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Research Article

Biological and Phytochemical Screening of *Tephrosia purpurea* extract on Chemically Induced Hepatocellular Carcinoma with Reference to Biochemical Parameters

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ABSTRACT
Tephrosia purpurea possesses hepatoprotective activity as evidenced by the significant and dose dependent restoring the activities of entire liver cancer marker enzymes, diminution in tumor incidence, decrease in lipid peroxidation (LPO) and increase in the level of antioxidant enzymes (GSH, CAT, SOD, GPx and GST) through scavenging of free radicals, or by enhancing the activity of antioxidant, which then detoxify free radicals. These factors protect cells from ROS damage in NDEA and CCl4-induced hepatocarcinogenesis. Histopathological observations of liver tissues too correlated with the biochemical observations. Thus, present investigation suggested that the *Tephrosia purpurea* would exert a chemoprotective effect by reversing the oxidant-antioxidant imbalance during hepatocarcinogenesis induced by NDEA and CCl4. Besides *Tephrosia purpurea* is very much effective in preventing NDEA-induced multistage hepatocarcinogenesis possibly through antioxidant and antigenotoxic nature, which was confirmed by various liver injury and biochemical tumour markers enzymes. The hepatoprotective activity of a*Tephrosia purpurea* of 50 % ethanolic extract was studied using rats. The animals received a single intraperitoneal injection of N-nitrosodiethylamine 200mg/kg body wt followed by subcutaneous injection of CCl4 in a dose of 3 ml/kg body wt.*Tephrosia purpurea*extract dose dependently and significantly the increase in serum hepatic enzyme levels after NDEA& CCl4 treatment compared to the toxin control group. The results of this study confirmed the antioxidant and hepatoprotective activity of the *Tephrosia purpurea* extract against carbon tetrachloride& N-nitrosodiethylamine induced hepatotoxicity in rats.

Keywords: Hepatocarcinogenesis, Antigenotoxic, Hepatoprotective,Carbon tetrachloride, N-nitrosodiethylamine**,** Hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) or liver cancer is the sixth most common cancer and the third leading cause of cancer mortality in the world.[1] The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as increasingly an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets. Liver cancer in men/ women is the fifth/seventh most frequently diagnosed cancer worldwide but the second/sixth most frequent cause of cancer death. An estimated 748,300 new liver cancer cases and 695,900 cancer deaths occurred worldwide in 2008.[2] Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver cancer.[3] Human liver is the major site in the body that metabolizes ingested material. Moreover, due to high tolerance of liver, HCC is seldom detected at an early stage and once detected treatment faces a poor prognosis in most cases.[4]

Essentially all proliferating tissues in the body are susceptible to the formation of cancer presenting challenges to both prevention and ultimate cure. Cancer is a genetic disease because it can be traced to alteration within specific genes, but in most cases, it is not an inherited disease. The genetic alterations that lead to most cancers arise in the deoxyribonucleic acid (DNA) of a somatic cell during the lifetime of affected individual. Because of these genetic changes, cancer cells proliferate uncontrollably, producing malignant tumour that invade surrounding healthy tissue. As long as the growth of tumour remains localized, the disease can usually be treated and cured by surgical removal of tumour. But malignant tumours tend to metastases, that is, to spawn cells that break away from the parent mass, enter the lymphatic or vascular circulation, and spread to distant sites in the body where they establish lethal secondary

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Table 1: Preparation of 50% EtOH extract of *Tephrosia purpurea* Linn

S.No.	Botanical Name	Family	Part used	Solvent used for extraction	% yield (Extract)
1	*Tephrosia purpurea*	Leguminosae	Whole plant	50% EtOH	6.28%

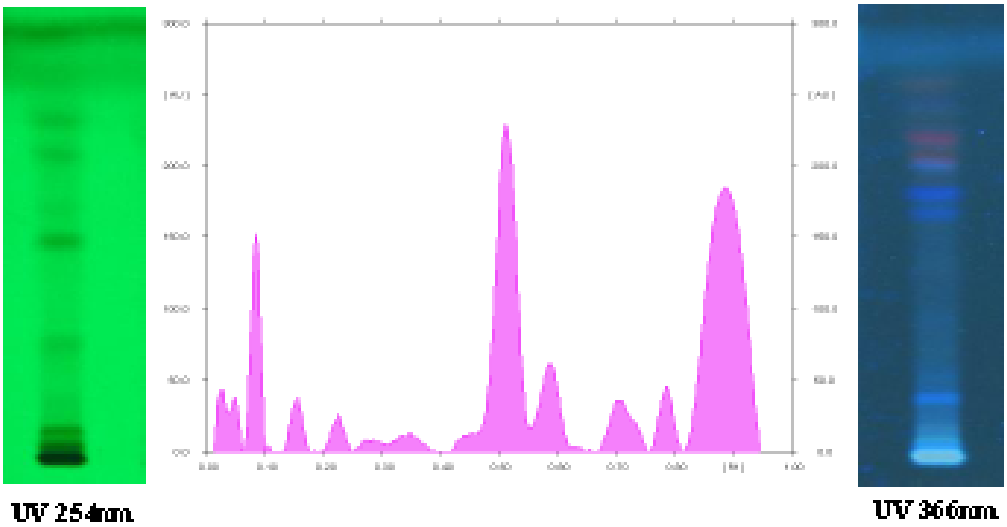

Figure 1: HPTLC finger print profile and densitometric scanning of 50%ethanolic extract of *Tephrosia purpurea* whole plant at 254 nm.
Solvent system: Ethyl acetate: Formic acid: Acetic acid: water (100:11:11:27)

Table 2: Rf values and the relative percentages of 13 spot in HPTLC

Peaks	Rf	% Area in spot
1	0.02	5.12
2	0.04	4.24
3	0.09	10.68
4	0.15	5.93
5	0.23	4.12
6	0.27	0.98
7	0.34	1.27
8	0.35	1.85
9	0.51	18.16
10	0.59	9.87
11	0.70	8.56
12	0.79	6.12
13	0.89	23.99

tumours (*metastases*) that are no longer amenable to surgical removal. Despite, various exogenous and endogenous factors are known to affect the normal pattern of cell growth, by which cell turns into a cancerous growth. The ratio of cell growth and cell death is strictly balanced through developmental and homeostatic processes in multi-cellular organisms.[5] Viruses and chemicals have also now been identified as the most important etiological factors associated with the development of human liver cancers and with the aid of animal models, exciting advances on the mechanism of hepatocarcinogenesis have begun to emerge. These advances may provide valuable insights into understanding and possibly preventing this disease.

Plant (*Tephrosia purpurea*)
Botanical name: *Tephrosia purpurea* Linn.
Family: Leguminosae
Part Used: Whole Plant

MATERIALS AND METHODS

Collection and identification of the plant materials

The whole plant of *Tephrosia purpurea* Linn. (Leguminosae) were collected from Botanical Garden of N.B.R.I. (National Botanical Research Institute), Lucknow, India. The plant material was identified and authenticated taxonomically by Dr. Priyanka Agnihotri, Scientist, Plant Diversity, Systematics & Herbarium Division, National Botanical Research Institute (CSIR), Lucknow, India. The herbarium, (LWG-80), was preserved at the department for future reference.

