Research Article

Chemopreventive effects of *Indigofera cassioides* on diethylnitrosamine induced and phenobarbital promoted rat liver carcinoma

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Abstract

Objective: In the present study, we evaluated the chemopreventive potential of methanolic leaf extract of *Indigofera cassioides* against diethylnitrosamine-induced phenobarbital promoted hepatocellular carcinoma in rats. **Materials and Methods:** Leaves of Indigofera cassioides (Fabaceae) was collected, shade dried and extracted with 80% v/v methanol by cold maceration process. The dried crude extract was used for investigation. Carcinogenesis was induced by a single intraperitoneal injection of diethylnitrosamine (200 mg/kg). Two weeks after DEN administration, carcinogenic effect was promoted by 0.05% Phenobarbital, which was supplemented to the animals through drinking water up to 16 successive weeks. After the completion of study period, body weight, tumour incidence, serum liver marker enzymes, antioxidant enzymes level, lipid peroxidation, nucleic acid levels and tissue protein content was analyzed. **Results:** A significant raise in liver weight, relative liver weight, tumour nodes, liver marker enzymes such as transaminases, lactate dehydrogenase, gamma glutamyl transpeptidase, prothrombin time, total cholesterol, total bilirubin level, lipid peroxidation and nucleic acids content were observed in tumour bearing animals. Alpha fetoprotein, carcinoembryonic antigen, vasculoendothelial growth factor and tumour necrosis factor-α level were also significantly increased. Animals treated with the extract at the dose of 200 and 400 mg/kg body weight significantly reduced the altered parameters to near normal. The elevated serum tumour marker levels were also significantly reduced by extract treatment. Chemoprevention of the extract was further confirmed by histopathological observation. **Conclusion:** From the data, the chemopreventive potential of methanolic leaf extract of *Indigofera cassioides* has been well established against diethylnitrosamine-induced phenobarbital promoted rat liver carcinogenesis.

Keywords: *Indigofera cassioides,* Diethylnitrosamine, hepatocellular carcinoma, tumour markers, chemoprevention

Introduction

Cancer incidence and its related mortality are increasing worldwide. Among the solid tumours, primary liver cancer which is known as Hepatocellular Carcinoma (HCC) is the fifth most common cancer and second leading cause of cancer mortality throughout the world (Jemal et al*.,* 2011). Exposure to environmental carcinogens such as aflatoxins, excessive consumption of alcohol, frequent consumption of processed meat, iron over load, obesity, environmental pollution, chronic infection with hepatitis B and C are the risk factors associated with HCC (Shih et al*.,* 2009; Kumar et al*.,* 2011). Currently there is no proven effective systemic chemotherapy for HCC and surgical resection is the treatment of choice of HCC in non-cirrhotic patients, but the relapse rates are as high as 50% within several years of surgery (Bishayee et al*.,* 2010). Considering the limited treatment options and prognosis of HCC, chemoprevention has considered being the best strategy in lowering the morbidity and mortality associated with this ailment. Chemoprevention is the recent strategy to control liver cancer and by definition it is the mode of cancer management in which the occurrence of the disease can be prevented, delayed or reversed by the administration of one or more natural and/or synthetic agent.

Diethylnitrosamine (DEN) is a chemical belongs to a family of carcinogenic N-nitroso compounds and is a wellknown hepatocarcinogenic agent that present in cigarette smoke,

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ground water, alcoholic beverages, cosmetics, pharmaceutical products, agricultural chemicals and occupational settings. It causes degenerative, proliferative and cancerous lesions in liver (Sivaramakrishnan et al., 2008; Gupta et al., 2010). It can alkylate DNA molecule with itself being converted to highly reactive molecule by Cytochrome P-450 dependent oxygenases and generates reactive oxygen species (ROS) causing oxidative stress (Mandal et al., 2008; Li et al., 2005). DEN forms alkyl DNA adducts which induces chromosomal abbreration, micronuclei and chromatid exchanges in the liver. These mutations induced by DEN are responsible for the development of HCC. The rat model of DEN induced HCC is considered as one of the most accepted and widely used experimental models to study HCC. Human liver metabolize DEN similar to that of rat liver and also exhibit considerable similarities with regard to genetic alteration, morphology and gene expression (Jagadeesh et al., 2009). Hence this model was chosen for the present study.

Indigofera cassioides Rottl. Ex.DC. belongs to the family Fabaceae, is a large shrub, distributed though out the hills of India. Traditionally this plant has been used to treat various disorders like inflammation, pain, jaundice and arthritis. Earlier studies in our laboratory revealed that the methanolic leaf extract of *I. cassioides* have potent antioxidant, free radical scavenging, anticancer, anti-inflammatory and anti-nociceptive activities (Kumar et al., 2011; Kumar et al., 2012; Kumar et al., 2013). In continuation of our previous works, the present study is aimed to investigate the chemopreventive effects of hydro-methanolic leaf extract of *I. cassioides* (MEIC) on DEN induced phenobarbital promoted liver tumour model in male Wistar rats.

