Chemoprevention of N-Nitrosodiethylamine Induced Phenobarbitol Promoted Liver Tumors in Rat by Extract of Indigofera aspalathoides

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The chemopreventive effect of ethanol extract of Indigofera aspalathoides (EIA) on N-nitrosodiethylamine (DEN, 200 mg/kg)-induced experimental liver tumor was investigated in male Wistar rats. Oral administration of ethanol extract of Indigofera aspalathoides (250 mg/kg) effectively suppressed liver tumor induced with DEN as revealed by decrease in the levels of extent of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), total bilirubin, gamma glutamate transpeptidase (GGTP), lipid peroxidase (LPO), glutathione peroxidase (Gpx) and glutathione S-transferase (GST) with a concomitant increase in enzymatic antioxidant (superoxide dismutase and catalase) levels when compared to those in liver tumor bearing rats. The histopathological changes of liver sample were compared with respective control. Our results show a significant chemopreventive effect of EIA against DEN induced liver tumor.

Key words chemoprevention; N-nitrosodiethylamine; Indigofera aspalathoides; biochemical parameter; antioxidant; histopathology

The major area of cancer chemoprevention that has been intensively studied in recent years is biologic modifiers of cancer cells. These agents are designed to retard the proliferation of cancer cells and to induce differentiation of these cells to a quiescent, non-dividing stage and/or to promote cell death.1)

Cancer is the leading cause of mortality worldwide and the failure of conventional chemotherapy to effect major reduction in the mortality indicates that new approaches are critically needed. The new and recent approach of chemoprevention serves as an alternative treatment against control malignancy.2) This is a pharmacological intervention aimed to arrest or reverse the process of carcinogenesis.3) In experimental chemoprevention studies, attempts are made to identify agents which could exhibit any or combination of the following characteristics: (i) prevent the initiation of tumors (ii) delay or arrest the development of tumors (iii) extend the cancer latency periods (iv) reduce in cancer metastasis and mortality and (v) prevention of recurrence of secondary tumors. The major focus of research in chemoprevention of cancer in recent times includes the identification, characterization and development of new and safe cancer chemopreventive agents.4)

Liver plays an important role in the metabolism and disposition of a large number of foreign chemicals to which vertebrates are continuously exposed. Liver damage commonly results by viral and protozoal infection, toxicity due to drugs, food additives and fungal toxins. Several investigations have provided convincing evidence that N-nitrosamines cause a wide range of tumors in all animal species. These compounds are considered to be effective health hazards to man.5) Exposure of man to preformed N-nitrosamines occur through the diet, in certain occupational settings and also due to the use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals.6) N-Nitrosodiethylamine (DEN), one of the most important environmental carcinogen of this class, primarily induces liver tumor. It is widely accepted that metabolic activation of nitrosamines by cytochrome P450 enzymes to reactive electrophiles is required for their cytotoxic, mutagenic and carcinogenic activity. Because of its relatively simple metabolic pathway and potent carcinogenicity. DEN has been used as an effective experimental model in the field of carcinogenesis and chemoprevention.

A large number of agents including natural and synthetic compounds have been identified as having some potential cancer chemopreventive value.4) Plants and plant products have been shown to play an important role in the management of various liver disorders. Indigofera aspalathoides Vahl, a plant belonging to the family of Papilionaceae, is a low under shrub with copiously spreading, terete branches. It is found in South India and Ceylon and is traditionally used for treating various skin disorders and tumours.7) It is found to be active against inflammation and transplantable tumors.8) In continuation of our previous work on this plant this study attempts has been made to evaluate the chemopreventive effect of ethanolic extract of I. aspalathoides (EIA) against DEN induced hepatic carcinogenesis in rats.

MATERIALS AND METHODS

Plant Material and Extraction Stems of Indigofera aspalathoides were collected in and around Salem district in the month of December 2002 and authenticated by Dr. G. Murthy, Botanical Survey of India, Coimbatore, Tamilnadu, India. The stems were dried in shade and pulverized. The powder was treated with petroleum ether for dewaxing and removal of chlorophyll. Later, it was packed (250 g) in a Soxhlet apparatus and subjected to hot continuous percolation for 8 h using 450 ml of ethanol (95% v/v) as solvent. The extract was concentrated under vacuum, dried in a desiccator (yield, 4.5% w/w) and suspended in 5% gum acacia for the study.

