2D Janine Place, New Brunswick, NJ 08901, USA for financial support to carry out the study.

References


3 Huang MT, Osawa T, Ho CT, Rosen RT (eds) Food Phytochemicals for Cancer Prevention I Fruits and Vegetables ACS Symposium 547. Washington DC.