Materials and methods

Chemicals

Diethylnitrosamine (DEN) and Phenobarbital (PB) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Silymarin was procured from Micro Labs, Hosur, India. All other chemicals used for the experiments were of analytical grade.

Animals

Male wistar rats (150-160g) were purchased from Venkateshwara Enterprises, Bangalore, India. The animals were housed in microloan cages in controlled temperature (25 ± 2˚C) and light cycle (12h dark/light) and fed with standard laboratory pellet diet with water ad libitum. The study was conducted after obtaining Institutional Animal Ethical Committee (IAEC) clearance.

Plant material and extraction

Leaves of *I. cassioides* were collected from Yercaud Hills and it was authenticated by the botanist, Botanical Survey of India, Coimbatore, India. The shade dried, powdered plant material was extracted with 80% v/v methanol by cold maceration process for 72h. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (50˚ - 60˚C) in a rotary evaporator. The dark brown solid mass obtained was stored in a vacuum desiccator until further use.

Experimental design

The rats were divided into five groups, each group consisting of six animals. Liver tumour was induced in group II-V with single intraperitoneal injection of DEN at a dose of 200 mg/kg body weight in saline. Two weeks after DEN administration, carcinogenic effect was promoted by 0.05% Phenobarbital, which was supplemented to the animals through drinking water up to 16 successive weeks (Rajkapoor et al., 2005; Singh et al., 2009). Group I animals were served as normal control which received 1 ml/kg body weight of 0.3% Carboxymethyl cellulose (CMC) through oral route. Group II animals served as DEN control group, received vehicle. Group III animals were treated with standard drug silymarin at a dose of 200 mg/kg by oral route, whereas group IV and V animals were treated with MEIC at a dose of 200 and 400 mg/kg by oral route, respectively (Kumar et al., 2011; Kumar et al., 2013). All the treatments were given for 16 weeks after the administration of DEN on 5 days per week.

At the end of 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 grossly examined for the tumour nodes and tumour incidence. A small portion of liver was fixed in 10% formalin for histopathological studies.

Serum biochemical and tumour markers estimation

Serum was analysed for serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total protein, total bilirubin, total cholesterol, gamma glutamate transpeptidase (GGTP), lactate dehydrogenase (LDH) and prothrombin time (PT) by using commercial kits (Agappe Diagnostics and Ecoline Diagnostic Kits, India) according to the manufacturer's instruction.

Liver tumour markers such as Alpha fetoprotein and Carcinoembryonic antigen in rat serum was estimated by using commercially available ELISA kits (USCN Life Science Inc. Wuhan, China). Vasculoendothelial growth factor (VEGF) and Tumour Necrosis Factor - α (TNF-α) in rat serum was estimated by using commercially available ELISA kit (RayBio, RayBiotech, Inc. USA) according to the manufacturer's instruction.

A 10% homogenate of liver tissue was used for analysis of lipid peroxidation (LPO) (Devasagayam and Tarachand, 1987), catalase (CAT) (Sinha, 1972), superoxide dismutase (SOD) (Marklund and Marklund, 1974), glutathione peroxidase (GPx) (Rotruk et al., 1973) and glutathione S-transferase (GST) (Habig et al., 1974). The liver homogenate was also used for the estimation of DNA, RNA (Schneider, 1957; Burton, 1956) and protein content (Lowry et al., 1951) in liver tissues.

Histopathological examination

The portion of the liver previously fixed in formalin was embedded in paraffin, sectioned at 5 µm and stained with haemotoxylin-eosin. Light microscopy was used to evaluate pathological changes of liver.

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 value < 0.05 was considered as significant.

Results

Effect of MEIC on body weight

This study results revealed that the body weight was significantly (P<0.001) decreased in DEN treated group as compared to the normal control. However, the extract and silymarin treatments showed significant (P<0.001) protection in body weight when compared to DEN control group. No significant difference in body weight was observed between the extract treatment and silymarin treatment. This indicates that the extract at the tested dose levels are equally potent to silymarin (Table 1).

Table 1. Effect of MEIC on body weight of DEN induced hepatocellular carcinoma

Design of treatment	Body weight (g)	
	Initial	Final
Control	152.5 ± 4.43	206.7 ± 5.11
DEN control	156.67 ± 3.1	135 ± 2.24[a]
Silymarin	159.17 ± 2.39	198.3 ± 3.3[b]
MEIC 200	152.33 ± 2.12	206 ± 3.16[b]
MEIC 400	154.32 ± 1.67	209.17 ± 5.97[b]

N=6; Data are expressed as Mean ± SEM; [a] p<0.001 vs control; [b] p<0.001 vs DEN control. Data were analysed by using Tukey-Kramer multiple comparison test.