Animals Male Wistar rats (100—125 g) were procured...
from Tamilnadu Veterinary College, Chennai, India. They were housed in standard microlon boxes with standard laboratory diet and water *ad libitum*. The protocol was approved by Institutional animal ethics committee constituted for the purpose.

**Chemicals**  N-Nitrosodiethylamine (DEN) was purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.), 1-Chloro2,4-dinitro benzoic acid (CDNB), 5,5-dithio-bis-2-nitro benzoic acid (DTNB), reduced glutathion (GSH) and glutathion were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Thiobarbituric acid was purchased from E-Merck, India. All other chemical used were of analytical grade.

**Experimental Design**  The rats were divided into three groups and each group consisting of six animals. Liver tumor was induced in group 2 and 3 with single intraperitoneal injection of DEN at a dose of 200 mg/kg body weight in saline. Two weeks after DEN administration, the carcinoenic effect was promoted by 0.05% phenobarbitol, which was supplemented to the experimental animals through drinking water for up to 16 successive weeks.10)

Group 1: Normal-control animals
Group 2: DEN-treated animals
Group 3: DEN-treated animals given EIA (250 mg/kg, *p.o.*)11) for 16 weeks after the administration of DEN on 5 d per week.

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

**Biochemical Estimation**  Serum was analysed for the following biochemical parameters: serum glutamate oxaloacetate transaminase (SGOT),12) serum glutamate pyruvate transaminase (SGPT),13) alkaline phosphatase,14) total bilirubin15) and gamma glutamate transeptidase (GGTP).15) A 10% homogenate of the tissue was used for the analysis of lipid peroxidation (LPO),16) superoxide dismutase (SOD),17) catalase,18) glutathione peroxidase (GPx)19) and glutathione S-transferase (GST).20)

**Histopathological Examination**  The portions of the liver embedded in paraffin, sectioned at 5 μ and were stained with haematoxylin–eosin. Light microscopy was used to evaluated pathological changes of liver.

**Statistical Analysis**  The values were expressed as mean±S.E.M. Statistical analysis were performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison test. *p* value <0.05 were considered as significant.

**RESULTS**  The preliminary phytochemical studies have revealed the presence of alkaloids, tannins, phytoesters and flavonoids. All the animals treated with DEN had a significant incidence of liver tumor at the end of 16 week as evidenced by: (i) Increase in liver weight (Fig. 1), (ii) Increased hepatic enzymes such as SGPT, SGOT, ALP and also total bilirubin (Table 1), (iii) Increased level of GGTP, GPx, GST and LPO (Table 2), (iv) Decrease in SOD and catalase (Table 2) and (v) morphological changes. All these changes were reverted back to normal by the EIA indicating a strong inhibition of hepatocellular carcinogenesis induced by DEN.

The liver weight increased nearly two folds in those animals which received DEN, the carcinogen. The liver weight of normal animals was 4.1±0.10 g/100 g body wt and it increased to 7.8±0.12 g in those which received DEN. Administration of the EIA brought down the weight to 5.6±0.10 g (Fig. 1) and the reduction is significant (*p*<0.001).

DEN treatment increased the levels of liver enzymes SGOT, SGPT, ALP as also that of total bilirubin. These lev-

![Graph](image)

**Fig. 1. Effect of EIA on Liver Weight Variation of Control and Experimental Groups**

Values are expressed as mean±S.E.M. (n=6). a, b) *p*<0.001 vs. control and DEN treated rats.

