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Original Article

Exploring the Protective Effects of *Abrus precatorius* in HepG2 and N-Nitrosodiethylamine-Induced Hepatocellular Carcinoma in Swiss Albino Rats

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Abstract

This study was designed to explore the protective effects of *Abrus precatorius* L. (Leguminosae) (AP) in HepG2 cells and *N*-nitrosodiethylamine (NDEA) induced hepatocellular carcinoma in Swiss albino rats. The effects of aqueous/ethanolic (50%) extract of AP on hepatic markers, haematological and histopathological parameters, and antioxidant enzymes were evaluated in NDEA (200 mg/kg and CCl_4, 3 ml/kg body weight) induced experimental hepatocarcinogenesis in Swiss albino rats. In addition, cytotoxicity of the extract and its effect on the expression on p53 were studied in human hepatoma cell line (HepG2). Results obtained from cytotoxicity studies showed that the AP extract has strong cytotoxic effects on HepG2 cells. The expression of p53 was markedly increased and maintained at high level from 6-12 hr with 100 µg/ml of AP extract. A decrease in the mean and relative liver weights in AP extract treated group at a dose of 100 and 200 mg/kg was observed compared to the control group. It was also demonstrated that AP extract provided significant protection against hepatic lipid peroxidation and increased antioxidant enzymes' activities such as superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and reduced glutathione levels. In a dose-dependent manner, the AP extract reduced the NDEA-induced elevated levels of various hepatic markers such as serum glutamate pyruvate transaminase, serum glutamate oxaloacetate transaminase, alkaline phosphatase, total bilirubin and gamma glutamate transpeptidase. The haematological paremater viz. RBC, WBC and haemaglobin was restored upon treatment with AP extract at 100 and 200 mg/kg. Histopathology of the liver was also carried out to mark the pathological changes in groups under study. The results of these studies demonstrate the protective effect of AP extract against NDEA induced hepatocarcinogenesis in Swiss albino rats and in HepG2 cell.

Keywords: Abrus precatorius; Antioxidant Enzymes; Cytotoxicity; HepG2; *N*-Nitrosodiethylamine; p53.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers with poor prognosis, and there are about 500,000 to 1,000,000 new cases per year [1]. The role in carcinogenesis of prolonged cellular damage by viral, bacterial or chemical-related chronic inflammations has been widely recognized [2]. *N*-Nitroso compounds are important chemical carcinogens that pose a significant human health hazard [3]. The presences of these compounds like N-nitrosodiethylamine (NDEA), *N*-nitrosodimethylamine, *N*-nitrosopyrrolidine and *N*-nitrosopiperidine has been widely reported in various foodstuffs such as milk products, meat products, soft drinks and alcoholic beverages [4]. Furthermore, tobacco smoke accounts for one of the biggest risks for individual exposure to these nitrosamines [5]. Cellular and molecular changes induced by some of these compounds in animals have been shown to be very similar to those in human tissues [6]. NDEA has been suggested to cause oxidative stress and cellular injury due to the involvement of free radicals that include partially reduced oxygen species ($O_2^{-\circ}$), hydrogen peroxidase (H_2O_2), hydroxyl radical (°OH) and singlet oxygen [7, 8]. It has been shown that these free radicals may also contribute to progressive decline in immune function [9]. Protection from damaged by free radicals provided by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), lipid peroxidase (LPO), glutathione peroxidase (GPx), glutathione S-transferase (GST) and reduced glutathione have been extensively studied *in vivo* [10].

Experimental models for the study of hepatocellular carcinoma have been prepared by induction of carcinogenesis by compounds such as NDEA, which is a well known hepatocarcinogen [11]. Mechanisms of NDEA induced carcinogenesis include DNA adduct formation, cytotoxicity following regenerative proliferation and oxidative stress by impairment of mitochondrial respiration by free radicals [12].

The Indian Ayurvedic system treasures a host of medicinal formulations that have been shown to possess cytotoxic and cytostatic effects on tumor cell lines [13]. However, there are few experimental studies, which validate the possible antitumor properties of plants [14]. Several medicinal plants have been screened based on the integrative approaches on drug development from Ayurveda [15]. One such plant which has

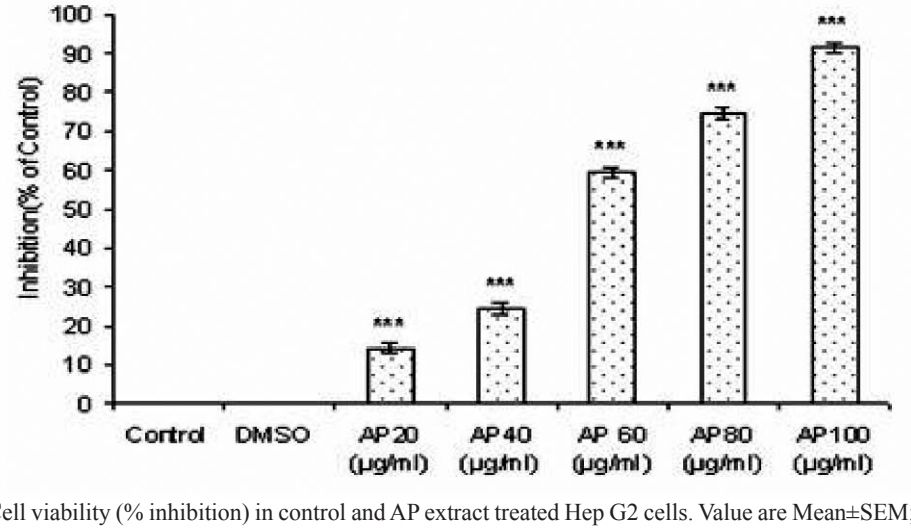

Figure 1. Cell viability (% inhibition) in control and AP extract treated Hep G2 cells. Value are Mean±SEM; n=6. ***P<0.001 vs Control. Control, DMSO -1% (v/v), Doses AP20, AP40, AP60, AP80, and AP100 represents administration of 20, 40, 60, 80, and 100 µg/ml of AP.

Table 1. Effects of aqueous/ethanolic extract of *A. precatorius* on the expression of p53 in HepG2 cells.

Treatment	p53 levels (pg/ 10^6 cells)			
	6 hr	12 hr	24 hr	48 hr
Control (DMSO) 0.1%	415.12±2.34	419.22±2.41	418.24±2.38	422.32±2.36
A. precatorius (100 µg/ml)	632.32±3.48[a]	842.32±3.48[a]	782.24±3.52[a]	768.42±3.44[a]

Data shown are Mean±SEM of two independent experiments.[a]Significantly different from control (p<0.05).

played a wide functional property is *Abrus precatorius*.

Abrus precatorius L. (Leguminosae) (AP) has been reported to posses anti-inflammatory [16], immunostimulant [17], anti-motility [18], uterotonic [19] and anti-diarrhoeal activities [20]. Abrin, one of its constituents, also exhibited antitumor activity in mice [21]. AP seed extract also showed protective effects following alcohol-induced renal damage [22]. In our study, we determined the effect of AP extract on NDEA induced hepatocellular carcinoma by studying haematological, antioxidant, hepatic markers and histopathology and expression of p53 and cell-viability in human hepatoma cell line (HepG2).

