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Research Article

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

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ABSTRACT

Bombax malabaricum (Bombacacaeae), 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 diethylamine (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 diethylamine (DEN) the carcinogenic effect of DEN is potentiated by administering phenobarbital in water. Various serum biochemical and histophaological 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*-Nitrosodiethylamine, Hepatocellular carcinoma, DEN, Ethanolic and aqueous extracts of bark.

INTRODUCTION

Bombax malabaricum (Bombacacaeae) is a tall and deciduous tree at a height of 20-25m, with smooth or butressed trunk with pyramidal spreading branches, gray or brown bark covered with hard, black, sharp, conical spines[1]. 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 (bombamalones A–D, bombamaloside,), and four known compounds (isohemigossypol-1-methyl ester, 2-*O*-methylisohemigossylic acid lactone, bombaxquinone B, and lacinilene C).The above compounds were evaluated against the HGC-27 human gastrointestinal cancer cell line[2]. A new Napthoquinone together with 7-hydroxycadalene and 8-formyl-7-hydroxy-5-isopropyl-2-methoxy-3-methyl-1,4-naphthoquinone were isolated from the heartwood of *Bombax malabaricum*[3]. 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 dasha-mulkwatha.Generally the plant exudates gum, light brown to opaque called as 'mochras' or 'semul gum' is used in vata diseases[4]. Bark is astringent, diuretic, demulcent, diuretic, healing of abscesses, wounds and other skin eruptions. Leaves are anti-inflammatory, roots are aphrodisiac, anti diarr-heal. Flowers are diuretic and laxative, gum is used in hemoptysis. Seeds are used in gonorrhea[5]. 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[6]. A large number of agents including natural and synthetic compounds have been shown to possess chemopreventive value[7]. ***N*-**nitrosodiethylamine (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[8]. An attempt has been made in the present study to evaluate the chemopreventive effect of bark extract of *Bombax malabaricum* againest DEN induced hepatpocellular 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

Preparation of various extracts of *Bombax Malabaricum*

Preparation of Ethanol Extract

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 acclimatize 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*-**nitrosodiethylamine (DEN) was purchased from sigma chemicals co (St, Louis, MO, USA). 1-Chloro 2, 4-dinitro benzoic acid (CDNB), 5, 5-dithio-bis-2-nitro benzoic acid (DTNB), reduced glutathione (GSH) and glutathione were purchased from Sisco Research Laboratories

Pvt. 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 phospahatase[12], total protein[13], total bilurubin[14] and gamma glutamate 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 (GP_X) [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* againest DEN induced hepatpocellular carcinoma. The results of the present study were presented in table1, 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 treatement 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 transference 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 treatement 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±0.189g/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

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

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

Values are expressed as mean ± SEM.

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 GGPT and Antioxidants Effect in DEN Treated Rats

Treatement group	Dose (mg/kg)	GGPT U/L	Lipid peroxidase µ Moles of MDA/ min/mg protein	GP_X µ Moles of GSH oxidized/min/ mg protein	GST µ Moles of CDNB conjugation formed/min/ mg protein	SOD Units/min/ mg protein	Catalase µ Moles of H_2O_2 consumed/min/ mg protein
Control	------	48.3 ± 1.30	6.9 ± 0.08	6.58 ± 0.20	0.18 ± 0.005	1.35 ± 0.08	72.5 ± 1.0
DEN	200	88.2 ± 3.6[a]	11.7 ± 0.20[a]	18.3 ± 0.84[a]	0.28 ± 0.008[a]	0.96 ± 0.02[a]	50.41 ± 1.2[a]
DEN+ Ethanolic Extract	250	52.6 ± 3.12[b]	8.05 ± 0.18[b]	14.2 ± 0.45[b]	0.17 ± 0.004[b]	1.24 ± 0.06[c]	73.5 ± 1.6[b]
DEN+ Aqueous extract	250	58.8 ± 1.4[b]	8.1 ± 0.12[b]	14.5 ± 0.4[b]	0.19 ± 0.002[b]	1.20 ± 0.06[c]	68.5 ± 2.1[b]

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

Values are expressed as mean ± SEM.

