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Evaluation of hepatoprotective potential of *Cassia fistula* in N-Diethylnitrosamine induced hepatocarcinogenesis in Wistar rats

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ABSTRACT

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Protective effect of *Cassia fistula* against N-Diethylnitrosamine (DEN) induced hepatocarcinogenesis was studied in rats. Ninety six male Wistar albino rats were randomly allotted to four groups of 24 rats each. Hepatocarcinogenesis and the protective effect of *C. fistula* were studied by administering 0.01% of DEN in drinking water *ad libitum* and *C. fistula* 500 mg/kg BW *per os* either alone or in combination for 120 days. After DEN administration, hepato to cellularpathology was observed grossly and histologically. In addition, elevated serum ALT, AST and GGT levels, hypoalbuminaemia, increased BUN and creatinine were observed in DEN treated rats. A significant ($P<0.05$) decrease in the CAT and SOD values was observed in the DEN and DEN+ *C. fistula* groups when compared to the other groups of rats. There was a significant ($P<0.05$) increase in the GSH, GPx and GGT values in the DEN and DEN+ *C. fistula* treated groups when compared to control and *C. fistula* groups. Liver showed significant ($P<0.05$) increase in lipid peroxidation values throughout the trial period. Concurrent administration of *C. fistula* with DEN significantly alleviated the effects of DEN on serum AST level, BUN value and relative liver weight. There was a significant ($P<0.05$) increase in the liver lipid peroxidation in the DEN and DEN+ *C. fistula* groups when compared to the other groups. It was concluded that *C. fistula* possess significant hepatoprotective potential against DEN induced hepatocarcinogenesis.

Key words: *Cassia fistula*, hepatocarcinogenesis, lipid peroxidation, pathology

INTRODUCTION

Nitrosamines are compounds formed by the combination of amines and nitrates or nitrites. Nitrosamines can be formed in the gastric juice of the human stomach by a process commonly referred to as endogenous nitrosation. The bacteria in the mouth chemically reduce nitrate found in many vegetables to nitrite, which in turn can form nitrosating agents. Many foods that contain amines can react with these nitrosating agents in the acidic environment of the stomach to form nitrosamines[1]. The presence of diethylnitrosamine (DEN) in wide varieties of foods, such as cheese, soya bean, smoked, salted and dried fish, cured meat, alcoholic beverages and ground water having high level of nitrates makes the human population vulnerable to its exposure. DEN induces oxidative stress possibly due to the generation of reactive oxygen species (ROS), which are capable of initiating peroxidative damage to the cell[2].

In recent years, much has been learnt about the multistep and multifactorial pathogenesis of neoplasia in the liver. Many studies have defined a correlation between human and rodent carcinogenesis in that both are multistage processes and stages of initiation, promotion and progression of persistent benign lesions to full malignancy can be identified in experimental models[3].

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Cassia fistula, a medium sized deciduous tree with beautiful bunches of yellow flowers, belonging to the family Leguminosae. It has been extensively used in Ayurvedic system of medicine for various ailments[4]. *C. fistula* is widely used for its antitumor[5], hepatoprotective[6] and antioxidant[7,8] activities. The present investigation was designed to evaluate the hepatoprotective and antioxidant properties of the ethanolic leaf extract of *C. fistula* against hepatocarcinogenicity induced by N-diethylnitrosamine in male wistar rats.

MATERIALS AND METHODS

Animals

Ninety six male Wistar albino rats of 7-9 weeks age weighing around 120-150 g were randomly allotted into four groups of 24 rats each. Group I was kept as control and maintained on normal feed and water. Group II served as tumor control and treated with Diethylnitrosamine (0.01%) in drinking water *ad libitum.* Group III was treated with Ethanolic leaf Extract (ELE) in combination with DEN (0.01%) and Group IV was given only ELE. ELE of *C. fistula* (500mg/kg BW *per os*) was administered either alone or in combination with DEN for 120 days. Three rats from each group were sacrificed fortnightly and blood samples and liver tissue were collected for haematobiochemical and antioxidative analysis. The animal experimentation was carried out as

per the guidelines and approval of Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Chemicals

N-Nitrosodiethylamine (Isopac, 1g, Product No. 0258) was obtained from M/s Sigma Chemical Co. USA. Serum biochemical kits were purchased from M/s Agappe Diagnostics Pvt. Ltd., Cochin. The ethanolic leaf extract of *Cassia fistula* was obtained from M/s Stanes Herbal Division, Phyto-Pharma Testing Lab, Coimbatore and it was certified by them.