2. Materials and methods

2.1. Preparation of extracts

Seeds of *Abrus precatorius* were identified and authenticated and the voucher specimens (NAB 200496) were deposited in the departmental herbarium and also in the institutional museum for future reference at the National Botanical Research Institute, Lucknow, India. These seeds were washed with distilled water to remove dirt and soil, and dried in shade. Dried material was powdered and 500 g of the powder was extracted thrice with ethanol (50%, v/v). The extracts were filtered, pooled and concentrated at reduced temperature (-5 °C) on a rotary evaporator (Buchi, USA), and then freeze-dried (Freezone® 4.5, Labconco, USA) at -40 °C in high vacuum (133×10^{-3} mbar) giving a yield of 12.6%, w/w. The extract was stored at 4-8 °C and was re-suspended in double distilled water containing carboxymethylcellulose (CMC; 1% w/v) when required.

2.2. Experimental animals.

Swiss albino rats of weighing 140-160 g were procured from the National Laboratory Animal Centre (NLAC), Central Drug Research Institute, Lucknow, India. They were kept in the departmental animal house for 1 week before and during the experiments,

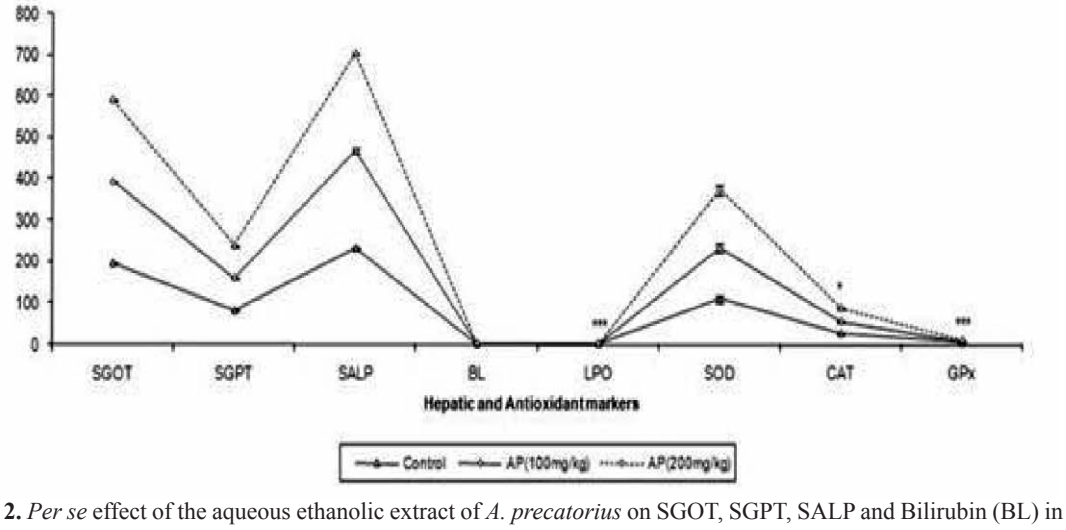

Figure 2. *Per se* effect of the aqueous ethanolic extract of *A. precatorius* on SGOT, SGPT, SALP and Bilirubin (BL) in serum and SOD, CAT, GPX and LPO in the liver of rat. Value are Mean±SEM; n=6, *p<0.05; and ***p<0.001 compared with control group.

in cross ventilated room at 27±2 °C with relative humidity of 44-56%, light and dark cycles of 10 and 14 h, respectively. Animals were fed on standard rodent pellet diet (Amrut, Lucknow, India) and food was withdrawn 18-24 h, though water was allowed *ad libitum*. All experiments conducted were in accordance with the institutional ethical committee and the Institutional Animal Care Committee, CPCSEA, India (Reg. No. 222/2000/CPCSEA).

2.3. Determination of cell viability assay.

Inhibitory effect of AP extract on proliferation of HepG2 cells was assessed by measuring cell viability study using trypan blue exclusion method [23]. HepG2 cells were grown as monolayers in RPMI- 1640 medium, supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), antibiotics (Penicillin 100 U/ml, Streptomycin 10µg/ml) and 1 mM sodium pyruvate under standard conditions (37 °C, 5% CO_2) in a controlled humidified atmosphere. The medium was changed every four days. AP was dissolved in 1% DMSO (final concentration of the DMSO was not exceeded 1% (v/v) and did not affect the cell survival) prepared in serum free RPMI medium and filtered by 0.3 mm syringe filter and stored at 4 ˚C. For determination of cell viability, monolayer of HepG2 cells were trypsinized and seeded at a density of 5×10^4 cells/well. After 24 h, the medium was replaced with the serum-free medium (RPMI-1640 medium, supplemented with antibiotics (Penicillin 100 U/ml, Streptomycin 10 µg/ml, 1 mM sodium pyruvate) and the cells were cultured for 24 h to arrest the cell growth and the monolayer of HepG2 were treated with various concentrations of AP (40, 60, 80,100 µg/ml) for 48 h and cells incubated with 1% DMSO as solvent control. Both attached and floating cells were collected by trypsinization, and an aliquot of cells were mixed with an equal volume of trypan blue dye. The cells excluding dye (viable cells) and those taking up dye (dead cells) were counted in duplicate using a hemocytometer and the numbers of these were expressed as the percent of total cell number.

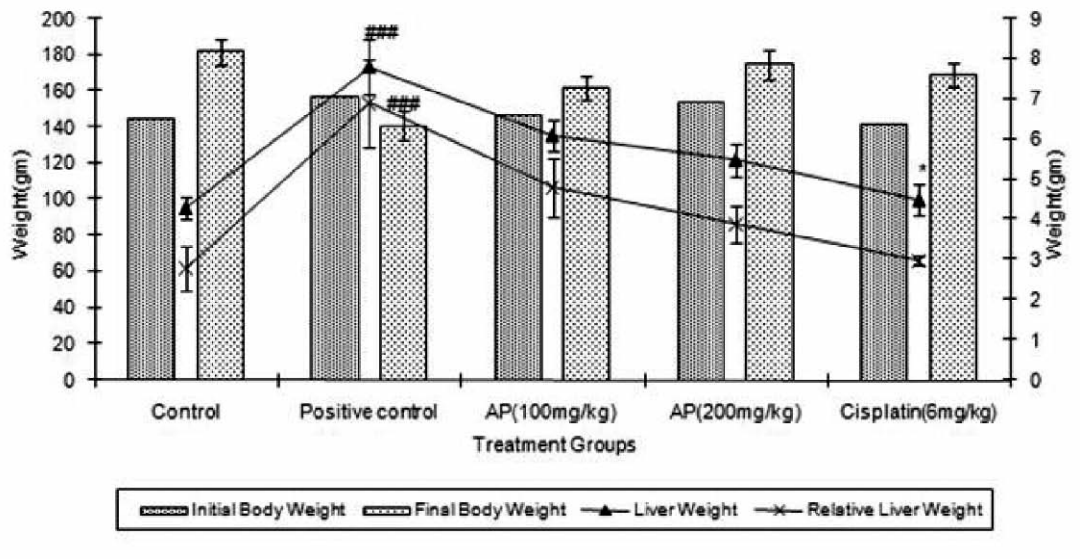

Figure 3. Effect of the AP extract on body weight, mean and relative liver weights in (NDEA+CCl_4) induced HCC rats. Values are mean±S.E.; n=6; *p<0.01 as compared with group II (NDEA+CCl_4 treated group). ###p<0.001, as compared with respective control group. Group I represent controls, Group III represent AP(100 mg/kg), Group IV represent AP (200 mg/kg) which depicts groups receiving oral doses of 100, 200 mg/kg b.w of AP extract. Group II represent positive control which represent 200 mg/kg and 3 ml/kg b.w. of NDEA and CCl_4. Group V represent cisplatin (6 mg/kgb.w).

