

RESEARCH COMMUNICATION

Anti-carcinogenic Potentials of a Plant Extract (*Hydrastis canadensis*): I. Evidence from In Vivo Studies in Mice (*Mus musculus*)

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Abstract

Ethanollic extract of *Hydrastis canadensis* has been tested for its possible anti-cancer potentials against p-dimethylaminoazobenzene (p-DAB) induced hepatocarcinogenesis in mice. Mice were chronically fed p-dimethylaminoazobenzene (p-DAB) and phenobarbital (PB), two hepato-carcinogens for 1, 2, 3 and 4 months, respectively, and were divided into sub-groups: i) fed normal low protein diet (Gr. I, normal control); ii) fed diet mixed with 0.06% p-DAB at a daily dose of 165 mg/kg b.w. per mouse plus 0.05% PB plus 0.06 ml 90% alcohol (vehicle of the crude extract) (Gr. II, carcinogen treated); iii) fed diet mixed with p-DAB and PB at the same daily dose plus crude extract of *Hydrastis canadensis* (Gr. III, drug treated). Several biochemical parameters like acid and alkaline phosphatases, alanine amino-, aspartate amino-, and gamma glutamyl-transferases, lipid peroxidation, reduced glutathione content, lactate dehydrogenase, catalase and glucose-6-phosphate dehydrogenase activities and electron microscopy of liver in different groups of treated and control mice were studied. A critical analysis of results of these studies suggested anti-cancer potentials of the drug suitable for use as a supportive complementary medicine in liver cancer.

Keywords: Hydrastis - anti-carcinogenesis - bio-markers - p-dimethylaminoazobenzene - phenobarbital

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Introduction

Hepatocarcinogenesis in mice induced by azo-dye has been extensively used as a tool in various toxicological studies earlier (Daoust and Molnar 1964; IARC 1987; Nesnow et al., 1987; Ohnishi et al. 2001) including in CAM practices (Biswas and Khuda-Bukhsh 2002; Kuzeyli et al. 2006). p-dimethylaminoazobenzene (p-DAB) is a potent carcinogen (initiator) and phenobarbital (PB) is a promoter of hepatic tumors (Kitagawa and Sugano 1977). When these two chemicals are chronically fed to mice or rats, liver tumors develop after 60 days, which subsequently transform into neoplastic growth (Ohnishi et al. 2001). Chronic feeding of both p-DAB and PB has been reported to generate cytogenetical damage manifested as chromosomal aberrations (CA), various nuclear anomalies including induction of micronuclei (MN), and sperm head anomaly (SHA) (Biswas et al 2004; Biswas and Khuda-Bukhsh 2005).

There has been extensive use of *Hydrastis* extract as popular herbal medicine in some traditional systems like Ayurved, Unani, etc apart from its use in homeopathy, generally in micro doses. Earlier studies on *Hydrastis canadensis* (Family: Ranunculaceae) revealed various berberine and protoberberine alkaloids (NTP 2007) in

the crude extract. In fact, in *Hydrastis canadensis* three major alkaloids, namely, "canadine", which stimulate uterine muscles, "hydrastine" and "berberine", which are known to have anti-spasmodic and anti-bacterial effects are of major importance in rendering various biological activities. Of these, berberine has been reported to induce cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line (Lin et al 2006).

Crude ethanolic extract of *Hydrastis canadensis* is routinely used in CAM generally for ameliorating various liver ailments (Scazzocchio et al 2001; Kim et al 2001; Birdsall and Kelly, 1997) but to our knowledge, micro doses of the homeopathically used mother tincture of *Hydrastis canadensis* had not been scientifically tested so far for its possible anti-carcinogenic effect in any mammalian model in vivo or in vitro. Hence, the present study was undertaken on the mammalian model mice (*Mus musculus*) in vivo and on in vitro system by deploying a human melanoma cell line A375. In this communication, we report results of in vivo studies only.

