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Protective Effect of *Prosopis cineraria* Against N-Nitrosodiethylamine Induced Liver Tumor by Modulating Membrane Bound Enzymes and Glycoproteins

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ABSTRACT

Purpose: The objective of the present study was to evaluate the protective effect of methanol extract of *Prosopis cineraria* (MPC) against N-nitrosodiethylamine (DEN, 200mg/kg) induced Phenobarbital promoted experimental liver tumors in male Wistar rats. ***Methods:*** The rats were divided into four groups, each group consisting of six animals. Group 1 served as control animals. Liver tumor was induced in group 2, 3, and 4 and Group 3 animals received MPC 200mg/kg and Group 4 animals received MPC 400mg/kg. ***Results:*** Administration of DEN has brought down the levels of membrane bound enzymes like Na^+/ K^+ ATPase, Mg^{2+} ATPase and Ca^{2+}ATPase which were later found to be increased by the administration of *Prosopis cineraria* (200 and 400mg/kg) in dose dependent manner. The MPC extract also suppressed the levels of glycoproteins like Hexose, Hexosamine and Sialic acid when compared to liver tumor bearing animals. ***Conclusions:*** Our study suggests that MPC may extend its protective role by modulating the levels of membrane bound enzymes and suppressing glycoprotein levels.

Introduction

Hepatocellularcarcinoma (HCC) 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]

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. 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 *Prosopis cineraria* against N-nitrosodiethylamine induced liver tumors on modulating membrane bound enzymes and glycoproteins.

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.

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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. Groups 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.

Na^+ / K^+ ATPase was evaluated by the method of Bonting.[7] The activity of Mg^{2+} ATPase was assayed by the method of Ohinishi *et al.*[8] The activity of Ca^{2+} ATPase was assayed by the method of Hjerten and Pan.[9] Hexose level was estimated by the method of Niebes.[10] Hexosamine content was estimated by the method of Wagner.[11] Sialic acid level was determined by the method of Warren.[12]

Statistical analysis

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

Results

Membrane ATPases activities

Table 1 shows the activities of ATPases in liver of control and experimental animals. The decrease ($p<0.001$) in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase activities were observed in group 2 tumor bearing animals when compared with group 1 control animals. MPC treated (group 3 and 4) animals showed a significant rise in the activities of ATPases, when compared with group 2 tumor bearing animals. MPC treatment both in group 3 and group 4 animals reverted these changes to near normal on dose dependent manner but in group 4 (400 mg/kg) it was found more effective than in group 3 (200 mg/kg) animals.

Glycoproteins

Table 2 represent the effect of MPC on the levels of liver glycoproteins in control and experimental animals. The glycoproteins studied were hexose, hexosamine and sialic acid. There was a rise ($p<0.001$) in the levels of all the three glycoproteins in Group 2 tumor bearing animals, when compared with control Group 1. This rise was significantly decreased ($p<0.001$) in groups 3 and 4 MPC treated animals when compared to Group 2 animals, thus reverting their levels to near normal values. It was found in the present study that MPC was more effective during the 400 mg/kg treated group (Group 4) than the 200 mg/kg treated (Group 3).

Table 1. Effect of MPC on Membrane bound enzymes in the liver of control and experimental animals

Treatment	Na^+/K^+ ATPase	Mg^{2+} ATPase	Ca^{2+} ATPase
Group 1 (Control)	4.16 ± 0.32	12.8 ± 0.23	7.5 ± 0.54
Group 2 (Tumor bearing animals)	2.35 ± 0.24 [a]	6.9 ± 0.48 [a]	3.06 ± 0.21 [a]
Group 3 (MPC 200 mg/kg)	2.79 ± 0.25 [b]	7.8 ± 0.84 [a]	4.03 ± 0.33 [a]
Group 4 (MPC 400 mg/kg)	3.32 ± 0.16 [d]	9.2 ± 0.56 [b]	5.52 ± 0.19 [b, e]

N=6 animals in each group; each value is expressed as mean ± SEM.
[a]P<0.001; [b]P<0.01; Vs Control
[d]P<0.05; [e]P<0.001 Vs Cancer bearing animals
Units: µmoles of Pi liberated/min/mg protein.
Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 2. Effect of MPC on the levels of liver glycoproteins in control and experimental animals