% Inhibition=No. of viable cells–No. of viable cells after treatment/No. of viable cells without treatment.

2.4. Assay on expression of p53

To detect the expression of p53 proteins, p53 pans ELISA (Roche molecular Biochemical, Germany) were used. Briefly, cells were treated with 100 µg/ml of the aqueous/ethanolic extract of AP for 6, 12, 24 and 48 h. The protein concentration of cell lysates was measured by the Bradford reagent (Sigma Chemical Co., St Louis, MO, USA). Samples with the same protein levels were placed together with the biotinylated specific detector antibody in 96-well microtiter plates, which were coated with monoclonal antibodies of p53. They were then incubated at room temperature for 2 h. After removing the unbound material by washing with Phosphate Buffer Saline (PBS), horse radish peroxidase conjugated streptavidin was added to bind the specific biotinylated detector antibodies. The horseradish peroxidase catalyzes the conversion of a chromogenic substrate (tetramethylbenzidine) to coloured solution with the color intensity proportional to the amount of protein (p53) present in the sample. The absorbance of each well was measured at 450 nm. The concentration of p53 was determined by interpolating from the standard curves obtained with known concentration of standard proteins [24].

2.5. In vivo study on NDEA induced HCC in Swiss albino rats

Five groups of 6 Swiss albino rats each were included in this study. Group I and II were normal/placebo control and positive control groups, respectively, while groups III and IV were experimental treated (AP at 100 and 200 mg/kg b.w) groups. Group V received standard drug cisplatin (6 mg/kg b.w, i.p. once a weekly for 3 weeks). All groups except group I were given intraperitoneal injections of NDEA (200 mg/kg b.w) followed by subcutaneous injections of carbon tetrachloride (3 ml/kg b.w) once a week for 6 weeks as described [25] with slight modifications. After 20 weeks post exposure to NDEA and CCl4, rats in groups III and IV were orally administered with a daily dose of

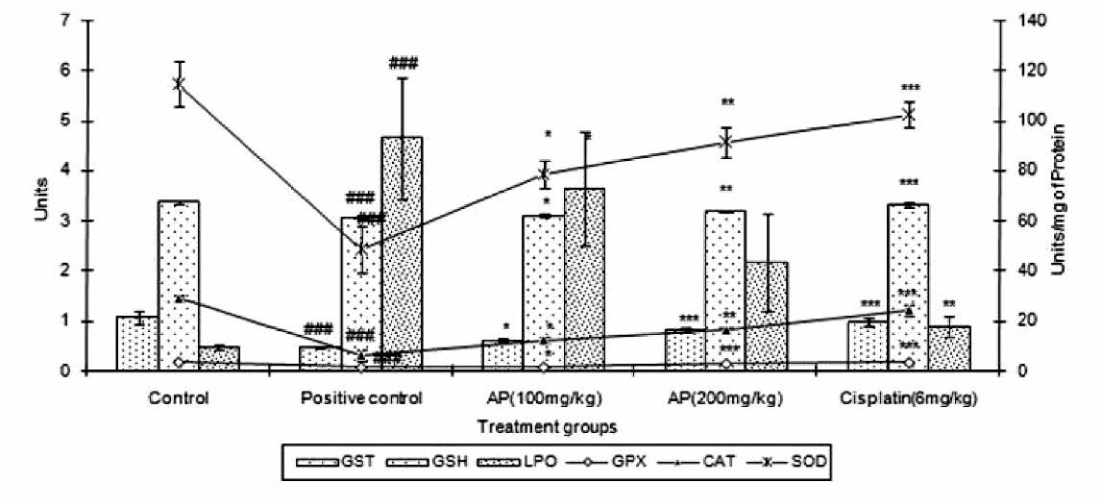

Figure 4. Effect of AP extract on LPO, GST, GSH, GPx, SOD and CAT activity *in vivo* in hepatic tissue of rats treated with NDEA and CCl4. Values are mean±S.E.; n=6. Significance level: ###p<0.001 compared with respective control group; *p<0.05, **p<0.01, ***p<0.001 compared with respective NDEA group. Results are expressed for LPO (MDA nmoles/mg of protein), GST and GSH (µg/mg of protein), GPx (µg/mg), SOD and CAT (units/mg of protein). Group I represent controls, Group III represent AP (100mg/kg), Group IV represent AP (200 mg/kg) which depicts groups receiving oral doses of 100, 200 mg/kg b.w of AP extract, Group II represent positive control which represent 200 mg/kg and 3 ml/kg b.w. of NDEA and CCl4. Group V represent cisplatin (6mg/kg,b.w).

100 and 200 mg/kg b.w. of AP extract for 4 consecutive weeks. The standard orogastric cannula was used for oral administration. At the end of 24 weeks, all of the rats were killed by cervical dislocation after an overnight fast. Blood was collected to determine levels of tumor markers and haematological study and the liver used for histopathology and studying antioxidant enzyme levels.

2.6. Determination of antioxidant enzymes

Hepatic tissues were homogenized in phosphate buffer (10 mM, pH 7.4) which contained potassium chloride (1.15%) and ethylene-diaminetetraaceticacid (1.15%, pH 7.4) and centrifuged at 12,000×g for 20 min to obtaine post mitochondrial supernatant (PMS), to measure glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) estimation.

2.6.1. Assay of lipid peroxidation activity

The concentration of thiobarbituric acid reactive substances (TBARS) was measured in liver using the method of Ohkawa [26]. One ml of the sample was mixed with 0.2 ml 4% (w/v) sodium dodecyl sulfate, 1.5 ml 20% acetic acid in 0.27 M hydrochloric acid (pH 3.5) and 15 ml of 0.8% thiobarbituric acid (TBA, pH 7.4). The mixture was heated in a hot water bath at 85 ˚C for 1 h. The intensity of the pink color developed was read against a reagent blank at 532 nm following centrifugation at 1200 g for 10 min. The concentration was expressed as n moles of MDA per mg of protein using 1, 1, 3, 3-tetra-ethoxypropane as the standard.

2.6.2. Assay of superoxide dismutase (SOD) activity

SOD was estimated according to the method of Kakkar [27]. Assay mixture contained sodium phosphate buffer (0.052 M, pH 8.3), phenazine methasulfate (PMS, 6.2 M), nitroblue tetrazolium (NBT, 30 M), potassium cyanide (KCN, 10 µM, pH 7.0) and 0.2 ml of sample fraction. Samples were preincubated for 5 min at 36 ˚C prior to the addition of reduced nicotinamide adenine dinucleotide (NADH, 52 µM). Mixture was further incubated for 120 sec at 37 ˚C in a water bath and the reaction was stopped by adding 1 ml glacial acetic acid (17.4 M). The