Animals Used

Wistar albino rats (150-170 g) and Swiss albino mice (25-30 g) were kept in the departmental animal house of Azad Institute of Pharmacy and Research, Lucknow, India. The animals were housed separately in polypropylene cage at temperature of 23 ± 2ºC and 50-60% relative humidity, with a 12 h light/dark cycle respectively, for one week before and during the commencement of experiment. Animals were allowed to access standard rodent pellet diet (Dayal animal feed, Barabankhi, U.P., India) and drinking water. Food was withdrawn 18-24 h before the experiment, though water was allowed *ad libitum* and allocated to different experimental groups. All experimental procedures involving animals were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA**)**. The study protocols were approved by Institutional Animal Ethics Committee (IAEC) of Azad Institute of Pharmacy and Research, Lucknow, India (Reg. No. 1146/PO/ERe/08/CPCSEA).

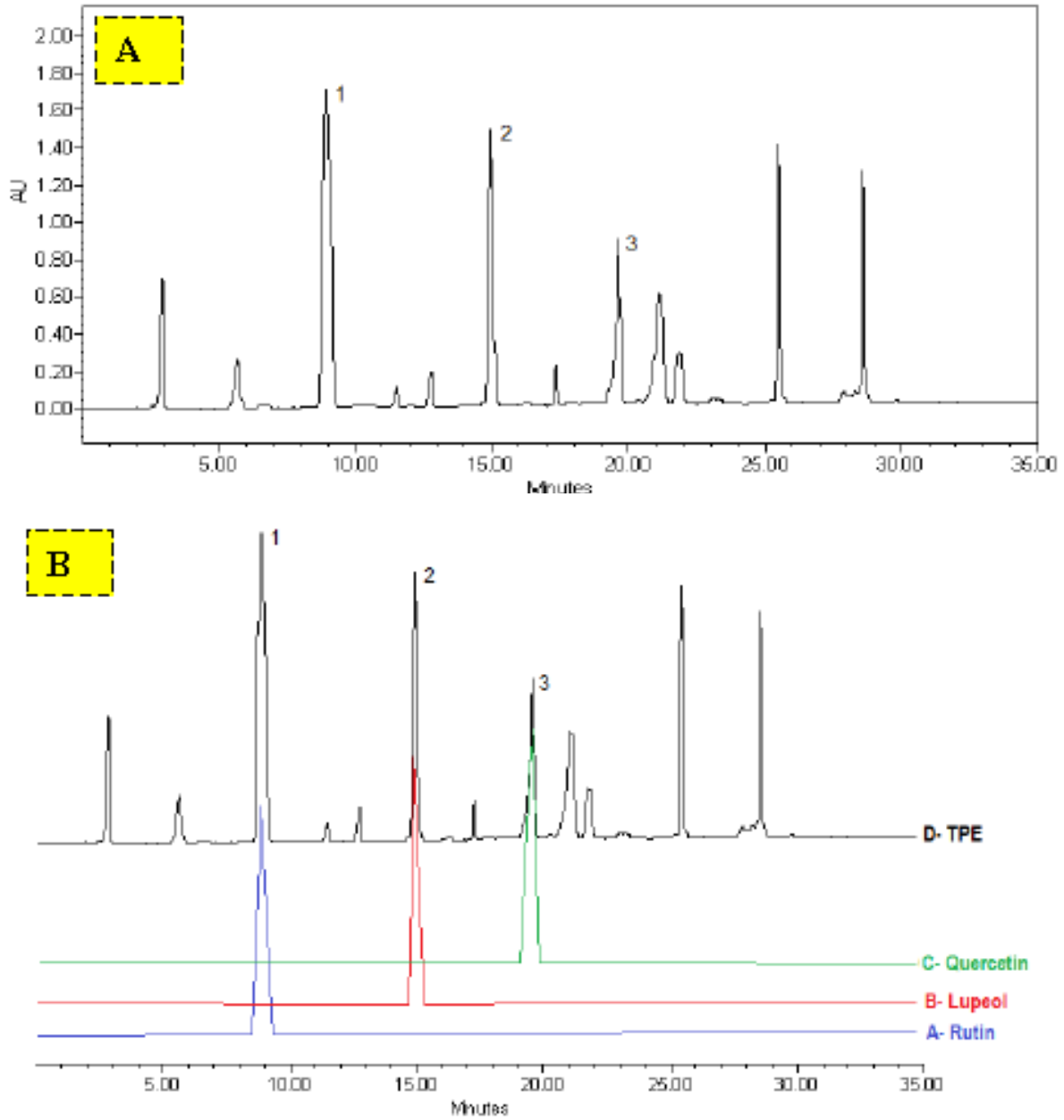

Figure 2: A- HPLC chromatograms of *Tephrosia purpurea* extract (TPE).
B - Chromatographic overlay and identification of compounds were obtainedby comparison of peak (254 nm) of the sample with that of standards, rutin(1, Rt = 9.12), lupeol (2, Rt = 5.14) and quercetin (3, Rt =19.51).

Phytochemical studies

Preparation of 50% EtOH extract of Tephrosia purpurea

The freshly collected plant materials of *Tephrosia purpurea* (4 kg) were washed with tap water to remove dirt and were shade dried. The dried materials were powdered and passed through a 10-mesh sieve. The coarse powdered materials (1 kg) were extracted with petroleum ether thrice to remove the fatty material and further marcs were exhaustively macerated thrice with ethanol (50%, v/v) by cold percolation method at room temperature. The extracts were separated by filtration and concentrated at 40 ± 1◦C on rotavapour (Buchi, USA) and then dried in lyophilizer (Labconco, USA) under reduced pressure. The yield for *Tephrosia purpurea* extract (TPE) 6.28% (w/w). The extract obtained was subjected to preliminary phytochemical analysis, toxicological and pharmacological investigations.

Preliminary phytochemical screening

Ethanolic extract (50%) of *Tephrosia purpurea* were subjected to qualitative tests for the identification of various active constituents viz. carbohydrate, glycoside, alkaloid, amino acids, flavanoids, fixed oil, tannins, gum and mucilage, phytosterols etc.[6]

Preliminary phytochemical fingerprint (HPTLC) analysis of 50% ethanolic extracts of Tephrosia purpurea.

Quantification and Documentation

The plates were visualized under UV 254 and Visible and Video documented Desaga video documentation unit. The plates were scanned densitometrically using CAMAG TLC scanner 3. CAMAG TLC scanner 3 was

Table 3: Effect of 50% ethanolic extract of *Tephrosia purpurea* on body weight of rats in subacute toxicity

Treatment/Dose	Average body weight (g)				
	Initial	1 week	2 week	3 week	4 week
Control	151.7±11.3	158.3±10.2	165.5±11.7	172.8±13.1	181.4±12.6
Tephrosia purpurea 200 mg/kg	154.3±12.6	162.1±11.6	169.7±10.6	176.3±12.5	184.7±13.2
Tephrosia purpurea 400 mg/kg	153.6±11.4	161.6±12.4	170.3±11.2	178.4±12.5	186.1±12.3

Values are expressed as Mean SEM of 10 rats in each group
The increase in body weight of extract treated groups are not significantly different from control group at the end of study (28 days).

Table 4: Effect of *Tephrosia purpurea* extract (TPE) on urine analysis of rats in subacute toxicity.

Treatment/Dose	Volume	SP. Gravity	Protein	Glucose	Ketone	Blood
Control	5.11±0.81	1.016±0.004	NIL	NIL	NIL	NIL
Tephrosia purpurea 200 mg/kg	5.18±0.73	1.017±0.003	NIL	NIL	NIL	NIL
Tephrosia purpurea 400 mg/kg	5.26±0.78	1.015±0.005	NIL	NIL	NIL	NIL

Values are expressed as Mean SEM of 10 rats in each group
The values observed in urine parameters in extract treated groups are not significantly different from control group at the end of study (28 days).

used for the quantification. Densitometry is *in situ* instrumental measurement of visible; the scanner converts the band on the layer in to a chromatogram consisting of peaks similar in appearance to HPLC. The portion of the scanned peaks on the recorder chart is related to Rf values of the spots on the layer and the peak height or area is related to the concentration of the substance on the spot.

