Original article

Hepato-Protective Activity of Plumbago Zeylanica against Diethylnitrosamine Induced Liver Damage in Male Wistar Albino Rats- A Chronic Study

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Abstract

Plumbago zeylanica, a widely used oriental herb in traditional medical systems across Asia was tested for its hepato-protective activity against diethylnitrosamine (DENA) induced liver damage in male wistar albino rats. DENA induced liver damage/hepatocarcinogensis is well accepted model to assess the in vivo anticancer activity of drugs and herbal extracts. For experimental purpose the rats were divided into four groups of six animals each. Liver damage was induced in a group of rats (positive control) by administering 200 ppm DENA in drinking water for 90 consecutive days. Treatment group received both DENA in drinking water and alcoholic root extract of *Plumbago zeylanica* (500 mg/kg b.wt.) by oral gavage for 90 days. Another group of animals received only the root extract and the animals that left untreated served as negative control. Results of this study shown a significant increase in tumor markers such as alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and 5' nucleotidase in serum of DENA alone treated animals on comparison to negative control. Contrarily, the treatment group showed lower levels of tumor markers in serum when compared with positive control. Results were comparable between the animals that received only root extract and untreated animals. Conclusively, this study demonstrated the hepato-protective nature of alcoholic root extract of *Plumbago zeylanica* against chemical carcinogen induced liver damage.

Keywords: DENA, Diethylnitrosamine, AFP, CEA, Liver damage, Hepatocellular carcinoma, *Plumbago zeylanica*, Chemical carcinogen.

INTRODUCTION

Hepatocellular carcinoma (HCC) can be induced in the livers of laboratory animals by wide variety of chemicals[1]. Among them diethylnitrosamine (DENA) induced HCC is very well studied and extensively used as a model to assess the anticancer activity of different drugs and herbal extracts[1]. DENA is an environmental carcinogen which is present in air, water, tobacco smoke and processed foods[2,3]. It was also detected in vegetable oils, alcoholic drinks, steamed and fried fish[4,5]. Its exposure was reported to result in massive proliferation of oval cell population (believed to be cancer stem cells) in the liver. Not all carcinogens induce oval cell proliferation, whereas DENA does[6]. Removal of carcinogen results in rapid decline in oval cell numbers[6]. Hence it is apparent that the oval cells proliferate in response to carcinogen exposure. Further these cells are responsible for an initial increase in serum alpha-fetoprotein

(AFP) levels in HCC[7]. Thus increase in serum AFP levels correlates well to the proliferation of oval cells and onset of hepatocarcinogenesis.

AFP is an oncofetal protein that is abundantly synthesized in the fetal liver and HCC. It is used as a marker for early detection of HCC[8]. In addition to AFP, carcinoembryonic antigen (CEA) and 5'Nucleotidase were also used as markers for detection of HCC initiation and progression[9,10]. Hence, in this present investigation, the levels of tumor marker enzymes such as AFP, CEA and 5'Nucleotidase were analyzed to evaluate the potency of DENA (200 ppm in drinking water) to induce HCC and to monitor the therapeutic efficacy of alcoholic root extract of an oriental herb *Plumbago zeylanica* (P.z) in male wistar albino rats. P.z is extensively used in various traditional medical systems in Asia to treat different pathological conditions[11,12].

In this study tumor marker enzymes were found to be elevated in the serum of DENA (200 ppm) alone received animals in drinking water for 90 days. Animals co-administered with both DENA and P.z (500 mg/kg b.wt.) by oral gavage showed significantly lower levels of the markers in the serum on comparison to DENA alone treated animals, indicating that the plant extract could protect the liver against the chemical carcinogen. Plant extract alone treated animals showed insignificant difference when compared with untreated animals.

MATERIAL AND METHODS

EXTRACT PREPARATION

Dried roots of P.z were purchased in bulk from Bhaleswari Ayurvedic Company, Chennai, India. The plant material was authenticated by a pharmacognist from K.M. College of Pharmacy, Madurai, Tamil Nadu, India. The roots were powdered and defatted by soaking in petroleum ether for 14 days and then the solvent was removed at low pressure. Post defatting 95% ethanol (EtOH) was added and pharmacological components were extracted by cool percolation method. Then the extract was concentrated in a water bath under reduced pressure. The concentrate was then dried in a vacuum dessicator over anhydrous calcium chloride to yield a deep brownish black residue which was used as a therapeutic agent.

CHEMICALS

DENA was purchased from Sigma Chemical Company, USA and all other chemicals were of analytical grade.