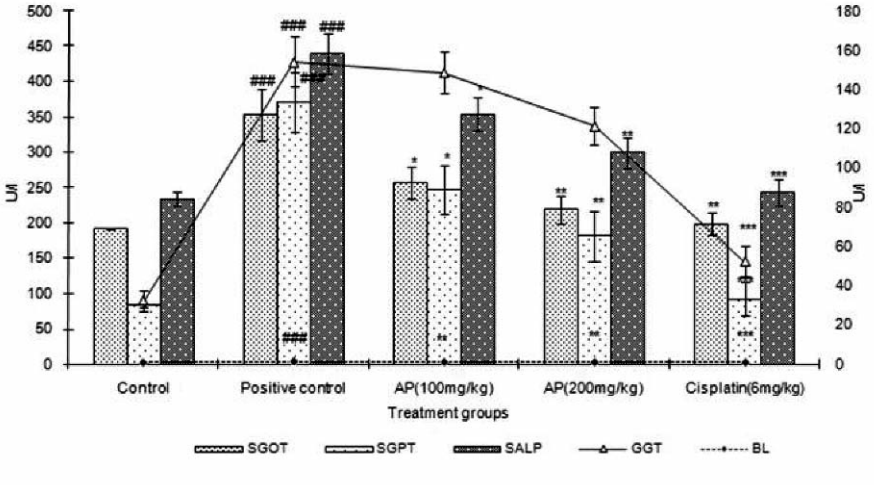

Figure 5. Effect of the AP extract on SGOT (U/l), SGPT (U/l), SALP (U/l), GGT (U/l) and BL (U/l) in serum of rat. Values are mean±S.E.; n=6. ###p<0.001, as compared with respective control group. *p<0.05, **p<0.01, ***p<0.001, as compared with group II (NDEA+CCl_4 treated group). Group I represent controls, Group III represent AP (100 mg/kg), Group IV represent AP (200 mg/kg) which depicts groups receiving oral doses of 100, 200 mg/kg b.w of AP extract, Group II represent positive control which represent 200 mg/kg and 3 ml/kg b.w. of NDEA and CCl_4. Group V represent cisplatin (6 mg/kg b.w).

violet color developed was extracted in 4.0 ml of n-butanol reagent blank. The activity was measured at 560 nm and the results are expressed as units (U) of SOD activity/mg protein.

2.6.3. Assay of catalase activity

Catalase (CAT) was estimated by method of Aebi [28]. Decomposition of H_2O_2 in the presence of CAT was followed at 240 nm. A 50 µl sample was added to buffered substrate (50 mM phosphate buffer, pH 7.0 containing 10 mM H_2O_2) to make total volume of 3 ml and decrease in the absorbance was monitored at 37 ˚C for 2.5 min. at an interval of 15 sec. The activity was calculated using extinction coefficient of H_2O_2, 0.041/µmole/cm^2 at 240 nm. Results are expressed as units (U) of CAT activity/mg protein.

2.6.4. Assay of glutathione S-transferase activity

The enzyme glutathione S-transferase was measured according to the method of Habig and Jacoby [29]. To 0.1 ml of liver homogenate 1.0 ml PBS buffer, 1.7 ml double distilled water, and 0.1 ml CDNB reagent were added. The tubes were incubated at 37°C for 15 min. Then, 0.1 ml of GSH was added and change in optical density was read at 340 nm from 0 to 3.0 min. in a Shimazdu UV-visible spectrophotometer. The activity of GST was expressed as µg/mg of protein.

2.6.5. Assay of reduced glutathione

Reduced glutathione was measured according to the method of Ellman [30]. Equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithio-bis-(2-nitrobenzoic acid) and 0.4 ml double distilled water was added. Mixture was vortexes and the absorbance read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg/mg of protein.

2.6.6. Assay of glutathione peroxidase activity

Glutathione peroxidase activity was measured according to the method of Rotruck [31]. Briefly, 0.2 ml of EDTA, sodium azide, reduced glutathione, H_2O_2; 0.4 ml of buffer and 0.1 ml of enzyme (liver homogenate) were mixed and incubated at 37 ˚C for 10 min. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged.

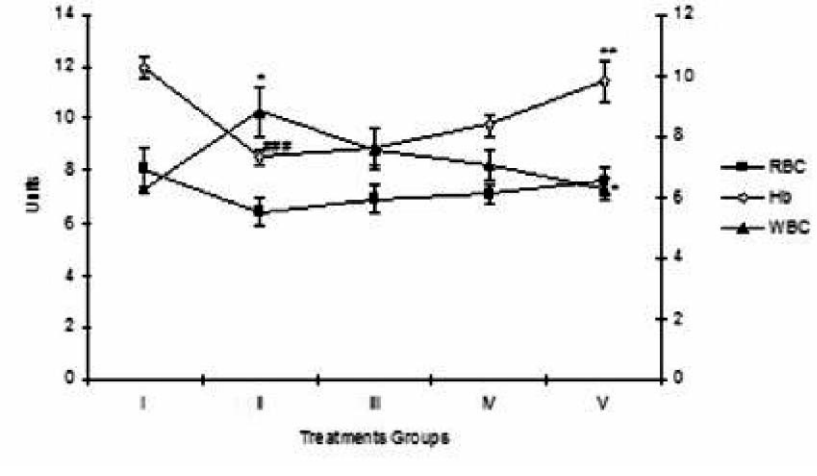

Figure 6. Effect of the AP extract on RBC (million/mm3), WBC (million/mm3) and Hb (g/dl) in rat. Values are mean±S.E.; n=6. ###p<0.001, as compared with respective control group. *p<0.05, **p<0.01 as compared with group II (NDEA + CCl4 treated group). Group I represent controls, Group III represent AP (100 mg/kg), Group IV represent AP (200 mg/kg) which depicts groups receiving oral doses of 100, 200 mg/kg b.w of AP extract, Group II represent positive control which represent 200 mg/kg and 3 ml/kg b.w. of NDEA and CCl_4. Group V represent cisplatin (6 mg/kgb.w).

To 0.5 ml of supernatant, 3.0 ml of sodium hydrogen phosphate and 1.0 ml of DTNB were added and the color developed was read at 412 nm immediately in a Shimazdu UV-visible spectrophotometer. Glutathione peroxidase activity in liver homogenate is expressed as µg/mg tissue.

2.7. Assay of serum transaminases activity

Serum transaminases (SGOT and SGPT) were determined by the method of Reitman and Frankel [32]. Each substrate (0.5 ml) [either α-L-alanine (200 mM) or L-aspartate (200 mM) with 2 mM α-ketoglutarate] was incubated for 5 min. at 37 °C. A 0.1 ml sample of serum was added and the volume was adjusted to 1 ml with sodium phosphate buffer (pH 7.4; 0.1 M). The reaction mixture was incubated for 30 and 60 min. for SGPT and SGOT, respectively. 0.5 ml of 2, 4-dinitrophenyl hydrazine (1 mM) was added to the reaction mixture and left for 30 min. at room temperature. Finally, the color as developed by the addition of 5 ml NaOH (0.4 N) and the product formed was read at 505 nm. Data were expressed as U/l.

