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Tephrosia Purpurea Modulates Hepatic Markers and Antioxidant Enzymes Against Two-Step Hepatocarcinogenesis Induced by N-Nitrosodiethylamine and Carbon Tetrachloride in Swiss Albino Rats

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Abstract

Tephrosia purpurea (Leguminosae) has been used as hepatoprotective and is used to treat liver disorders. The study was aimed to investigate the amelioration effect of T. purpurea extract (TPE) against two-step hepatocarcinogenesis induced by N-nitrosodiethylamine (NDEA) and tumor promoter, carbon tetrachloride (CCl4) in Swiss albino rats and the mechanism of its hepatocarcinogenesis suppression effect. The hepatic function markers viz. SGPT, SGOT, SALP, gamma glutamyl transpeptidase (GGT), bilirubin, Protein and antioxidant enzymes viz. catalase, superoxide dismutase, reduced glutathione, glutathione peroxidase and glutathione-s-transferase, lipid peroxidation (LPO) were assayed. The hematological and nucleic acid viz. WBC, RBC, Hb, DNA and RNA were also assayed. Administration of TPE effectively suppressed hepatic marker and oxidative stress marker as revealed by decrease in NDEA and CCl4 induced elevated levels in hepatic marker, WBC and LPO and significant elevation in the level of antioxidant enzymes, protein, Hb and RBC. The body weight, mean and relative liver weight were assessed. The histopathology of liver revealed that TPE reduced the incidence of liver lesions. These results suggest that an extract of Tephrosia purpurea is able to alleviate the hepatic and oxidative stress markers, which signify its amelioration effect against two-step NDEA and CCl4-induced hepatocarcinogenesis in swiss albino rats.

Keywords: Tephrosia Purpurea (Leguminosae), N-Nitrosodiethlyamine, CCl4, Hepatic Markers,Antioxidant Markers, DNA, RNA, Hematological Parameter

1. Introduction

Hepatocellular carcinoma is the world's fifth most prevalent malignancy. Hepatocellular carcinoma accounts for over 90% of all liver cancers and is the fourth most common cause of cancer mortality, accounting for more than 4% of all cancer occurrences globally [1]. Long-term cellular damage caused by viral, bacterial, or chemical-related chronic inflammations is well understood to play a role in carcinogenesis [2]. N-nitroso compounds are potent chemical carcinogens that endanger human health [3]. The occurrence of these chemicals, such as N-nitrosodiethylamine, N-nitrosodimethylamine, N-nitrosopyrrolidine, and N-nitrosopiperidine, has been commonly observed in a variety of foods, including milk, meat, soft drinks, and alcoholic beverages [4]. Furthermore, tobacco smoke is one of the leading causes of individual exposure to nitrosamines [5]. N-nitrosodiethylamine, a hepatocarcinogen, has been shown to produce promutagenic

adducts such as O6-ethyl deoxy guanosine and O4- and O6-ethyl deoxy thymidine, which may induce liver carcinogenesis [6]. The mechanism of NDEA-induced carcinogenesis includes DNA adduct production followed by gene mutation, cytolethality following regenerative growth, and oxidative stress or damage caused by free radicals impairing mitochondrial respiration [7,8]. It is widely documented that oxidative stress is a causal factor during carcinogenesis [9]. Reactive oxygen species (ROS) are a significant inducer of both tissue injury and DNA damage [10]. The Indian Ayurvedic tradition contains a plethora of pharmaceutical formulas for many maladies, including liver issues and cancer [11,12]. However, these natural remedies have not received scientific validation. Nonetheless, a few experimental investigations were carried out to confirm the potential of herbal medicines against tumours [13]. *Tephrosia Purpurea* (L.) Pers. (Family: Leguminosae) has been used to treat acute and chronic inflammation, liver diseases, and hepatoprotection ulcer and it inhibits the tumor-promoting impact of croton oil (phorbol ester) in mouse skin [14,16]. It also has considerable hydroxyl radical scavenging action in vitro and prevents benzoyl peroxide-induced cutaneous oxidative stress and toxicity [17].

Aside from being a skin antioxidant, T. Purpurea is an effective chemopreventive agent against renal oxidative stress and carcinogenesis caused by N-diethylnitrosamine and KBrO3 [18]. Tephrosia purpurea L. aerial parts and Tecomella undulate stem bark were evaluated for hepatoprotective potential. *T.pupurea* contains flavanols, tephrosin pongaglabol and semiglabrin [19,21]. In the present study, we investigated the amelioration capacity of Tephrosia purpurea by modulating the hepatic and antioxidant marker against N-nitrosodiethylamine and promoter CCl4 induced hepatocarcinogenesis in swiss albino rat by studying hepatic function marker, oxidative stress marker, free radical scavenging enzymes, haematological and nucleic acid parameter, and changes in the morphology of liver cells and sought to determine how Tephrosia purpurea b In a nutshell, the current study sought to assess the protective effects of Tephrosia purpurea against two-step hepatocarcinogenesis caused by NDEA (initiator) and CCl4 (promoter) [22].

