CHEMOPREVENTIVE EFFECT OF BOMBAX MALABARICUM DC AGAINST N-NITROSO DIETHYLAMINE INDUCED HEPATOCELLULAR CARCINOMA

SURENDER RP, HARI KL, ASHOK RV AND NARSIMHA RY*

Department of Pharmacology and Toxidology, University College of Pharmaceutical Sciences; Kakatiya University, Warangal - 506 009, Andhra Pradesh, India Email: surenderparupati@gmail.com

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Abstract

Bombax malabaricum (Bombacaceae), generally the plant exudates gum, light brown to opaque called as ‘mochras’ or ‘semul gum’ is used in vata diseases. Bark is astringent, diuretic, demulcent, healing of abscesses, wounds and other skin eruptions. Leaves are anti-inflammatory; roots are aphrodisiac, anti diarrheal. Flowers are diuretic and laxative, gum is used in hemoptysis. Seeds are used in gonorrhea. Traditionally the decoction of the bark is used externally in inflammations, in fomenting, sealing of secondary infection, healing of wounds and skin eruptions in the form of paste and leaves of this plant are ground and mixed with milk are given for strangury and inflammations. The aim of the present study is to evaluate the chemopreventive effect of bark extracts of Bombax malabaricum against N-nitroso diethyamine (DEN) induced Hepatocellular carcinoma. The chemopreventive effect of the plant extracts were evaluated by inducing Hepatocellular carcinoma in rat by giving single dose (200 mg/kg) of N-nitroso diethyamine (DEN) the carcinogenic effect of DEN is potentiated by administering phenobarbital in water. Various serum biochemical and histopathological studies were done to determine the effect of plant extracts on Hepatocellular carcinoma. The results of the serum biochemical estimations demonstrated that the Hepatocellular carcinoma was successfully induced by the DEN whereas the effect of DEN was reversed by the administration of the bark extracts. The antioxidant effect of the plant extracts were proved by estimating various parameters of the liver tissue homogenate. All these results indicate that the Ethanolic and Aqueous extract of Bombax Malabaricum bark have chemopreventive effect against DEN induced Hepatocellular carcinoma.

Keywords: Chemoprevention, N-Nitroso diethyamine, Hepatocellular carcinoma, DEN, Ethanolic and aqueous extracts of bark.

INTRODUCTION

Bombax malabaricum (Bombacaceae) is a tall and deciduous tree at a height of 20-25m, with smooth or buttressed trunk with pyramidal spreading branches, gray or brown bark covered with hard, black, sharp, conical spines. Flowers are red with 5 petals appear in the spring before the new foliage. It produces a capsule which, when ripe, contains white fibers like cotton. Phytochemical investigation of the chemical constituents of bark and roots of Bombax malabaricum has cadinane sesquiterpenoids, including five new compounds (bombamanones A-D, bombamaloxide, ), and four known compounds (isohemigossypol-1-methyl ester, 2,0-methylisohemigossylic acid lactone, bombaxquinone B, and lacnilen C). The above compounds were evaluated against the HGC-27 human gastrointestinal cancer cell line. A new Napthoquinone together with 7-hydroxyxycadakene and 8-formyl-7-hydroxy-5-isopropyl-2-methoxy-3-methyl-1,4-naphthoquinone were isolated from the heartwood of Bombax malabaricum. In Indian system of medicine ‘Ayurveda’, the plant is popularly known as Rakta shalmali ( Sanskrit ). This drug is a rasayana. It is a component of dashamulkwath. Generally the plant exudates gum, light brown to opaque called as ‘mochras’ or ‘semul gum’ is used in vata diseases. Bark is astringent, diuretic, demulcent, diuretic, healing of abscesses, wounds and other skin eruptions. Leaves are anti-inflammatory, roots are aphrodisiac, anti diarrheal. Flowers are diuretic and laxative, gum is used in hemoptysis. Seeds are used in gonorrhea. Traditionally the decoction of the bark is used externally in inflammations, in fomenting, sealing of secondary infection, healing of wounds and skin eruptions in the form of paste and leaves of this plant are ground and mixed with milk are given for strangury and inflammations. Despite the traditional use of this plant, no scientific report is focused on the biological activity of Bombax malabaricum. Cancer chemoprevention is a major area that has been intensively investigated in recent years. A large number of agents including natural and synthetic compounds have been shown to possess chemopreventive value. N-nitroso diethyamine (DEN) is an important carcinogen and it primarily induces liver tumor. DEN has been used as an effective experimental model in the field of carcinogenesis and chemoprevention. An attempt has been made in the present study to evaluate the chemopreventive effect of bark extract of Bombax malabaricum against DEN induced hepatocellular carcinoma.