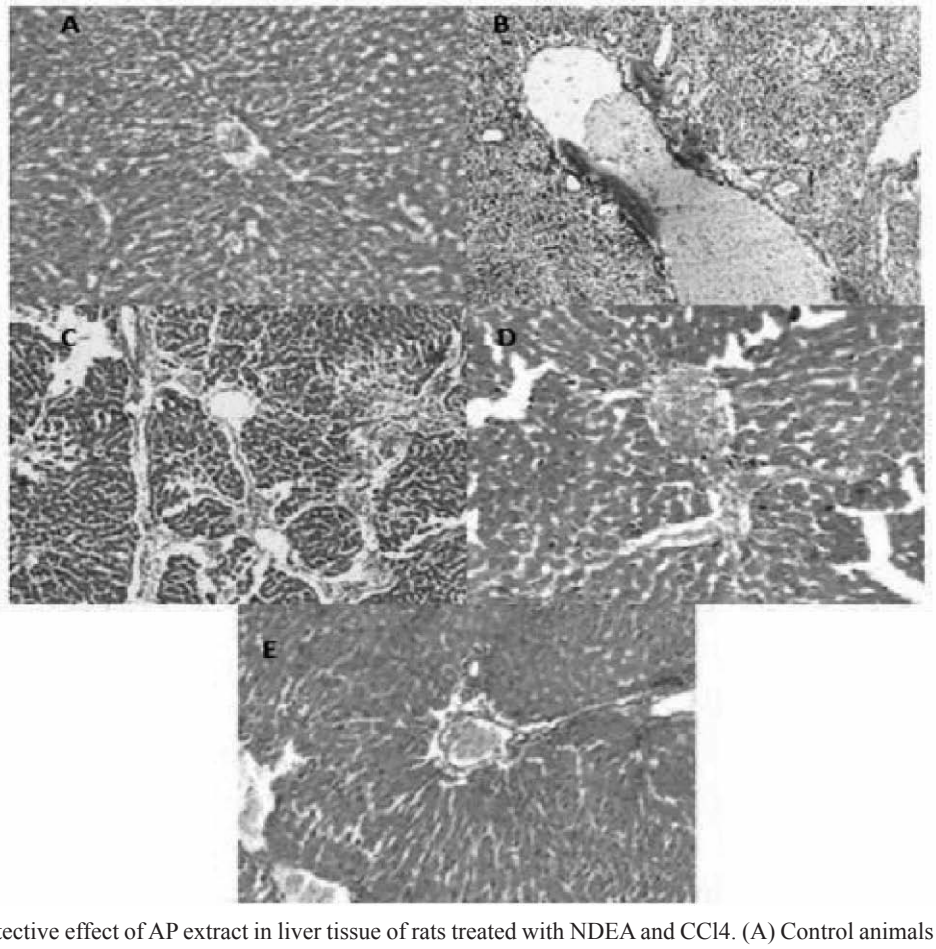

Figure 7. Protective effect of AP extract in liver tissue of rats treated with NDEA and CCl4. (A) Control animals show normal architecture. The hepatocytes are polygonal in shape with well-defined border and eosinophillic finely granular cytoplasm arranged in cords with sinusoids lined by endothelial cells. (B) Low power magnification of NDEA induced cancer bearing animal shows neoplastic cells are arranged in lobules separated by fibrous septa with inflammatory collection and small bile duct proliferation. The malignant (Neoplastic) hepatocytes. Binucleate with prominent nucleoli. (C) Administration of 50 % ethanolic extract (100 mg/kg) of *A. precatorius* extract on hepatocellular carcinoma bearing animals. Liver shows cirrhosis with sinusoidal dilation. (D) Administration of 50% ethanolic extract (200 mg/kg) of *A. precatorius* extract on hepatocellular carcinoma bearing animals. It shows mild congestion of central vein. The structure of hepa tocytes is near to normal architecture. (E) Administration of cisplatin (6 mg/kg) on hepatocellular carcinoma bearing animals shows normal architecture with some hepatocytes show anisokaryosis minimal inflammatory cell infiltration is seen around the portal triads.

2.8. Assay of serum alkaline phosphatase activity

The alkaline phosphatase level was determined by method of King and Armstrong [33]. Buffered substrate (0.5 ml) was incubated for 3 min. at 37 °C. 0.05 ml of serum was added and the reaction mixture was mixed well and incubated for 15 min. A 1.0 ml of chromogen reagent was added to the reaction mixture, the color as developed and the product formed was read at 510 nm. Data were expressed as U/l.

2.9. Assay of gamma glutamyl transpeptidase activity

The gamma glutamyl transpeptidase (GGT) activity was determined according to the method of Szas [34]. 1ml of the working reagents (reconstituted reagents Tris buffer 182 mM, pH 8.25 and L-γ-glutamyl-3-carboxy-4-nitroanillide 2.97 mM containing 85 mM glycylglycine) was mixed with 0.1 ml serum. After 1 min., changes in absorbance were measured per minutes for 3 min. at 405 nm using distilled water blank. Data were expressed as U/l.

2.10. Assay of serum bilirubin content

The bilirubin levels in serum were determined by modified DMSO method Walter and Gerard [35]. To 1.0 ml total bilirubin reagent, 0.02 ml of activator and 0.1 ml of serum were added, mixed well and incubated for exactly 5 min. at room temperature. The absorbance of each sample blank and test were measured at 532-546 nm against distilled water blank. A total bilirubin level in serum was expressed as U/l.

2.11. Histopathological investigation

Histopathological studies were performed as per the standard protocol described by Luna [36]. Briefly, the tissues were kept in Bouins solution for 12 h. The liver tissues were dehydrated with variable concentration of alcohol. After keeping the tissues in 90% alcohol overnight, they were transferred to the xylene bath and were kept for at least 4 h. The tissues were then impregnated with wax at 58 °C and paraffin fixed. The sections were cut at 5 µm (Automatic Tissue Processor, Lipshaw) in a rotary microtome and slides were prepared. The dried slides were stained by hematoxylin and eosin dyes and mounted with Canada balsam. The histopathological slides were examined and photographs were taken with a digital stereomicroscope (Olympus, B061).

2.12. Statistical analysis

Data are expressed as mean±SEM (standard error of mean). The difference among means has been analysed by unpaired student's *t*-test [37].

3. Results

The results of p53 protein expression study on HepG2 cells are shown in Table 1. The p53-positive HepG2 cells were treated with 100 µg/ml of AP extract for 6, 12, 24 and 48 h. The results indicated that the expression of p53 markedly increased after 6 h of incubation, and was maintained at high level from 6 to 12 h after 100µg/ml of AP extract treatment. Figure 1 shows that the AP extract demonstrated marked inhibitory effect on HepG2 proliferation at least 48 h post dosage at 20, 40, 60, 80, and 100 µg/ml of AP as compared with the control group. Further, decreased in viability of the HepG2 were noted in a dose dependent manner.

The results of the per se effect showed that the AP extract was relatively safe for oral administration at dose of 100 and 200 mg/kg b.w given to the animals for 28 days. There was no gross apparent effect on general appearance, general motor activity, muscular weakness, fecal output or feeding behavior, as observed during this study as well no change in the level of hepatic marker viz. SGOT, SGPT and ALP as well as antioxidant enzymes SOD, CAT and GPX but decrease in LPO which suggest free radical scavenging

activity and showed no toxicity of plant extract which is shown in Figure 2.

The study also showed that the liver weights were significantly increased in NDEA and CCl_4 group. However, aqueous ethanolic extracts of AP showed a dose dependent and significant reduction in liver weight as shown in Figure 3. The group treated with standard drug cisplatin showed a reduction in the liver weight. The relative liver weight increased in NDEA and CCl_4 group which were reduced upon treatment with 100 and 200 mg/kg b.w of AP extract. The relative liver weight was also reduced in group treated with standard drug, cisplatin. The final body weight of control versus positive control shows a significant decrease following treatment with NDEA and CCl_4. The final bodyweight was relatively increased in group treated with 100 and 200 mg/kg b.w of AP extract and in group treated with cisplatin which is shown in Figure 3. Administration of NDEA and CCl_4 led to increase in the levels of LPO and decrease in SOD, CAT, GPX, GST and GSH levels in the 5% w/v liver homogenate. Treatment with aqueous/ethanolic of AP at doses of 100 and 200 mg/kg and cisplatin markedly prevented the NDEA and CCl_4 induced alterations of various antioxidant parameters as shown in Figure 4. It is clearly evident from Figure 5 that NDEA and CCl_4 caused a significant elevation in the level of serum markers viz. SGOT, SGPT, SALP, BL and GGT. In contrast, the groups treated with AP extract at doses of 100 and 200 mg/kg showed a decrease in the levels of the enzymes in a dose related manner.