Treatment	Hexose	Hexosamine	Sialic acid
Group 1 (Control)	1.36 ± 0.09	2.3 ± 0.06	1.9 ± 0.04
Group 2 (Cancer bearing animals)	4.52 ± 0.27 [a]	3.86 ± 0.19 [a]	3.08 ± 0.05 [a]
Group 3 (MPC 200 mg/kg)	3.16 ± 0.12 [a, d]	3.14 ± 0.12 [b]	2.76 ± 0.15 [a]
Group 4 (MPC 400 mg/kg)	2.10 ± 0.21 [c]	2.73 ± 0.16 [c]	2.24 ± 0.07 [b, c]

N=6 animals in each group; each value is expressed as mean ± SEM.
[a]P<0.001; [b]P<0.05 Vs Control
[c]P<0.001; [d]P<0.01 Vs Tumor bearing animals
Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test
Values are expressed as mg/g of defatted tissue.

Discussion

Protecting the membrane bound enzymes is essentially important in the treatment of diseases. Tumour bearing animals found to be shown inhibited activities of all the three enzymes. The activity of Mg^{2+} ATPase in normal liver cell is 140% higher than that in hepatoma cells, as cited by Ohnishi *et al.*[8] The transport of sodium/potassium, magnesium and calcium ions across the cell membranes mediated by the membrane bound enzymes such as Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase at the expense of ATP by hydrolysis.[13] Ca^{2+}-ATPase is the enzyme which is responsible for active calcium transport and is inhibited by hydro peroxides. The peroxidative stress, which may act on the sulphydryl groups present in the active sites of the Ca^{2+} ATPase, impairs the enzyme.[14] In the present study, reduction in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase were found in liver tumour bearing animals. MPC (200 and 400 mg/kg) treated animals found to bring back near normal values of all the three ATPases.

Glycoproteins are important groups of compounds involved in the cellular function. They play a significant role in contributing to the surface properties of the cells and also important role in tumorigenesis and as mediators of immunological specificity. Carbohydrates moieties of glycoproteins have also been implicated in the transport of metabolites across cell membranes and also observed a direct relationship between glycoproteins and tumorigenesis. The biochemical markers, hexose, hexosamine and sialic acid, have been measured in the liver tissues. Many chemical changes in the host's lung and liver are detectable before the onset of secondary physiological and nutritional changes that may be associated with the condition of tumor-bearing host.

Glycoproteins exert key role in mediating cell surface function, such as cell–cell recognition, cellular adhesion, binding and clearance of serum glycoproteins and metabolic transport among others. Elevations of glycoprotein contents are useful indicators of carcinogenic process and these changes alter the rigidity of cell membrane. Malignant transformation of normal cell may be accompanied by changes in the carbohydrate composition of glycoproteins viz. hexose, hexosamine and sialic acid in liver tissue. Rachesky *et al*[15] have been reported an increased level of glycoproteins in animals exposed to carcinogen be significantly elevated in liver cancer bearing animals. These reported changes in surface carbohydrates during cellular differentiation and neoplastic transformation suggest their importance in physiology and behaviour of the cells. Such changes have long been implicated in malignant transformation. A second inference regarding the increased cell proliferation observed in DEN induced liver tumor bearing rat can be that it may lead to promotion of reactive oxygen-initiated cells, thereby enhancing the possibility of neoplastic changes. Elevations of glycoprotein components serve as a classical marker and as an indicator in the progression

of tumour growth. The crucial roles of cell surface and membrane constituents in neoplastic behaviour and changes in normal serum glycoconjugates have long been associated with malignancies. Sialic acid is the most important glycocomponent of glycoproteins, and its level was found to be significantly reduced after treatment with MPC.

This study shows that MPC (200 and 400 mg/kg) administration can inhibit glycoprotein synthesis in tumor cells (Table 2). It was found in the present study that MPC was more effective during the initiation treated groups (Group III and IV). This may be due to the inhibitory action of MPC on the initiation of DEN activation/detoxification process.

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. Flavanoids have been found to possess antimutagenic and antimalignant effects.[16] 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.[17] The antitumor properties of the MPC extract may be due to the presence of flavonoids. All these observations clearly indicate a significant protective activity of methanol extract of *Prosopis cineraria*.

Conflict of Interest

There is no conflict of interest in this study.

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