Physical Parameters

The liver damage was evaluated by parameters like decrease in body weight and increase in liver weight. After sacrificing, the relative liver weight was determined.

Biochemical profile

Three rats from each group were sacrificed at fortnightly intervals. Detailed necropsy was conducted on rats that died during the experimental period and on those sacrificed at regular intervals. Animals were subjected to mild diethyl ether anesthesia and blood was collected from the retro orbital plexus for serum separation (3000 rpm for 15 min at 4°C). Animals were then sacrificed by decapitation and the livers were excised, washed in ice cold saline and blotted to dryness and weighed. A 10% homogenate of the liver tissue was prepared in Tris-HCl buffer (0.1M; pH 7.4), centrifuged (1000 rpm for 10 min at 4°C) to pellet the cell debris and the clear supernatant used for biochemical assays. A piece of the liver tissue was also fixed in 10% neutral buffered formalin and stained with hematoxylin-eosin for histopathological analysis.

Samples of blood collected from the rats on days of sacrifice were allowed to clot and centrifuged at 1500 rpm for 30 min to separate the sera. Serum ALT and AST were estimated by IFCC (International Federation of Clinical Chemistry) method. Serum GGT was estimated by Szasz kinetic method, blood urea nitrogen (BUN) by glutamate dehydrogenase (GLDH) method and creatinine by Jaffe's kinetic method[9]. Total protein and albumin were estimated by Biuret and Duma's method. Lipid peroxidation assay was determined as thiobarbituric acid reactive substances (TBARs) by the method of Yagi (1976). Catalase (CAT) was assayed by the method of Caliborne[10]. Superoxide dismutase (SOD) was measured by the method of Marklund and Marklund[11]. Glutathione peroxidase (GPx) was measured by the method of Rotruck *et al*.[12]. Reduced Glutathione (GSH) and glutathione S transferase (GST) were estimated by the method of Meron *et al*.[13]. Enzyme activity of (GGT) was assayed by the modified Meister and Orlowski procedure using gamma-glutamyl-p-nitroanilide as substrate[14].

Histopathological study

The processing of the tissues was done following the procedure described by Drury and Wallington[15]. Paraffin sections of 5 mm thickness were obtained with the help of microtome on the slide using Mayer's egg albumin as adhesive. Staining was done using Lillie Mayer's haematoxyline and alcoholic eosin by routine procedure.

Statistical Analysis

The data obtained from different parameters were subjected to one-way analysis of variance using SPSS (Version 16.0 for windows) statistical software.

RESULTS

Body weight and relative liver weight

A significant (P<0.05) decrease in the body weight gain was observed in group II and III. Relative liver weight values increased significantly (P<0.05) in group I when compared to the other groups. There was a significant (P<0.05) increase in the overall relative liver weights in group II and III (Table 1).

Biochemical profile and antioxidant assay

A significant (P<0.05) increase in the levels of ALT, AST and GGT was observed in groups II and III. There was a significant (P<0.05) decrease in the overall AST value of group III when compared to group II (Table 2). There was a significant (P<0.05) increase in the BUN and creatinine values in the groups II and III when compared to the control. There was a significant (P<0.05) decrease in the overall BUN value of group III when compared to group II. There was no significant difference in the total

Table 1. Effect on body weight and relative liver weight following daily oral administration of *C. fistula* extract with or without DEN induced carcinogenesis in rats (n=24).