Figure 6 shows the levels of red blood cells (RBC), white blood cells (WBC) and haemoglobin (Hb). However, the Hb and RBC levels were decreased with a concomitant increase in WBC with respect to control. In contrast, the groups treated with AP extract at dose of 100 and 200 mg/kg were protected in a dose related manner. Histopathological observations were found to support the findings of tumor marker analysis and hepatic antioxidant enzyme assays (Figure 7). The structural integrity of liver cells in control and malignant state were studied by staining tissue sections with hematoxylin-eosin. In the control group, the hepatocytes had normal architecture, with portal triads, portal veins, and hepatic artery and vein clearly visible with stable integrity. In contrast, in NDEA and CCl_4 induced carcinogenic groups, there was an extensive loss of hepatic architecture. Neoplastic cells were arranged in lobules separated by fibrous septa with inflammatory collection and small bile duct proliferation. The characteristic bi-nucleated feature of malignant (neoplastic) hepatocytes was also prominent with a large amount of cell damage. However, in the case of rats treated with AP extract, the liver retained almost normal hepatic architecture, with much less pathological changes. Administration of cisplatin (6 mg/kg) on hepatocellular carcinoma bearing animals shows normal architecture with some hepatocytes show anisokaryosis minimal inflammatory cell infiltration is seen around the portal triads. The above results clearly indicate that AP extract inhibits NDEA and CCl_4-induced hepatocellular carcinoma and supressed proliferation by cell viability and expression of p53 in HepG2 cells.

4. Discussion

The results of the present study showed that the extract of *Abrus Precatorius* had marked inhibitory effect on hepatoma cell line (HepG2) proliferation and in NDEA-induced hepatocellular carcinoma in Swiss albino rats.

AP extracts markedly reduced cell viability in a concentration dependent manner. The suppression of cell growth induced by extract may be due to induction of cell death rather than the inhibition of cell proliferation. Thus, the inhibitory activity of extract provides evidence for *in vitro* cytotoxicity. Extensive reports were documented on medicinal plant extract induced cytotoxicity on cancer cells

[39].

The p53 acts as a guardian of the genome and is one of the major factors controlling cell proliferation, suppression growth and transformation. Also p53-mediated tumour suppression appears to be critical for therapeutic potential in the treatment of tumour [40]. HepG2 cells have been used extensively as a model to study p53 protein expression because of several key characteristics that make them distinct from many other hepatic cell lines [41]. It was reported in HepG2, saikosaponin d, a saponin derivative from species of *Bupleurumi* increased the expression of p53 resulting in the cell cycle arrest (G2/M phase) and apoptosis in HepG2 cells [42]. In the present study, AP extract increased the expression of p53 markedly after 6 hr of incubation and was maintained at high levels from 6 to 12 hr after treatment with 100 µg/ml of the extract. The effect was pronounced at 100 µg/ml of extract. Therefore, it is suggested that p53 may at least in part be involved in extract-mediated anti-proliferation, cell cycle arrest (G2/M) by attenuating the tumour suppressor gene p53 and affects the sensitivity of liver cancer cells to extracts under study.

In the present study, the aqueous/ethanolic extracts of AP showed a dose dependent antioxidant activity as evidenced by their effects on elevated levels of SOD, CAT, GPX and depleted levels of LPO in the liver homogenate. Further, there was no change observed in the biochemical tumor markers *viz.*, SGOT, SGPT and ALP. This indicates that AP extracts exert their antioxidant defense mechanism probably by metabolizing lipid peroxides and scavenging endogenous peroxides. The previous studies suggest that the protection offered by several herbal drugs in treatment of various chronic incurable diseases may be because of the involvement of several endogenous mediators [38].

The changes brought about by NDEA-induced carcinogenesis can be marked by various biochemical serum markers [43] as well as levels of hepatic antioxidant enzymes such as LPO, SOD, CAT, GST, reduced glutathione and GPx [10, 44]. It is believed that oxidative stress by production of free radicals is one of the main reasons paving the way for carcinogenesis in liver [8, 12, 45]. Antioxidant enzymes, thus, play a crucial role in the first line defense against oxidative injury [38, 46]. Many plant secondary metabolites have been shown to act as potent antioxidants [15, 47]. AP is one of the plants that contain various phytoconstituents such as glycyrrhizin, precol, abrol, abrasine, precasine, abrine, hypaphorine, choline, trigonelline, precatorine, stigmasterol, β-sitosterol, 5-β-cholanic acids and gallic acid, that have free radical scavenging activity [48, 49]. However, to our knowledge, the potentiation of AP against hepatic cancer has not been studied by modulating antioxidant and tumor markers. Lipid peroxidation is one of the most important biologically relevant free radical producing chain reactions [50]. NDEA and other nitrosoamines have been shown to increase LPO levels and eventually free radical production in experimental animals [51]. We had similar observations in our study, however, the AP extract significantly reduced levels of LPO that were elevated by NDEA given to the experimental animals. SOD speeds up superoxide dismutase and thus eliminates superoxide radicals from the system [52]. CAT is a crucial cellular antioxidant enzyme that degrades hydrogen peroxide. Another important peroxide removing enzyme, perhaps having higher efficiency than CAT is GPx [53]. GSTs are a family of detoxification enzymes involved in protecting the cells against cytotoxicity and carcinogenic chemicals by conjugating with GSH [54]. GSH forms a well known non-enzymatic antioxidant defense system detoxifying endogenous and exogenous compounds [55]. In the present study, we found that the levels of these antioxidant enzymes were markedly reduced in

experimental rats following induction of carcinogenesis with NDEA and CCl_4 which had been reported from various other studies [56-58]. The administration of AP extract significantly brought back the levels of the reduced enzymes to near normal levels is however dose dependent as observed in this study. It is probable that the various phytoconstituents of the plant are involved in scavenging the free radicals from the tissues, thus, reducing oxidative stress. In addition to this effect, administration of AP extract also normalized the elevated levels of various hepatic markers such as SGOT, SGPT, SALP, GGT and TBIL in Swiss albino rats. Elevated levels of these hepatic markers are regarded diagnostic and to a certain extent indicate the severity of the disease condition [59-63]. Results similar to our findings were also reported [25, 14, 64, 65] in NDEA-induced HCC in rats. This is clearly evident from histological investigation. Proliferation of cells in the liver tissue induced by NDEA and CCl_4 was evident from the increase in liver weights as observed in our study. Similar findings have been reported [64]. However, with the administration of AP extract, a significant reduction in liver weights which suggests cytotoxic or apoptotic effect of the AP extract on tumor tissue as compared with the carcinogenic group of rats was observed in our study. The extract, thus, neutralizes the effects of NDEA-induced proliferation of cells, thus, suppressing carcinogenesis. In the present study, the administration of NDEA produced a significant decrease in RBC count, Hb content; with concomitant increase in WBC count. The decreased RBC count may be due to destruction of erythrocytes or the results of adverse effect of NDEA on the erythropoietic tissue namely the bone marrow. Further, the reduction in RBC correlates well with the decreased Hb content. Decreased level of RBC count and Hb content are the indicative factors for anemic condition [66]. In general, anaemia is known to result from decreased rate of production or increased destruction of RBC [67]. In summary, The AP extracts express p53 and decrease viability in HepG2 cells. AP stabilizes and increases all the components of the antioxidant defense system that were examined in this study viz., GSH, CAT, SOD, GPx and GST. These factors protect cells from ROS damage in NDEA-induced hepatocellular carcinogenesis, as AP abolishes the activities of liver injury and tumor markers by decreasing LPO. We propose that AP abolishes the oxidative state induced by the initiator NDEA, and it does so by interacting directly with ROS (e.g., •OH), as well as indirectly by activating the antioxidant defense system. We conclude that AP counteracts LPO induced by increased ROS generation during NDEA-induced hepatocarcinogenesis and promote the enzymatic and non-enzymatic antioxidant defense system and potential chemoprevention might be due to synergistic effect of the phytomolecules present in the extract.

