Evaluation of Hepato-protective and Nephron-protective Potential of *Euphorbia nivulia* Buch.-Ham. Against Carbon Tetrachloride-induced Toxicity in Sprague Dawley Rats

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**Authors’ contributions**

This work was carried out in collaboration among all authors. Author MY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MMH and KA managed the analyses of the study. Author IH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Euphorbia nivulia Buch.-Ham. (En) is one of the members of Euphorbiaceae family that is rich in phytochemicals including flavonoids, triterpenes and polyphenolics.

Purpose: To evaluate hepato-nephroprotective potential of Euphorbia nivulia.

Study Design: Sprague Dawley rats were used as animal models in the study.

Methods: En hydro alcoholic extract was standardized and managed in high dose (300 mgkg⁻¹ body weight (BW) and low dose (150 mgkg⁻¹ BW) to Sprague Dawley rats, administered with CCl₄ (1mlkg⁻¹BW). Silymarin (50 mgkg⁻¹ BW) was taken as positive control. The treatments were given thrice a week. Consequently, blood and hepatic homogenates were collected after 4 weeks of treatment. While the situation of kidney was explored through measurement of serum creatinine, serum urea, sodium and albumin levels. Hepatic and renal samples of rats treated with both 150 and 300 mg/kg of the extract were used for tissue pathological study.

Results: En extract revealed dose dependent moderate level of shelter against CCl₄ intoxicated hepato-nephrotoxicity as directed from the acquired results. The decrease of the albumin levels by the maximum dose of the extract exceeded similar to that attained with Silymarin, and the protecting effects of the extract against oxidative destruction were evaluated. Examination of serum show significant (p < 0.05) elevation in the level of aspartate transaminase(AST), alkaline phosphatase(ALP), alanine transaminase(ALT), whereas decline were noted for albumin in CCl₄ treated rats. Histopathological cuts and damages were seen in hepatic cells and kidney of rats managed by CCl₄. But, co-administration of En extract, dose dependently, improved the CCl₄-carried hepatic harms in these limits.

Conclusion: These effects propose that the phyto-ingredients of En extract with known polyphenols were able to improve the oxidative stress brought along with CCl₄ and may be a useful healing mediator to manage oxidative stress related disorders like hepato-nephro toxicity.

Keywords: Euphorbia nivulia; oxidative stress; aspartate transaminase; alkaline phosphatase; ccl₄ induced toxicity.

ABBREVIATIONS

| ALP  | Alkaline Phosphatase |
| ALT  | Alanine Transaminase |
| AST  | Aspartate Transaminase |
| CCl₄ | Carbon Tetra Chloride |
| DPPH | Diphenyl-1-picrylhydrazy |
| En   | Euphorbia nivulia |
| ROS  | Reactive Oxygen Species |
| TPC  | Total Phenolic Content |
| TFC  | Total Flavonoid Content |

1. INTRODUCTION

Hepatic and kidney infections are the major health concerns in the world due to medications, toxic chemicals and reactive oxygen species (ROS) related oxidative stress [1]. Management of these life-threatening liver and kidney diseases is a major challenge to modern medicine [2]. Corticosteroids and immunosuppressive agents that are the only available drugs, exert several adverse effects. Several researches have proved that antioxidants can inhibit hepato-nephro toxicity by countering ROS [3-4] and offer protection against chemical induced renal failure and mitochondrial function maintenance [5] and may be useful as liver protective agents. Moreover, these antioxidants can search reactive oxygen species (ROS) that are the most usual cause of hepatic disorders [6], singlet oxygen [1] and superoxide anion [7]. Similarly, CCl₄ may be a reason for increasing the production of free radicals within liver including H₂O₂, which requires ferritin and hemoglobin for its conversion into OH ions. Decreased Hb level endorses excessive consumption of ferric ions for conversion of H₂O₂ into OH ions. Increased WBC count and ESR is a highly frequent finding in inflammation due to oxidative stress [8-9]. Systemically administered CCl₄ in rats is distributed at higher concentrations in the kidney than in the liver [10]. Kidney has high affinity for CCl₄ [11] and contains cytochrome P450 predominantly in the cortex [12-13], thus CCl₄ is extensively metabolized in the kidney generating more reactive species. CCl₄ exposure causes damage to the kidney due to enhanced production of reactive oxygen species. It has also been reported that chronic administration of CCl₄ (0.15 ml/kg, sc, in olive oil) three times a week for seven weeks in rats caused various degrees of tubular and glomerular changes, interstitial...
mononuclear cell proliferation and fibrosis in the kidney [14]. Silymarin is a natural flavonoid complex having potent anti-oxidative as well as anti-inflammatory effects. It could be used as standard nephroprotective drug for kidney diseases [15]. Many studies have shown the efficacy of silymarin for drug/chemical nephrotoxicity [16] and diabetic nephropathy [17]. Silymarin is found to be effective as a complementary treatment for inflammatory conditions of liver as well [18]. Many synthetic anti-oxidants are being used in drugs but are carcinogenic. Thus, potent antioxidants and anti-hepato-nephrotoxic drugs of natural origin against liver and renal diseases are needed. This has led to increased dependency on alternative especially plant-based medicines [19]. Various medicinal plants/herbs belonging to Euphorbiaceae family have been reported to possess antioxidant bioactive molecules like triterpenes and flavonoids, both of which are reported to have hepato-protective activity [20]. Euphorbia nivulia-Buch. –Ham.is one of the members of Euphorbiaceae family that is rich in phytoconstituents including flavonoids and polyphenolics but no scientific validation has been done on its hepatic-nephroprotective potential. The current study was aimed to evaluate Euphorbia nivulia-Buch. –Ham’s hepato-nephroprotective potentials against CCl₄ induced toxicity in Sprague Dawley rats.

