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Role of *Prosopis cineraria* against N-nitrosodiethylamine-induced liver tumor in rats with reference to marker enzymes and nucleic acid contents

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Abstract

The effect of methanol extract of *Prosopis cineraria* against experimental liver tumor in rats was studied. Liver tumor was induced by the administration of N-nitrosodiethylamine (200 mg/kg) and it was promoted by phenobarbital administration. Methanol extract (200 and 400 mg/kg) was administered to determine the protective activity. Administration of methanol extract suppressed the liver tumor effectively as revealed by the decrease in elevated levels of aryl hydrocarbon hydroxylase, lactate dehydrogenase, γ -glutamyl transpeptidase (γ -GTP), 5'nucleotidase, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). We found that methanol extract may extend its protective role by modifying the levels of marker enzymes and nucleic acid contents.

Introduction

Hepatocellular carcinoma (HCC) is one of the ten most common human cancers, with a worldwide incidence of over one million cases every year (Arya et al., 1988). 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 *P. cineraria* against liver tumor. Hence, our present study is aimed

to evaluate the protective activity of methanol extract against n-nitroso diethylamine (DEN)-induced phenobarbital promoted liver tumor by regulating the levels of marker enzymes and nucleic acid contents in rats.

Materials and Methods

Collection of the plant material

P. 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 *P. 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 methanol extract was subjected to preliminary phytochemical investigations (Kokate et al., 1991) 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 and maintained in a controlled environmental condition of temperature ($23 \pm 2^\circ\text{C}$) and relative humidity (50-70%) on alternatively 12 hours 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 constituted for the purpose.

Acute toxicity studies (LD₅₀)

The oral acute toxicity study of the extract was carried out in Swiss albino mice using up and down procedure as per OECD guidelines (OECD, 2001). 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 hours after dosing. Finally, the number of survivors was noticed after 24 hours. In the toxicity study, no mortality occurred within 24 hours under the tested doses of methanol extract.

Sources of chemicals

DEN, bovine serum albumin and 2,4,6-trinitrobenzene 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 and Glaxo Laboratories, Mumbai.

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 (Yoshiji et al., 1991).

Whereas Group 2 animals receive DEN alone, Group 3 animals were treated with methanol extract (200 mg/kg, dissolved in 0.3% carboxymethyl cellulose) simultaneously for 20 weeks from the first dose of DEN and Group 4 animals treated with methanol extract (400 mg/kg, dissolved in 0.3% carboxymethyl cellulose) 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. At the end of experimental period of 20 weeks biochemical parameters were analyzed.

Measurement of marker enzymes

The aryl hydrocarbon hydroxylase assayed by the modified method of Cantrell et al. (1973). The activity of lactate dehydrogenase was assayed by the method of King (1965). The activity of γ -glutamyl transpeptidase (γ -GTP) was evaluated by using the method of Orłowski and Meister (1965). 5'-Nucleotidase was estimated by the method of Belfield and Goldberg (1969).

Measurement of nucleic acid contents

The nucleic acids were extracted by the method of Schneider (1957). Deoxy ribonucleic acid (DNA) was estimated by the method of Burton (1956) and Ribonucleic acid (RNA) was estimated by the method of Rawal et al. (1977).

Statistical analysis

The values were expressed as mean \pm 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

The activities of the marker enzymes (arylhydrocarbon hydroxylase, lactate dehydrogenase, γ -GTP and 5'-nucleotidase) in the liver of control and experimental animals were found to be a significant ($p < 0.001$) rise in the enzyme activities in tumor bearing animals when compared with control (Table I). The rise ($p < 0.001$) in the activities of these marker enzymes found in Group 2 tumor bearing animals was significantly decreased in Group 3 and 4 methanol extract-treated animals respectively on dose-dependent manner when compared with tumor bearing group.