Materials and Methods

Inbred strain of Swiss albino mice (*Mus musculus*) were reared and maintained in the animal house of

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the Department of Zoology (with clearance from the University Ethical Committee and under the supervision of the Animal Welfare Committee), University of Kalyani, for the investigation. Mice were provided food and water ad libitum and kept in hygienic condition. For the formation of hepatic nodules and development of subsequent hepatocarcinoma, the chronic dietary feeding method used by several workers earlier (Doust and Molnar, 1964; Ohnishi et al., 2001; Biswas and Khuda-Bukhsh, 2002, 2004, 2005; Biswas et al., 2004) was adopted. The general diet was prepared from wheat, gram and powdered milk without any animal protein supplement. A group of 21 healthy mice weighing between 20 and 27 grams were used for each treatment series, namely, i) normal, ii) p-dimethylaminoazobenzene (p-DAB) + PB+ Alc, iii) p-DAB + PB + Hydrastis-Q series for each of four fixation intervals, namely, at 1 month, 2, 3 and 4 months.

Source and dose of the drug

The crude ethanolic (90%) extract of *Hydrastis canadensis*, was procured from "HAPCO", 165, Bipin Behari Ganguly Street, Kolkata.

Treatment of drug and placebo

Each mouse was fed 1 drop (0.06 ml) of stock solution of *Hydrastis* mother tincture (i.e. at 7 A.M., 1 P.M. and 7 P.M.) or 90% ethyl alcohol (being the vehicle of *Hydrastis* mother tincture or *Hydrastis* Q), as the case may be, with the aid of a fine pipette.

Methodology

Biochemical assays:

From Tissue Samples

Mice were sacrificed and their liver, spleen and kidney tissues were quickly isolated. 50 mg each of liver and kidney tissues and 20 mg of spleen tissue diluted in 20 ml of phosphate buffer saline were homogenized separately and centrifuged at 3000 g for 20 min in a cooling centrifuge (C24, Remi Instruments). Before carrying out the enzymatic estimations the quantitative estimation of total protein was made by the method of Lowry et al. (1951) with the help of a UV-spectrophotometer (Shimadzu, Double beam Spectrophotometer UV-1700, Japan).

Standardized methods were followed for the study of : acid and alkaline phosphatases (Walter and Schutt;1974), alanine amino- and aspartate amino-transferases (ALT and AST, respectively) activities (Bergmeyer and Brent, 1974), lipid peroxidation (LPO) (Buege and Aust, 1984), reduced glutathione (GSH) (Ellman (1959).

From Serum Sample

Blood was drawn by ventricular puncture of etherized (approximately 2.5ml from each mouse) mice by the routine procedure using sterile disposable syringe and needle. Blood was collected in vials without EDTA. Serum was obtained by centrifugation for the estimation of lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT) and catalase activity.

LDH activity was assayed by the UV-Kinetic method of Gay et al. (1968) and the GGT activity was assayed by

the method of Szasz (1976). Reagent kits were supplied by Reckon Diagnostics P. Limited, Gorwa, Baroda, India (Code-64X014 and 6LX010 respectively).

Catalase activity was measured using the method of Chance and Maehly (1955).

From whole blood

Blood was drawn by the method described earlier and was collected in vials containing EDTA (anti-coagulant).

G-6-PDH activity was assayed by the reagent kit (UV-Kinetic method) supplied by Reckon Diagnostics P. Limited, Gorwa, Baroda, India (Code-6KX009).

Scoring of data (blinding) and statistical analysis

Results of both the control and treated series were analyzed and the mean and standard errors of values determined. Statistical analysis of the data in different series was done by Students t-test.

During analysis of the different parameters the "observer" was kept "blinded" of the animal belonged to "treated" or "control" group in order to remove any "bias" in observation and thereby uniformity was maintained in scoring data of both treated and control sets of mice.

Electron Microscopic Studies:

Sample preparation for scanning and transmission electron microscope was done by the method of David et al. (1973). After the sample preparation the ultra structural parts of both the control and treated liver samples were observed under Scanning Electron Microscope (LEO, 435VP, United Kingdom) and transmission electron microscope (Morgagni 268D Model, Fei Company, The Netherlands).

Tumor Incidence and Tissue weight /Body weight Ratio:

The numbers of mice showing the nodule in the liver in each series of all the fixation intervals were studied. The total body weight and the sexes of the mice were recorded before they were sacrificed. The total body weight of each mouse was recorded with the help of a Pan balance. After the mice were sacrificed the liver, spleen and kidney were dissected out immediately and cleaned properly with the help of a clean forceps and tissue paper. Then each organ was weighed with the help of a digital Petit balance (supplied by – Adair Dutt & Co. (I) Pvt. Ltd., Kolkata, India). Then tissue weight and body weight ratio was calculated by the formula: $TW/BW = \text{Individual Organ (Tissue) Weight} / \text{Total Body Weight}$.