Effect of MEIC on liver weight, relative liver weight and tumour incidence

The study showed that the liver weights were significantly increased in DEN control group. However, treatment with MEIC showed a dose dependent and significant protection in liver weight (P<0.001 to P<0.01). Whereas, standard drug silymarin do not show significant reduction in liver weight compared to DEN control group. No significant difference was observed between silymarin and extract treated groups in relative liver weight. Number of tumour nodes present in DEN treated animals was found to be very high, whereas it was found to be very less in extract treated groups. Treatment with methanol extract of *I. cassioides* significantly reduced the number of tumour nodes per animal and drastically reduces the tumour incidence. This indicates the chemopreventive potential of methanol extract of *I. cassioides*. The results are presented in table 2.

Effect of MEIC on serum hepatic parameters

It is clearly evident from the table 3 that DEN caused significant elevation of liver serum markers. In the DEN treated group, the level of SGOT, SGPT, ALP, GGTP, LDH, Total cholesterol, Total bilirubin and Total protein were significantly elevated (P<0.001 to P<0.05). A significant increase in prothrombin time was also observed in DEN treated animals. In contrast, the groups treated with methanol extract of *I. cassioides* at dose of 200 and 400 mg/kg once daily for 16 weeks prevented the cancer in a dose related manner. The chemoprevention of extract was

Table 2. Effect of MEIC on liver weight, tumour nodes and tumour incidence of DEN induced hepatocellular carcinoma

Design of treatment	Liver Weight (g)	Relative liver weight (g/100 g)	Total No. of tumour nodes	Tumour Nodes/Animal	Tumour Incidence (%)
Control	4.50 ± 0.18	2.18 ± 0.07	-	-	-
DEN control	7.91 ± 0.34[a]	5.1 ± 0.21[a]	87	14.5 ± 1.06	6/6 (100)
Silymarin	6.36 ± 0.22[a,d]	3.72 ± 0.15[a,c]	31	5.17 ± 1.4[c]	5/6 (83.3)
MEIC 200	6.54 ± 0.22[a,d]	3.33 ± 0.12[a,c]	5	0.83 ± 0.54[c,f]	2/6 (33.3)
MEIC 400	5.22 ± 0.05[c,f,g]	3.07 ± 0.14[b,c,f]	2	0.33 ± 0.3[c,e]	1/6 (16.67)

N=6; Data are expressed as Mean ± SEM; [a] p<0.001; [b] p<0.01 vs control; [c] p<0.001; [d] p<0.01 vs DEN control; [e] p<0.01; [f] p<0.05 vs silymarin; [g] p<0.01 vs MEIC 200. Data were analysed by using Tukey-Kramer multiple comparison test.

confirmed by the alterations in the serum hepatic parameters. The extract treatment was able to reverse all the elevated serum hepatic parameters to near normal and the results were comparable to that of standard silymarin treated group.

Effect of MEIC on antioxidant enzyme levels

Table 4 illustrated the lipid peroxidation and the enzymic antioxidant level in liver of experimental animals. Administration of DEN led to increase in the levels of lipid peroxidation and decrease in catalase, superoxide dismutase, glutathione peroxidase and glutathione-s-transferase levels in the 10 %w/v liver homogenate. Treatment of rats with methanolic extract of *I. cassioides* at a dose of 200 and 400 mg/kg markedly prevented the DEN induced alterations of various parameters such as lipid peroxidation, catalase, superoxide dismutase, glutathione peroxidase and glutathione-s-transferase respectively in dose dependent manner (P<0.001 to P<0.05). The protection produced by the extract was comparable with that of the standard silymarin.

Effect of MEIC on liver tumour markers, VEGF and TNF-α

Alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) are the specific liver tumour markers, which were found to be significantly increased in DEN treated animals (P<0.001). Vasculoendothelial growth factor (VEGF) and tumour necrosis factor alpha (TNF-α) levels were also found to be increased significantly (P<0.001) when compared to control group. Treatment with methanol extract of *I. cassioides* at the tested dose levels significantly reduces the elevated levels of tumour specific markers and cytokine levels in dose dependent manner (P<0.001). The results obtained were comparable with standard silymarin. The tested plant extract at higher dose level significantly reduce elevated levels of these biomarkers than silymarin. The values were tabulated and presented in table 5.

Effect of MEIC on DNA, RNA and protein content

It is clearly evident from the table 6 that DEN caused significant elevation of DNA and RNA and decrease in protein level. In contrast, the groups treated with methanol extract of *I. cassioides* at the dose of 200 and 400 mg/kg decreased the elevated levels of DNA and RNA and increase the protein level towards normalization. The results produced by the extract were almost comparable with standard silymarin. The histological observations also basically support the results obtained and the results are displayed in figure 1(A) to figure 1(E).