Tumor bearing animals showed a significantly increased nucleic acid contents (DNA and RNA) in liver tissues ($p < 0.001$; Figure 1). Methanol extract (200 and 400 mg/kg) treatment resulted in a significant decrease in the levels of nucleic acid contents in Group 3 and Group 4 animals. Methanol extract-treated

Table I

Effect of methanol extract of *Prosopis cineraria* on the activities of some marker enzymes in the liver of control and experimental rats

Group	Treatment	Arylhydrocarbon hydroxylase	Lactate dehydrogenase ^a	γ -Glutamyl transpeptidase ^a	5'nucleotidase ^a
1	Control	0.7 \pm 0.0	1.4 \pm 0.0	6.9 \pm 0.2	1.9 \pm 0.1
2	Tumor bearing	1.1 \pm 0.0 ^a	2.6 \pm 0.1 ^a	12.8 \pm 0.2 ^a	3.7 \pm 0.3 ^a
3	Methanol extract 200 mg/kg	0.9 \pm 0.0 ^{a,b}	2.3 \pm 0.1 ^{a,b}	10.5 \pm 0.3 ^{a,b}	3.0 \pm 0.1 ^{a,c}
4	Methanol extract 400 mg/kg	0.9 \pm 0.0 ^{a,b}	1.8 \pm 0.0 ^{a,b}	8.7 \pm 0.2 ^{a,b}	2.2 \pm 0.1 ^b

n = 6 animals in each group; Each value is expressed as mean \pm SEM; ^ap<0.001 Vs control; ^bp<0.001; ^cp<0.01 Vs tumor bearing animals; Data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test; ^aUnits: Aryl hydrocarbon hydroxylase- mmoles of fluorescent phenolic metabolites formed/min/mg/protein, Lactate dehydrogenase- mmoles of pyruvate liberated/min/mg/protein, γ -GTP- nmoles of p-nitroaniline formed/min/mg/protein, 5'nucleotidase-nmoles of Pi liberated/min/mg/protein

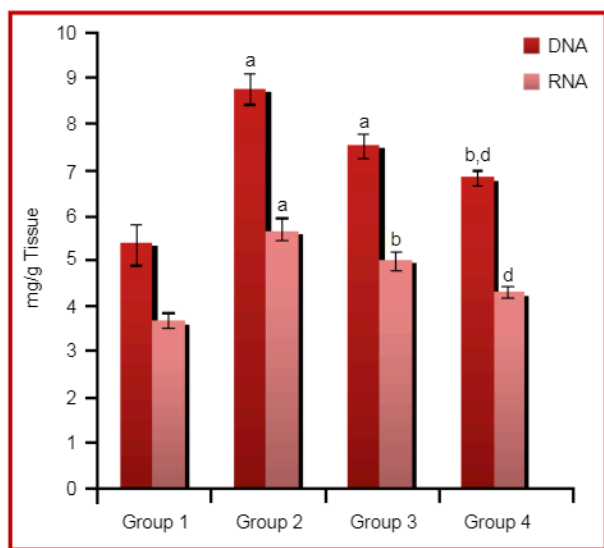


Figure 1: Effect of methanol extract of *P. cineraria* on activities of DNA and RNA in liver tissues against DEN-induced liver cancer in rats

n = 6; Each value is expressed as mean \pm SEM; Group 1: control animals, Group 2: liver tumor bearing animals, Group 3 and 4: methanol extract 200 and 400 mg/kg treated. ^ap<0.001; ^bp<0.01 Vs control; ^cp<0.001 Vs tumor bearing animals; Data were analysed by one-way ANOVA followed by Tukey-Kramer multiple comparison test

Group 4 shows more restoration than treated Group 3.

Discussion

Change in metabolism that occurs during malignancy (Stefanini, 1985) reflected by the abnormal variation in the marker enzymes. Cancer marker enzymes functioning as an indicator of cancer response to therapy. The marker enzymes such as aryl hydrocarbon hydroxylase, γ -GTP, 5'-nucleotidase and lactate dehydrogenase are specific indicators of tissue damage (Durak et al; 1993 and Ferrigno et al; 1994). The increase in the activities of these enzymes may be due

to the increased tumor incidence. Aryl hydrocarbon hydroxylase is a large group of cytochrome P₄₅₀ mono-oxygenases that complex with NAD (P) H-flavin oxidoreductase in numerous mixed-function oxidations of aromatic compounds. They are functioning as catalysts in hydroxylation of a broad spectrum of substrates and are important in the metabolism of steroids, drugs, and toxins such as phenobarbital, carcinogens.