Results

Biochemical

Chronic feeding of the carcinogens showed an increase in ACP, ALKP, ALT, AST, lipid peroxidation (LPO), LDH, GGT activities and a decrease in GSH content, catalase, G-6-PDH activities in mice fed with the carcinogen as well as alcohol (Figure 1). The activities of ACP, ALKP, ALT, AST, lipid peroxidation (LPO), LDH, GGT were decreased and GSH content, catalase, and G-6-PDH activities were considerably increased in p-DAB+PB+Hyc-M fed series at different fixation

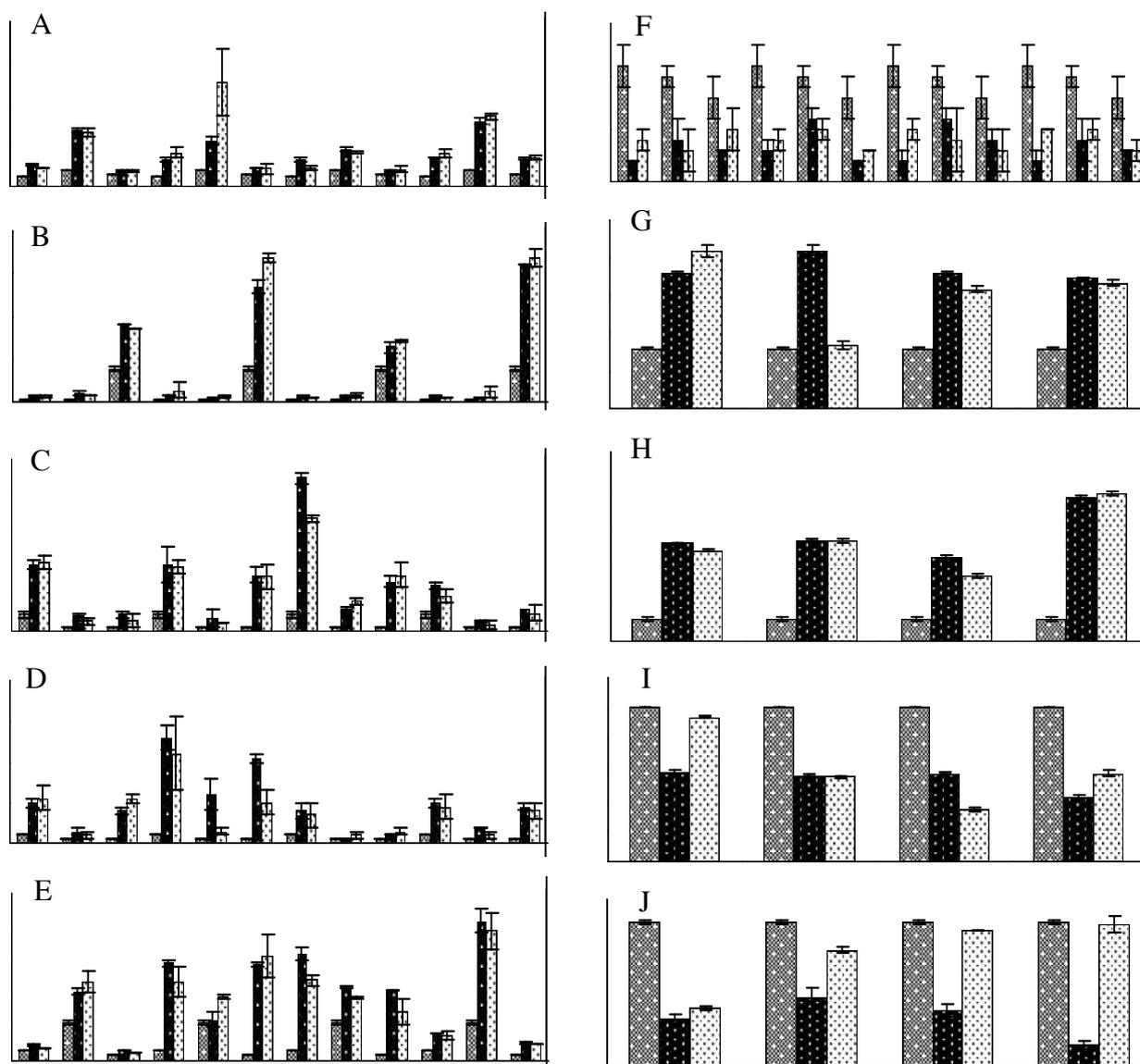


Figure 1. Activities of Different Toxicity Biomarker Enzymes in Different Tissues (liver=L, spleen=S and kidney=K), Serum and Whole Blood of Mice of Treated and Control Series at Different Fixation Intervals (1 month=1M, 2 months=2M, 3 months=3M and 4 months=4M). (A) Acid phosphatase (ACP). (B) Alkaline phosphatase (ALKP). (C) Alanine amino transferase (ALT). (D) Aspartate amino transferase (AST). (E) Lipid peroxidation (LPO). (F) Reduced glutathione (GSH) content. (G) Lactate dehydrogenase (LDH). (H) Gamma glutamyl-transferases (GGT). (I) Catalase. (J) Glucose-6-phosphate dehydrogenase (G-6-PDH).

intervals (i.e. 1 month, 2 months, 3 months and 4 months) as compared to that of p-DAB+PB+Alc. fed series with some exceptions (especially for ALKP, ALT and AST activity).

Electron Microscopic Studies:

Scanning Electron Microscopic (SEM) Studies