2. MATERIALS AND METHODS

2.1 Plant Collection

The aerial parts of fresh & well grown En species were collected in March & April 2015 from Hasilpur Road and close areas of Bahawalpur region, Pakistan. The validation of plant was done by taxonomist, Glalam Sarwar, Department of Botany, The Islamic University of Bahawalpur. Voucher specimen (EN-AP-05-12-041) was deposited at the herbarium of Pharmacology Research Lab, Faculty of Pharmacy, The Islamic University of Bahawalpur. The collected parts of plant were cut into pieces, spread on filter paper at room temperature and dried under shade. Dried parts were then converted into fine particles by mean of electric grinder, and No.60 mesh was used to sieve. The powder was kept in closed amber colored glass bottle till used for the extraction.

2.2 Extraction Procedure

Dried 10 kg ground plant material was soaked at room temperature in 12 L of 70% ethanol for 2 weeks with intermittent stirring. A rotary evaporator was used to vaporize the filtrate under controlled pressure (-760mmHg) and temperature (45-50°C). Oven was used to get a thick & semisolid, dark brown gummy mass. The mass was weighed, marked and stored at 4°C in fridge in air tight container. The percentage yield was calculated. For further experimentation the condensed extract was used [21].

2.3 Preliminary Phytochemical Analysis

Different phyto-constituents like flavonoids, alkaloids, glycosides, tannins, saponins and phenols, etc., were determined and results have been published in our recent article [22].

2.3.1 Antioxidant activity

By diphenyl-1-picrylhydrazyl (DPPH) reagent method [23] anti-oxidant activity of the extract was seen. Different concentrations (25–250 mg/ml) were used for IC₅₀ calculations. The total inhibition percentage of DPPH radicals was calculated by:

\[ \text{Inhibition} \% = \left( \frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank}} \right) \times 100 \]

Using EZ-Fit Enzyme Kinetics Software (Pirelli Scientific Inc. Amherst, (USA) IC₅₀ values were calculated.

2.3.2 Total assay antioxidant capacity / power reducing assay

Fe-reducing potency of plant extract was checked by the technique used by Nile and Park with slight modifications [24]. The calibration curve was created using Troop (100–2000 elm) and the effects were conveyed as 3M Troop equivalent (TE)/g fresh mass.

2.3.3 Total Phenolic content

Total phenolic content (TPC) was calculated by doing the slight modifications in Folin & Ciocalteu’s colorimetric methods [25]. TPC was designed using the usual calibration curve (ranging from 0-200 µg/mL) and data was conveyed as milligram Gallic acid equivalent per gram of dry extract (mg of GAE/g of DE).

2.3.4 Total flavonoid content

Total flavonoid content (TFC) was determined by modified colorimetric method [26]. TFC was
designed by the calibration curve equation and stated as milligram Quercetin equivalent per gram of dried extract (mg of QE/g of DE).

2.3.5 HPLC Analysis of phenolic compounds

HPLC of En was done in Central Hi-Tech Laboratory, University of Agriculture, Faisalabad, Pakistan as defined before [27].