Groups	Treatments	Initial Body weight	Body weight (10^{th} week)	% increase in body weight	Relative liver weight
I	Control	123.12±5.05	292.07^{b}±5.84	57.87%	3.72^{a}±0.13
II	DEN	123.38±6.31	196.78^{a}±6.43	37.24%	5.26^{c}±0.20
III	DEN+*C. fistula*	123.25±6.31	202.33^{a}±9.21	39.10%	4.61^{b}±0.22
IV	*C. fistula*	123.04±4.68	289.18^{b}±9.04	57.43%	4.13^{ab}±0.21

Table 2. Effect on biochemical profile following daily oral administration of *C. fistula* extract with or without DEN induced carcinogenesis in rats (n=24).

Groups	Treatments	AST (I.U/L)	ALT (I.U/L)	GGT (I.U/L)	BUN (mg/dL)	Creatinine (mg/dL)
I	Control	177.27^{a}±10.34	82.04^{a}±12.36	4.68^{a}±0.30	23.33^{a}±0.64	0.49^{a}±0.01
II	DEN	266.33^{c}±11.43	228.38^{b}±13.66	66.44^{b}±12.29	40.63^{c}±1.13	0.64^{b}±0.02
III	DEN+*C. fistula*	234.94^{b}±11.43	222.66^{b}±13.66	55.05^{b}±10.25	34.54^{b}±1.17	0.62^{b}±0.02
IV	*C. fistula*	200.25^{a}±10.84	96.85^{a}±12.96	5.55^{a}±0.46	24.46^{a}±0.77	0.50^{a}±0.01