Normal hepatic cells were polyhedral in shape; arranged in chords; they consist of interconnecting plates of hepatocytes that radiate towards a central vein. Kupffer cells were few in number (Figure 2A, 2D). In carcinogen fed series Hepatic cells appeared to be unhealthy and damaged. Phagocytes were also found in large numbers as compared to normal group. Blood liver barrier did not appear to be intact. Scattered RBCs were found at 3 months and 4 months of fixation intervals (Figure 2B, 2E). In Hydrastis extract fed series apparent decrease of necrosis was evident. Blood liver barrier appeared to be

restituted. Blood vessel was present in its normal shape. Hepatic cells appeared to be healthy and arranged properly. Fibrosis appeared to decrease in comparison to carcinogen fed series (Figure 2C, 2F).

Transmission Electron Microscopic (TEM) Studies

Normal hepatic cells were with a distinct cell outline. Nuclear membrane was continuous. Intracellular organelles were normal with distinct membrane. Mitochondria appeared to be normal without any swelling and prominent cristae. Endoplasmic reticulum was continuous with ribosomes bound to it. Lipid droplets were absent. Kupffer cells were present but less in number and they were not activated (Figure 3A, 3D). In carcinogen fed series at 3 months and 4 months of fixation intervals damage to intracellular organelles was evident. Endoplasmic reticulum was broken at places. A few Kupffer cells were activated. Nucleus appeared to have

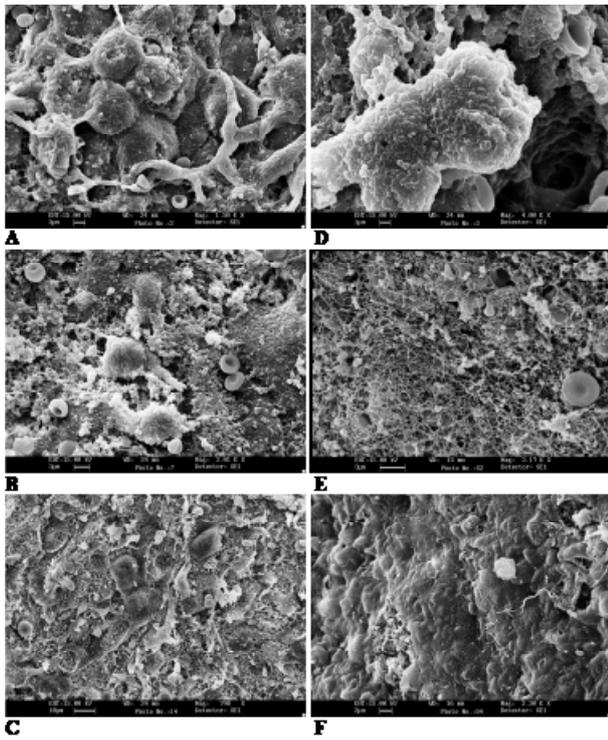


Figure 2. Scanning (SEM) Electron Photomicrographs of Liver in Different Series of Treated and Control (positive and negative) Mice at 3 Months (2A-C) and 4 Months (2D-F) of Fixation Intervals. A, D - Normal; B, E - pDAB+PB+Alc; C, F - pDAB+PB+Hyc-M