2.4 Animals and Treatment

2.4.1 Acute toxicity assessment (In vivo)

Acute toxicity of En (70% aq. ethanol) extract was examined on Sprague-Dawley rats. There were six rats in each group (4 male, 2 females); (and these rats were orally controlled during morning (12 hrs fasting) with the extract at 150, 300, 500, 1000, 2000 mg/kg doses). Extracts of 150, 300, 500, 1000, 2000 mg/kg doses were orally administered to the rats. Advices of Organisation for Economic Cooperation and Development (OECD) 425 were monitored to perform toxicity studies.

2.5 Experimental Study Design

12-24 weeks old (adults) 48 male Sprague-Dawley rats weighing 180g to 200g were used for the study. Strategies of National Institute of Health, Islamabad were firmly monitored to conduct the trial. The following eight experimental groups, each with 6 animals, were studied.

Group-I: Control (Untreated; only standard food supply was offered)
Group-II: Vehicle (10% DMSO in olive oil 1 ml/kg body weight)
Group-III: Disease model (1 ml/kg 30% CCl₄ (in olive oil))
Group-IV: Drug control (CCl₄ + silymarin 50 mg/kg)
Group-V: CCl₄ + En (150 mg/Kg)
Group-VI: CCl₄ + En (300 mg/Kg)
Group-VII: En (150 mg/Kg)
Group-VIII: En (300 mg/Kg)

CCl₄ was inserted intraperitoneally (ip), while vehicle and Silymarin were administered using gastric cannula. Doses of extract and positive standard were administered orally through gastric tube 30 minutes after the administration of CCl₄. The treatments of CCl₄ and En (samples) were given in the early morning on alternate days (from day 1), thrice a week for 4 weeks.

2.6 Biochemical Analysis

2.6.1 Collection of blood sample and organs for biochemical investigation

After the final treatment, rats were fasted for 24 hours. Blood samples were collected after sedation with chloroform for hematological studies and serum analysis. Small portion of excised hepatic cells was stored in 10% formalin solution for tissue pathological studies.

2.6.2 Hematological studies and serum analysis

Neubauer hemocytometer (Feinoptik, Germany) used to estimate platelets, red blood corpuscles and white blood cells; Sahli’s haemoglobin meter was used to count hemoglobin content. Modified Westergren method was used to measure erythrocyte sedimentation rate. Serum replicates analysis was done for alkaline phosphatase, alanine transaminase, aspartate transaminase, albumin and bilirubin by means of AMP diagnostic kits (Graz, Austria). Bradford method was used to regulate protein concentration [28].

2.6.3 Renal function tests and serum analysis

Serum albumin, urea and creatinine levels were estimated calorimetrically using commercial diagnostic kits. Then, creatinine/albumin ratio (C/A) was measured. After decapitation, kidneys were excised. Small portion of excised kidney was used for histopathological and biochemical studies.

2.6.4 Histopathological studies of liver/kidney

Specimen were fixed in paraffin and saved on hard blocks and divided in thin layers of 3–4μm, followed by staining with hematoxylin and eosin. Then light microscope (DIALUX 20 EB) at 40× power was used to examine these slides and photographed using HDCE-50B camera. All tissue pathological abnormalities were classified by using altered signs showing marked changes.

2.6.5 Statistical analysis

Tukey’s multiple assessment tests were used to evaluate important changes among in vivo treatment groups using computer software Statistix 8.1. Statistical significance for demeanors was done at p < 0.05. Graph was created using software GraphPad5.
3. RESULTS

3.1 Extraction

% age yield of En extract was 7.14.

3.2 Preliminary Phytochemical Analysis

The results for various phytochemicals confirmed presence of alkaloids, flavonoids, glycosides, phenols, tannins and saponins in En, and have been shown in Table 1.

3.3 Antioxidant, Total Phenolic & flavonoids Contents

DPPH activity shown by En was 91±0.13% inhibition at 1.0mg/mL (IC\textsubscript{50} 0.14±0.83 (mg/mL); while FRAP was 708.32 3M. Moreover, En showed (125.6 mg/g GA) TPC (Total Phenolic Content); and (69.8 mg/g Quercetin) TFC (Total Flavonoids Content).

3.3.1 HPLC analysis

Quantitative sketching and chromatographic finger printing confirmed presence of polyphenols: gallic acid, quercetin, caffeic acid, benzoic acid, vanillic acid, chlorogenic acid, ferulic acid and syringic acid in the extract. These polyphenols were present in quantities of 1.47, 0.99, 0.43, 0.24, 0.19, 1.25 and 0.11 ppm/mg extract, respectively Fig. 1 & Table 2.