2. Materials and Methods

2.1. Chemicals

Chemicals utilised in the studies, including NDEA and carbon tetrachloride (CCl4), were acquired from Sigma Chemicals St. Louis, USA, while all other chemicals were purchased from SD Fine Chem Ltd, Mumbai, India, and were of the highest purity grade.

2.2. Plant Material and the Extraction Process

Tephrosia purpurea was obtained as a complete plant from the National Botanical Research Institute in Lucknow, India in November 2005. Dr Sayeeda Khatoon, chemotaxonomist, certified the plant materials and placed the voucher specimen (NAB 200497) in the departmental herbarium for future reference. The plants were cleaned with distilled water to remove dirt and soil, then dried in the shade and finely pulverised.

The powdered substance (1000 g) was extracted three times with 50% ethanol (v/v). The extracts were filtered, pooled, and concentrated at 50°C using a rotary evaporator (Buchi, USA). They were subsequently freeze-dried (Freezone 4.5, Labconco, USA) at high vacuum (133103 mbar) and low temperature (-402°C). The yield reached at least 6.28% (w/w). The TPE extract was kept at 4-8°C and resuspended in double distilled water with 1% carboxymethylcellulose (CMC, w/v) at the time of administration.

2.3. Animals

Swiss albino rats of either sex weighing 140-160 g were obtained from the National Laboratory Animal Centre (NLAC), Central Drug Research Institute in Lucknow, India. The animals were housed in the Departmental Animal House for a week before and during the trials. They were kept in a cross-ventilated room with a temperature of 27±2°C, relative humidity of 44-56%, and light and dark cycles of 10 and 14 hours respectively. The animals were fed a conventional rat pellet diet (Amrut; Lucknow, India). The experimental groups of rats were fasted for 18-24 hours prior to the experiment and given unlimited access to water. All studies were carried out in conformity with the institutional ethical committee and the Institutional Animal Care Committee at CPCSEA, India (Reg. No. 222/2000/CPCSEA).

2.4. Experimental Design

The study involved five groups of six Swiss albino rats each. Groups I and II were normal/placebo control and carcinogen control, respectively, whereas groups III, IV, and V were experimental. Except for group I, all groups received single 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 by22 with minor variations. After 20 weeks of exposure to NDEA and CCl4, rats in groups III, IV, and V were orally treated twice daily with 100, 200, and 400 mg/kg b.w of TPE extract in CMC (vehicle) for four weeks. Groups II and I received CMC (1ml/kg, p.o). For oral administration, a conventional orogastric cannula was employed. After an overnight fast, all 24 week-old rats were killed by cervical dislocation. Blood was drawn to evaluate hepatic and oxidative stress markers, and the liver was used for histology, antioxidant Enzyme analyses and haematological parameters.

2.5. Determination of Serum Transaminases (SGOT and SGPT)

Serum transaminases (SGOT and SGPT) were analyzed [23]. Each substrate (0.5 mL) [200 mM α-L-alanine or 200 mM L-aspartate with 2 mM α-ketoglutarate] was incubated for 5 minutes at 37°C. 0.1ml of serum was added, and the volume was adjusted to 1ml with sodium phosphate buffer (pH 7.4; 0.1M). The reaction mixture was incubated for 30 and 60 minutes for SGPT and SGOT, respectively. The reaction mixture was treated with 0.5ml of 2,4-dinitrophenyl hydrazine (1 mM) and kept at room temperature for 30 minutes. Finally, the colour acquired by the addition of 5ml NaOH (0.4 N) and the resulting product were measured at 505 nm. The data were expressed as U/l.

2.6. Determination of Serum Alkaline phosphatase (SALP)

The alkaline phosphatase levels were determined. 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 1.0ml of chromogen reagent was added to the reaction mixture, the color as developed and the product formed were read at 510 nm. Data were expressed as U/l.

2.7. Determination of Gamma Glutamyl Tranpeptidase (GGT)

The gamma glutamyl transpeptidase (GGT) activity was determined by method [24]. 1ml of the working reagents (reconstituted reagents tris buffer 182mM, pH 8.25 and L-gamma-glutamyl-3-carboxy-4-nitroanillide 2.97mM containing 85mm glycylglycine) was mixed with 0.1ml serum. After 1 min, changes in absorbance were measured per minutes for 3 min at 405nm using distilled water as blank. Data were expressed as U/l.