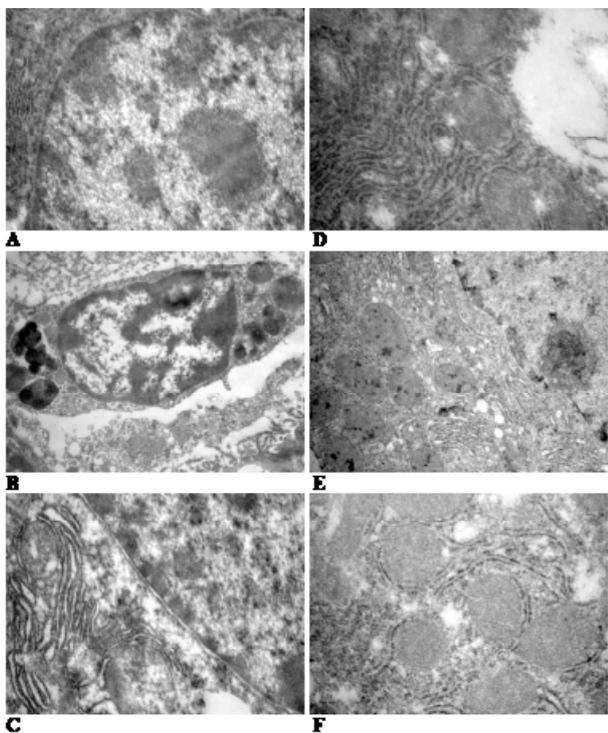


Figure 3. Transmission (TEM) Electron Photomicrographs of Liver in Different Series of Treated and Control (positive and negative) Mice at 3 Months (3A-C) and 4 Months (3D-F) of Fixation intervals. A, D - Normal; B, E - pDAB+PB+Alc; C, F - pDAB+PB+Hyc-M

broken nuclear membrane and dispersed nucleoplasm. Mitochondria were small and numerous with abnormal or

no cristae. Few lipid droplets were present (Figure 3B, 3E). In the Hydrastis extract fed series Endoplasmic reticulum was broken at places but much less in comparison to carcinogen fed series. Nucleus had broken nuclear membrane. Mitochondria were few in number, some having cristae more or less like the normal. Kupffer cells were less activated. Lipid droplets were present but less than the carcinogen fed series (Figure 3C, 3F).

The development of tumor

In normal control mice fed with standard low protein diet, out of 28 mice, 7 mice each sacrificed and dissected at 1 month, 2 months, 3 months, and 4 months, none had shown any incidence of liver tumor. The same was true for the group of 28 mice that were fed normal diet and alcohol (in prescribed dose). Further, another set of control maintained earlier in the laboratory that was fed p-DAB+PB showed high incidence of liver tumor (21 out of 28 mice; data not shown).

However, for saving lives of mice and cost this set of control was not maintained in the present study. Instead, as the vehicle of the drug was alcohol the p-DAB+PB+Alc. fed mice served as positive control. Out of 28 mice in this group all 7 out of 7 mice showed liver tumor on autopsy at 2 months onward. In the p-DAB+PB+Hyc-Q fed series 2 out of 7, 4 out of 7, and 3 out of 7 showed tumors at 1 month, 2 months and 3 months respectively.

Tissue weight /Body weight Ratio

The comparative data of tissue weight-body weight ratio as obtained from the present investigation are summarized in the table. The differences which were statistically significant are denoted in the table (Table 1).