Table 3. Effect of MEIC on liver parameters of DEN induced hepatocellular carcinoma

Design of treatment	SGOT	SGPT	ALP	TPL	TBL	TCL	GGTP	LDH	PT
Control	110.7 ± 16.38	68.37 ± 2.71	9.11 ± 0.79	5.96 ± 0.24	0.52 ± 0.05	95.33 ± 5.58	10.45 ± 1.13	72 ± 2.08	15.3 ± 1.12
DEN control	230.7 ± 13.5[a]	188.3 ± 3.4[a]	15.37 ± 2.07[a]	2.27 ± 0.21[a]	2.96 ± 0.24[a]	157.3 ± 7.98[a]	28.67 ± 1.69[a]	151.32 ± 10.22[a]	48.3 ± 2.75[a]
Silymarin	170.83 ± 7.91[b,d]	67 ± 2.9[c]	8.33 ± 0.74[c]	5.27 ± 0.23[c]	0.88 ± 0.05[c]	101 ± 3.75[c]	12.53 ± 1.27[c]	86.08 ± 3.2[c]	35 ± 0.73[a,c]
MEIC 200	125.25 ± 5.1[c]	75.38 ± 6.67[c]	9.59 ± 0.25[d]	4.49 ± 0.19[c]	0.57 ± 0.05[c]	102.7 ± 7.26[c]	13.48 ± 0.76[c]	76.08 ± 3.57[c]	28.17 ± 1.52[a,c]
MEIC 400	121.42 ± 6.36[c]	69.95 ± 3.56[c]	7.47 ± 0.67[c]	5.46 ± 0.32[c]	0.52 ± 0.03[c]	109.3 ± 4.34[c]	13 ± 0.43[c]	65.33 ± 3.58[c]	28.83 ± 2.68[a,c]

N=6; Data are expressed as Mean ± SEM; [a] p<0.001; [b] p<0.01 vs control. [c] p<0.001; [d] P<0.01 vs DEN control. [f]p<0.001; Data were analysed by using Tukey-Kramer multiple comparison test.

Table 4. Effect of MEIC on antioxidant enzyme levels and lipid peroxidation of DEN induced hepatocellular carcinoma

Design of	Antioxidant enzymes				LPO
treatment	SOD	CAT	GPx	GST	
Control	2.61 ± 0.15	69.42 ± 1.21	26.18 ± 0.47	107.82 ± 1.45	8.6 ± 0.48
DEN control	0.54 ± 0.03[a]	16.57 ± 0.31[a]	7.83 ± 0.29[a]	52.61 ± 3.04[a]	26.87 ± 1.39[a]
Silymarin	1.56 ± 0.1[a,d]	44.18 ± 1.25[a,d]	14.62 ± 0.89[a,d]	81.95 ± 2.75[a,d]	12.42 ± 0.57[c,d]
MEIC 200	1.23 ± 0.07[a,d]	46.99 ± 0.73[a,d]	9.02 ± 0.53[a,f]	62.81 ± 2.07[a,e,f]	13.64 ± 0.99[b,d]
MEIC 400	1.61 ± 0.1[a,d]	55.82 ± 0.92[a,d,f,g]	15.78 ± 0.59[a,d,g]	83.94 ± 2.34[a,d,g]	10.01 ± 0.44[d,h]

N=6; Data are expressed as Mean ± SEM; [a] p<0.001; [b] p<0.01; [c]p<0.05 vs control. [d] p<0.001; [e] P<0.05 vs DEN control. [f]p<0.001 vs silymarin; [g]p<0.001; [h]p<0.05 vs MEIC 200. Data were analysed by using Tukey-Kramer multiple comparison test.

Table 5. Effect of MEIC on liver tumour markers of DEN induced hepatocellular carcinoma

Design of Treatment	AFP (ng/ml)	VEGF (pg/ml)	CEA (ng/ml)	TNF-α (pg/ml)
Control	1.03 ± 0.05	8.57 ± 0.29	1.21 ± 0.08	683.3 ± 47.91
DEN control	12.24 ± 0.41[a]	37.79 ± 0.61[a]	7.85 ± 0.24[a]	3774.3 ± 105.58[a]
Silymarin	7.29 ± 0.54[a,c]	19.55 ± 0.37[a,c]	5.42 ± 0.12[a,c]	2320.17 ± 65.7[a,c]
MEIC 200	5.86 ± 0.17[a,c,e]	17.79 ± 0.42[a,c,e]	3.41 ± 0.09[a,c,d]	2969.42 ± 39.26[a,c,d]
MEIC 400	3.29 ± 0.13[a,c,d,f]	10.56 ± 0.23[b,c,d,f]	2.12 ± 0.07[a,c,d,f]	2507.7 ± 18.52[a,c,f]

N=6; Data are expressed as Mean ± SEM; [a] p<0.001; [b] P<0.05 vs control. [c] p<0.001 vs DEN control. [d]p<0.001; [e]p<0.05 vs silymarin; [f]p<0.001 vs MEIC 200. Data were analyzed by using Tukey-Kramer multiple comparison test.

Table 6. Effect of MEIC on DNA, RNA and protein content of DEN induced hepatocellular carcinoma

Design of Treatment	DNA (mg/g of wet tissue)	RNA (mg/g of wet tissue)	Proteins (mg/g of wet tissue)
Control	6.31 ± 0.28	8.02 ± 0.41	8.76 ± 0.32
DEN control	9.10 ± 0.39[a]	10.49 ± 0.72[b]	5.09 ± 0.3[a]
Silymarin	5.95 ± 0.2[d]	7.2 ± 0.39[d]	7.24 ± 0.35[c,d]
MEIC 200	7.01 ± 0.19[d]	8.64 ± 0.29	6.59 ± 0.20[a,e]
MEIC 400	6.57 ± 0.16[d]	7.22 ± 0.21[d]	8.30 ± 0.35[d]

N=6; Data are expressed as Mean ± SEM; [a]p<0.001, [b]p<0.01, [c]P<0.05 vs control; [d]p<0.001, [e]P<0.05 vs DEN control. Data were analysed by using Tukey-Kramer multiple comparison test.

