Anticancer potential of *Polyalthia longifolia* fruits in DEN/PB induced hepatocellular carcinoma (HCC) in rats.

A.J.M.Christina1*, Jayaraman Rajangam2, Bibhu Prasad Panda1

1Taylors University, Malaysia.
2Karpagam University, Coimbatore, Tamil Nadu, India.

*Corresponding author: A.J.M.Christina,*
E-mail id: JosephineMariaChristina.Arokiasamy@taylors.edu.my

**ABSTRACT**

This study was designed to evaluate the anticancer potential of methanolic extract of fruits of *Polyalthia longifolia* (MEFPL) in N-nitrosodiethylamine (DEN) induced and phenobarbital (PB) promoted hepatocellular carcinoma (HCC) in male albino Wistar rats. A single intraperitoneal injection of N-nitrosodiethylamine (DEN, 200 mg/kg) was administered. After 14 days, phenobarbital was given orally for up to 14 weeks to promote the liver cancer. The hepatocarcinoma induced rats were treated with MEFPL (200 and 400 mg/kg) for 28 days. After the experimental period, the liver was examined for the number and size of nodules present. Also the serum level of tumour marker, Alpha-fetoprotein (AFP) and DNA and RNA content in liver were assessed. The number of nodules in liver, serum Alpha-fetoprotein, DNA and RNA content in liver were reduced by the extract. Histopathology of liver revealed improvement in the architecture that was damaged by DEN and PB. To conclude, results of present study strongly support the anticancer potential of MEFPL against DEN/PB induced hepatocarcinogenesis.

**Keywords:** *Polyalthia longifolia*, N-nitrosodiethylamine, Phenobarbital, hepatocellular carcinoma, Antioxidants.

**Abbreviations:** HCC, hepatocellular carcinoma; DEN, N-nitrosodiethylamine; P. longifolia, Alpha-fetoprotein.

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is one of the most common life threatening malignancies which accounts for nearly 85% of the primary malignant tumours of the liver and is the third most common cause of cancer related death worldwide. HCC is more common in men than women, with an incidence ratio around 3:1. Earlier studies suggested that, the incidence of HCC is multifactorial which includes infections, nutritional, metabolic, endocrine factors and exposure to hepatocarcinogens like DEN and alcohol etc.

Literature supports the use of DEN as a carcinogen and/or lesion initiator in animal models. Diethyl nitrosamine has been shown to be metabolized to its active ethyl radical metabolite, that causes DNA mutation. Literature reveals that DNA mutations
are induced by oxidative stress also. Hence plants containing antioxidant principles are being investigated against cancers resulting from DNA mutations. One such plant is *Polyalthia longifolia*. *Polyalthia longifolia* (family: Annonaceae) is a lofty evergreen tree found in India and Sri Lanka, commonly planted for its effectiveness in alleviating noise pollution. In India, the seeds and bark of the plant are used as febrifuge in the Balasore district of Orissa. The extract of stem bark of the plant and the alkaloids isolated from it have been reported for antibacterial and antifungal activities. Its aqueous extract stimulates the isolated ileum and uterus, depresses heart rate, decrease blood pressure and respiration rate in experimental animals. The crude extracts of the seeds of the plant have also shown remarkable antibacterial activities and plants of Annonaceae family contain antitumor and anti-cancer active principles. Moreover, the various parts of *Polyalthia longifolia* yielded more than twenty cytotoxic compounds along with flavonoids, triterpinoids and phenolic compounds. In view of the above, *Polyalthia longifolia* was chosen to study its anticancer role against DEN (initiator) and PB (promoter) induced HCC in rats.

**MATERIAL AND METHODS**

**Plant material**

*Polyalthia longifolia* fruits were collected from Irumbulikurichi, Ariyalur district, Tamilnadu, India and authenticated by G.V.S Murthy, botanical survey of India (BSI), southern circle, Coimbatore, Tamilnadu, India (BSI/SC/5/23/11-12/Tech-1759).

**Preparation of methanolic extract from the fruits of Polyalthia longifolia**

The chopped and shade dried fruits were powdered and passed through a 40-mesh sieve, then subjected to extraction with methanol in a Soxhlet apparatus. The solvent from the methanolic extract was completely removed and concentrated to dryness at 40°C under reduced pressure in a rotary vacuum evaporator. The *Polyalthia longifolia* fruits yielded brown semisolid residue of methanolic extract, weighing 9.0% w/w with respect to the dried starting material.