<table>
<thead>
<tr>
<th>Design of treatment</th>
<th>Dose (mg/kg)</th>
<th>GGTP U/l</th>
<th>Lipid peroxidase (μmol of MDA/min/mg protein)</th>
<th>GPx (μmol of GSH oxidised/min/mg protein)</th>
<th>GST (μmol of CDNB conjugation formed/min/mg protein)</th>
<th>SOD (Units/min/mg protein)</th>
<th>Catalase (μmol of H2O2 consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>48.3±1.3</td>
<td>6.9±0.08</td>
<td>6.5±0.20</td>
<td>0.18±0.005</td>
<td>1.35±0.08</td>
<td>72.5±1.0</td>
</tr>
<tr>
<td>DEN</td>
<td>200</td>
<td>88.2±3.6</td>
<td>11.7±0.29</td>
<td>18.3±0.84</td>
<td>0.28±0.008</td>
<td>0.96±0.02</td>
<td>50.4±1.2</td>
</tr>
<tr>
<td>EIA+DEN</td>
<td>250</td>
<td>52.6±3.1</td>
<td>8.05±0.18</td>
<td>14.2±0.45</td>
<td>0.17±0.004</td>
<td>1.24±0.06</td>
<td>73.5±1.6</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.E.M. (n=6). a, b) *p*<0.001 vs. control and DEN treated rats; c) *p*<0.05 vs. DEN treated rats.
els were markedly lowered (p<0.001) by the administration of EIA (Table 1).

The level of GGTP, GPx, GST and lipid peroxides were significantly elevated (p<0.001) by the administration of DEN. These elevated levels were lowered by the administration of EIA (Table 2).

The levels of SOD and catalase antioxidants were lowered by DEN and they were returned back and raised to normal levels by the EIA. DEN induced histopathological changes in liver such as fatty infiltration, variation in mitotic figures and focal necrosis. These changes are indicative of hepatocellular carcinoma. All these histopathological changes were reversed by the administration of EIA.

DISCUSSION

In recent times, there is an increased risk of malignancy because of environmental pollution such as exposure to genotoxic and carcinogenic chemicals. This has created awareness to prevent the harmful effect of these chemical agents. This has lead to the development of several preventive agents. These agents significantly reduce tumor incidence, delay tumor onset and also have minimal long-term toxicity.

Any natural or synthetic agents which exhibits any or combination of these characteristics will qualify as a cancer chemopreventive agent. The present study was undertaken to establish the cancer chemopreventive efficacy of EIA against DEN induced malignancy of liver.

Treatment with the EIA 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 GGTP, GPx and GST. All these indicate an induction of hepatocellular carcinoma by DEN. Treatment with the EIA reduced the levels of all these tumor markers.

Increased activity of GGTP is responsible for the increased levels of GPx and GST in DEN treated group of animals. This increased level of GST and GPx likely to be the key mediator of drug resistance in cancer chemotherapy. The decreased level of these two enzymes in the EIA treated groups compared to those treated with DEN is indicative of its antimalignant potency. A high level of GST occurs in neoplastic and preneoplastic lesions induced by hepatic chemical carcinogens. The low level of GST in animals receiving the extract plus DEN only indicates the ability of the EIA to inhibit tumor progression.

DEN has been shown to be metabolized by microsomal mixed function oxidase system to its active ethyl radical metabolites. These reactive radicals interact with DNA producing mutation and oncogenesis. In hepatocellular carcinoma there is a disequilibrium between oxidant and antioxidant balance which is tilted towards oxidant side. This oxidative stress may be the reason for the elevated LPO level in the liver of DEN treated animals. LPO may lead to the formation of several toxic by-products such as 4-hydroxyxone-nal and melaconaldehyde which can attack cellular targets including DNA, inducing mutagenicity and carcinogenicity. Treatment with the extract after the administration of DEN significantly reduced the level of LPO and SOD. Further, histopathological studies showed a significant reduction of mitotic level and hyperplasia in the livers of animals treated with the extract and DEN those treated with DEN alone.

All these observations clearly indicate a chemopreventive function of the extract previous studies conducted by us the extract has antitumor activity but no sub acute toxicity. Preliminary phytochemical studies have shown the presence of alkaloids and flavonoids in EIA. Flavonoids are known to possess antimutagenic and antimalignant effects. Moreover, flavonoids have a chemopreventive role in cancer through the induction of enzymes affecting carcinogen metabolism and inhibit various activities of tumor promoters, which are involved in the process of carcinogenesis. Chemopreventive effect of the EIA may be due to the presence of these compounds. Our results clearly indicate a significant chemopreventive effect of EIA. Further studies to characterise the active principles and to elucidate the mechanism of action of EIA are in progress.

REFERENCES