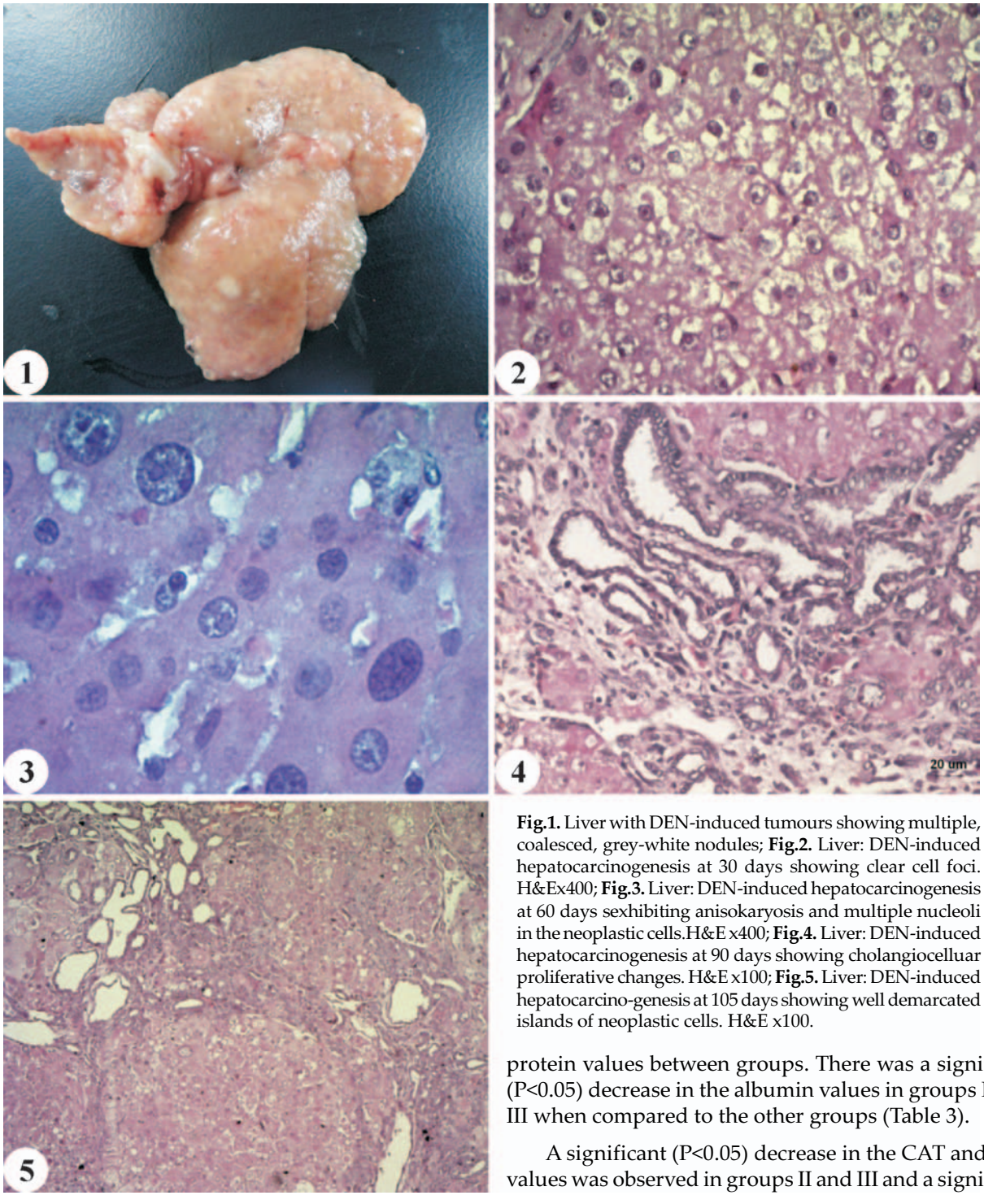

Fig.1. Liver with DEN-induced tumours showing multiple, coalesced, grey-white nodules; **Fig.2.** Liver: DEN-induced hepatocarcinogenesis at 30 days showing clear cell foci. H&Ex400; **Fig.3.** Liver: DEN-induced hepatocarcinogenesis at 60 days sexhibiting anisokaryosis and multiple nucleoli in the neoplastic cells.H&E x400; **Fig.4.** Liver: DEN-induced hepatocarcinogenesis at 90 days showing cholangiocelluar proliferative changes. H&E x100; **Fig.5.** Liver: DEN-induced hepatocarcino-genesis at 105 days showing well demarcated islands of neoplastic cells. H&E x100.

protein values between groups. There was a significant (P<0.05) decrease in the albumin values in groups II and III when compared to the other groups (Table 3).

A significant (P<0.05) decrease in the CAT and SOD values was observed in groups II and III and a significant

Table 3. Effect on protein profile following daily oral administration of *C. fistula* extract with or without DEN induced carcinogenesis in rats (n=24).

Groups	Treatments	TP (g/dL)	Albumin(g/dL)
I	Control	6.76±0.15	3.01±0.06[b]
II	DEN	6.71±0.17	2.77±0.07[a]
III	DEN+*C. fistula*	6.37±6.80	2.69±0.07[a]
IV	*C. fistula*	6.80±0.16	3.16±0.07[b]

Table 4. Effect on lipid peroxidation and antioxidant assay following daily oral administration of *C. fistula* extract with or without DEN induced carcinogenesis in rats (n=24).

Groups	Treatments	Enzymatic antioxidants			Non-enzymatic antioxidants	Tissue GGT (μmoles/h/g)
		CAT (μg/unit/g)	SOD (μg/unit/g)	GPx (mg/g)	GSH (mg/g)	
I	Control	5.21[b]±0.27	1.46[b]±0.06	628.63[a]±12.62	162.10[a]±6.71	65.50[a]±14.21
II	DEN	1.56[a]±0.27	0.77[a]±0.06	796.66[c]±12.62	245.86[b]±6.71	176.84[b]±14.20
III	DEN+*C. fistula*	1.76[a]±0.28	0.7[a]±0.06	776.49[c]±12.94	241.4[b]±6.88	172.60[b]±14.58
IV	*C. fistula*	4.77[b]±0.27	1.28[b]±0.06	667.94[b]±12.31	177.15[a]±6.59	72.46[a]±13.8

($P<0.05$) increase in the GSH, GPx and GGT values in groups II and III when compared to control and only *C. fistula* treated group (Table 4).

Pathology

Grossly, the liver from groups II and III showed the development of multiple raised nodules from 45th day of treatment. By 60 days, DEN produced multiple coalescing cystic neoplastic nodules of variable sizes in the liver. By 120 days, the liver was completely distorted with multiple, large sized, coalesced, grey white or dark red nodules (Fig 1).