2.8. Determination of Serum Bilirubin (BL)

The bilirubin level in serum was determined by modified DMSO method [25]. 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 as blank. A total bilirubin level in serum was expressed as U/l.

2.9. Histopathological Studies

Histopathological studies were performed as per the standard protocol [26]. 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 5µm (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).

2.10. Biochemical Estimation of Markers for Oxidative Stress

2.10.1. Tissue Preparation

Hepatic tissues were homogenized in KCl [10 mM] phosphate buffer (1.15%) with ethylene-diamine tetra acetic acid (EDTA; pH 7.4) and centrifuged at 12,000×g for 60 min. The supernatant was used for assay of the marker enzymes like glutathione peroxidase, glutathione-S-transferase, superoxide dismutase and catalase, reduced glutathione and thiobarbituric acid reactive substances (TBARS) estimation.

2.10.2. Determination of Lipid Peroxidation (LPO)

The concentration of thiobarbituric acid reactive substances (TBARS) was measured (lipid peroxidation product maondialdehyde (MDA) was estimated) in liver [27]. 1 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.

2.10.3. Determination of Superoxide Dismutase (SOD)

SOD was estimated as described [28]. 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). Mixtures were 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 violet color developed was extracted in 4.0 ml of n-butanol reagent blank. The activity was measured at 560 nm and the results have been expressed as units (U) of SOD activity/mg protein.

2.10.4. Determination of Catalase (CAT)

CAT was estimated as described [29]. Decomposition of H_2O_2 in presence of catalase was followed at 240 nm. A 50µm sample was added to buffered substrate (50 mM phosphate buffer, pH 7.0 containing 10 mM H_2O_2) to make total volume 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.

2.10.5. Determination of Glutathione-S- Transferase (GST)

The enzyme glutathione-S-transferase was measured [30]. To 0.1 ml of liver homogenate, 1.0 ml buffer, 1.7 ml double distilled water, and 0.1 ml CDNB reagent were added. The tubes were incubated at 37°C for 15 minutes. 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.

2.10.6. Determination of Reduced Glutathione (GSH)

Reduced glutathione was measured. Equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01ml 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.

2.10.7. Determination of Glutathione Peroxidase (GPx)

Glutathione peroxidase was measured [31]. A 0.2 ml each of EDTA, sodium azide, reduced glutathione, H2O2; 0.4 ml of buffer and 0.1 ml of enzyme (liver homogenate) were mixed and incubated at 37˚C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged. To 0.5 ml of supernatant, 3.0ml 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. Graded concentrations of the standard were also treated similarly. Glutathione peroxidase activity in liver homogenate is expressed as µg/mg tissue.

2.11. Hematological Parameter

The hematological parameter viz. Red blood cells (RBC), White blood cells (WBC) and Haemoglobin (Hb) were estimated with the help of hematology analyzer (Medonic CA620, Boule, Sweden). The RBC and WBC were expressed as million/mm3 of blood and Hb as g/dl of blood.

2.12. Extraction of Nucleic Acids

Liver tissues of known amount were homogenized in 5.0ml of ice-cold distilled water using a potter – Elvehjem homogenizer with a Teflon pestle. 5.0 ml of 5% TCA was added to the homogenate and this was kept in ice for 30 minutes to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice-cold 10%TCA. Then it was treated with 95% ethanol to remove lipids. The final precipitate was heated at 900C for 15 minutes with occasional shaking, which facilitated the quantitative separation of nucleic acids from protein. The supernatant after centrifugation was used for the estimation of DNA and RNA.

2.12.1. Estimation of Deoxyribonucleic Acid (DNA)

A known volume of the nucleic acid extract (DNA) was made upto 3.0 ml with 1N perchloric acid. This was mixed with 2.0 ml of diphenylamine reagent. A reagent blank and standard was also carried out concurrently. This was kept in a boiling water bath for 10 minutes and the blue colour developed was read at 640 nm in a spectrophotometer. The values are expressed as mg/g wet tissue.

2.12.2. Estimation of Ribonucleic Acid (RNA)

Aliquots of nucleic acid extract were made upto 2.0 ml with 5% TCA. To this 3.0 ml of orcinol-ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 minutes. Reagent blank and standards were also treated in the same way. The tubes were cooled and the colour developed was measured at 640 nm using spectrophotometer. The values are expressed as mg/g tissue.

2.13. Determination of Serum total Protein

Serum protein was determined by using (Span diagnostics kit). The reagents used was from Span diagnostics kit. 1ml of working reagent was mixed with 0.01 ml of serum and absorbed at 530nm. The reagent blank, 0.01ml of standard solution was treated in same way.

$$\text{Serum total protein (g/dl)} = \frac{\text{O.D Treated} \times 6}{\text{O.D Standard}}$$

2.14. Statistical Analysis

Values were represented as mean + standard error (SEM) for six rats. Analysis of variance (ANOVA) test was followed by individual comparison by Newmann Keuls test for the determination of level of significance.