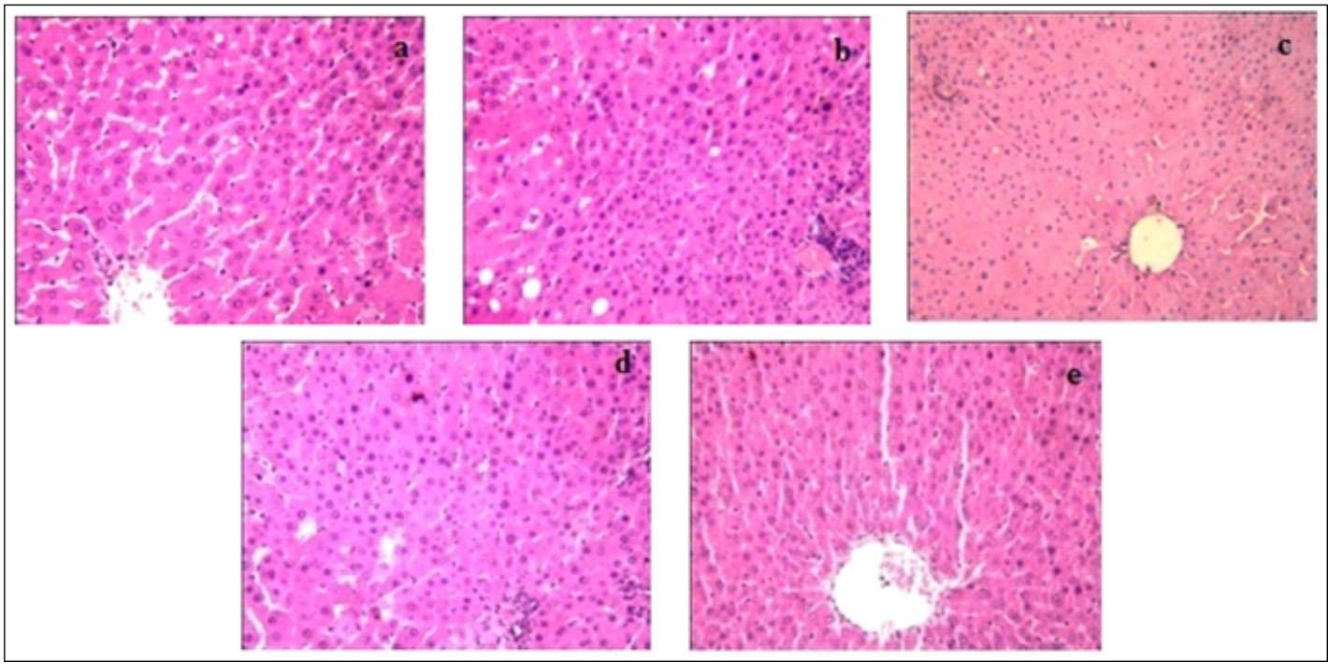

Figure 1. Figures (a) to (e) represent the histological examination of liver viewed under light microscope in control and experimental animals (Hematoxyllin and Eosin staining x 400) (a) Liver from normal control group revealing normal architectural pattern with central vein and hepatic cords. (b) Liver from tumour control group revealing monotonous sheet arrangement of neoplastic, multi-nucleated, highly proliferating hepatocytes. (c) Liver from Silymarin treated group showing lack of inter-hepatic cord space and pleomorphic hepatocytes. (d) Liver Hepatocytes of MEIC 200mg treated group showing mild pleomorphism. (e) Liver from MEIC 400 mg treated group revealing almost normal radiating hepatic cords

Discussion

Hepatocellular carcinoma (HCC) is one of the most dreadful cancers with a worldwide incidence of over one million cases every year (Jemal et al., 2011). It is induced by toxic industrial chemicals, air pollutants, water contaminants, food additives and fungal toxins (Park et al., 2009). DEN predominantly induces liver tumours in various species. It is widely accepted that metabolic activation of nitrosamines by cytochrome P450 enzyme to reactive electrophiles is required for their cytotoxic mutagenic and carcinogenic activity. 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 (Borbath et al., 2010). Hence in this study hepatocellular carcinoma was successfully induced in rats by using DEN. Plants and plant products have been shown to play an important role in the management of various liver disorders. The present study was undertaken to establish the cancer chemopreventive effect of MEIC against DEN induced liver tumour.

There is an appreciable loss in body weight in HCC bearing rat as compared to control rats (Glory and Thiruvengadam, 2012). The body weights steadily increased after treatment with extract and silymarin in HCC bearing animal when compared with tumor control which indicate that the plant extract reduces the tumour incidence and changes in energy metabolism and also shows anticancer potency.

