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Activity of *Prosopis cineraria* against N-nitrosodiethylamine induced liver tumors by regulating the levels of Tumor marker, Lipid peroxidation and Antioxidants

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ABSTRACT

In this study, the activity of methanol extract of *Prosopis cineraria* (MPC) was evaluated in N-nitrosodiethylamine (DEN, 200mg/kg) induced experimental liver tumor in male Wistar rats. It was found that the administration of MPC (200 and 400mg/kg) effectively suppressed liver tumor induced by N-nitrosodiethylamine (DEN) as revealed by decrease in N-nitrosodiethylamine (DEN) induced elevated levels of alfa feto protein (AFP) and lipid peroxidation (LPO). The MPC extract also produced an increase in enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and non enzymatic antioxidants [Reduced Glutathione (GSH), Vitamin C and vitamin E] levels when compared to liver tumor bearing animals. Our data suggests that MPC may extend its chemopreventive effect by modulating the levels of alfa feto protein and lipid peroxidation and augmenting antioxidant defense system.

Keywords: Alpha feto protein; Antioxidants; Lipid Peroxidation; N-nitrosodiethylamine; *Prosopis cineraria.*

INTRODUCTION

Hepatocellularcarcinoma is a major problem not only in developed countries but also in most undeveloped countries. It is induced by toxic industrial chemicals, air and water pollutants and also, food additives and fungal toxins[1]. Since the liver is the major site of metabolism of ingested materials, it is more susceptible to carcinogenic insult. Moreover, due to the high tolerance of liver, hepatocellularcarcinoma is seldom detected at the early stage and once detected treatment has a poor prognosis in most cases[2].

Hepatocellularcarcinoma (HCC) is one of the ten most common human cancers, with a worldwide incidence of over one million cases every year[3]. It accounts for about 90% of all primary liver cancers. Hepatocellularcarcinoma (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 diseased condition. A large number of agents including natural and synthetic compounds have been identified as having

some potential cancer chemopreventive value. Plants and plant products have been shown to play an important role in the management of various liver disorders.

Prosopis cineraria Linn (Leguminosae) is a small tree found in dry and arid regions of Arabia and in regions of India mainly Rajasthan, Haryana, Punjab, Gujarat, Western Uttar Pradesh and drier parts of Deccan and extends as far as South in Tuticorin.

This plant is used for the treatment of several ailments, including safeguard against miscarriage and inflammation. The literature survey has shown that there is no work being done on the protective effect of *Prosopis cineraria* against liver tumor. Hence, our present study is aimed to evaluate the protective activity of MPC against DEN induced phenobarbital promoted liver tumor by regulating the levels of alfa feto protein (Tumor marker), lipid peroxidation and enzymic and non enzymic antioxidants in male Wistar rats.

MATERIALS AND METHODS

Collection of the plant material

Prosopis cineraria (Leguminosae) collected in the month of November 2009 from kolli hills, Tamilnadu, India and identified by Botanical Survey of India, Coimbatore, and Tamilnadu, India. A voucher specimen has been kept in our laboratory for future reference.

Preparation of extract

The leaves of *Prosopis cineraria* were dried under shade and then powdered with a mechanical grinder. The powder was passed through sieve No 40 and treated with petroleum ether for dewaxing as well as to remove chlorophyll and it was later packed into soxhlet apparatus with methanol and subjected to hot continuous percolation using Soxhlet apparatus. After the completion of extraction, it was filtered and the solvent was removed by distillation under reduced pressure. The extract was stored in desiccator.

Phytochemical Screening

The MPC extract was subjected to preliminary phytochemical investigations[4] and was found with the presence of various constituents like Alkaloids, Carbohydrates, Glycosides, Phenolic compounds, Tannins and Flavanoids.