3. Results

3.1. Effect on Body Weight, mean and Relative liver Weight in Control, NDEA +CCl4 induced and TPE Treated Rats

The mean and relative liver weights of the control group of rats was found to be 4.3 g and 3.1 g, respectively; whereas, that of the NDEA and CCl4 induced carcinogenic group was found to be 7.8 g and 6.4 g, respectively. The mean and relative liver weight of group of rats receiving a dose of 100 mg/kg, 200 mg/kg and 400 mg/kg of TPE were studied. It was noted that the TPE extract dose-dependently reduced the mean and relative liver weights of the carcinogenic groups. The doses at 100, 200 and 400 mg/kg reduced the mean liver weights from initial 7.8 g to 6.1 g ($p<0.05$), 5.5 g ($p<0.05$) and 4.8 g ($p<0.01$), respectively; and also the relative liver weights from 6.4 g to 4.34 g ($p<0.05$), 3.82 g ($p< 0.05$) and 3.54 g ($p<0.05$), respectively Figure1.

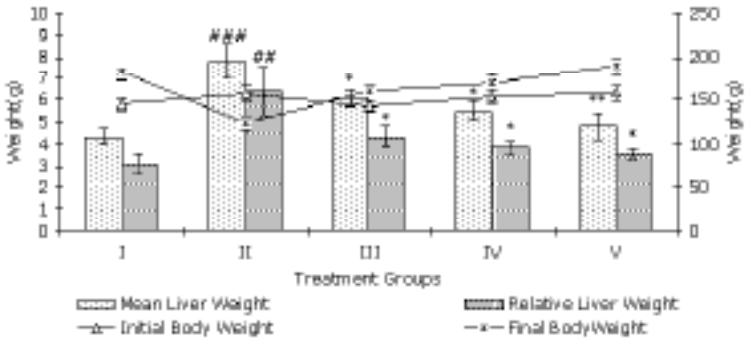

Figure 1: Effect of the Extract of *Tephrosia purpurea* (TPE) on Body Weight, Mean liver weight and Relative Liver Weight in Control, NDEA+CCl4 Induced and TPE Treated Rat

Values are mean ± SEM.; n = 6. P values: *P<0.05, **P<0.01 as compared with group II (NDEA+CCl4 treated group). P values: ##P<0.01, ###P<0.001 as compared with respective control group. Group I represent Control, Group III, IV, V represent oral doses of 100, 200, 400 mg kg^{-1} body weight of the extract of *Tephrosia purpurea,* Group II represent 200 mg kg-1 and 3ml kg-1 body weight of NDEA and CCl4 respectively. The fig. shows the body weight(initial and final)in normal and experimental groups of rats.

In group 2, there was an appreciable loss in the body weight as compared to control rats. The reduction in body weight correlates well with the decreased food intake observed in group 2 rats. Moreover, a significant increase in the liver weight was observed. The body and liver weight were not affected by the treatment with NDEA/CCl4 in animals which received the TPE extract in the mean time with a noxious agent.

3.2. Effect on Liver Tumor Markers Enzymes in Control, NDEA +CCl4 Induced and TPE Treated Rats

It is clearly evident that NDEA and CCl_4 caused significant elevation ($p<0.001$) in the levels of tumor markers such as SGOT, SGPT, SALP and GGT in serum as compared with normal levels of the control group Table1.

Treatments	SGOT(U/l)	SGPT(U/l)	SALP (U/l)	BL(U/l)	GGT(U/l)
Normal (Control)	198.22 ± 1.52	86.22 ± 1.64	236.12 ± 8.29	0.78 ± 0.04	33.4 ± 5.9
NDEA+CCl4	364.62 ± 24.39###	386.48 ± 28.26###	442.39 ± 19.34###	1.34 ± 0.04###	161.4 ± 9.1###
TPE (100mg/kg)	284.36 ± 21.52*	281.31 ± 26.24**	382.29 ± 22.32*	1.09 ± 0.05*	138.2 ± 8.2*
TPE (200mg/kg)	248.32 ± 23.31**	194.11 ± 29.28***	352.39 ± 20.52**	0.92 ± 0.09**	116.9± 4.1***
TPE (400mg/kg)	204.72 ± 21.96***	102.02 ± 30.39***	248.86 ± 22.24***	0.80 ± 0.11***	59.4 ± 3.5***

Table 1: Effect of the Extract of *Tephrosia purpurea* (TPE) on SGOT, SGPT, SALP, BL and GGT in Serum of Normal and NDEA+CCl4 Induced and TPE Treated Rats

Values are mean ± SEM. of six rats in each group.
P values: ###<0.001 compared with respective control group.
P values: *<0.05, **<0.01, ***<0.001 compared with group II (NDEA +CCl4).