ANIMALS

Male wistar rats weighing approximately 130-150 g were obtained from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. Animals were housed in polypropylene cages, in rooms with controlled temperature (24 ± 4 ºC), relative humidity (60 ± 5%) and proper lighting; and fed with standard pelleted diet (Hindustan Lever Limited, Bangalore, India) and water *ad libitum.* The animals were used for the study on approval by Institutional Animal Ethical Committee (IAEC no.01/007/02).

EXPERIMENTAL PROTOCOL

For experimental purpose rats were divided in to 4 groups with 6 animals in each group.
Group I - Untreated (control)
Group II - Received DENA (200 ppm) in drinking water, *ad libitum* for 90 consecutive days.
Group III - DENA 200 ppm in drinking water + P.z root extract (500 mg/kg b.wt.). P.z extract administered orally using gastric intubation, once daily for 90 days.
Group IV - Received only P.z extract (500 mg/kg/b.wt./day for 90 days) as mentioned in group III.

BIOCHEMICAL INVESTIGATIONS

At the end of experimental period, animals were sacrificed by cervical decapitation and the serum was collected to analyze AFP, CEA and 5' nucleotidase as reported earlier [9,13,14] and the procedure was briefly described as follows.

AFP ESTIMATION

AFP levels in serum was quantified based on the solid phase enzyme linked immunosorbent assay (ELISA) using the UBI MAGIWELL (USA) enzyme -immunoassay kit which includes the microwell strips coated with anti-AFP antibody (ab) and an enzyme conjugate - anti-AFP ab conjugated to horseradish peroxidase (HRP).

SAMPLE DILUENT REFERENCE

Standard set: Calibrated to 0, 3, 10, 25,100 and 200 IU / ml against WHO IS12/388.
Solution A: Buffer solution containing hydrogen peroxide
Solution B: Tetramethyl benzidine

PROCEDURE

Dispensed 25 µl of serum samples, controls and standards into the assigned wells. Then added 100 µl of UB10 IU/ml of AFP standard. The mixture was incubated for 30 min at room temperature (RT). Then the incubation mixture was removed and the wells were washed with distilled water for 5 times. Post washing 100 µl of enzyme conjugate was added to each well and incubated for 30 min at RT. After incubation, the enzyme conjugate was removed and the wells were washed as mentioned above. Then, solutions -A and –B (100 µl) were added to each well and incubated for 10 min at RT. The reaction was arrested by adding 50 µl of 1N H_2SO_4 to each well and optical density (OD) was measured at 450 nm with a microwell reader (Biotech Instruments, EL 308, USA). The values were expressed as ng/ml serum.

CEA ESTIMATION

Quantitative estimation of CEA was done based on the solid phase enzyme linked immunosorbent assay (ELISA) using the UBI MAGIWELL (USA) enzyme immunoassay kit.

REAGENTS

1. Micro-well strips with anti-CEA (primary ab) coated in each well.
2. Enzyme conjugate: anti-CEA ab conjugated to HRP (secondary ab)
3. Reference standard calibrated to 1.5, 3, 6, 15 and 30 ng/ml in the sample diluents.
4. Chromogen-substrate: Buffer solution containing hydrogen peroxide (H_2O_2)-tetramethyl benzidine.
5. 1 N sulphuric acid (H_2SO_4).

PROCEDURE

Twenty five micro liter of serum sample or standard or controls were added into appropriate wells followed by 100 µl of enzyme conjugate into each well and incubated for 60 min at RT. Post incubation wells were washed (5 x) with distilled water. Then to each well chromogen-substrate solution (100 µl) was added and incubated for 30 min at RT. The reaction was then stopped by adding 50 µl of 1N H_2SO_4 to each well. OD was measured at 450 nm as mentioned above. The values are expressed as ng/ml serum.

ASSAY OF 5'-NUCLEOTIDASE (5'-RIBONUCLEOTIDE PHOSPHOHYDROLASE)

REAGENTS

1. Tris-HCl buffer: 0.009 M, pH 7.5
2. Magnesium sulphate ($MgSO_4$) - 0.005 M
3. Potassium chloride (KCl) - 0.65 M
4. Ethylenediaminetetraacetic acid (EDTA) - 0.001 M
5. Trichloroacetic acid (TCA) - 10%.
6. Adenosine 5' monophosphate - 0.004 M.