HPLC analysis of Tephrosia purpurea extract

Sample preparation

For the HPLC analysis, *Tephrosia purpurea* powder was extracted with 6 ml of 25% hydrochloric acid and 20 ml methanol for 1 h. The obtained extract was filtered to a volumetric flask. The residue was heated twice with 20 ml of methanol for 20 min to obtain the extract. The combined extract (100 mg) was diluted with methanol to 100 ml. 5 ml portion of the solution was filtered and transferred to a volumetric flask and diluted with 10 ml of methanol. The sample (10 µl) was injected into the HPLC apparatus.

Chromatographic conditions

Separation was achieved with a two pump linear gradient program for pump A (Water containing 1% acetic acid) and pump B (Acetonitrile). Initially started with a gradient of 20% B changing to 30% in 10.0 min and finally to 50% in 35 min. The flow rate was 1 ml/min. The standard compounds such as rutin, lupeol and quercetin were run for comparative detection and optimization. All the samples were injected in triplicate and results (mg/g dry wt.) were obtained by comparison of peak areas (254 nm) of the samples with that of standards.

Toxicological studies

Acute toxicity study

Acute oral toxicity of the 50% aqueous ethanolic extract of *Tephrosia purpurea* were evaluated in Swiss albino mice of either sex (25–30 g), as per OECD guideline (Organization for Economic Co-operation and Development, Guideline-423, adopted on 17th December, 2001) with slight modifications.

Twenty four animals were equally divided into four groups (n = 6) as per sex for each plant extract. The extract was administered in 0.3% carboxy-methyl cellulose (CMC) suspension at doses of 50, 300 and 2000 mg/kg, whereas the control group received the CMC suspension only. Food or water was withheld for a further 1-2 hours after drug administration. Mice were observed for the initial 4h after the administrations, and then once daily during the following days. The behavioural changes observed for were: hyperactivity, ataxia, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Total observation period for eventual mortality was 14 days. One-tenth and one-fifth of the maximum tolerated dose of the extract (2000 mg/kg) tested for acute toxicity was selected for the subacute toxicity study i.e., 200 and 400 mg/kg.

Bio-chemical parameters investigated

Serum was analyzed for the following parameters as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphate (ALP), total cholesterol (TC), total protein (TP) and albumin (ALB) glucose, creatinine, blood urea nitrogen (BUN), total billirrubin (TB).

Determination of Serum glutamic oxaloacetic transaminase (SGOT)/

aspartate transaminase (AST)

The SGOT activity was determined according to the method of Reitman and Frankel, (1957)[7] method using SGOT.

Determination of Serum glutamate pyruvate transaminase (SGPT)/ alanine transaminase (ALT)

The SGPT level was estimated by Reitman and Frankel, (1957)[7] method using SGPT.

Determination of serum alkaline phosphatase (SALP)/ Alkaline phosphatase (ALP)

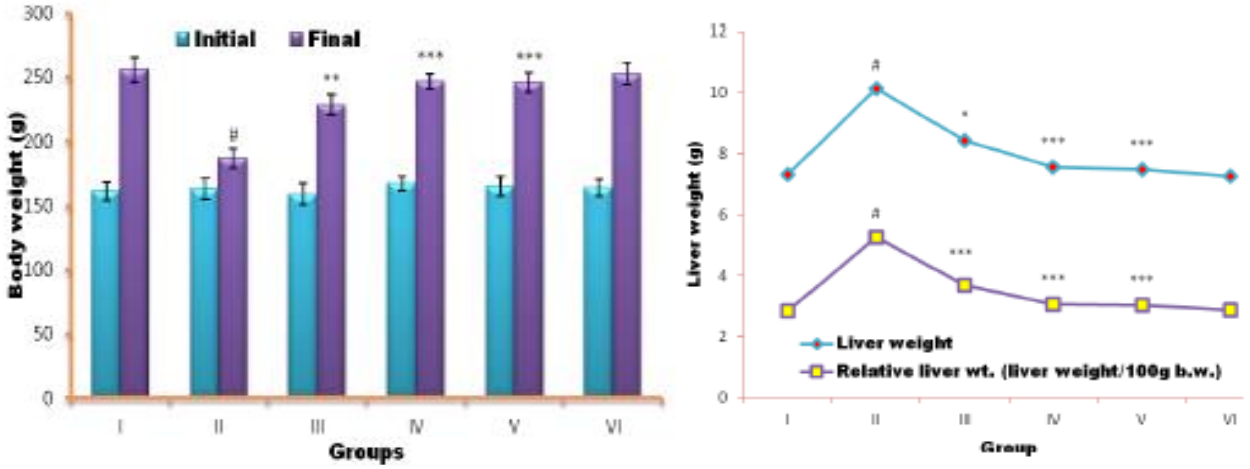

Figure 3: Effect of *Tephrosia purpurea* extract on body weight, liver weight and relative liver weight of control andNDEA induced HCC in rats.
Values are expressed as mean ± SEM of 6 rats in each group.
P values : #<0.001 compared with respective control group I.
P values : *<0.05, **<0.01 and *** <0.001 compared with group II (NDEA +CCl4)

Table 5: Effect of *Tephrosia purpurea* extract on the development of nodules in the liver of control and NDEA induced HCC in rats.

Groups	Treatment	No of rats with nodules/ total no of animals	Nodules incidence (%)	Total no of nodules	Average no of nodules/ nodules bearing liver
I	Control	0/6	0.00	00	00
II	NDEA (200 mg/kg) + CCl4 (3ml/kg)	6/6	100	51	8.5
III	TPE (200 mg/kg) + NDEA + CCl4	4/6	66.67	22	5.5
IV	TPE (400 mg/kg) + NDEA + CCl4	2/6	33.33	10	5.0
V	Silymarin (200 mg/kg) + NDEA + CCl4	2/6	33.33	09	4.5
VI	TPE alone (400 mg/kg)	0/6	0.00	00	00

Values are expressed as mean of 6 rats in each group.

The alkaline phosphates level was estimated by King, (1965)[8] method alkaline phosphatase.

Determination of serum γ glutamyl transferase (γ-GT) activity

Serum γ-GT activity was determined according to the method of Szas, (1974)[9].

Determination of serum bilirubin

The bilirubin level in serum was determined by Dangerfield and Finlayson, (1953)[10].

Total protein (TP) and albumin (ALB)

Total cholesterol

The total cholesterol level was estimated by Zlatkis, (1953)[11].

Estimation of serum creatinine

Glucose estimation

The collected serum samples of different study group were subjected to the serum glucose level estimation by enzymatic GOD-POD method by using (Braham and Trinder, 1972)[12]

Estimation of Alfa- Fetoprotein (AFP)

AFP is a glycoprotein with a molecular weight of between 65,000 and 70,000 daltons including 4% of carbohydrate. During fetal development, AFP maintains high levels in the serum and drops to very low levels throughout the remainder of life. AFP is elevated in the malignant diseases of hepatocellular, testicular nonseminomatous origin, and occasionally of other entodermal origin.