Interestingly, the carcinogenic groups treated with TPE extract at variable doses (100 mg/kg, 200 mg/kg and 400 mg/kg) showed reduction in the levels of these tumor markers, in a dose dependent manner. The ranges of protection for liver tumor markers in the serum were found to be statistically significant ($p<0.01$ to $p<0.001$) for SGPT and ($p<0.05$-$p<0.001$) for SGOT and SALP and ($p<0.05$ to $p<0.001$) for GGT.

3.3. Effect on Serum Bilirubin in Control, NDEA +CCl4 Induced and TPE Treated Rats

Similar was the case with serum bilirubin which was found to be elevated ($p<0.001$) in NDEA and CCl_4 induced carcinogenic group versus the control group. Treatment with TPE extract at the 3 different doses showed significant reduction in the levels of serum bilirubin in a dose dependent manner ($p<0.05$-$p<0.001$).

3.4. Effect on Antioxidant Enzymes Levels in Control, NDEA +CCl4 Induced and TPE Treated Rats

The levels of different enzymes in the liver such as LPO, SOD, CAT, GST, GSH and GPx were analyzed prior to administration of the extract in the NDEA and CCl¬4 induced carcinogenic group of rats and compared with the control group. Except the LPO levels, the levels of SOD, CAT, GST, reduced glutathione and GPx were significantly reduced ($p<0.001$) in the NDEA and CCl¬4 induced carcinogenic group as compared with the controls. The LPO level on the other hand was found to be significantly increased ($p<0.01$) in the carcinogenic group as compared with the control group Table.2.

Parameters	SOD (units/mg of protein)	CAT (units/mg of protein)	LPO (MDA nmoles/ mg of protein)	GPx (µg/mg)	GST (GST (µg/mg of protein)	GSH (µg/mg of protein)
Normal (Control)	116.8 ± 8.1	29.4 ± 1.6	0.51 ± 0.03	4.14 ± 0.02	1.24 ± 0.18	3.56 ± 0.02
NDEA+CCl4	38.43 ± 8.22###	5.38 ± 2.4###	5.96 ± 1.19###	1.37 ± 0.01###	0.34 ± 0.04###	2.98 ± 0.01###
TPE (100mg/kg)	62.24 ± 5.37*	12.89 ± 1.4*	3.48 ± 1.09*	2.81 ± 0.04***	0.68 ± 0.02*	3.08 ± 0.02**
TPE (200mg/kg)	79.89 ± 7.56**	15.95 ± 2.75**	2.18 ± 0.93**	2.94 ± 0.02***	0.81 ± 0.05**	3.26 ± 0.03***
TPE (400mg/kg)	93.58 ± 8.24***	23.32 ± 1.91***	1.54 ± 0.68***	3.68 ± 0.01***	1.04 ± 0.04***	3.37 ± 0.02***

Table 2: Effect of the Extract of *Tephrosia purpurea* (TPE) on Superoxide Dismutase (SOD), Catalase (CAT), lipid Peroxidase(LPO), Glutathione peroxidase(GPx), Glutathione-s-Transferase(GST) and Reduced Glutathione(GSH) in Liver of Normal, NDEA+CCl4 Induced and TPE Treated Rats

Values are mean ± SEM. of six rats in each group.
P values : ###<0.001 compared with respective control group.
P values : *<0.05, **<0.01, ***<0.001 compared with group II (NDEA +CCl4).

However, upon treatment with the TPE extract to the carcinogenic group, significant improvement in the levels of all the antioxidant enzymes was noted. At 400 mg/kg b.w., the TPE extract significantly ($p<0.001$) corrected the levels of LPO, SOD, CAT, GST, reduced glutathione and GPx as observed during the study. A dose-dependent response in correction of the values of the enzymes to normal levels was noted when the TPE extract was administered at 100 mg/kg and 200 mg/kg giving 'p' values of $p<0.05$, $p<0.01$, and $p<0.001$ respectively.

3.5. Histological Examination

Changes in the architecture of liver cells were observed in NDEA induced changes in liver as evidenced by fatty acid infiltration, variation in mitotic figures and focal necrosis Figure 2. These changes are indicative of hepatocellular carcinoma. However, in the case of carcinogenic rats treated with TPE extract, the liver retained almost normal hepatic architecture, with much less pathological changes. These results clearly indicate that TPE extract provides protection against NDEA-induced hepatic damage.