Ethanolic and Aqueous extract of bark when administered with DEN show an antioxident effect as shown by changes in enzyme levels studied (increase in GGPT, LPO, GP_X and GST and decreases in SOD and Catalase)

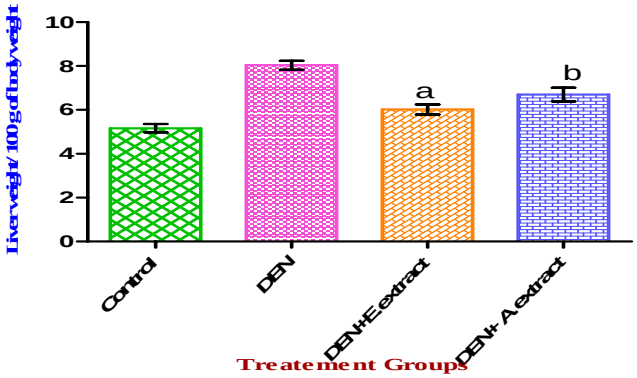

Fig. 1: Effect of Ethanolic and Aqueous extract of bark on variation of liver weight in control and experimental animal groups.

[a,b]*p< 0.001* Vs Control and DEN treated rat

Etahnolic or Aqueous extract when administered with DEN decreases liver weight, which is increased by administration of DEN alone.

DISCUSSIONS

Hepatocellular carcinoma (HCC)

Hepatpocarcinoma 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[21]. Since the liver is the major site of metabolism of ingested materials, it is more susceptible to carcinogenic insult. Moreover, due to high tolerance of liver hepatocarcinoma is seldom detected at the early stage and once detected treatement has a poor prognosis in most cases[21]

HCC is one of the ten most common human cancers, with a worldwide incidence of over one million cases every year[23]. It accounts for about 90% of all primary liver cancers.

HCC, a fatal malignancy represents 4% of all malignant tumors. Liver plays a significant important intriguing site in the study of neoplastic diseases. As abnormal metabolism represents cancer, the liver being 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[24]. These (Nitroso) compounds can also be formed *in vivo* in physiological conditions[25].

N-nitrosodiethylamine (DEN), one of the most important environmental carcinogens in this class, primarily induces tumors of liver. DEN is predominantly inducing liver tumors in various species, but other nitrosamine may have varied effects. It is widely accepted that metabolic activation of nitrosamines by cytochrome P_{450} enzyme to reactive elctrophiles is required for their cytotoxic mutagenic and carcinogenic activity[26]. Because of its relatively simple metabolic pathway and potent carcinogenic activity, DEN has found widespread use as an experimental model in the field of carcinogenesis and in chemoprevention[27]. A single administration of DEN induced liver tumor is evidenced by the increase in liver weight increased level of hepatic enzyme like SGPT, SGOT, ALP, total bilirubin and decrease in total proteins and increased levels of GGPT, GP_X, GST and LPO (oxidant enzymes), decrease in SOD and catalase (free radical scavengers) and morphological changes noted by physical examination. All these changes were reverted back to normal by the extract of *Bombax Malabaricum* treatement indicating a strong inhibition of Hepatocellular carcinogenesis induced by DEN. As shown in the fig 1 the liver weight of normal animals was 4.4±0.10g/100g body weight and it increase to 7.9 ± 0.12 g in DEN treated animals. Whereas bark extract of *B.Malabaricum* treatement brought down the liver weight to 5.4 ± 0.10 and 5.8 ± 0.12 and the reduction is significant (p< 0.001). The effect of DEN on liver enzymes was shown in the table 1. As far as the effect in liver enzymes concerned, DEN treatment increased the levels of SGOT, SGPT, ALP, total bilirubin and decreased total proteins. These levels were reversed back to normal (p< 0.001) by the administration of ethanolic and aqueous extract of *B.Malabaricum.*

The levels of GGPT (liver enzyme) and those of GP_X, 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 treatement increased the levels of GGPT and GST. All these indicate an induction of Hepatocellular carcinoma induced by DEN. Treatement 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[28]. It has been reported that free radical play an important role in the complex course of multistep carcinogens[29]. Increased activity of GGPT is responsible for the increased levels of GP_X and GST in DEN treated group of animals. This increased levels of GST and GP_X 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[31] several authors have cited decreased activities of SOD and Catalase in various types of tumor cells when compared to

normal cells[29]. On treatement 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 malanodialdehyde which can attack cellular targets including DNA, inducing mutagencity and carcinogenicity[29]. Treatement 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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