PROCEDURE

Briefly 100 µl of plasma was added to the reaction mixture containing 1 mL of Tris-HCl buffer and 100 µl each of $MgSO_4$, KCl, EDTA, substrate and water and incubated at 37 °C for 15 min. Post incubation, the reaction was inhibited by adding 2 ml of 10% TCA and then

centrifuged. The phosphorus liberated in the supernatant was measured as briefly mentioned below using the method of Fiske and Subbarow[15]. Enzyme activity is expressed as nmoles of inorganic phosphorus liberated/min/mg protein in tissue.

PHOSPHORUS ESTIMATION IN THE SUPERNATANT

REAGENTS

1. Ammonium molybdate solution: 2.5 g ammonium molybdate was dissolved in 100 ml of 5N H_2SO_4.
2. 2, 4-Aminonaphthol sulphonic acid (ANSA): 500 mg of aminonaphthol sulphonic acid was dissolved in 195 ml of 15% sodium bisulphate and 5 ml of 20% sodium sulphite was added to it. The solution was filtered and stored in a brown bottle.

PROCEDURE

The supernatant along with aliquots of standards in the range of 8-40 µg were made up to 4.3 ml with distilled water. Ammonium molybdate (1 ml) and 400 µl ANSA were added and mixed by vortexing thoroughly and made up to 10 ml with distilled water. The standard and sample mixture was incubated for 20 min at RT. The color developed was measured at 600 nm against the reagent blank in Shimadzu spectrophotometer. The levels of inorganic phosphorus were expressed as nmoles/mg protein.

STATISTICAL ANALYSIS

Data were analyzed using SPSS/7.5 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of <0.05 were considered to indicate statistical significance and the results were expressed as mean ± S.D for six animals in each group.

RESULTS AND DISCUSSION

AFP is synthesized by tumors of the corresponding tissues, i.e. germ cell origin, particularly embryonic carcinomas and yolk sac tumours, hepatoblastomas, hepatocellularcarcinomas and very rarely gastrointestinal tumours[16]. Exposure to hepatocarcinogens was reported to increase this protein and are often used as marker for HCC[7]. In line with this, serum from DENA alone received animals shown significantly higher levels of AFP when compared with untreated animals (Figure 1). Since AFP has high specificity for HCC, its significantly low levels observed in animals co-treated with DENA and P.z extract on comparison to DENA in this study, indicates that the plant extract could protect the liver against the chemical carcinogen (Figure 1). The animals treated only with P.z extract did not increase the AFP levels and its levels were comparable with the untreated animals (Figure 1).

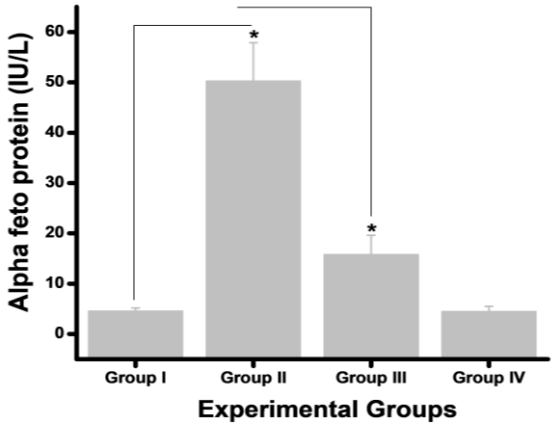

Figure 1. Serum AFP levels in male wistar rats. Group I – rat's left untreated (negative control). Group II – animals received only 200 ppm DENA in drinking water daily for 90 days (positive control). Group III – both DENA (200 ppm) in drinking water and P.z extract (500 mg/kg b.wt.) co-administered by oral gavage once daily for 90 days. Group IV – animals received only P.z (500 mg/kg b.wt.) for 90 days. AFP levels were significantly increased in DENA alone received animals. Its levels were significantly decreased in treatment group on comparison to positive control. Data were expressed as mean ±S.D; n=6; *p values < 0.05 considered statistically significant.

CEA, a member of the immunoglobulin supergene family is 180,000 Dalton glycoprotein that is elevated in various cancers[17]. In this study, CEA levels were found elevated in DENA received animals on comparison to untreated controls (group I; Figure 2). Increased CEA levels in circulation was controlled by rate of cancer cell proliferation, tumor location, stage and size and it is frequently associated with liver

metastases and also used as tumor marker[9,18]. Hence the observed increase in CEA on exposure to chemical carcinogen for 90 days via drinking water in this study indicates that consistent exposure could initiate and progress the carcinogenesis in experimental animals. Contrarily, daily administration of P.z extract by oral gavage for 90 days offered protection to the liver against the DENA induced hepatic insult. The CEA levels were found to be significantly lowered in animals co-treated with DENA and P.z when compared with DENA alone administered animals. P.z alone had no influence on the CEA production and it was comparable with the untreated animals (Figure 2).