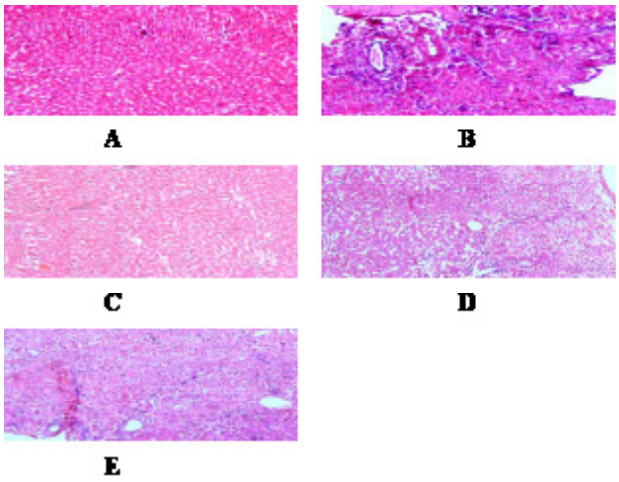

Figure 2: Histopathological Investigation of liver Architecture in Normal (control), NDEA+ CCl4 Induced and *Tephrosia purpurea* Extract Treated Groups

A- Control animals' shows normal architecture of liver. B- NDEA + CCL4 induced HCC bearing animal shows neoplastic cells. Some bile duct proliferation is seen. It shows the malignant hepatocytes. C- Administration of 50 % ethanolic extract (100mg/kg) of *T.purpurea* (TPE) on hepatocellular carcinoma bearing animals. There is the presence an island of malignant hepatocytes surrounded by necrosis. D-Administration of 50 % ethanolic extract (200mg/kg) of *T.purpurea* (TPE) on hepatocellular carcinoma bearing animals, revealing the structure of liver cells with necrosis but with few malignant hepatocytes. E- Administration of 50 % ethanolic extract (400mg/kg) of *T.purpurea* (TPE) on hepatocellular carcinoma bearing animals. Upon treatment the liver shows the structure nearer to normal architecture of hepatocytes.

3.6. Effect on Hematological Parameter Levels in Control, NDEA +CCl4 Induced and TPE Treated Rats

The Figure 3 shows the level of Hb RBC counts, all of which were significantly decreased (11.23 - 7.54, $p<0.01$) and (8.39 – 5.62, $p<0.05$) and with simultaneous increase in WBC (6.32 - 9.76, $p<0.01$) with respect to control. In contrast, the groups treated with *T.purpurea* extract at dose of 100,200, 400 mg/kg once daily for 28 days prevented the cancer in a dose related manner. The range of protection in the Hb, RBC and WBC shows (7.94 - 9.87, $p<0.05$), (6.67 -7.82) and (8.51 - 6.89, $p<0.05$ to $p<0.01$).

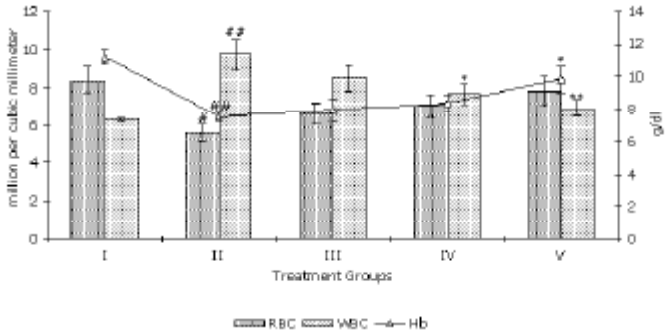

Figure 3: Effect of the Extract of *Tephrosia Purpurea* (TPE) on Red Blood Corpusule (RBC), White Blood Corpusule (WBC) and Hemoglobin (Hb) in Control, NDEA+CCl4 Induced and TPE Treated Rat

Values are mean ± SEM.; n = 6. P values: *P<0.05, **P<0.01 as compared with group II (NDEA+CCl_4 treated group). P values: #P<0.05, ##P<0.01 as compared with respective control group. Group I represent Control, Group III, IV, V represent oral doses of 100, 200, 400 mg kg^{-1} body weight of the extract of *Tephrosia purpurea,* Group II represent 200 mg kg^{-1} and 3ml kg^{-1} body weight of NDEA and CCl_4 respectively.

3.7. Effect on DNA, RNA and Protein Levels in Control, NDEA +CCl_4 Induced and TPE Treated Rats

It is clearly evident from the Figure4 that NDEA+ CCl_4 caused significant elevation of DNA and RNA and decrease in Protein level. In the NDEA+ CCl_4 group, the level of DNA, RNA were increased to (4.98 - 8.56, p<0.001) and (6.99 - 9.92, p<0.001) and protein decreased to (8.01 – 6.87) respectively. In contrast, the groups treated with *T.purpurea* at 100,200, 400 mg/kg decreased altered the levels of DNA (7.46 - 5.49, p<0.05 to p<0.001) and RNA (8.67 - 7.52, p<0.05 p<0.01) and increase the protein level towards normalization (6.97 - 7.57).