Animals

Healthy Male Wistar albino rats (6-8 weeks old) were used throughout the study. The animals were purchased from King Institute of Preventive Medicine, Chennai-600 034 and maintained in a controlled environmental condition of temperature (23 ± 2°C) and relative humidity (50-70%) on alternatively 12 hr light/dark cycles. All animals were fed standard pellet diet and water *ad libitum*. The research has followed the national ethical standards for the care and use of laboratory animals and it was approved by the Institutional Animal Ethics Committee (IAEC) constituted for the purpose.

Acute toxicity studies (LD_{50})

The oral acute toxicity study of the extract was carried out in Swiss albino mice using up and down procedure as per OECD, 2001[5]. Mice received methanol extract at various doses (500-2,000 mg/Kg) orally by gavage. They were observed for toxic symptoms continuously for the first 4 h after dosing. Finally, the number of survivors was noticed after 24 h. In the toxicity study, no mortality occurred within 24 h under the tested doses of MPC.

Sources of Chemicals

N-Nitroso Diethylamine [DEN], bovine serum albumin and 2, 4, 6-Trinitro benzene sulfonate, was obtained from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Glaxo Laboratories, CDH division, Mumbai, India.

Experimental protocol

The rats were divided into four groups, each group consisting of six animals. Group 1 served as control animals and were treated with distilled water orally for 20 weeks. Liver tumor was induced in group 2, 3, and 4 using single intraperitoneal injection of DEN at a dose of 200 mg/kg body weight in saline. Two weeks after the DEN administration, the carcinogenic effect was promoted by 0.05% Phenobarbital, which was supplemented to the experimental animals through drinking water for up to 20 successive weeks[6]. Whereas Group 2 animals receive DEN alone, Group 3 animals were treated with MPC (200 mg/kg b.wt, dissolved in 0.3% cmc) simultaneously for 20 weeks from the first dose of DEN and Group 4 animals treated with MPC (400 mg/kg b.wt, dissolved in 0.3% cmc) simultaneously for 20 weeks from the first dose of DEN. 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 10% of liver homogenate was used for antioxidant studies.

Biochemical analysis

Serum α-feto protein (AFP) was estimated by method described by Premalatha & Sachdanandam[7]. A portion of 10% of liver homogenate was used for antioxidant studies such as lipid peroxidation by the method of Ohkawa *et al.* , superoxide dismutase (SOD) by the method of Marklund and Marklund[8], catalase (CAT) by the method of Sinha[9], Glutathione peroxidase (GPx) by the method of Rotruck *et al.* [10], Glutathione Reductase (GR) by the method of Staal *et al.* [11], reduced glutathione (GSH) by the method of Moron *et al.* [12], ascorbic acid (Vit C) by the method of Omaye *et al.* [13] and vitamin E by the method of Desai[14].

Statistical analysis

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

RESULTS

Serum α-feto protein:

Fig 1 shows the level of α-feto protein (AFP) in the serum of the control and experimental animals. The group II showed the highest level of AFP (2.86 ± 0.18) IU/ml. The other groups III and IV were significantly lowered AFP levels ($p<0.001$) on dose dependent manner when compared to the tumor bearing animals of group II. Among the MPC treated animals, the group IV (400 mg/kg) showed more pronounce activity than group III (200 mg/kg). This result suggests that MPC could reduce back the level of AFP in the blood of cancerous rats near to the normal value.

Lipid peroxidation

The level of LPO in liver tissues of control and experimental animals were depicted in Fig.2. There found to

be an increase in LPO in group II (p<0.001) tumor bearing rats when compared to control animals. These significant effects were reversed in MPC (200 and 400 mg/kg) treated group III and IV (p<0.001) on dose dependent manner.