AFP may be slightly elevated or persisted in the patients with large hepatic metastases or viral hepatitis. AFP measurement is widely accepted as tumor marker and for monitoring the therapeutic effectiveness of hepatocellular cancer and nonseminomatous testicular cancer.

Estimation of Carcinoembryonic Antigen (CEA)

Carcinoembroyonic antigen (CEA) is a cell-surface 200-kd glycoprotein. Increased levels of CEA are observed in patients with cancer of the lung, liver, pancreas, breast, colon, head or neck, bladder, cervix, and prostate. Elevated plasma levels are related to the stage and extent of the disease, the degree of differentiation of the tumor,

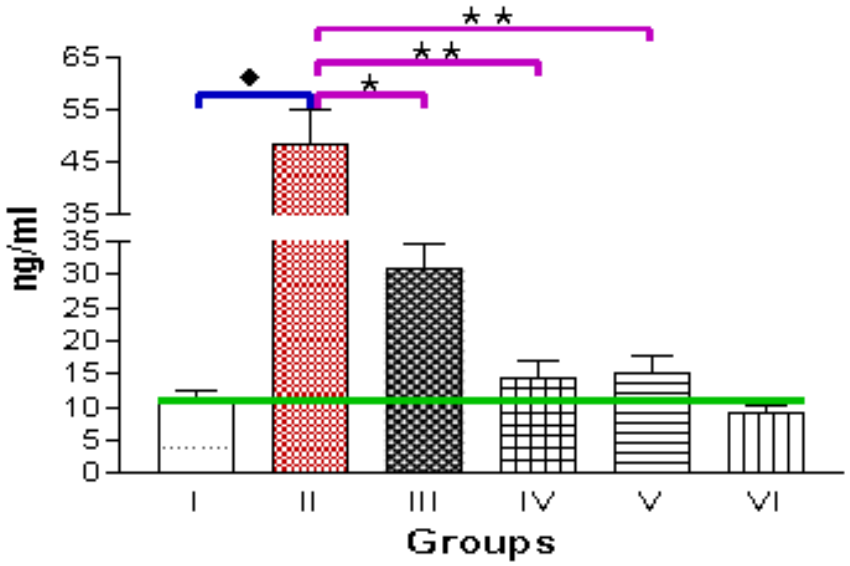

Figure 4: Effect of *Tephrosia purpurea* extract on the levels of α-feto protein (AFP) in the serum of control and NDEA induced HCC in rats.
Values are expressed as mean ± SEM of 6 rats in each group.
P values: <0.001 compared with respective control group I.
P values: <0.01 and <0.001 compared with group II (NDEA + CCl4).

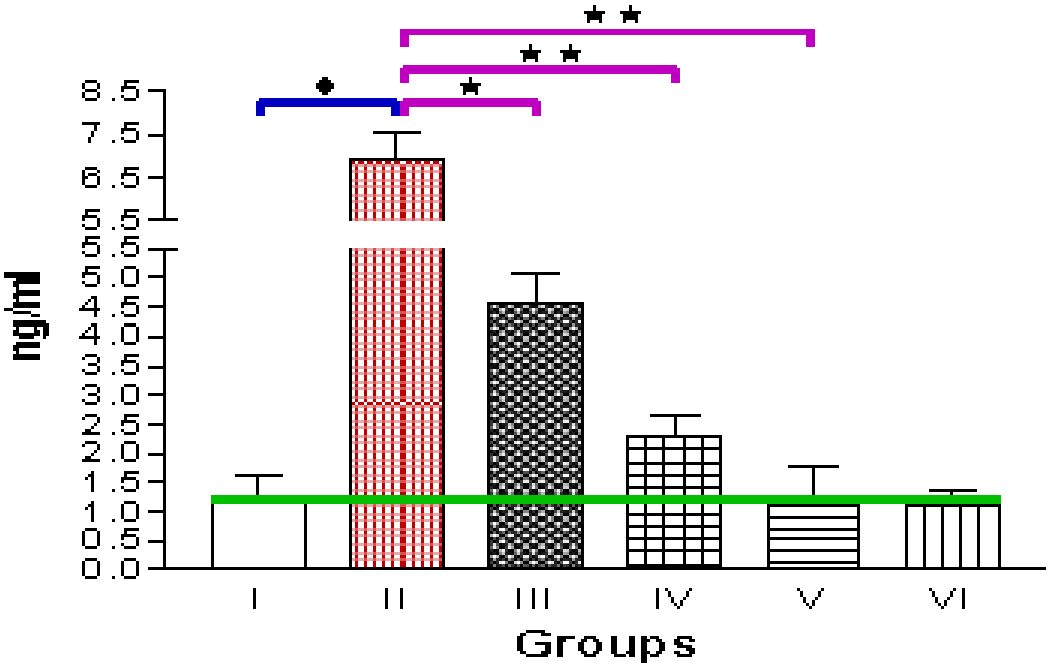

Figure 5: Effect of *Tephrosia purpurea* extract on the levels of carcinoembryonic antigen (CEA) in the serum of control and NDEA induced HCC in rats.
Values are expressed as mean ± SEM of 6 rats in each group.
P values: <0.001 compared with respective control group I.
P values: <0.05 and <0.001 compared with group II (NDEA + CCl4).

and the site of metastasis. CEA is also found in normal tissue.

Histopathological studies

The kidney and liver were dissected out and were fixed in 10% formalin solution. Paraffin sections were made and stained with haematoxylin and eosine for detailed histopathology study

Pharmacological studies

Experimental design and induction of hepatocellular carcinoma (HCC)

The experimental male Wistar rats were divided into six groups (for each plant), each group comprising of six animals (n = 6) for a study period of 20 weeks. Group I served as normal control and treated with 0.9% normal saline throughout the study. HCC was induced in groups II, III, IV and V with single intraperitoneal injection of NDEA in normal saline at a dose of 200 mg/kg body

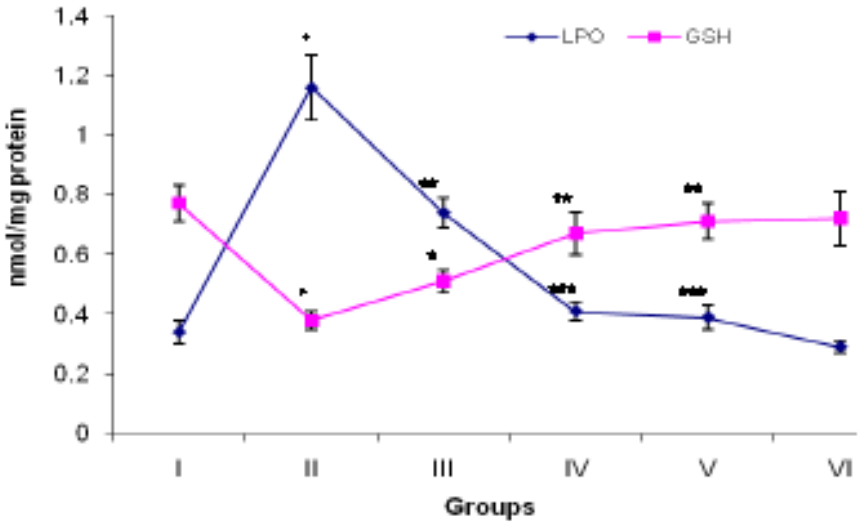

Figure 6**:** Effect of *Tephrosia purpurea* extract on the levels of lipid peroxide and glutathione in the PMS of control and NDEA induced HCC in rats.
Values are expressed as mean ± SEM of 6 rats in each group.
P values: ¥ <0.01 and <0.001 compared with respective control group I.
P values: <0.05, <0.01 and <0.001 compared with group II (NDEA + CCl4).