MATERIALS AND METHODS

Source of plant

The bark of Bombax malabaricum DC were collected from the Udupi district Karnataka in the month of December 2009 and authenticated by Dr. Surender Parupati, Department of Pharmacology and Toxidology, University College of Pharmaceutical Sciences; Kakatiya University, Warangal - 506 009, Andhra Pradesh, India.

Preparation of various extracts of Bombax Malabaricum

The bark of Bombax Malabaricum DC was shade dried and powdered coarsely. The drug powder was taken in the soxhlet extractor and extracted using ethanol for 24 hours. After extraction the solvent was recovered by distillation and concentrated in vacuum. The extract obtained stored in desiccator. The yield obtained was 4.8%.

Preparation of Aqueous Extract

The coarsely powdered shade dried bark of Bombax Malabaricum DC was extracted with water containing 1% of chloroform by cold maceration process for 7 days. Daily the extract was stirred once. After completion of extraction the marc was filtered through muslin cloth and concentrated in vacuum. The yield obtained was 11%.

Animals:

Adult male Wistar albino rats (Mahaveer Enterprises, Hyderabad, India) of 8 weeks old at study start (mean weights in the range of 200-225 grams) were selected and housed in polypropylene cages in a room where the congenial temperature was 27°C ±1°C and 12 hrs light and dark cycles were maintained. The animals were allowed to aclimatize to the environment for 7 days and supplied with a standard pellet diet and water ad libitum. All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee of Kakatiya University.

Chemicals:

N-nitroso diethyamine (DEN) was purchased from sigma chemicals co (St, Louis, MO, USA). 1-Chloro 2, 4-dinitro benzoic acid (CDNB), 5, 5-dihydro-bis-2-nitro benzoic acid (DTNB), reduced glutathione (GSH) and glutathione were purchased from Sisco Research Laboratories.
Pt. Ltd., Mumbai, India. Thiobarbituric acid was purchased from E-Merck, India. All other chemicals used were of analytical grade.

**Experimental Design**

The rats were divided into 4 groups, each group consisting of six animals. Liver tumor was induced in group 2, 3 and 4 with single intraperitoneal injection of DEN at a dose of 200 mg/kg body weight in saline. Two weeks after DEN administration, the carcinogenic effect was promoted by 0.05% Phenobarbital, which was supplemented to experimental animal through drinking water up to 16 successive weeks.9

Group 1 : Normal control animals

Group 2 : DEN- treated animals

Group 3 : DEN- treated animals given Ethanolic extract (250 mg/kg, p.o.) for 16 weeks after the administration of DEN on 5 days per week.

Group 4 : DEN- treated animals given aqueous extract (250 mg/kg, p.o.) for 16 weeks after the administration of DEN on 5 days per week.

At the end of the experiments, animals were fasted overnight and killed by cervical decapitation. Blood was collected and serum was separated out. The liver was immediately removed, weighed and suspended in ice cold saline. A small portion of liver was fixed in 10% formalin for histopathological studies.

**Biochemical estimation**

Serum was analyzed for the following biochemical parameters: serum glutamate oxaloacetate transaminase (SGOT) 10, serum glutamate pyruvate transaminase (SGPT) 11, alkaline phosphatase 12, total protein 13, total bilirubin 14 and gamma glutamal transpeptidase (γ-GTP) 15. A 10% homogenate of liver tissue was used for analysis of lipid peroxidation (LPO) 16, superoxide dismutase (SOD) 17, catalase 18, glutathione peroxidase (GPx) 19 and Glutathione S- transferase (GST).

**Statistical analysis**

The values were expressed as mean ± SEM. Statistical analysis were performed by one way analysis of variance (ANOVA) followed by tukey multiple comparison test. P values < 0.05 were considered as significant.

**RESULTS**

We carried out the present study to evaluate the chemopreventive effect of bark extracts of Bombax malabaricum against DEN induced hepatocellular carcinoma. The results of the present study were presented in table 1, table 2 and in fig 1.