Discussion

In the present study it was clearly revealed that the feeding of carcinogens and alcohol, the vehicle of the drug, induced cytotoxicity and liver tumors. The principal carcinogen is one of the aminoazo dyes that can produce reactive electrophyles (Ohnishi et al., 2001) and free radicals which subsequently form reactive oxygen species (ROS). ROS in turn react with cellular components which probably play an important role in the initiation and promotion of cells to neoplastic growth (Ho et al., 2001) and generate hepato-toxicity. In fact attack of a free radical on unsaturated lipids initiates the process of lipid peroxidation resulting in a breakdown of lipid into products like lipid, alcohol, aldehyde and malonaldehyde. Thus there is a cascade of peroxidative reaction which ultimately leads to the destruction of lipid and thus the liberation and quantification of malonaldehyde reflects the state of toxicity which may affect membrane structure (Biswas and Khuda-Bukhsh, 2004). The Hydrastis extract generally reduced lipid peroxidation produced by the carcinogens. The drug showed some protective activity not only in the target organ liver, but also to some extent in kidney and spleen as well. The positive modulation of cellular damage in liver induced by the chronic feeding of carcinogens was also evident through the electron microscopic studies, like scanning and transmission

Table 1. Tissue Weight/Body Weight Ratio of Mice of Treated and Control (positive and negative) Series at 1 Month, 2 Months, 3 Months and 4 Months of Fixation Interval

Series	1 Month				2 Months				3 Months				4 Months					
	LW/BW ±SE	Diff. in value	Male SW/BW ±SE	Diff. in value	KW/BW ±SE	Diff. in value	LW/BW ±SE	Diff. in value	Female SW/BW ±SE	Diff. in value	KW/BW ±SE	Diff. in value	LW/BW ±SE	Diff. in value	Female SW/BW ±SE	Diff. in value	KW/BW ±SE	Diff. in value
Normal	0.0381	-	0.0016	-	0.0119	-	0.0332	-	0.0021	-	0.0151	-	0.0332	-	0.0021	-	0.0151	-
	±0.001		±0.000		±0.001		±0.002		±0.001		±0.001		±0.002		±0.001		±0.001	
	0.0601	-	0.0150	-	0.0160	-	0.0646	-	0.0214	-	0.0100	-	0.0646	-	0.0214	-	0.0100	-
p-DAB+PB+Alcohol	±0.003		±0.003		±0.002		±0.000		±0.003		±0.001		±0.000		±0.003		±0.001	
	0.0563	0.0038n	0.0119	0.0031n	0.0128	0.0032n	0.0545	0.0101**	0.0146	0.0068n	0.0089	0.0011n	0.0545	0.0101**	0.0146	0.0068n	0.0089	0.0011n
	±0.007		±0.001		±0.003		±0.003		±0.004		±0.003		±0.003		±0.004		±0.003	
Normal	0.0381	-	0.0016	-	0.0119	-	0.0332	-	0.0021	-	0.0151	-	0.0332	-	0.0021	-	0.0151	-
	±0.003		±0.000		±0.003		±0.003		±0.001		±0.002		±0.003		±0.001		±0.002	
	0.0673	-	0.0095	-	0.0101	-	0.0562	-	0.0105	-	0.0137	-	0.0562	-	0.0105	-	0.0137	-
p-DAB+PB+Alcohol	±0.006		±0.001		±0.002		±0.003		±0.002		±0.004		±0.003		±0.002		±0.004	
	0.0541	0.0132n	0.0041	0.0054***	0.0095	0.0006n	0.0495	0.0067n	0.0032	0.0073*	0.0100	0.0037n	0.0495	0.0067n	0.0032	0.0073*	0.0100	0.0037n
	±0.001		±0.000		±0.001		±0.005		±0.001		±0.001		±0.005		±0.001		±0.001	
Normal	0.0381	-	0.0016	-	0.0119	-	0.0332	-	0.0021	-	0.0151	-	0.0332	-	0.0021	-	0.0151	-
	±0.004		±0.000		±0.002		±0.008		±0.000		±0.002		±0.008		±0.000		±0.002	
	0.1315	-	0.0131	-	0.0120	-	0.0645	-	0.0078	-	0.0073	-	0.0645	-	0.0078	-	0.0073	-
p-DAB+PB+Alcohol	±0.014		±0.002		±0.001		±0.006		±0.001		±0.001		±0.006		±0.001		±0.001	
	0.0782	0.0533**	0.0046	0.0085**	0.0114	0.0006n	0.0638	0.0007n	0.0064	0.0014n	0.0093	-0.0020*	0.0638	0.0007n	0.0064	0.0014n	0.0093	-0.0020*
	±0.004		±0.000		±0.003		±0.007		±0.001		±0.000		±0.007		±0.001		±0.000	
Normal	0.0381	-	0.0016	-	0.0119	-	0.0332	-	0.0021	-	0.0151	-	0.0332	-	0.0021	-	0.0151	-
	±0.004		±0.000		±0.002		±0.002		±0.000		±0.002		±0.002		±0.000		±0.002	
	0.0464	-	0.0128	-	0.0115	-	0.0686	-	0.0181	-	0.0149	-	0.0686	-	0.0181	-	0.0149	-
p-DAB+PB+Alcohol	±0.002		±0.002		±0.001		±0.007		±0.003		±0.004		±0.007		±0.003		±0.004	
	0.0654	-0.0190**	0.0130	-0.0002n	0.0123	-0.0008n	0.0514	0.0172n	0.0165	0.0016n	0.0101	0.0048n	0.0514	0.0172n	0.0165	0.0016n	0.0101	0.0048n
	±0.005		±0.001		±0.001		±0.003		±0.002		±0.000		±0.003		±0.002		±0.000	