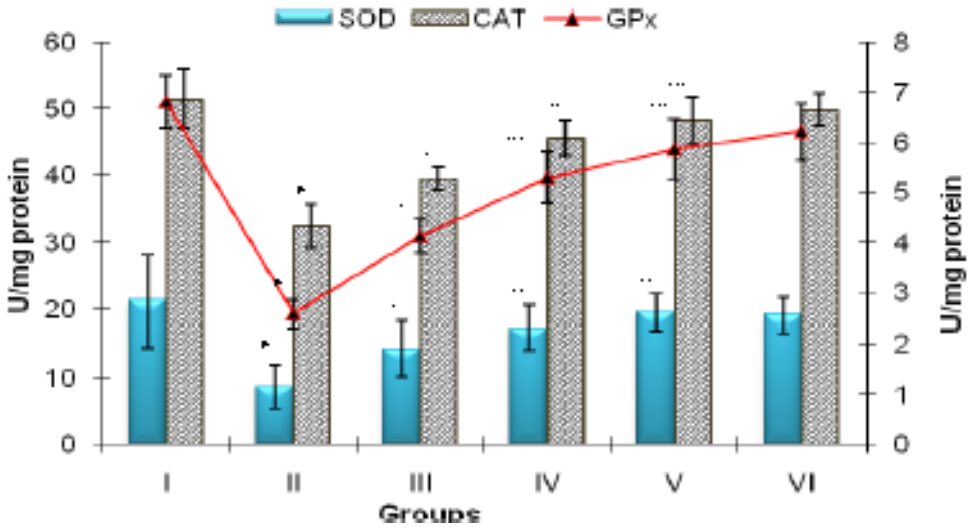

Figure 7: Effect of *Tephrosia purpurea* extract on the antioxidant activities of SOD (50% inhibition of chromogen/min/mg protein), CAT (µmol H_2O_2 consumed/min/mg protein) and GPx (µmol GSH utilized/min/mg protein) in the PMS of control and NDEA induced HCC in rats.
Values are expressed as mean ± SEM of 6 rats in each group.
P values: ¥ <0.001 compared with respective control group I.
P values: *<0.05, **<0.01 and ***<0.001 compared with group II (NDEA + CCl4).

weight followed by weekly subcutaneous injections of CCl_4 (3 ml/kg/week) for 6 weeks, as the promoter of carcinogenic effect.[13] After administration of NDEA, test groups III and IV were administered orally 400 mg/kg TPE, in the form of aqueous suspension daily once a day throughout the study. Group V received silymarin, the known hepatoprotective and anti-hepatocellular carcinoma compound at a dose of 200 mg/kg.[14] The dose of TPE (400 mg/kg) was administered orally to rats of group.

The experiment was terminated at the end of 20 weeks of experimental period, the body weight of each rat was taken before sacrifice. The overnight fasted animals were anaesthetized and sacrificed 48 h after the last dose of the drug. Blood was collected by retro-orbital plexus followed by heart puncture and allowed to clot before centrifugation at 2500 × g for 15 min at 4 ˚C to separate serum. The liver tissue was washed twice with ice cold saline, blotted, dried, observed for the presence of nodules and then weighed. The relative liver weight was calculated as the percentage ratio of liver weight to the body weight.

A small portion of the tissue was fixed in formalin for histological examination. The remaining tissues were stored at 20 ˚C for not more than 12 h before analysis.[4]

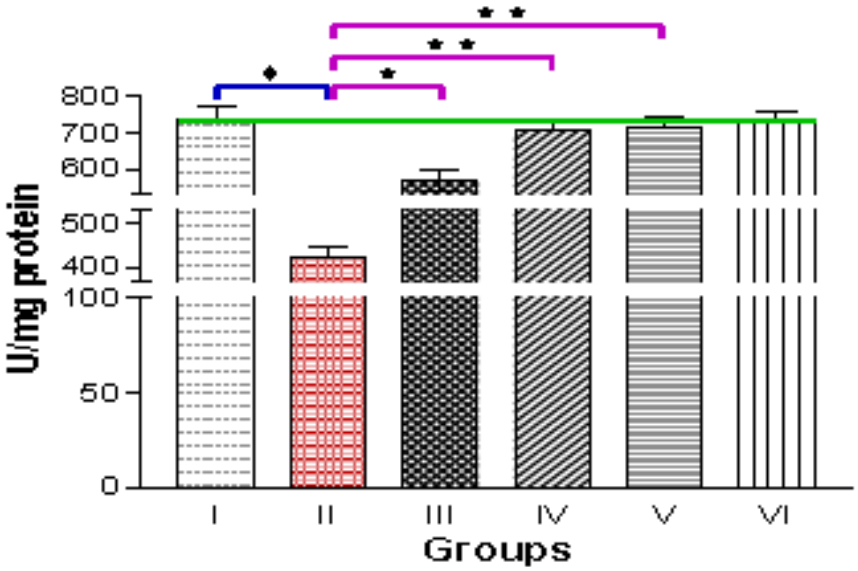

Figure 8: Effect of *Tephrosia purpurea* extract on the antioxidant activities of GST (1-chloro-2,4-dinitrobenzene nmol/min/mg protein) in the PMS of control and NDEA induced HCC in rats.
Values are expressed as mean ± SEM of 6 rats in each group.
P values: <0.001 compared with respective control group I.
P values: <0.01 and <0.001 compared with group II (NDEA + CCl4).