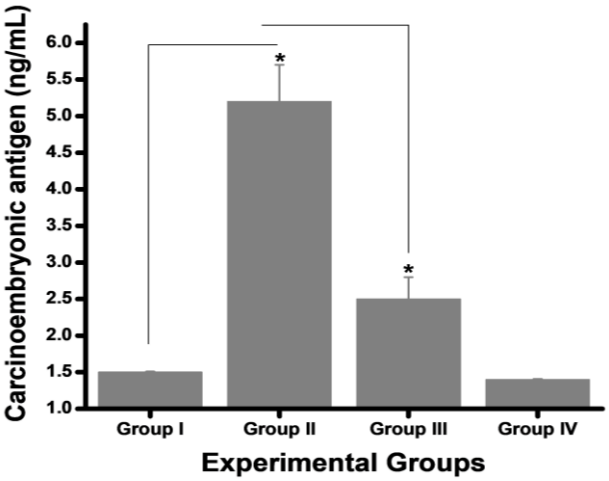

Figure 2. Influence of P.z extract administration on serum CEA levels in male wistar albino rats. Group I – negative control. Group II – positive control – received 200 ppm DENA in drinking water daily for 90 days. Group III – received DENA (200 ppm) in drinking water + P.z extract (500 mg/kg b.wt.) by oral gavage once daily for 90 days. Group IV – administered only P.z (500 mg/kg b.wt.) for 90 days. CEA levels were significantly increased positive control, while lower levels were observed in treated animals (group III). CEA levels were in different in group IV on comparison to negative control. Data expressed as mean ± S.D; n=6; *p values < 0.05 considered statistically significant.

Serum 5'- Nucleotidase, serves as the marker for liver injury due to bile duct obstruction or impaired bile flow[19]. Its activity was demonstrated to be raised in carcinoma of the liver, gastrointestinal tract and pancreas[19]. In this study, increased levels of 5' nucleotidase was detected in serum of animals that received only DENA for 90 days (group II) on comparison with its control (group I). Similar to other tumor markers discussed above, 5' nucleotidase levels were also found to be lowered in animals co-treated with DENA and P.z extract indicating the hepato-tonic nature of plant extract. Animals that received only P.z extract shown no significant difference in enzyme levels when compared with control group (Figure 3).

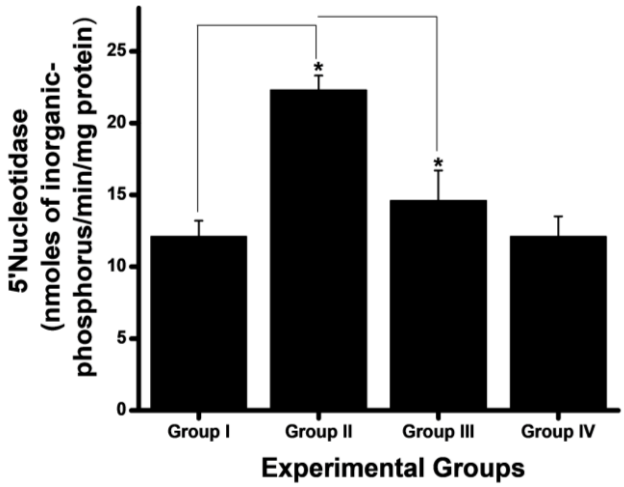

Figure 3. 5' nucleotidase levels in serum of male wistar albino rats treated with P.z extract and its respective controls. Group I – negative control. Group II – positive control – received 200 ppm DENA (200 ppm/day for 90 days) in drinking water. Group III – received DENA (200 ppm) in drinking water + P.z extract (500 mg/kg b.wt./day for 90 days) by oral gavage. Group IV – administered only P.z (500 mg/kg b.wt.) for 90 days. Significantly elevated enzyme levels were found in positive control; in contrast P.z treated group showed near normal levels of enzyme in serum. No significant changes in 5'nucleotidase levels were observed in P.z alone received animals when compared with negative control.

CONCLUSION

AFP, CEA and 5'Nucleotidase were generally found elevated in HCC. Increase of these tumor markers in serum of animals that received only 200 ppm DENA for 90 days in drinking water indicates, that with this dose regimen DENA could induce heptocarcinogenesis in experimental animals. Decreased levels of markers in serum of animals co-treated with DENA and P.z extract

indicates positive prognosis and hepato-protective nature of plant extract against chemical carcinogen.

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