γ -GTP is a transferase enzyme which catalyses the transfer of gamma glutamyl groups from a large variety of peptide donors to a wide range of aminoacids and peptide receptors (Valentich and Moris, 1992). γ -GTP activities were increased in cancer conditions. Chemical carcinogens entering the liver initiate some systematic effects which in turn induce γ -GTP synthesis. These elevations show the progress of carcinogenic process, since its ability is correlated with growth rate, histological differentiation and survival time of the host. An increased level of γ -GTP was observed in cancerous cells (Ngo and Nutler, 1994). This rise may indicate the presence of tumor and the reports show that γ -GTP activity in liver was significantly increased in tumor bearing rats than in control group.

Patients with solid tumors, reported to have altered 5'-nucleotidase. And the 5'-nucleotidase has been described as an important marker for differentiation of B-lymphocytes. In our study, 5'-nucleotidase has been increased significantly in the hepatoma bearing rats. It has been demonstrated that increased activity of 5'-nucleotidase in carcinoma of liver, gastrointestinal tract and pancreas (Rosi et al., 1998) also observed an increased activity of 5'-nucleotidase in leukemia patients. During methanol extract treatment 5'-Nucleotidase activity got reduced significantly on dose-dependent manner.

Lactate dehydrogenase is a cytoplasmic enzyme which is involved as a catalyst in the oxidation of lactate to pyruvate and vice versa. It is a marker for membrane integrity and is a regulator of many biochemical

reactions in the body tissues and fluids. It has been reported that an excessive activity of lactate dehydrogenase found in malignant cells which spreads through the organs of tumor bearing rats. Changes in permeability of cell membranes and the leakage to soluble enzymes caused by increased enzyme activity in the serum of patients with lung and ovarian cancer (Bose and Mukherjee, 1994). Enhanced glycolysis using the growth of tumor caused by elevated activity of Lactate dehydrogenase in tumor bearing rats. The treatment with methanol extract (200 and 400 mg/kg) causes controlled glycolysis which has reduced lactate dehydrogenase activity and protected the membrane integrity. In methanol extract-treated groups, these enzymes level were reverted to near normal level, attributed to the antimutagenic and anticarcinogenic activity of methanol extract.

Nucleic acid content of tumor is found to be an important indicator of prognosis, because it is well correlated with the size of the tumor in the cancerous condition (Gallagher, 1986). In diseased state, the degree of malignancy increases with the defective abnormalities in DNA. Reports reveal that abnormal amount of DNA was observed in various cancers including breast carcinoma, endometrial carcinoma and lung carcinoma (Ellis et al., 1991). In the present study, an increased activity was observed in DEN induced liver cancer animals and this may be due to the over expression of many enzymes which are necessary for DNA synthesis in tumor cells.

RNA levels were found to be increased in the cancerous condition as DNA and RNA are directly related to each other, an abnormally increased content of DNA may lead to an increased transcription, which in turn increased RNA content in tumor cells. The mechanisms by which tea polyphenols may act includes the inhibition of promutagen activation, the inactivation of mutagens and carcinogens, blocking and scavenging of reactive molecules, modulation of DNA replication or repair, inhibition of promotion, and inhibition of invasion and metastasis of tumor cells. These mechanisms are currently being progressively clarified. Most of the reports on mechanisms, however, still remain as suggestive or speculative (Kurado and Hara, 1999). In methanol extract (200 and 400 mg/kg) treated animals, the nucleic acid levels were decreased due to its inhibition of mutagenesis process.

Conclusion

The antitumor properties of the methanol extract may be due to the presence of flavanoids and all these observations clearly indicate a significant protective activity of methanol extract of *P. cineraria*.

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