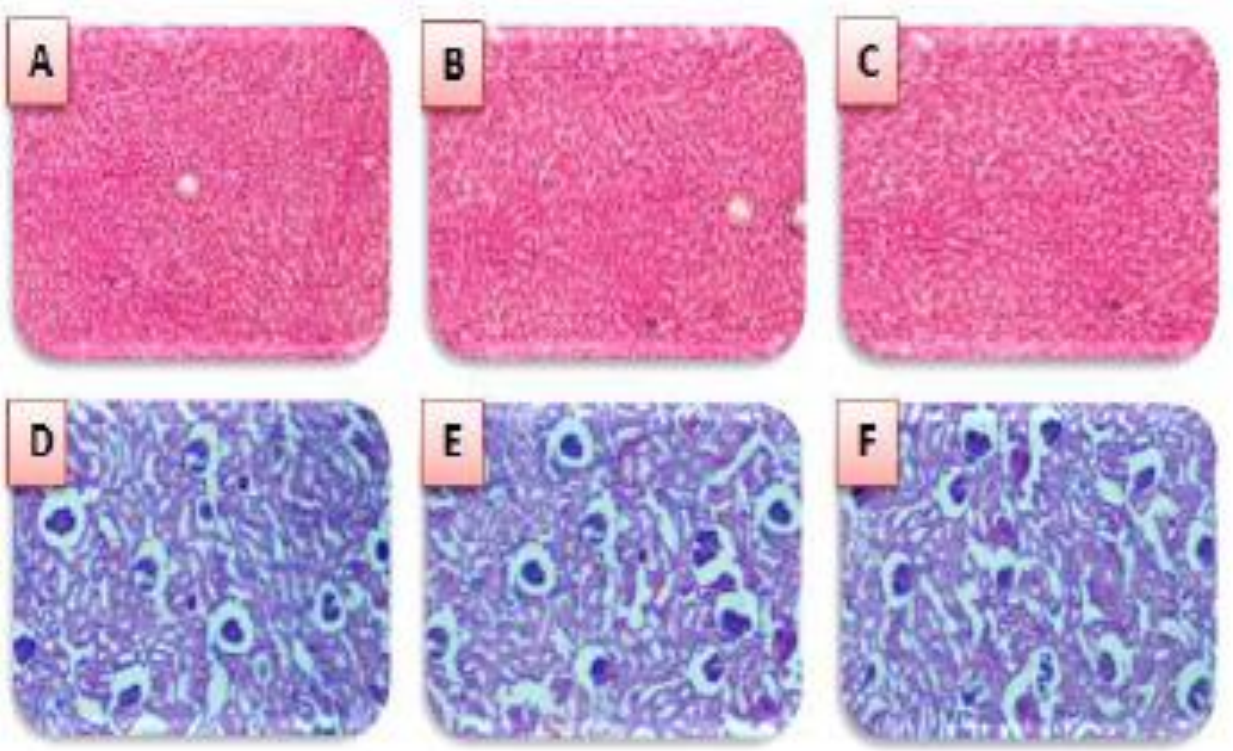

Figure 9: Histopathological picture of liver and kidney of control and experimental group of animals. (A) The section of liver from control animals revealed normal architecture and hepatic cells with granulated cytoplasm,(B) and (C) The section of liver from TPE 200 and 400 mg/kg exhibited normal architecture of hepatocytes indicating the safe nature of the extract.(D) The section of kidney from control animals showed normal size of glomeruli with normal tubules, (E) and (F) The section of kidney from TPE200 and 400 mg/kg exhibited within the limits of normalcy

Assessment of liver injury markers and liver tumor markers

The activities of biochemical parameters investigated in serum was, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), Total bilirubin level (TBL) and γ-glutamyl transferase (γ -GT) as well as quantitative estimation of serum hepatic tumor markers - α-feto protein (AFP) and carcinoembryonic

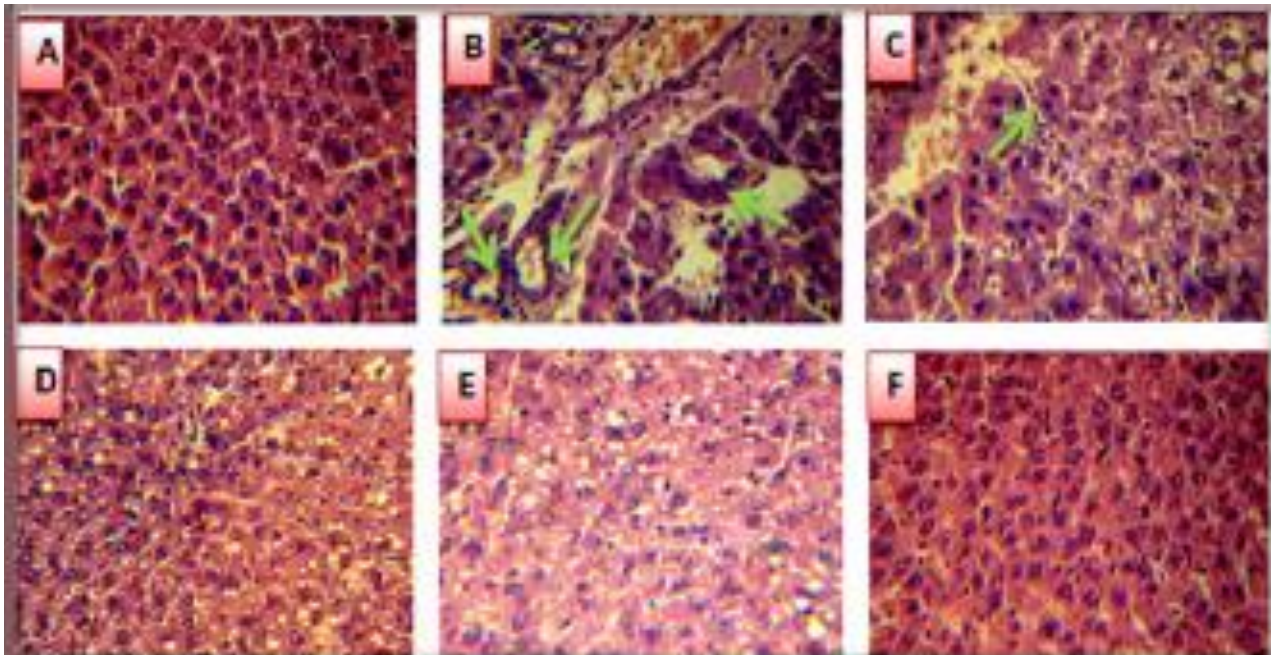

Figure 10: Histological study of liver tissue in control and experimental groups of rats. (A) (40x) H and E stained section of liver from control animals revealed normal architecture and hepatic cells with granulated cytoplasm, (B) (40x) H and E stained section of liver from NDEA induced carcinoma bearing animal showing neoplastic hepatocyte surrounded by necrosis and the cells are separated by fibrous septa with inflammatory collection as well as loss of architecture. Neoplastic cells were larger than normal cells with larger hyperchromatic nuclei and hyaline globules (arrow)that represent proteins produced by the tumor cells (C) (40x) H and E stained section of liver from 50% ethanolic extract of TPE (200 mg/kg) on HCC bearing animal hepatocyte showing minimal inflammatory cell infiltration around the portal triads and necrosis with few malignant hepatocytes. (D)(40x) H and E stained section of liver from 50% ethanolic extract of TPE(400 mg/kg) on HCC bearing animals exhibited hepatocyte maintaining nearnormal architecture. (E) (40x) H and E stained section of liver from silymarin (200 mg/kg) on HCC bearing animals shows the structure close to proximity of normal hepatocytes. (F) (40x) H and E stained section of liverfrom TPE (400 mg/kg) alone exhibited normal architecture of hepatocytes indicating the non-toxic nature of the extract.

antigen (CEA) were based on ELISA using assay kits from USCN LIFE science and technology (Wuhan, China).

Assessment of antioxidant parameters

Preparation of PMS (post mitochondrial supernatant)

Hepatic tissues of rats were homogenized (10%) in phosphate buffer (pH 7.4) with a Potter Elvenhjem glass homogenizer. The homogenate was centrifuged at 12,000 × g for 20 minutes at 4 ◦C to obtain post mitochondrial supernatant (PMS) and it was used for the estimation of the following parameters.

Assessment of lipid peroxidation (LPO)

A volume of the homogenate (0.2 ml) was transferred to a vial and was mixed with 0.2 ml of a 8.1% (w/v) sodium dodecyl sulphate solution, 1.5 ml of a 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 ml of a 0.8% (w/v) solution of thiobarbituric acid (TBA) and the final volume was adjusted to 4.0 ml with distilled water. Each vial was tightly capped and heated in a boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test samples and 10% trichloroacetic acid were transferred into a centrifuge tube and centrifuged at 1000 × g for 10 min. The absorbance of the supernatant fraction was measured at 532 nm (Beckman DU 650 spectrometer). Control experiment was processed using the same experimental procedure except the TBA solution was replaced with distilled water (Jamall and Smith, 1985)[15]. Malonyldialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen thiobarbituric acid reactive substance. 1,1, 3, 3-tetraethoxypropane was used as standard for calibration of the curve and is expressed as n mole/mg protein.