p-DAB: p-dimethylaminoazobenzene; PB: phenobarbital; Alc: alcohol; Hyd-Q: Mother tincture of Hydrastis; LW: Liver Weight; KW: Kidney weight; SW: Spleen weight; *p < 0.05, **p < 0.01, ***p < 0.001; n: non-significant

electron microscopies of liver, the target organ. There were clear evidences of the Hydrastis extract providing protective action to different sub-cellular organelles like mitochondria, golgibodies, endoplasmic reticulum etc.

Feeding of the drug resulted in less number of mice showing liver tumors, or slower tumor growth in mice that showed up tumors. Morphological and tissue-weight/body-weight correlation studies supplemented with the electron microscopic studies were supportive of this statement. There was less damage in the liver tissue of the drug fed mice or better recovery. Data of all the biomarkers would show dramatic changes in acid and alkaline phosphatases (ACP and ALKP), and different transferases like ALT, AST, GGT etc. in the drug fed mice. Acid and alkaline phosphatases have been directly implicated to the extent of cellular damage and toxicity (Plaa et al., 1991; Timbrell 1991; Aiso et al., 2005), particularly of liver and cardiac tissue. Since liver is the primary target of the carcinogens, the gradual increase in alkaline phosphatase levels in the carcinogens fed mice and their substantial reduction in activity in the drug fed mice would suggest that toxicity was considerably reduced by the ethanolic extract of Hydrastis canadensis. Activities of transferases like ALT, AST and GGT have been linked to hepatocellular injury or necrosis of some striated muscles (Valentine et al., 1990). In fact ALT activity and its positive modulation by the remedy could provide a sensitive indicator of the efficacy of the drug in bringing down the carcinogen induced toxicity. Similarly, reduced glutathione is involved in a number of reactions where it reduces several cellular components. It is required for stability and integrity of the red blood cells and it also plays an important role in regulation of cellular proliferation and cellular defense. It has a nucleophilic thiol group and it can modify substances in one of the three ways: i) By chemical reaction with a reactive metabolite to form a conjugate. ii) By donation of a proton or Hydrogen atom to reactive metabolites or free radicals, and iii) By conjugation catalyzed by a glutathione transferase or else other metabolites may chemically oxidize GSSG to GSH (Karasaki, 1975).

Therefore, the GSH depletion in intoxicated mice and repletion following drug administration is perfectly in conformity with the trend in enzymatic activities observed for enzymes including a peroxisomal enzyme, catalase. Catalase, a haem-containing redox enzyme of about 250 kDa, catalyses conversion of hydrogen peroxide to water and oxygen, and is a powerful tool in removing cellular toxicity (Lente and Pepoy, 1990; Centinkaya et al., 2006).

How the extract could bring about a multitude of positive changes observed in this study was not clearly understood. However, one possible explanation could be that the drug could possibly have some regulatory influence on certain relevant genes, either activating or de-activating them (Khuda-Bukhsh, 1997, 2003, 2006, 2009), rendering the required protection/repair leading to the recovery pathway.

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