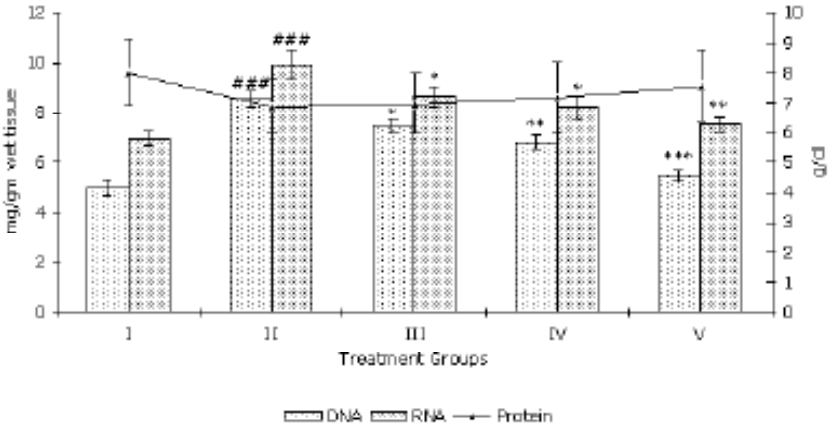

Figure 4: Effect of the Extract of *Tephrosia Purpurea* (TPE) on Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA) and Protein in Control, NDEA+CCl_4 Induced and TPE Treated Rat

Values are mean ± SEM.; n = 6. P values: *P<0.05, **P<0.01, ***P<0.001 as compared with group II (NDEA+CCl_4 treated group). P values: ###P<0.001 as compared with respective control group. Group I represent Control, Group III, IV, V represent oral doses of 100, 200, 400 mg kg^{-1} body weight of the extract of Tephrosia purpurea, Group II represent 200 mg kg-1 and 3ml kg-1 body weight of NDEA and CCl4 respectively.

4. Discussion

Hepatocellular carcinoma is one of the top causes of death in underdeveloped nations, owing to limited therapeutic options and poor treatment outcomes. Hepatocellular carcinoma (HCC) has emerged as the leading cause of cancer-related death in Asia. There is a growing recognition of the importance of considering the interaction of anticancer medications, particularly those encountered frequently, in order to establish an accurate estimate of the risk associated with exposure to genotoxic and carcinogenic environmental contaminants. Many naturally occurring plant compounds have anticarcinogenic and antimutagenic properties. Biochemical tumour indicators are useful diagnostic and prognostic markers that correlate with disease stage and malignancy severity. Increased liver weight in NDEA-treated rats may be associated with cell proliferation. The injection of *T.purpurea* extracts reduced liver weight, demonstrating the extracts' rehabilitative capabilities. The increase in the level of SGOT and SGPT (diagnostic tumour indicators) in the NDEA-treated rats was also found, which could be attributed to NDEA-induced damage on the liver and tumour stress and may also be due to the leakage of enzyme from neoplastic cells into the blood, the release of enzyme from normal tissue by tumour, or the possible effect of tumour or remote tissue leading to the loss of enzyme and release into the blood, according to the report of while also causing damage to the structural integrity of liver [32].

The higher level of SALP in serum was reported in NDEA-treated rats, which might be attributed to a disturbance in secretory activity or metabolite transport, or it could be due to altered production of a specific enzyme, as in previous hepatotoxic combinations [33,34]. The administration of *T.purpurea* extract may normalise the activity of these enzymes in serum, and a reduction in these enzyme levels is strongly associated with hepatoprotection. GGT is classified as an oncofetal protein because its activity changes with the development of cancer. Activity was found to be higher in a variety of malignancies and experimentally produced malignant lesions [35]. The administration of *T.purpurea* extract dramatically reduced GGT levels, indicating lower cancer cell growth. In the current study, serum bilirubin levels were elevated in the NDEA-induced group, as previously reported by which could be attributed to plasma membrane leakage and loss of functional integrity of cell membranes in the liver [36,37]. In groups treated with *T.purpurea* plant extract at varied doses of 100, 200, and 400 mg/kg, the increased levels were lowered, indicating that serum marker

enzymes can be restored to near normal levels. Furthermore, accelerated lipid peroxidation (LPO) expressed in terms of TBARS following NDEA treatment has been found to generate lipid peroxidation products in general and improve chemiluminescence, signifying the generation of activated oxygen species in preneoplastic nodules [38,39]. In the current investigation, there was an increase in the level of LPO in hepatoma-bearing mice, which returned to near normal after administration of *T.purpurea* extract. In the present investigation, the levels of SOD and CAT were dramatically decreased in hepatoma-bearing mice, which can be attributed to an elevated radical generation during NDEA and CCl4 metabolism, whereas these levels increased dramatically after treatment with TPE extract in a dose-dependent manner, demonstrating the extract's antioxidant activity. In the current investigation, depletion of GPx levels was caused by increased free radical formation during NDEA and CCl4 metabolism, as well as free radical-mediated enzyme inactivation. This is consistent with the findings of who reported lower levels of GPx in hepatoma [40].