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References

[1] Goshi S, Ken-ichi H, Masato I, Yasushi T, Michiko O, Shunsuke K, Hisao I, Hironaka K. Inhibition of hepatocellular carcinoma by glycyrrhizin in diethlynitrosamine-treated mice. *Carcinogenesis* 1999; 20: 59-63.

[2] Ohshima H, Bartsch H. Chronic infectious and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. *Mut Res* 1994; 305: 253-64.

[3] Knekt P, Jarvinen R, Dich J, Hakulinen T. Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and *N*-nitroso compounds: A follow-up study. *Int J Cancer* 1999; 80: 852-6.

[4] Levallois P, Ayotte P, Van Maanen JM, Desrosiers T, Gingras S, Dallinga JW. Excretion of volatile nitrosamines in a rural population in relation to

food and drinking water consumption. *Food Chem Toxicol* 2000; 38: 1013-9.

[5] Wu W, Zhang L, Jain RB, Ashley DL, Watson CH. Determination of carcinogenic tobacco-specific nitrosamines in mainstream smoke from US-brand and non-US-brand cigarettes from 14 countries. *Nicotine Tob Res* 2005; 7: 443-51.

[6] Bansal AK, Bhatnagar D, Soni GL. *In vitro* effect of N-nitrosodiethylamine on lipid peroxidation and antioxidant system in human erythrocytes. *Toxicol In Vitro* 1996; 10: 649-53.

[7] Bartsch H, Hietanen E, Malaveille C. Carcinogenic nitrosamines: Free radical aspects of their action. *Free Rad Biol Med* 1989; 7: 637-44.

[8] Halliwell B. Free radicals, antioxidants, and human diseases: Cause or consequences? *Lancet* 1994; 344: 721-4.

[9] Pike J, Chandra RK. Effect of vitamin and trace element supplementation on immune indices in healthy elderly. *Int J Vit Nutr Res* 1995; 65:117-20.

[10] Prince Vijeya Singh J, Selvendiran K, Mumtaz Banu S, Padmavati R, Sakthisekaran D. Protective role of apigenin on the status of lipid peroxidation and antioxidant defense against hepatocarcinogenesis in Wistar albino rats. *Phytomedicine* 2004; 11: 309-14.

[11] Mitali Basu C, Mahendra Kumar CB, Suresh KP, Ray RS, Roy S, Chatterjee M. Combined supplementation of vanadium and beta-carotene suppresses placental glutathione S-transferase-positive foci and enhances antioxidant functions during the inhibition of diethylnitrosamine-induced rat liver carcinogenesis. *J Gastroenterol Hepatol* 2004; 19: 683-93.

[12] Finkel T, Holbrook NJ. Oxidants, oxidative stress and biology of ageing. Nature 2000; 408: 239-49.

[13] Smit HF. Ayurvedic herbal drugs with possible cytostatic activity. *J Ethnopharmacol* 1995; 47: 75-84.

[14] Sreepriya M, Geetha B. Chemopreventive effects of embelin and curcumin against *N*-nitrosodiethylamine/phenobarbital-induced hepatocarcinogenesis in Wistar rats. *Fitoterapia* 2005; 76: 549-55.

[15] Wattenberg LW. Chemoprevention of cancer by naturally occurring and synthetic compounds. *Proc Am Assoc Cancer Res* 1990; 32: 461-3.

[16] Anam EM. Anti-inflammatory activity of compounds isolated from the aerial parts of *Abrus precatorius* (Fabaceae). *Phytomedicine* 2001; 8: 24-7.

[17] Tripathi S, Maiti TK. Stimulation of murine macrophages by native and heat denatured lectin from *Abrus precatorius*. *Int Immunopharmacol* 2003; 3: 375-81.

[18] Ratnasooriyaa WD, Amarasekerab AS, Pereraa NSD, Premakumaraa GAS. Sperm antimotility properties of a seed extract of *Abrus precatorius*. *J Ethnopharmacol* 1991; 33: 85-90.

[19] Nwodo OFC. Studies on *Abrus precatorius* seeds. I: Uterotonic activity of seed oil. *J Ethnopharmacol* 1991; 31: 391-4.

[20] Nwodo OFC, Alumanah EO. Studies on *Abrus precatorius* seeds. II: Antidiarrhoeal activity. *J Ethnopharmacol* 1991; 31: 395-8.

[21] Ramnath V, Kuttan G, Kuttan R. Immunopotentiating activity of abrin, a lectin from *Abrus precatorius* Linn. *Indian J Exp Biol* 2002; 40: 910-3.

[22] Ligha Ae, Jaja Bnr1, Numbere Nf. Protective effect of Abrus precatorius seed extract following alcohol induced renal damage. *Eur J Sci Res* 2009; 25: 428-36.

[23] Morita K, Arimochi H, Ohnishi Y. *In vitro* cytotoxicity of 4 methyl catechol in murine tumor cells: Induction of apoptotic cell death by extra cellular pro-oxidant action. *J Pharmacol Exp Ther* 2003; 306: 317-23.

[24] Ya-Ling Hsu, Po-Lin Kuo, Lien-Chai Chaing, Chun-Ching Lin. Involvement of p53, nuclear factor kB and Fas/Fas ligand in induction of apoptosis and cell cycle arrest by saikosaponin d in human hepatoma cell lines. *Cancer lett* 2004; 213: 213-21.

[25] Sundaresan S, Subramanian P. Evaluation of chemopreventive potential of garlic extract on *N*-nitrosodiethylamine-induced hepatocarcinoma in rats. *Pharma Biol* 2002; 40: 548-51.

[26] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. *Annal Biochem* 1979; 95: 51-8.

[27] Kakkar P, Das B, Viswanathan PN. Modified spectrophotometric assay of SOD. *Indian J Biochem Biophys* 1984; 2: 130-2.

[28] Aebi H. Catalase. In: Bergmeyer HV, eds., *Method in Enzymatic Analysis* vol 3. Academic Press Inc, NewYork, 1974; pp. 673-86.

[29] Habig WH, Jacoby WB. Assays for differentiation of glutathione S-transferases. *Methods in Enzymol* 1981; 77: 398-405.

[30] Ellman GI. Tissue sulphydryl groups. *Arch Biochem Biophys* 1959; 82: 70-7.

[31] Rotruck JT, Pope AL, Ganther HE, Hafeman DG, Hoekstra WG. Selenium: Biochemical role as a component of glutathione peroxidase. *Science* 1973; 179: 588-90.