Assessment of reduced glutathione (GSH)

Reduced glutathione (GSH) level was measured by the method of Ellman.[16] The PMS of rat liver (720µl) and 5% TCA were mixed to precipitate the protein content of the supernatant. After centrifugation at 10,000 × g for 5 min, the supernatant was taken. DTNB (5, 5´-dithio-bis (2-nitrobenzoic acid) Ellman's reagent was added to it and the absorbance was measured at 412 nm. A standard graph was drawn using different concentrations of a standard GSH solution. GSH contents were calculated in the PMS of rat liver and expressed as n mol/mg of protein.

Assessment of catalase (CAT)

Catalase activity was measured according to the method of Aebi.[17] One unit of catalase was defined as the amount of enzyme required to decompose 1.0 µM of hydrogen peroxide (H_2O_2) in 1 min. The reaction was initiated by the addition of freshly prepared 20 mM H2O2

(1.0 ml). The rate of decomposition of H2O2 was measured at 240 nm for 1 min, at 25 ◦C. The enzyme activity was expressed as U/mg of protein.

Assessment of superoxide dismutase (SOD)

The activity of superoxide dismutase in the PMS of liver was assayed[18] based on the oxidation of epinephrine adrenochrome transition by enzyme. The post-mitochondrial suspension of rat liver (0.5 ml) was diluted with distilled water (0.5 ml). To this, chilled ethanol (0.25 ml) and chloroform (0.15 ml) were added. The mixture was shaken for 1 min and centrifuged at 2000×g for 10min. The PMS (0.5 ml) was added with PBS buffer (pH 7.2; 1.5ml). The reaction initiated by the addition of epinephrine (0.4 ml) and change in optical density (O.D., min−1) was measured at 470 nm. SOD activity expressed as U/mg of protein. Change in O.D. (min−1) at 50% inhibition to adrenochrome transition by the enzyme was taken as one enzyme unit.

5.3.6 Assessment of Glutathione peroxidase (GPx)

Glutathione peroxidase activity was measured by the method described by Rotruck (1973)[19]. To 0.2 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of PMS were added. To that mixture, 0.2 ml of glutathione solution and 0.1 ml of H_2O_2 were added. The contents were mixed well and incubated at 37°C for 10 minutes along with the control tubes containing the entire reagent but no enzyme. After 10 minutes, the reaction was arrested by the addition of 0.4 ml of 10% TCA. Then 0.2 ml of PMS was added to the control tubes and the tubes were centrifuged. 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 spectrophotometer. Graded concentrations of the standard were also treated similarly. Glutathione peroxidase activity in liver homogenate was expressed as U/mg of protein.

Assessment of glutathione-S-transferase (GST)

The activity of glutathione-S-transferase was measured according to the method of Habig et al., (1974)[20]. The PMS of liver (0.1ml), PBS buffer (pH 7.4; 1.0 ml), double distilled water (1.7 ml), and 1-chloro-2,4-dinitrobenzene (CDNB; 0.1ml) reagent were taken in the screw cap tubes and incubated at 37 ◦C for 15 min. the reaction was started by the addition of GSH (1.0 ml) was added and increase in optical density was recorded at 340 nm from 0 to 3.0 minutes. The reaction mixture without the enzyme was used as blank. The activity of GST was expressed as U/mg of protein.

Histopathological assessment

At the end of each scheduled the control as well as treated rats were sacrificed by using cervical dislocation. For histologic studies, the liver sections were made immediately from the liver of different groups of rats, fixed in 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin wax. Sections were cut at 4μm thick (Automatic tissue processor, Lipshaw) in a rotary microtone and the pathological changes were observed microscopically after staining with hematoxylin and eosin (H & E).

Statistical analyses

The data were represented as mean ± S.E.M. for six rats. Analysis of variance (ANOVA) test was followed by individual comparison by Newman-Keuls test using Prism Pad software (Version 3.0) for the determination of level of significance. The value of probability less than 5% ($P < 0.05$) was considered statically significant.

RESULTS

Preliminary phytochemical studies

Extraction and Preliminary phytochemical screening of 50%EtOH extract of Tephrosia purpurea

Tephrosia purpurea was subjected to 50% ethanolic (EtOH) extractionand the percentage yield was 6.28% (w/w). Preliminary phytochemical screening of 50% EtOH extract of *Tephrosia purpurea* (TPE) gave positive tests for various phytoconstituent such as alkaloids, glycosides, flavonoids, carbohydrates, protein, amino acids, terpines and steroids.

HPTLC analysis of 50% EtOH extract of Tephrosia purpurea

The preliminary HPTLC studies revealed that the solvent system; Ethyl acetate: Formic acid: Acetic acid: water (100:11:11:27) was ideal forthe 50% ethanolic extract of *Tephrosia purpurea* and plates were visualized under UV 254 and visible. The plates were sprayed with anisaldehyde -sulfuric acid and heated at 110ºC for 10 min. The plates were scanned densitometrically using CAMAG TLC scanner at 254 nm wavelength. The Rf values and the relative percentage of area in each peak in the extract applied were also calculated.

HPLC analysis of Tephrosia purpurea

HPLC analysis of TPE revealed the presence of chromatographic peaks consistent with the pattern showed by the marker components such asrutin (1), lupeol (2) and quercetin (3). Quantitative HPLC analysis showed that rutin (32.7 mg/g of TPE), lupeol (24.3 mg/g of TPE) and quercetin (13.6mg/g of TPE) with retention times (Rt) of 9.12, 15.14 and 19.51 minutes respectively, were the main flavonoids in TPE.

Pharmacological studies

Effect of 50% ethanolic extract of Tephrosia purpurea on body weight, liver weight and relative liver weight in control and NDEA +CCl_4- induced HCC rats

The body weight (initial and final) and liver weights of control and experimental groups of animals. The final body weight of normal group I rats was 256 ± 9.6 g which significantly decreased to 187 ±7.3 g ($P < 0.001$) as compared to group II rats following NDEA treatment. In TPE-treated group III (200 mg/kg) and IV (400 mg/kg) rats, the final bodyweights became significantly increased 229 ± 7.6 ($P < 0.01$) and 247 ± 8.5 g($P < 0.001$) when compared to the NDEA group II, respectively. Moreover, NDEA treatment significantly increased the relative liver weight to 5.27 ±0.11/100 g ($P < 0.001$) body weight when compared to the control (group I,2.86 ± 0.04/100 g body weights). However, administration of 200 and 400mg/kg TPE significantly reduced ($P < 0.001$) the relative liver weight to 3.68± 0.08 and 3.06 ± 0.06/100 g body weights, respectively, compared to 5.27 ±0.11/100 g in NDEA treatment. TPE treated group IV activity was almost

similar to standard silymarin-treated group V rats at the concentration used. The animals treated with TPE alone (400 mg/kg) showed no significant change in the body weight and relative liver weight when compared to control group I.

Effect of 50% ethanolic extract of Tephrosia purpurea on the development of liver nodules in control and NDEA + CCl4- induced HCC rats

The total number of nodules, nodule incidence and average number of nodules per nodule bearing liver in the normal and experimental groups of animals. When rats were treated with NDEA (groupII), 100% developed nodules in the liver were observed, whereas administration of TPE 200 mg/kg (group III) and 400 mg/kg (group IV) showed marked decrease in the number and multiplicity of the nodules as compared to group II rats. The incidence of nodule growth was reduced to66.67% in group III and 33.33% in group IV, respectively. The nodule incidence of TPE treated group IV was almost comparable to standard silymarin treated group V rats. The occurrence of hepatic nodules was not observed in the normal group I and TPE alone treated group VI animals.