Several investigators reported that the increase in liver weights of HCC bearing animal might be due to increased cell proliferation largely due to protein degradation during tumour growth. Protein metabolic perturbations in the tissues of host causing tissue waste may also favour the growth of tumour (Bhattacharya and Chatterjee, 1998). Relative liver weight was significantly increased in DEN treated animals, which is due to the increase in liver weight without increase in body weight of the tumour bearing animals. The increase in relative liver weight could be due to hyperplasia, hypertrophy and induction of cirrhosis of liver by DEN (Roy and Gadad, 2016). The administration of MEIC and silymarin decreased the liver weight and relative liver weight which shows the rehabilitating capability of extract in respect with anticancer potency in comparison with the standard drug silymarin. Our result is in agreement with the previous reports (Ghosh et al., 2012).

Tumor nodules are the precursors of hepatic cancer and also severity of liver cancer may correlates with number and incidence of tumour nodules (Sell and Leffert, 2008). Several nodes were found in DEN treated animals. Treatment with MEIC significantly increases the nodule growth and reduction of tumour incidence which establish chemopreventive property of MEIC. The extract treatment also delays the tumour onset which was confirmed by reduced morphological changes establishes the evidence for cancer preventive effect against HCC.

The changes in the activities of liver marker enzymes reflect the effect of proliferation of cells with growth potential and its metabolic turnover. The rise in their activities is shown to be in good correlation with the number of transformed cells in cancer conditions. The carcinogenesis process in the liver also affecting the liver cells with subsequent breakdown in membrane architecture of the cells leads to their release in to sera where their levels goes high (Ramakrishnan et al., 2007).

In the present investigation, animals treated with DEN causes hepatocellular damage which was observed from significant increase in the serum liver marker enzymes like SGOT, SGPT, ALP, LDH and GGTP. The raised levels of these markers are significantly reduced to near normal in the extract and silymarin treatment. It shows the antineoplastic effect of plant extract as with the standard drug silymarin.

LDH activity is related to functional hepatocytes and a high rate of glycolysis is manifested in malignant conditions because highly proliferating cancer cells utilize more energy than required in normal state. This may lead to continuous oxidation of glucose in glycolysis and to an abnormal increase in LDH levels (Scatena et al., 2010). GGTP level was increased in HCC induced by several chemical carcinogens and it acts as an important marker for HCC. It is a plasma membrane combined glycoprotein which is known to break the gamma glutamyl bond of glutathione and the metabolism of glutathione xenobiotic conjugates (Rocchi et al., 1997). This enzyme is induced mainly due to hepatic damage. An increase in the activity of GGTP causes resorption of reduced glutathione by the neoplastic foci rich in GGTP that enhances the proliferation and tumour promotion and favours transformation of precancerous foci in neoplastic disorder. In the present investigation, both LDH and GGTP levels were significantly increased in DEN treated group. This elevated level was significantly restored in extract as well as silymarin treatment. Our results were good agreement with previous reports (Jahan et al., 2011).

The total bilirubin level is a biomarker for liver damage. The accumulation of bilirubin in the serum is a result of decreased biliary excretion after the conjugation of bilirubin in the liver rather than the result of an increased bilirubin load caused by hemolysis. In hepatic tumours haemolysis plus deranged liver function leads to hyperbilirubinemia. In the present investigation, the HCC

bearing animals showed an elevation in levels of serum bilirubin which may be due to the leakage of plasma membrane and loss of functional integrity of cell membranes in liver (Sivaramakrishnan et al., 2008; Chen et al., 2012). Also the elevated levels of lipids in tumour bearing animals may be due to decreased activities of lipid metabolizing enzymes Lyon et al., 1982. The groups treated with MEIC showed significant dose dependent restoration of bilirubin level as well as total cholesterol level. In the present investigation, a decline in protein content was observed in DEN treated animals and this may be due to the use of host protein for cancer development. MEIC administration increased the protein content which indicates that the plant extract is involved in the maintenance of macromolecules such as proteins.

When liver function is severely abnormal, the synthesis and secretion of clotting proteins into the blood is decreased. The determination of prothrombin time (PT) indicates the concentrations of some of the clotting factors made by the liver. In non-cholestatic chronic liver diseases, the prothrombin time is not elevated usually until cirrhosis and significant liver damage occur (Toyoda et al., 2015). In the present study, prothrombin time was increased in tumour control animals whereas it was brought back to normal in treatment groups. This indicates the hepatic chemoprotective effect of plant extract.