[32] Reitman S, Frankel SA. Colorimetric method for determination of serum glutamic oxaloacetic and

glutamic pyruvic transaminases. *Am J Clin Pathol* 1957; 28: 56-63.

[33] King EJ, Armstrong AR. *Method of King and Armstrong*. In: Varley, H., Gowenlock, AH, Bell, M. (Eds.), Practical Clinical Biochemistry, fifth ed. William Heinemann Medical Books Ltd, London, 1980; pp. 897-9.

[34] Szas G. Reaction rate method for gamma glutamyl transferase activity in serum. *Clinical Chem* 1976; 22: 2031-55.

[35] Walters MI, Gerarde HW. An ultramicromethod for the determination of conjugated and total bilirubin in serum or plasma. *Microchem J* 1970; 15: 231-43.

[36] Luna GL. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*.3rd edn, New York: McGraw-Hill Book Company, 1968; pp.1-35.

[37] Woolson RF. *Statistical methods for the analysis of biomedical data*, John Wiley and Sons Inc., New York. 1987.

[38] Rao GMM., Rao Ch V., Pushpangadan P, Shirwaikar A. Antioxidant and antihepatotoxic activities of *Hemidesmus indicus* R. Br. *Acta Pharmac Turc* 2005; 47: 73-8.

[39] Marina Galvez, Carmen Martin-Cordero, Miguel Lopez-Lazaro, Felipe Cortes, Maria Jesus Ayuso. Cytotoxic effect of Plantago SPP on cancer cell lines. *J Ethnopharmacol* 2003; 88: 125-30.

[40] Ryan KM, Phillips AD, Vousden KH. Regulation and function of the p53 tumour suppressor protein. *Curr Opin Cell Biol* 2001; 13: 332-7.

[41] Reeves SK, Fanzo JC, Arima K, Wu JYJ, Wang YR, Lei KY. Expression of the p53 tumour supresor gene is upregulated by depletion of intracellular zinc in HepG2 cells. *J Nutr* 2000; 130: 1688-94.

[42] Ya-Ling Hsu, Po-Lin Kuo, Lien-Chai Chaing, Chun-Ching Lin. Involvement of p53, nuclear factor kB and Fas/Fas ligand in induction of apoptosis and cell cycle arrest by saikosaponin d in human hepatoma cell lines. *Cancer lett* 2004; 213: 213-21.

[43] Kovalsky I, Schaff Lapis K, Jeney A. Marker enzymes of rat chemical hepatocarcinogenesis in human liver tumours. *Pathol Oncol Res* 1996; 2: 54-5.

[44] Gaurav M, Apminder Pal SB, Giridhar S. *N*-nitrosodiethylamine-induced toxicity in relation to oxidative stress and development of atherosclerosis in hypercholesterolemic diet-fed rabbits. *Exp and Toxicol Pathol* 2008; 59: 409-14.

[45] Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radical Biol Med* 1990; 8: 583-99.

[46] Buettner GR. The packing order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate. *Arch Biochem Biophys* 1993; 300: 533-45.

[47] Vimal V, Devaki T. Hepatoprotective effect of allicin on tissue defense system in galactosamine/endotoxin challenged rats. *J Ethnopharmacol* 1993; 90: 151-4.

[48] Wattenberg LW. Inhibition of carcinogenesis by minor nutrient constituents of the diet. *Proc Nutr Soc* 1990; 49: 173-83.

[49] Saleem M, Alam A, Sultana S. Asafoetida inhibits early events of carcinogenesis: A chemopreventive study. *Life Sci* 2001; 68: 1913-21.

[50] Banakar MC, Paramasivan SK, Chattopadhyay MB, Datta S, Chakraborty P, Chatterjee M, Kannan K, Thyagarajan E. 1, 25-Dihydroxyvitamin D3 prevents DNA damage and restores antioxidant enzymes in rat hepatocarcinogenesis induced by diethylnitrosamine and promoted by phenobarbital. *World J Gasteroenterol* 2004; 10: 1268-75.

[51] Ceruthi PA. Proxidant states and tumour promotion. *Science*, 1985; 227: 375-80.

[52] Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*, 2nd ed., Clarendon Press, Oxford, UK, 1989; pp. 543.

[53] Gaetani GF, Galiano S, Canepa L, Ferraris AM, Kirkman HN. Catalase and glutathione peroxidase are equally active in detoxification of hydrogen peroxide in human erythrocytes. *Blood* 1989; 73: 334-339.

[54] Hayes JD, Pulford DJ. The GST super gene family: Regulation of GST and the contribution of isoenzyme to cancer chemoprevention and resistance. *Crit Rev Biochem Mol Biol* 1995; 30: 445-600.

[55] Yu BP. Cellular defense against damage from reactive oxygen species. *Physiol Rev* 1994; 74: 136-62.

[56] Aniya Y, Anders MW. Alteration of hepatic glutathione S-transferase and release into serum after treatment with bromobenzene and carbon tetrachloride. *Biochem Pharmacol* 1985; 39: 4239-44.

[57] Thirunavukkarasu C, Prince Vijeya Singh J, Selvendiran K, Sakthisekaran D. Chemopreventive efficacy of selenium against N-nitrosodiethlyamine-induced hepatoma in albino rats. *Cell Biochem Funct* 2002; 19: 265-71.

[58] Peskin AV, Koen YM, Zbarsky IB. Superoxide dismutase and glutathione peroxidase activities in tumours. *FEBS Lett* 1977; 78: 41-5.

[59] Wallersteadt S, Olsson R, Walderstrom J. The diagnostic significance of high ASAT, ALAT ratio

in patients with very high serum aminotransferase level. *Acta Med Scand* 1974; 195: 227-9.

[60] Kobayashi T, Kawakubo T. Prospective investigation of tumour markers and risk assessment in early cancer screening. *Cancer* 1994; 73: 1946-53.

[61] Joy KL, Girija K, Ramadasan K. Antioxidant and anticarcinogenic activity of Lycovin – an indigenous herbal preparation. *Indian J Exp Biol* 1999; 37: 1177-81.

[62] Wills PJ, Suresh V, Arun M, Asha VV. Antiangiogenic effect of *Lygodium flexuosum* against *N*-nitrosodiethylamine-induced hepatotoxicity in rats. *Chemico-Biol Interactions* 2006; 164: 25-38.

[63] Gopalakrishnan R, Hanumantha Rao BR, Radhakrishnan V, Thiruvengadam D. Suppression of N-nitrosodiethylamine induced hepatocarcinogenesis by silymarin in rats. *Chemico-Biol Interactions* 2006; 161: 104-14.

[64] Jose Jeena, K, Joy KL, Ramadasan Kuttan. Effect of *Emblica officinalis, Phyllanthus amarus* and *Picrorrhiza kurroa* on *N*-nitrosodiethylamine induced hepatocarcinogenesis. *Cancer lett* 1999; 136: 11-6.

[65] Amit Singh Y, Bhatnagar D. Chemo-preventive effect of Star anise in *N*-nitrosodiethylamine initiated and phenobarbital promoted hepatocarcinogenesis. *Chemico-Biol Interactions* 2007; 169: 207-14.

[66] Harper, HA. In: *Review of physiological chemistry*, Harper,H.A., Rodwell, VM and Meyer, PA(ed), Lange Medical Publications, California, 1979; pp. 193-213.

[67] Perez, G.R.F., Terreblanca,RA., Bourges,RH and Garcia,GJ. *Technol Ailment*, 18, 1983; p. 4.