Enzymic and Non-Enzymic Antioxidants

Table 1 represents the changes of enzymic and non-enzymic antioxidants of liver tissues of control and experimental animals. The enzymic and nonenzymic antioxidants such as Superoxide dismutase, Catalase, Glutathione peroxidase, Glutathione reductase, Reduced glutathione, vitamin C and vitamin E were significantly (p<0.001) reduced in group II animals when compared with group I animals. MPC (200 and 400 mg/kg) treated (Group III and Group IV) animals, these changes were brought back to near normal but in group IV it was more effective than group III [(p<0.001 and p<0.01].

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 MPC against DEN induced malignancy of liver.

α - fetoprotein (AFP), molecule carry the onco-fetal specificity Tumor marker. AFP is a serum protein that is detected in elevated concentration in conditions like hepatocellular carcinoma. AFP is a serum protein similar in size, structure and amino acid composition to serum albumin, but it is detectable only in minute amounts in the serum of normal adults. Elevated serum concentrations of this protein can be achieved in the adult by exposure to hepatotoxic agents (or) hepatocarcinogens and are frequently associated with HCC. It is a 72 KDa α-1 globulin with an uncertain biological function, is synthesized during embryonic life by fetal yolk sac, liver and intestinal tract. AFP has high specificity for hepatocarcinoma. Its serum concentration can be used to confirm hepatocarcinoma and for the diagnosis of tumor response to therapy. More than 90% of patients with hepatic cancer have increased serum AFP levels.

In the present study, a decrease in the levels of AFP following MPC (200 and 400 mg/kg) treatment indicates a positive prognosis. The decrease in the levels on MPC co-treatment prevents the neoplastic growth and reduces hepatic disorder, indicating that it possesses anti carcinogenic properties (Fig 1).

Lipid peroxidation is regarded as one of the basic mechanisms of tissue damage caused by free radicals[15]. Administration of DEN has been reported to generate LPO products in general[16] and Phenobarbital enhanced the formation of the activated oxygen species in the preneoplastic nodules[17] in rat liver. Here the administration of DEN and Phenobarbital has shown to increase the level of liver tissue LPO during hepato carcinogenesis (Fig 2). This vigorous action may be lead by the uncompromised production of free radicals. It has been extensively reported that free radicals participated in DEN induced hepatocarcinogenesis[18, 19].

Nevertheless, by administration of MPC (200 and 400 mg/kg) in DEN induced and Phenobarbital promoted animals the level of LPO was decreased. LPO can be prevented at the initiation stage by free radical scavengers and antioxidants[20]. This may represent the antioxidant potency of MPC and it might be an effective inhibitor in reducing

TBARS formation. This scrutiny reveals that MPC is able to quench the LPO chain and is capable to shield the membrane from free radicals caused injuries.

Endogenous antioxidant system may counteract the ROS and reduce the oxidative stress with the enzymic antioxidants SOD, CAT and GPx. SOD accelerates the conversion of superoxide radical (O–2) to hydrogen peroxide while CAT or GPx converts H_2O_2 to H_2O. Depletion in the activity of these three antioxidant enzymes can be owed to an enhanced radical production during DEN and Phenobarbital metabolism. In this present observation an increase in MDA was presumably associated with increased free radicals, confirming the fact that these free radicals inhibited the activities of SOD, CAT and GPx. Here the super oxide radical itself is also capable to inhibit the activity of SOD and CAT[21]. This is supported by earlier studies that showed during the DEN induced and Phenobarbital promoted hepato carcinogenesis[22]. The observed reduction in enzyme activities may be attributed to ROS; here the ROS themselves can reduce the activities of enzymes[23]. Activities of the enzymic antioxidants are reverted to near normal in MPC (200 and 400 mg/kg) treated animals. This indicates the antioxidant potency of the drug and so preventing the inactivity of these enzymes from ROS.