DEN treatment increase the levels of lipid peroxidation in carcinogenic process may be due to abnormal levels of reactive oxygen species (ROS) that react with membrane lipids (Singh et al., 2004). Measurement of tissue malondialdehyde (MDA), one of the end products of lipid peroxidation, is commonly used as an indirect index for assessing the extent of lipid peroxidation in tissues (Pracheta et al., 2011). The treatment with MEIC lowered the malanoaldehyde (MDA) levels in comparison with HCC bearing animals. Therefore it is convenient to suggest that the extract definitely have beneficial effect on N-nitosodiethylamine induced HCC. The presence of flavonoids in *I. cassioides* may contribute this effect because they are proved to be potent inhibitors of conjugated dienes and are able to inhibit lipid peroxidation. Phenobarbital is a tumour promoter and it has a strong inhibiting effect on cellular antioxidant defense system like SOD, catalase, GST and GPx (Yadav and Bhatnagar, 2007). A sharp fall in these antioxidant enzymes can be due to increased free radicals production during the metabolism of DEN and Phenobarbital. Upon treatment with MEIC, a dose dependent increase in the levels of antioxidant enzymes and the results were almost equal to the standard drug silymarin. In addition, our research group found that *I. cassioides* acts as a potent free radical scavenging, antioxidant enzyme inducing and having antitumour properties (Kumar et al., 2011; Kumar et al., 2012). The plant extract also contains rich in flavonoids, phenolics, ascorbic acid and tannins which might modulate the oxidative status via free radical scavenging and antioxidant enhancing activities in rat liver.

The most commonly used tumour markers for the diagnosis of hepatocellular carcinoma (HCC) is Alpha fetoprotein (AFP) which is a unique immunomodulatory glycoprotein. Approximately 70 % of the patients with primary liver cancer, the serum AFP level may be markedly elevated. An increased level of AFP has also been noted in patients with gastric, colonic and pancreatic cancer. Detection of AFP during monitoring of liver cancer treatment is well accepted in patients (Zamcheck and Pusztaszeri, 1975). It has been recognized that exposure of animals with DEN increases the circulating AFP levels. In our study, the results showing significant rise in levels of AFP in tumour bearing animals and that were found to be reduced in extract treated animals and our results are more consistent with the previous studies (Singh et al., 2009; Sivaramakrishnan et al., 2008; El Miniawy et al., 2014).

Carcinoembryonic antigen (CEA) is an oncofetal glycoprotein, which is expressed in normal mucosal cells and is over expressed in liver and colon cancers. It is frequently detected in high concentrations in the serum of individuals with liver tumours (Maeda et al., 1988). CEA acts as an adhesion molecule that can form aggregates between cells. It is cleared from circulation by the liver with traces taken up by the spleen and lungs. In the present study, a significant increase in serum CEA levels following DEN treatment was associated with production rate of tumours, their location, stage, size, vascularity and differentiation. Reduction in CEA expression in extract treated groups indicates the decreased proliferation rate of liver tumours. Our findings are in good agreement with earlier reports (Jagan et al., 2008; Ramakrishnan et al., 2007).

Tumour angiogenesis is the development of new vasculature from previous existing blood vessels. Physiologically, under normal circumstances angiogenesis does not occur. Under certain circumstances, such as tumour formation or wound healing, the positive regulation of angiogenesis predominates and the endothelium becomes activated. Vascular endothelial growth factor (VEGF) and angiogenin (ANG) are important angiogenic factors of neoangiogenesis. VEGF is a primary stimulant of angiogenesis. It is a multifunctional cytokine, induced by hypoxia and oncogenic mutation (Zaghloul et al., 2006). Excessive production of VEGF and ANG in HCC may contribute to angiogenesis, suggesting the potential role for the use of their antagonists in treating HCC. Antiangiogenesis has recently become the focus of the study for chemotherapy and chemoprevention. This is because antiangiogenic drugs inhibit the new blood vessels growth that provides the

tumour with nutrients and oxygen which are essential for the growth of tumour cells. Antiangiogenic agents are the inhibitors of growth factors and their receptors are promising therapeutic targets (Poon et al., 2001; Wills et al., 2006). Keeping this in mind, in the present study, the serum VEGF levels were estimated in liver tumour bearing animals. A significant rise in serum VEGF was observed in liver tumour bearing animals. Treatment with MEIC significantly reduces the VEGF levels to near normal. Many flavonoids such as genistein, kaempferol, apigenin, quercetin, luteolin, rutin and naringin have shown strong inhibition to cell proliferation and VEGF expression (Luo et al., 2008; Oak et al., 2005). Antiangiogenic activity of the plant extract may be due to the presence of rich amount of flavonoids present in the extract.

The microenvironment of any tumour is composed of fibroblasts, endothelial cells and pericytes of capillary walls, smooth muscles, immune and inflammatory cells. This elaborate infrastructure is instrumental in the growth, invasion and metastasis of cancer. The tumour microenvironment might influence tumour cell behavior. Tumour Necrosis Factor – Alpha (TNF-α) is a cytokine mainly produced by macrophages. It is highly expressed in tumours and thought to be pro-angiogenic. Interestingly, it is also a potent anti-vascular cytokine at higher doses and can be used clinically to destroy tumour vasculature. Unfortunately, TNF-α has powerful and toxic systemic side effects. Overexpression of TNF-α lead to upregulation of VEGF and induce angiogenesis and tumour metastasis. The role of TNF-α may be responsible for initiation and progression of HCC. Elevated circulating TNF-α has been observed in patients with HCC (Burton and Libutti, 2009; Yang, 2011) In the present study, a significant rise in the level of TNF-α was observed in tumour bearing animals. MEIC treatment significantly reduces the elevated TNF-α level. This indicates the complementary effect to the antiangiogenic activity of the plant extract.