This study was carried out on adult Wistar male albino rats (150-180g). They were housed in room temperature of 25±1°C and fed with rodent pellet diet. The food was withdrawn 18-24 h before the experiment though water was allowed ad libitum. The experimental protocols were approved by institutional animal ethics committee (IAEC KMCRET/Pharm/06/2012) and conducted according to the CPCSEA guidelines for the use and care of experimental animals, New Delhi, India.

**Experimental Design**

The rats were divided into six groups, each group consisting of six animals. DEN was administered at a dose of 200 mg/kg body weight in saline to induce cancer in the animals of groups II to V. Two weeks after administration of DEN, Phenobarbital at a concentration of 0.05% was incorporated into rat chow for up to 14 weeks to promote the hepatocarcinogenesis. After the induction period, group III and group IV were treated with MEFPL orally at a dose of 200 and 400 mg/kg respectively for 28 days and group V animals received standard drug silymarin the known hepatoprotective and antihepatocellular carcinoma compound at a dose of 200 mg/kg. Finally, group VI animals which have not been treated with DEN received MEPL extract alone at a dose of 400 mg/kg, p.o to check for any adverse effect of the extract on the animals.

**Treatment Protocol**

- **Groups I** - Control- Normal saline (0.9%)
- **Group II** - DEN [200 mg/kg + PB 0.05% for 14 weeks]
- **Group III** - HCC bearing rats+ MEPL Extract 200 mg/kg.p.o for 28 Days
- **Group IV** - HCC bearing rats+ MEPL Extract 400 mg/kg.p.o for 28 Days
- **Group V** - DEN+ Sylimarin200 mg/kg
- **Group VI** - MEPL Alone 400 mg/kg.p.o for 28 Days

**Measurement of tumor marker-AFP**

At the end of week 20 starting from the first day of DEN administration, blood was collected from retro orbital plexus and serum was separated for the
estimation of Alpha-fetoprotein (AFP). Serum AFP was estimated using ELISA kit supplied by Coral Clinical Systems, India.

Morphological observation of liver
After collection of blood, the animals were sacrificed by cervical dislocation and the abdominal cavity of rats was dissected immediately and the liver was rapidly removed, washed in ice-cold saline, weighed and blotted dry. Weight of liver was also noted. The weight of liver relative to body weight was also calculated. Also the livers were morphologically observed for the presence of nodules and the number and size of the nodules were recorded.

Estimation of nucleic acid from liver
The amount of nucleic acids viz DNA and RNA of liver were estimated following standard methods.

Statistical analysis
Data were expressed as the mean ± S.E.M. Means were compared by one way analysis of variance (ANOVA) followed by Dunnett test. A value of p < 0.05 was considered as significant.

RESULTS AND DISCUSSION

Results

Alpha fetoprotein protein activity (AFP)
The effect of MEFPL on AFP is given in Table No 1. The level of α-fetoprotein in serum significantly increased in DEN treated animals (0.85±0.05, P<0.01) as compared to control group (0.31±0.02). Treatment with MEFPL 200 and MEFPL 400 mg/kg b.w. showed significant decrease in level (P<0.001) of AFP as compared to DEN treated group. The animals treated with standard drug silymarin at a dose level of 200 mg/kg b.w. showed significant decrease (P<0.05) in the level of serum AFP activity as compared to DEN treated group. This reduction was more the two doses of the extract. Administration of MEFPL at the doses of 200 and 400 mg/kg remarkably reduced the level of AFP in a dose dependent manner.

Effect of MEFPL on body and liver weight
The measurement of the body weight was done on weekly basis for 28 days. The weight of liver, and its weight relative to body weight by the end of the study in all experimental groups were listed in Table No 1. The DEN received animals showed significant decrease (159±8.23) in the final body weight when compared with normal saline received control group (192±12.39). The animals subjected to the treatment with either MEFPL extract (200 and 400 mg/kg.p.o; Group III & IV) and silymarin (Group V) exhibited significant improvement in the final body weight when compared with DEN treated animals. The results suggest that MEFPL had practically beneficial effect on the growth response of the animals.