As shown in table 1 SGOT, SGPT, ALP and total bilirubin levels were increased total protein level was decreased in DEN control group whereas the treatment with the ethanolic and aqueous extract of Bombax malabaricum reverse these levels more are less to the normal.

The tissue homogenate levels of Lipid peroxidase, Glutathione peroxidase, and Glutathione transferase were increased where as the levels of the Catalase and superoxide dismutase levels were decreased as shown in table 2 but these levels were bring back to the normal levels by the treatment with the plant extracts. The liver weight (fig 1) was increased almost equal to double the weight when compared with the normal in DEN treated animals. Normal rat liver weight was found to be 5.16±0.189 g/100g, DEN treated animal liver weight was 8.01±0.203 g and the ethanolic extract treated group animals liver weight was found to be 6.11±0.223 whereas aqueous extract treated animals liver weight was 6.7±0.314.

**Table 1: Effect of Ethanolic and Aqueous extracts on Serum GPT, GOT, ALP, Total proteins and Total bilirubin in DEN Treated Rats**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg)</th>
<th>SGPT U/L</th>
<th>SGOT mg%</th>
<th>ALP U/L</th>
<th>Total Proteins Mg%</th>
<th>Total Bilirubin Mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>38.33±1.10</td>
<td>84.16±2.20</td>
<td>179.83±3.8</td>
<td>6.4±0.10</td>
<td>0.38±0.03</td>
</tr>
<tr>
<td>DEN</td>
<td>200</td>
<td>210±6.9a</td>
<td>286±5.34a</td>
<td>397±7.7a</td>
<td>5.1±0.4b</td>
<td>2.21±0.20a</td>
</tr>
<tr>
<td>DEN+ Ethanolic Extract</td>
<td>250</td>
<td>86±2.72c</td>
<td>99.66±2.1c</td>
<td>195±4.6c</td>
<td>6.4±0.35a</td>
<td>0.96±0.01c</td>
</tr>
<tr>
<td>DEN+ Aqueous extract</td>
<td>250</td>
<td>94±2.20c</td>
<td>105.4±2.7c</td>
<td>210±3.2c</td>
<td>6.3±0.32c</td>
<td>1.0±0.02c</td>
</tr>
</tbody>
</table>

N= 6 animals in each group; a,b,c,p<0.001; 0.01 Vs control; d,p<0.001; 0.05 Vs DEN treated rats; e,p<0.01 Vs DEN treated rats.

Administration of DEN alone increases the level of liver enzymes and decreases total protein. DEN when administered with Ethanolic and Aqueous extracts reverses these changes i.e. decrease liver enzymes and increases total protein.

**Table 2: Effect of Ethanolic and Aqueous extracts on GGT and Antioxidants Effect in DEN Treated Rats**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg)</th>
<th>GGPT µMoles of MDA/ min/mg protein</th>
<th>Lipid peroxidase µMoles of MDA/ min/mg protein</th>
<th>GST µMoles of CdNB conjugation formed/min/ mg protein</th>
<th>Catalase µMoles of H2O2 consumed/min/ mg protein</th>
<th>GPx µMoles of GSH oxidized/min/ mg protein</th>
<th>SOD Units/min/ mg protein</th>
<th>Total Proteins Mg%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>48.3±1.30</td>
<td>6.9±0.08</td>
<td>0.18±0.005</td>
<td>72.5±1.0</td>
<td>6.58±0.20</td>
<td>1.35±0.08</td>
<td>72.5±1.0</td>
</tr>
<tr>
<td>DEN</td>
<td>200</td>
<td>88.2±3.6a</td>
<td>11.7±0.20a</td>
<td>18.3±0.84a</td>
<td>5.04±1.2a</td>
<td>0.28±0.008</td>
<td>0.96±0.02</td>
<td>5.04±1.2a</td>
</tr>
<tr>
<td>DEN+ Ethanolic Extract</td>
<td>250</td>
<td>52.6±3.12a</td>
<td>8.05±0.18b</td>
<td>14.2±0.45b</td>
<td>7.35±1.6b</td>
<td>0.17±0.004</td>
<td>1.24±0.06</td>
<td>0.17±0.004</td>
</tr>
<tr>
<td>DEN+ Aqueous extract</td>
<td>250</td>
<td>58.8±1.4b</td>
<td>8.1±0.12b</td>
<td>14.5±0.4b</td>
<td>6.85±2.1b</td>
<td>0.19±0.002</td>
<td>1.20±0.06</td>
<td>6.85±2.1b</td>
</tr>
</tbody>
</table>

N= 6 animals in each group; a,b,c,p<0.001; 0.01 Vs control; d,p<0.001; 0.05 Vs DEN treated rats; e,p<0.01 Vs DEN treated rats.