Reduction of GPx in hepatoma circumstances is predicted to have serious repercussions. Treatment with extracts produces a dose-dependent increase in GPx activity, indicating radical scavenging activity by increasing GPx levels. When HCC-bearing rats were given with TPE extract at dosage concentrations (100,200, and 400 mg/kg), the level of GST increased, whereas enzyme activity decreased in the NDEA-induced animals. This potentiation of plant extract at the enzyme level could be attributed to reducing and/or blocking lipid peroxide generation, which is one of the primary activities in the carcinogenic process. In the current investigation, GSH levels were reduced in hepatoma-bearing mice [41]. An earlier report, in accordance with the findings of stated that the GSH, Non-enzymatic antioxidant enzymes were reduced in hepatoma-bearing animals. Treatment with *T.purpurea* extract (100, 200, and 400 mg/kg) results in a reversion to near-normal levels, indicating that these antioxidants are being used to ameliorate free radical-induced oxidative stress by NDEA. In the current investigation, the injection of NDEA resulted in a considerable drop in RBC count and Hb content while simultaneously increasing WBC count. The lower RBC count could be attributed to erythrocyte destruction or the deleterious effects of NDEA on erythropoietic tissue, specifically bone marrow. Furthermore, the drop in RBC corresponds closely with the decrease in Hb content. Decreased RBC count and Hb content are indicators of anemia. It is generally thought to be caused by a decrease in RBC production or a rise in its destruction [42, 43]. Nucleic acids have a critical function in neoplastic transformation. T. purpurea's effects on macromolecular production in hepatoma were investigated by studying deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It has been discovered that tumour growth is associated with increased amounts of DNA and RNA production. Cisplatin reduces DNA and RNA synthesis. Cisplatin's anticancer activity is thought to be owing to its interaction with DNA in proliferating tissues [44,45]. Cisplatin inhibited DNA, RNA, and protein production [46]. Taking the action of cisplatin into consideration the phytoconstituent present in the *T.purpurea* may act like a mechanism of cisplatin and interact with the nucleic acid and showed the effect in a dose graded manner.

The level of DNA and RNA of liver found to be progressively increased in hepatocellular carcinoma bearing animals. Among the nucleic acid DNA exhibited prominent increase than RNA. The increased nucleic acid synthesis in tumour animals was found to decrease when the animals were treated with *T.purpurea* (100,200 and 400mg/kg) in a dose graded manner. Protein levels in malignant tissues are reduced. In the current study, lower protein levels in plasma of hepatoma-bearing mice were detected, which appears to be due to poor hepatic function caused by tumour infiltration. The liver is an essential location of protein synthesis because it produces the most tissue proteins. Cancer cachexia drastically affects the organism's total protein mass. Protein waste indicates an underlying metabolic imbalance that is manifested by an increase in apparent synthesis rate. Reduced liver protein has been documented in Morris hepatoma-bearing mice and Walker 256 carcinom [47]. Other reports have also revealed increased protein degradation13. Amino acid recycling has been reduced in tumour circumstances, resulting in increased outflow from the tissues. Thus, in response to increased tumour load, the host increases tissue protein degradation. Hepatoma patients have hypoproteinemia. The administration of T. purpurea at 100, 200, and 400 mg/kg to the HCC-bearing group restored the protein level to near normal.

5. Conclusion

In conclusion, TPE's inhibitory effects on two-step NDEA-CCl4-induced hepatocarcinogenesis were verified in a rat model, including well-documented liver markers and antioxidative actions mediated by overexpression of antioxidant enzymes in the liver. Recently, great emphasis has been paid to the preventive biochemical effects of naturally occurring antioxidants, as well as the inhibition of hepatic markers and targets into nucleic acid in biological systems, and their mechanisms. This study presents biological data supporting the use of TPE to treat hepatocarcinogenesis. Thus, based on our first biochemical data, we propose that Tephrosia purpurea extract may inhibit NDEA/CCl4-induced hepatocarcinogenesis in Swiss albino rats. The precise mechanism of action has yet to be understood. The effect of *T.purpurea* chemical isolates on two-stage hepatocarcinogenesis is now being investigated.

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