Vitamin E, Vitamin C and GSH are well known non enzymic antioxidant defense system of cells. Among these vitamin E is a well recognized, important biological free radical scavenger in the cell membrane[24]. It has been shown to provide protection against superoxides as well as H_2O_2 [25] and it contributes to membrane stability[26]. In hepatoma bearing animals the level of vitamin E was decreased considerably. Vitamin C is water soluble, antioxidant vitamin and can react with vitamin E radicals to regenerate vitamin E[27]. GSH, a non protein thiol is involved in many cellular processes including the detoxification of endogenous and exogenous compounds[28]. Accordingly GSH might be depleted partly by the GPx mediated excess utilization of GSH. These three non-enzymic antioxidants are inter related by recycling processes.

Earlier report reveals that the levels of these non-enzymic antioxidants were also decreased in hepatoma bearing animals[18]. This observed reduction might be attributed to the utilization of these antioxidants to alleviate free radical induced oxidative stress. The increase in the level of these antioxidants after the administration of MPC (200 and 400 mg/kg) may be due to the direct reaction of MPC with ROS.

CONCLUSION

The active principle of the MPC extract was isolated by using Column chromatographic technique and the isolated compound was identified as 5, 4′-dihydroxy 6, 8-dimethoxy flavone skeleton by using UV, ^{1}H-NMR, ^{13}C-NMR and EIMS spectral studies. Flavonoids have been found to possess antimutagenic and antimalignant effects[29]. 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[30]. The antitumor properties of the MPC extract may be due to the presence of flavanoids.

All these observations clearly indicate a significant protective activity of methanol extract of *Prosopis cineraria*. Further studies to characterize the active principles and to elucidate mechanism of action are in progress.

Table 1. Effect of MPP on enzymic and non-enzymic antioxidants in liver of control and experimental animals

Treatment	SOD	CAT	GPx	GR	GSH	Vit -C	Vit -E
Group I (Control)	7.14 ± 0.12	145 ± 3.10	23.45 ± 1.16	3.26 ± 0.14	1.94± 0.06	0.75 ± 0.005	0.45 ± 0.002
Group II (Tumor bearing animals)	4.35 ± 0.17 [a]	83 ± 2.36 [a]	12.65 ± 1.05 [a]	1.95 ± 0.17 [a]	1.06 ± 0.10 [a]	0.31 ± 0.002 [a]	0.12 ± 0.003 [a]
Group III (MPC 200 mg/kg)	5.58 ± 0.14 [a,d]	92 ± 1.24 [a]	17.06 ± 1.15 [c]	2.32 ± 0.08 [a]	1.21 ± 0.10 [a]	0.42 ± 0.003 [a,d]	0.21 ± 0.007 [a,d]
Group IV (MPC 400 mg/kg)	6.26 ± 0.16 [b,d]	118 ± 2.12 [a,d]	20.73 ± 1.32 [d]	2.89 ± 0.12 [d]	1.49 ± 0.06 [c,e]	0.53 ± 0.007 [a,d]	0.30 ± 0.005 [a,d]

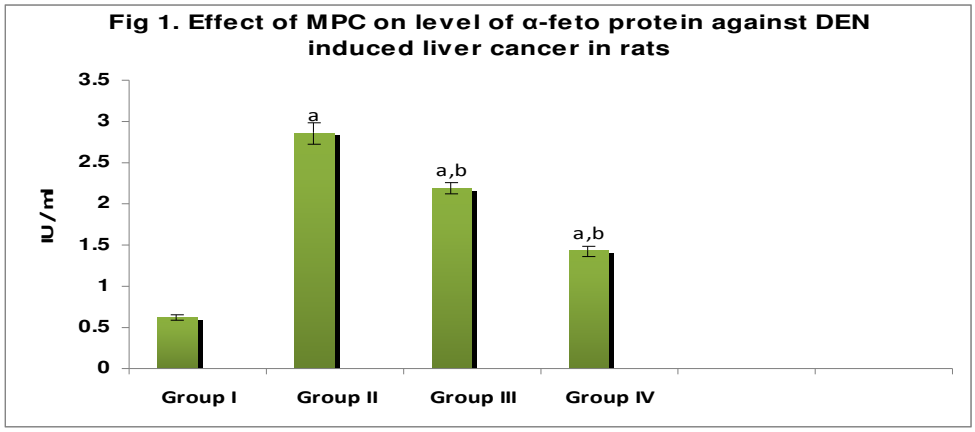
N=6; Each value is expressed as mean ± S.E.M.