Morphological observations of Liver

Effect of MEPL on hepatic nodule incidence
The Table No 2 shows the nodule incidence and total number of nodules caused by the carcinogen DEN followed by the efficacy of MEFPL 200,400 mg/kg.p.o and silymarin treatment. DEN treated groups (DEN + PB) showed 100% nodule incidence whereas Group III and Group IV (DEN + PB + MEFPL 200,400 mg/kg.p.o) animals showed a significant decrease (67% and 33% respectively) in tumour incidence compared to DEN received groups.

Effect of MEPL on the size of hepatic nodule
The average number of nodules with nodular sizes in millimetre in animals that received DEN and MEPL are shown in Table No 3. The MEFPL 200,400 mg/kg.p.o treated groups III and IV and silymarin treated group showed a significant decrease in the average number of nodules in tumour induced animals as 10.50±1.13, 7.16±0.97, 4.90±0.78 (P<0.05) respectively when compared with DEN treated animals (18.16±1.27). The nodular size was also significantly reduced in MEFPL treated animals compared to DEN induced group animals. However this reduction is statistically insignificant.

Estimation of nucleic acids
Table 4represents the levels of nucleic acids DNA and RNA in liver of control and experimental animals. In DEN received animals, the levels DNA and RNA were significantly elevated (P<0.001) when compared to control groups whereas MEFPL treatment, significantly reduced the elevated hepatic levels of DNA and RNA in a dose dependent manner.
DISCUSSION

Many studies support the use of DEN as a hepatocarcinogen. It has been reported that DEN by itself can induce hepatic neoplastic lesions. Earlier studies report that in DEN induced and Phenobarbital promoted hepatoma carcinoma tumor initiation and promotion is evident from increased incidence of nodules in liver. In the present study, tumor initiation and promotion is confirmed from increased number of nodules, and size of nodules in the liver of DEN received groups. All the animals that were treated with DEN developed nodules in liver which clearly indicates the total incidence of nodulogenesis. Inhibition of nodulogenesis by both the doses of MEFPL (200 and 400 mg/kg b.w) indicates its anticancer potential. However there was no significant difference as far as the size of the nodules is concerned. However the anticancer potential is further evidenced by the fall in the level of AFP. AFP is reported to be clear tumor marker of hepatic carcinoma and its level markedly rises in hepatic carcinomas. The effect of the extract on AFP further substantiates the anticancer potential. Moreover the extract produced useful effects through its action on DNA and RNA. During carcinogenesis there will be cell proliferation and hence increase in DNA level. In the present study, there was an increase in the amount of DNA content in tumor bearing animals when compared to control animals. However the extract reduced the levels of DNA & RNA further substantiating the anticancer potential.

CONCLUSION

The present study clearly indicates the anticancer potential of MEFPL from its modifying effect on tumor marker enzyme and nucleic acid levels. Further it inhibited the nodulogenesis in liver.

However, additional studies are warranted to explore further the mechanism of anticancer potential of MEFPL as a protective agent against DEN/PB induced hepatocarcinogenesis.

Table 1. Effect of MEFPL on body weight, liver and AFP in DEN/PB treated groups of rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial body Weight (g)</th>
<th>Final body weight (g)</th>
<th>Liver weight (g)</th>
<th>Relative weight of liver (Liver/100 g b.w)</th>
<th>AFP (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (NS-0.9%)</td>
<td>174±9.28</td>
<td>192±12.39</td>
<td>8.07±2.24</td>
<td>4.20±0.21</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>DEN + PB (200 mg/kg.p.o)</td>
<td>168±6.27</td>
<td>159±8.23</td>
<td>10.78±1.82</td>
<td>6.77±1.31</td>
<td>0.85±0.05^a</td>
</tr>
<tr>
<td>DEN + MEFPL (200 mg/kg.p.o)</td>
<td>170±1.27</td>
<td>187±2.31</td>
<td>9.34±0.48</td>
<td>4.99±0.34</td>
<td>0.63±0.03^b</td>
</tr>
<tr>
<td>DEN + MEFPL (400mg/kg.p.o)</td>
<td>167±1.28</td>
<td>188±2.26</td>
<td>8.54±0.52</td>
<td>4.47±0.13</td>
<td>0.53±0.03^b</td>
</tr>
<tr>
<td>DEN+Silymarin(200 mg/kg )</td>
<td>164±5.89</td>
<td>200±2.23</td>
<td>8.35±0.34</td>
<td>4.54±0.23</td>
<td>0.38±0.02^b</td>
</tr>
<tr>
<td>MEFPL alone (400 mg/kg.p.o)</td>
<td>170±1.33</td>
<td>180±1.71</td>
<td>9.27±0.52</td>
<td>5.15±0.24</td>
<td>0.32±0.02</td>
</tr>
</tbody>
</table>