Values are expressed as mean ± SEM.
DEN treated animals. Whereas bark extract of *B.Malabaricum* a strong inhibition of Hepatocellular carcinogenesis induced by DEN. Since the liver is the major site of toxic industrial chemicals, air and air pollutants as also, food additives and fungal toxins. The liver is the major vital metabolic organ, the structural and functional abnormalities represent the disease condition. N-nitrosodiethylamine is a widely occurring nitrosamine which is present in tobacco and various processed foods. These (Nitroso) compounds can also be formed in vivo in physiological conditions.

**DISCUSSIONS**

**Hepatocellular carcinoma (HCC)**

Hepatocarcinoma is a major problem not only in developed countries but also in most undeveloped countries. It is induced by toxic industrial chemicals, air and air pollutants as also, food additives and fungal toxins. Hepatocarcinoma is seldom detected at the early stage and once treated with the Ethanolic and aqueous extract of *B.Malabaricum*. The levels of GGPT (liver enzyme) and those of GPs, GST and lipid peroxidase (oxidant enzyme) were significantly elevated (p < 0.001) by the administration of DEN. These elevated levels were lowered by the administration of bark extracts of *B.Malabaricum*. The levels of SOD and catalase, the two antioxidants were lowered by DEN and they were returned back to normal levels by the Ethanolic and Aqueous extracts. DEN induced Histopathological changes in liver as shown in fig 1 such as fatty infiltration, focal necrosis and hepatocytes having hyperchromatic nuclei. These changes are indicative of Hepatocellular carcinoma. All these Histopathological changes were reversed by the administration of extracts.

Treatement with the Ethanolic and aqueous extract of *Bombax Malabaricum* bark produced a significant reduction in tumor incidence as revealed by reduction of morphological changes. Elevated serum levels of SGOT, SGPT, ALP and total bilirubin are indicative of poor hepatic function in DEN treated animals. Also DEN treatment increased the levels of GGPT and GST. All these indicate an induction of Hepatocellular carcinoma induced by DEN. Treatment with the Ethanolic and aqueous extract of *Bombax Malabaricum* bark reduced the levels of all tumor markers.

**Role of antioxidant effect of *Bombax Malabaricum* in chemoprevention**

Antioxidants have the capacity to scavenge free radical directly or to interfere with the generation of free radical events which results in the inhibition of neoplastic process. It has been reported that free radical play an important role in the complex course of multistep carcinogens. Increased activity of GGPT is responsible for the increased levels of GPs and GST in DEN treated group of animals. This increased levels of GST and GPs, likely to be the key mediator of drug resistance in cancer chemotherapy. The decreased level of these two enzymes in the extract treated groups compared to those treated with DEN is indicative of its antimalignant potency. Antioxidant enzymes are altered during carcinogenesis or after tumor formation several authors have cited decreased activities of SOD and Catalase in various types of tumor cells when compared to

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*Fig. 1: Effect of Ethanolic and Aqueous extract of bark on variation of liver weight in control and experimental animal groups.*

*p < 0.001 Vs Control and DEN treated rat*

Ethanolic or Aqueous extract when administered with DEN decreases liver weight, which is increased by administration of DEN alone.
normal cells. On treatment with the both extracts reversed the level of these enzymes to normal.

LPO may lead to formation of several toxic by-products such as 4-hydroxynoneal and malondialdehyde which can attack cellular targets including DNA, inducing mutagenity and carcinogenicity. Treatment with the extract after administration of DEN significantly reduced the levels of LPO. Thus inhibiting peroxidative changes which are a clear proof for antioxidant effect.

All these results indicate that the Ethanolic and Aqueous extract of Bombax Malabaricum bark have chemopreventive effect against DEN induced liver tumor.

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