Nucleic acids play an important role during neoplastic transformation. The determination of nucleic acid content was more important with regards to biological and functional aspects of the tumour. Additionally their content is found to be an independent factor of prognosis, since the size of the tumour often correlates with the nucleic acid content of tumour. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis was studied in order to determine the effects of silymarin and MEIC on macromolecular synthesis in HCC. The level of DNA and RNA of liver found to be progressively increased in hepatocellular carcinoma bearing animals. Among the nucleic acids, RNA exhibited prominent increase than DNA. The increased nucleic acid synthesis in tumour animals was found to decrease when the animals were treated with MEIC in a dose dependent manner and also shown the result effective when compared with the standard drug silymarin.

In the present investigation it has also been observed that decreased protein contents in plasma of HCC bearing animals appears which could be attributed to the impaired hepatic function resulting from infiltration with tumour. The liver is an important site of protein synthesis and it has the highest rate of synthesis of tissue proteins. Recycling of amino acids has been decreased in tumour conditions resulting in enhanced efflux of these amino acids from the tissues. Thus, the host responds to increased tumour load by increasing tissue protein breakdown. In HCC condition they exhibit hypoproteinemia. The administration of MEIC to the HCC bearing group resumed the protein level to near normal and also in comparison with the standard drug silymarin.

DEN treatment induced histological changes in liver such as fatty infiltration, focal necrosis and hepatocytes having hyperchromatic nuclei. These changes are indicative of hepatocellular carcinoma. Further, histopathological studies showed normal architecture, mild congested sinusoids and absence of hepatocarcinoma cells in the livers of animals treated with the extract and DEN compared to those treated with DEN alone. All these results indicate that MEIC have chemopreventive effect against DEN induced liver tumour.

Our previous studies on this plant has been revealed that the plant extract was rich on many phytochemical compounds such as sterols, tannins, phenolics, flavonoids, alkaloids, terpenoids and saponins. Many such compounds are known to possess potent antitumor properties (Kintzios, 2006). The plant extract also exerts good radical scavenging and antioxidant activities in various in vitro models (Kumar et al., 2012). The extract also has potent antitumour activity in animal models and it enhances the antioxidant enzyme system in tumour bearing animals (Kumar et al., 2011). The plant extract is also having potent anti-inflammatory and analgesic activity and this activity is mediated through the inhibition of inflammatory mediators which are known to play an important role in carcinogenesis process (Kumar et al., 2013).

Flavonoids are known to possess antimutagenic and antimalignancy effect (Hertog et al., 1992). In addition to antineoplastic activity, flavonoids exert growth inhibitory effects on several malignant tumour cell lines *in vitro* (Markaverich et al., 1988; Yoshida et al., 1990). Flavonoids may inhibit carcinogenesis by a) Inhibiting the metabolic activation of the carcinogen to its reactive intermediate b) Inducing the enzymes involved in the detoxification of the carcinogen c) Binding to reactive forms of carcinogens, thereby preventing their interaction with critical cellular targets such as DNA, RNA and proteins (Wattenberg,

1985). In addition, plant flavonoids could also inhibit tumour promotional events as mentioned above. It is likely that flavonoids may emerge as a distinct group of antitumour agents. Previous studies have shown that the structural feature essential for antitumour activity of flavonoids in the presence of hydroxyl group in 5th position of 'A' ring and 4' position of the 'B' ring (Middleton et al., 2000). Most of the flavonoids have this structural feature and because of this structural feature, the flavonoids exhibit the antitumour activity. Saponins have been found beneficial targeted on inhibition of tumor angiogenesis by suppressing its inducer in the epithelial cells of blood vessels and then on adhering, invasion and metastasis of tumor cells. They also exhibit the antitumor effect by cell cycle arrest and apoptosis (Man et al., 2010). Plants from the *Indigofera* genus contains Indirubin, is a purple 3.2'-bis indole. It binds to and subsequently inhibits cyclin-dependent kinases (CDKs), glycogen synthase kinase 3 (GSK3) and the aryl hydrocarbon receptor and thus suppress the growth of various cell types through cell cycle arrest (Knockaert et al., 2004; Sugihara et al., 2004). Antineoplastic and chemopreventive effects of the extract may be due to these phytochemical constituents. Hence, the chemopreventive potential of methaolic leaf extract of *I. cassioides* may be due to its antioxidant, free radical scavenging and reduction of inflammatory response and elevation of antioxidant defense system.

Conclusion

The present study clearly revealed that the methanolic extract of *Indigofera cassioides* showed potent chemopreventive effects against chemical-induced hepatocarcinogenesis. This result provides a scientific validation for the use of *I. cassioides* in traditional medicine.

Conflict of interests

None.

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