[a]P<0.001; [b]P<0.05; [c]P<0.01 Vs Control

[d]P<0.001; [e]P<0.01 Vs Tumor bearing animals

Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test

Units: SOD-1U=amount of enzyme that inhibits the antioxidants of pyrogallol by 50%; CAT- µmoles of H_2O_2 consumed/min/mg protein; GPx-µmoles of GSH oxidized/min/mg protein; GSH-µg/mg protein; GR-nmoles of NADPH oxidized/min/mg protein; Vitamin C and Vitamin E- µg/mg protein



N=6; Each value is expressed as mean ± S.E.M.

Group I: control animals,

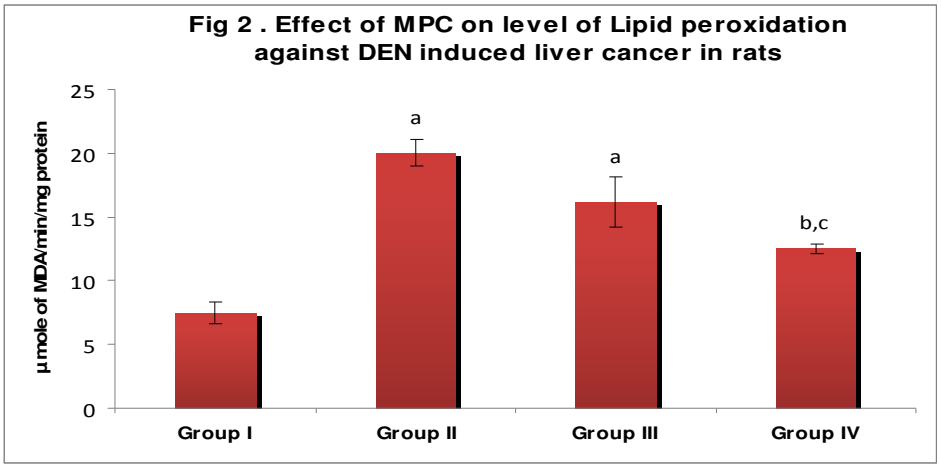
Group II: Liver Tumor bearing animals,

Group III & IV: MPC 200 and 400 mg/kg treated.

[a]P<0.001 Vs Control;

[b]P<0.001 Vs Tumor bearing animals

Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test



N=6; Each value is expressed as mean ± S.E.M.

Group I: control animals,

Group II: Liver Tumor bearing animals,

Group III & IV: MPC 200 and 400 mg/kg treated.

[a]P<0.001; [b]P<0.05 Vs Control

[c]P<0.001 Vs Tumor bearing animals

Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test

Fig 1 & 2. Effect of MPC on the levels of AFP & LPO

Treatment	Alfa Feto Protein IU/ml	LPO (µ mole of MDA/min /mg protein)
Group I (Control)	0.62 ± 0.04	7.5 ± 0.83
Group II (Tumor bearing animals)	2.86 ± 0.13 [a]	20.0 ± 1.06 [a]
Group III (MPC 200 mg/kg)	2.19 ± 0.09 [a,b]	16.2 ± 0.86 [a]
Group IV (MPC 400 mg/kg)	1.43 ± 0.05 [a,b]	12.5 ± 0.67 [b,c]

N=6; Each value is expressed as mean ± S.E.M.

L P O

[a]P<0.001; [b]P<0.05 Vs Control

[c]P<0.001 Vs Tumor bearing animals

A F P

[a]P<0.001 Vs Control;

[b]P<0.001 Vs Tumor bearing animals

Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test

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