Effect of 50% ethanolic extract of Tephrosia purpurea on liverinjury and cancer markers in control and NDEA + CCl4 –induced HCC in rats

The effect of TPE on liver injury is shown. NDEA treated group II rats showed increased serum AST (372.64 ± 26.14 U/l, P <0.001), ALT (212.13 ± 17.81 U/l, P < 0.001), ALP (148.23 ± 13.51U/l, P <0.001), γGT (128.83 ± 14.65 U/l, P < 0.001) and TBL (2.09 ± 0.27 mg/dl, P< 0.001) compared to control group I rats (101.28 ± 17.11 U/l, 36.83 ± 7.16U/l, 62.15 ± 7.22 U/l, 32.11 ± 3.39 U/l and 0.69 ± 0.06 mg/dl), respectively. In contrast, the TPE treated groups III and IV rats at 200 and 400 mg/kg significantly decreased AST (251.77 ± 18.73 and 117.62 ± 16.57 U/l, P <0.001), ALT (124.45 ± 13.27 and 49.83 ± 9.83U/l, P < 0.001), ALP (103.67± 10.14 and 67.38 ± 7.62 U/l, P < 0.01 and P < 0.001), γGT (89.72 ± 6.86and 40.55 ± 4.88 U/l, P < 0.01 and P < 0.001) and TBL (1.37 ± 0.22 and 0.87± 0.13 mg/ml, P < 0.01 and P < 0.001), respectively compared to group II animals. The levels of the tumor markers α-feto protein (AFP) and carcinoembryonic antigen (CEA). Their levels were found to be increased significantly (AFP, 48.32 ± 6.57 mg/ml; CEA, 6.93 ± 0.64 mg/ml, P< 0.001) in NDEA-induced rats whereas they were significantly (AFP, 30.66± 3.87 and 14.47 ± 2.35 mg/ml, P < 0.01 and P < 0.001; CEA, 4.56 ± 0.53and 2.29 ± 0.38 mg/ml, P < 0.05 and P < 0.001) decreased on treatment with200 and 400 mg/kg of TPE.

DISCUSSION

Hepatocellular carcinoma (HCC) one of the leading causes of death in developing countries. Hepatocellular carcinoma (HCC) is one of the most common cancers with poor prognosis. However, various exogenous and endogenous factors are known to affect the normal pattern of cell growth, by which cell becomes a cancerous. N-nitrosodiethylamine (NDEA) is the most important carcinogen among nitrosamines and primarily induces tumor of liver. The liver is one of the largest organs in the body. It plays an important role as a neutralizer of exo and endotoxins. Recent studies during the last few years have shown new hopes in the treatment of this dreadful diseases by measures aimed at the close relationship between free radical production and hepatic cancer.[21] Among other functions it serves as an interface that processes absorbed nutrients into chemicals that are nontoxic for the organism and can safely be utilized by other tissues and organs. Indeed, curative treatment such as tumor resection and orthotopic liver transplantation are not feasible in advanced stages of Hepatocellular carcinoma (HCC). Therefore, searching for effective chemotherapeutic agents is important to improve the survival rate of patients with advanced or recurrent HCC.

In addition, HCC is well known for multi-drug resistance and its poor response to current chemotherapeutic agents.[22] On the other hand, Ayurveda, an indigenous system of medicine has long been used for treating liver disorders based on traditional knowledge.[23] However, traditional medicine, especially the herbal medicine plays a vital role in the management of various liver disorders. The relative importance of *Tephrosia purpurea* (TPE) is considered useful in the treatment of various ailment as well as liver disorders and tumour. Hence, it is usually intensive to chemotherapeutic drugs currently used in clinical setting, and there is an urgent need for the evaluation of new active drugs against HCC.

In the present investigation, biochemical parameters were observed in animal of hepatoma (NDEA and CCl_4) induced hepatocellular carcinoma in the rats. In the present study, 50% ethanolic extract of the plant tested showed significant improvement in biochemical parameters shows the potentiating the level of back to normal and also likely show the level of these enzymes near to the level when treated with the standard drug which indicates , the anti carcinogenic effects of plant in (NDEA and CCl_4) treated hepatoma rats. In groups treated with 50% ethanolic extract of the plant showed significant results, reducing the levels of these elevated levels in a dose dependent manner, indicates the restoring serum marker enzymes back to normal. To prove the anticancer activity of *Tephrosia purpurea* (TPE), histopathological studies were carried out. In the present investigation, noticeable changes were observed in the architecture of liver of cancer bearing animals. These indicates the presence of neoplastic conditions following NDEA and CCl_4 administration. In drug treated animals, the NDEA and CCl_4 damage was recovered due to anticancer potency of *Tephrosia purpurea* (TPE). The regression of the tumours in liver may be due to the protective effect of *Tephrosia purpurea* (TPE).

CONCLUSION

Hepatocellular carcinoma (HCC) is one of the common cancers in the world. The most important part is the evolution of experimental hepatocarcinogenesis and its importance as an animal model in treatment of disease relating to human efficacy. The promoter which is as

important as carcinogen which boost the process of malignancy and decrease the latency period of occurrence of malignancy. NDEA and CCl_4 is been widely used as a study for chemical induced hepatocellular carcinoma and its mechanism of action is also well illustrated. Based on this, potentiation of plant for the treatment of dreaded disease like cancer may shows a ray of light for better protocol for further treatment of hepatocellular carcinoma. Recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of research on medicinal plant.

In the acute toxicity study, TPE produced neither mortality nor changes in behavior in mice. In subacute toxicity study, TPE did not produce any significant difference in their body weight, food consumption and water intake, hematological and biochemical parameters in experimental groups of rats. Histopathological study revealed normal architecture of kidney and liver of TPE treated rats. These finding demonstrated that there is a wide margin of safety for the therapeutic use of *Tephrosia purpurea*. Thus further corroborated the traditional use of these extract as an anti hepatocarcinogenic agent.

The efficacy of *Tephrosia purpurea* in experimental liver cancer described in the present investigation offer the potential for reaching on understanding of anticancer potency. The administration of *Tephrosia purpurea* extract and Silymarin show the rehabilitating capability of extracts in respect with anticancer potency in comparison with the standard drug Silymarin.

These finding suggested that the oral administration of *Tephrosia purpurea* possesses antihepatocellular carcinoma activity as evidenced by the significant and dose dependent restoring the activities of entire liver cancer marker enzymes, diminution in tumor incidence, decrease in lipid peroxidation (LPO) and increase in the level of antioxidant enzymes (GSH, CAT, SOD, GPx and GST) through scavenging of free radicals, or by enhancing the activity of antioxidant, which then detoxify free radicals. These factors protect cells from ROS damage in NDEA and CCl4-induced hepatocarcinogenesis. Histopathological observations of liver tissues too correlated with the biochemical observations. Thus, present investigation suggested that the *Tephrosia purpurea* would exert a chemoprotective effect by reversing the oxidant-antioxidant imbalance during hepatocarcinogenesis induced by NDEA and CCl_4. Besides *Tephrosia purpurea* is very much effective in preventing NDEA-induced multistage hepatocarcinogenesis possibly through antioxidant and antigenotoxic nature, which was confirmed by various liver injury and biochemical tumour markers enzymes.

These observations and description of mechanism of *Tephrosia purpurea*, which interplay with cancer biology and pharmacology lead to rapid development in cancer treatment. In addition to this, studies on molecular aspect of cancer therapy will give mechanistic information in cancer therapy and also critical balance should be there between the animal model and clinical research. This holds great promise for future research in human beings. The anticancer properties of *Tephrosia purpurea* should provide useful information in the possible application in cancer prevention and cancer therapy.

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