Histopathologically, in groups II and III, mild bile duct hyperplasia, mitotic figures and multifocal areas of mild lymphocytic infiltration were observed on 15th day. By 30th day, clear cell foci, areas of vacuolar degeneration of hepatocytes and eosinophilic foci were seen in the DEN treated rats (Fig. 2). On 45th day, cells showed hepatocytomegaly, karyomegaly, hyperchromasia, multinucleate cells and extensive oval cell proliferation in addition to previous findings. On 60th day, carcinoma cells showed anisokaryosis, altered foci, clear cell foci, eosinophilic foci and oval cell proliferation in periportal areas forming immature ductules (Fig. 3). Islands of neoplastic cells, hepatocellular and cholangiocellular carcinoma were the consistent findings observed from 75 days to 120 days (Fig. 4, 5).

DISCUSSION

Hepatoprotective and antioxidant properties of *C. fistula* were studied against hepatocarcinogenesis induced by N-diethylnitrosamine in rats. A significant increase in the relative liver weights between DEN treated groups was found, when compared to control[16]. The protective effect of *C. fistula* on DEN induced increase in relative liver weight noticed could be attributed to free radical scavenging activity of *C. fistula,* thus protecting the hepatocytes from free radical injury[17]. The reason of increase in the activities of serum ALT and AST could be the activation of hepatospecificenzymes which is a secondary event following DEN induced lipid peroxidation of hepatocyte membranes, resulting in consequent leakage of ALT, GGT and ALP from liver tissues[18,19]. The significant decrease in the serum GGT levels in group III (DEN+*C. fistula*) group as compared to the group II (DEN) is an indicator of possible hepatoprotective property of *C. fistula* which concurred with the observation of earlier workers[20]. An elevated creatinine level is attributed to vasoconstriction of the renal circulation as a result of increased portal venous system pressure affecting the renal circulation[21]. Increased BUN value is a manifestation of decreased glomerular filtration rate due to the above cause, or an increased load of urea for excretion from the diet or tissue metabolism[22]. In this case, excessive muscle catabolism might have caused elevation of BUN value. Reduction in albumin could be ascribed to the development of hepatic lesions observed in the present study affecting the protein synthesis in concurrence with the previous findings[3].

The lipid peroxidation values for group III showed a significant difference with DEN alone treated group from 15th to 45th day and on 105th day. This finding indicated the protective effect of *C. fistula* on DEN induced cell membrane damage. The phytochemicals present in the ELE supplemented as exogenous antioxidants by scavenging reactive oxygen species thus preventing peroxidative damage to hepatocytes[23]. The increase in the GPx and GSH could be attributed to the adaptive

response of tumor cells to escape intrinsic oxidative stress[24]. The liver antioxidants in DEN group which showed significant decrease in the SOD and CAT levels and increase in the GSH and GPx levels throughout the trial were consistent with the findings of Sundaresan and Subramanian[25].

Gross and histopathological findings of hepatic tumourscomprising carcinogenic changes in terms of nuclear and cellular morphology, number and differentiation of the tumour mass were similar to the observations in other studies[26,27]. Treatment with *C. fistula* extract showed a mild hepatoprotective effect, where although a complete prevention was not evident, a delay in progression of tumour was obvious. The possible mechanisms of actions of preventing tumour development may be by inducing tumour cell apoptosis, or by inhibiting DNA topoisomerase II and p53 down regulation, or by causing mitochondrial toxicity, which initiates mitochondrial apoptosis[28]. Anti-tumour activity of *C. fistula* seed extract based on cytological studies revealed that a reduction in the mitotic activity can be the leading mechanism of action against tumorigenesis. Indeed, the appearance of membrane blebbing and intracytoplasmic vacuoles in the treated tumour cells suggest that these pathways may account for the reduction in tumour volume[5].

It was inferred from the study that ELE of *C. fistula* reduced the carcinogenic effect of DEN in the liver of rats, suggesting that it may be an alleviating factor by inhibiting the production of free radicles in the liver. The process involves many other factors and further research is needed.

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