Values are presented as mean±S.E.M. of six rats in each group. ^P<0.001 as compared with control group. ^P<0.001 as compared with DEN treated group.
Table 2. Effect of MEFPL on the incidence of hepatic nodules in DEN/PB induced hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. of rats</th>
<th>No. of rats with nodules</th>
<th>Average no. of Nodules</th>
<th>Nodule incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (NS-0.9%)</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DEN + PB (200 +0.05%)</td>
<td>6</td>
<td>6</td>
<td>18.16±1.27</td>
<td>100</td>
</tr>
<tr>
<td>DEN + MEFPL (200 mg/kg.p.o)</td>
<td>6</td>
<td>4</td>
<td>10.50±1.13*</td>
<td>67</td>
</tr>
<tr>
<td>DEN + MEFPL (400mg/kg.p.o)</td>
<td>6</td>
<td>2</td>
<td>7.16±0.97*</td>
<td>33</td>
</tr>
<tr>
<td>DEN+Silymarin (200 mg/kg)</td>
<td>6</td>
<td>1</td>
<td>4.90±0.78*</td>
<td>17</td>
</tr>
<tr>
<td>MEFPL alone (400 mg/kg.p.o)</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The percentage was calculated by dividing the number of rats with hepatic nodules over the total number of rats per groups.

* represents significance against DEN+PB treated group at p<0.05.

Table 3. Effect of MEFPL on number and size of hepatocellular nodules during DEN/PB induced hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Size (% of total number)</th>
<th>&lt;1mm</th>
<th>&gt;1mm&lt;3mm</th>
<th>&gt;3mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (NS-0.9%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DEN + PB (200 +0.05%)</td>
<td>56.0±1.27</td>
<td>34.0±2.04</td>
<td>15.0±2.46</td>
<td></td>
</tr>
<tr>
<td>DEN + MEFPL (200 mg/kg.p.o)</td>
<td>54.0±1.31</td>
<td>30.1±1.76</td>
<td>16±2.04</td>
<td></td>
</tr>
<tr>
<td>DEN + MEFPL (400mg/kg.p.o)</td>
<td>53.4±0.82</td>
<td>53.4±1.34</td>
<td>16.2±1.54</td>
<td></td>
</tr>
<tr>
<td>DEN+Silymarin (200 mg/kg)</td>
<td>48.2±0.93</td>
<td>34.4±1.56</td>
<td>17.2±1.69</td>
<td></td>
</tr>
<tr>
<td>MEFPL alone (400 mg/kg.p.o)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are presented as mean±S.E.M. of six rats in each group. Values were insignificant as compared to DEN+PB treated group.

Table 4. Effect of MEFPL on nucleic acids in liver and kidney experimental animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver mg/g of wet tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
</tr>
<tr>
<td>CONTROL- NS (0.9%)</td>
<td>6.48±0.21</td>
</tr>
<tr>
<td>DEN + PB (200 +0.05%)</td>
<td>8.56±0.24a</td>
</tr>
<tr>
<td>DEN + MEFPL (200 mg/kg.p.o)</td>
<td>8.26±0.31ns</td>
</tr>
<tr>
<td>DEN + MEFPL (400mg/kg.p.o)</td>
<td>6.81±0.27b</td>
</tr>
<tr>
<td>DEN+Silymarin (200 mg/kg)</td>
<td>6.67±0.31b</td>
</tr>
<tr>
<td>MEFPL alone (400 mg/kg.p.o)</td>
<td>6.36±0.22</td>
</tr>
</tbody>
</table>

Values are presented as mean±S.E.M. of six rats in each group.

ap<0.001 as compared with control groups.
bp<0.001 as compared with DEN treated groups.
REFERENCES