3.3.2 Acute toxicity assessment \((\text{In vivo})\)

In acute toxicity assessment, all animals were observed carefully for development of any toxic signs or symptoms at different time intervals of 0, 30 min, 1, 2, 4, 6, 8, 12 h. and then daily for a period of 3 days. There was no toxic signs like lacrimation, salivation, piloerection, drowsiness, tremors, convulsions. Moreover, body weight, food consumption and water consumption was normal, and no mortality was observed in clinical parameters during acute study. So, it indicates that the LD50 of En extract is greater than 2000 mg/kg/day BW.

3.3.3 Hepato-/Nephro-protective activity

The current study demonstrated hepato-nephroprotective role of En in CCl\textsubscript{4} induced hepatic-nephrotoxicity at different doses (150 mg and 300 mg/kg BW).

3.3.4 Hematological parameters

Effects of En and CCl\textsubscript{4} treatments are shown in Table 3, Fig. 2. Important decline was observed in Hb level, RBCs & platelet count, while raised levels of WBCs observed in CCl\textsubscript{4} intoxicated group (III) as compared to the control group (I). Silymarin treated group (IV) restored toxic alteration induced via CCl\textsubscript{4} in contrast to the control group (I). Treatment with high (300 mg/kg) and low (150 mg/kg) doses of En (groups V–VI) significantly restored CCl\textsubscript{4} intoxicated irregularities of hematological parameters towards their normal values. Maximal restoration was observed by high dose of the extract (group V) with 15.1 ± 0.03 g/dl Hb level, 8.03 ± 0.26 (×10\textsuperscript{5})/μl RBCs, 12.80 ± 0.33 (×10\textsuperscript{5})/μl platelet count and 9.70 ±0.50 (×10\textsuperscript{3})/μl WBCs. But, no major difference in blood profile observed when En (150 and 300 mg/kg; groups VII and VIII) was given alone; thereby, validating the safety profile of the extract.

| Test                        | Observation                   | Inference                   |
|-----------------------------|-------------------------------|-----------------------------|
| Alkaloids                   |                               |                             |
| Hager's test                | Yellow ppt                    | Alkaloids present           |
| Mayer's test                | Creamy ppt                    | Alkaloids present           |
| Wagner's test               | Reddish brown ppt             | Alkaloids present           |
| Glycosides                  | Brown ring (at junction)      | Glycosides present          |
| Keller-Killani test         |                               |                             |
| Tannins                     | Blackish color                | Tannins present             |
| Fe Cl\textsubscript{3} test | Dark yellow color             | Flavonoids present          |
| Flavonoids                  |                               |                             |
| Saponins                    | Froth formation               | Saponins present            |
| Froth test                  |                               |                             |
| Phenolic Contents           | Blackish color                | Phenolic contents present   |
| Fe Cl\textsubscript{3} test |                               |                             |

Table 1. Phytochemical evaluation of Euphorbia nivulia
Table 2. Qualitative and quantitative analysis of phenolic compounds in *Euphorbia nivulia* using HPLC

| Sr# | Compounds     | Retention time (min) | Area (mV.s) | Phenolic content (ppm/mg) |
|-----|---------------|----------------------|-------------|--------------------------|
| 1   | Quercetin     | 3.36                 | 27.85       | 1.47                     |
| 2   | Gallic acid   | 4.58                 | 27.59       | 0.99                     |
| 3   | Caffeic acid  | 12.85                | 9.55        | 0.43                     |
| 4   | Syringic acid | 16.25                | 9.78        | 0.24                     |
| 5   | m-coumeric acid | 20.14            | 16.67       | 0.19                     |
| 6   | Ferulic acid  | 22.15                | 17.43       | 1.25                     |
| 7   | Cinnamic acid | 25.38                | 2.97        | 0.11                     |

Fig. 1. HPLC chromatogram of *Euphorbia nivulia*

Table 3. Effect of *Euphorbia nivulia* on hematological parameters

| Groups           | Dose       | RBCs (×10^6)/μl | WBCs (×10^3)/μl | Platelets (×10^5)/μl | Hb (g/dl)  |
|------------------|------------|-----------------|-----------------|----------------------|------------|
| Control          | -          | 8.00±1.15       | 8.80±0.21       | 11.22±0.06           | 14.50±0.33 |
| Vehicle control  | 1ml/kg     | 8.59±0.09       | 8.70±0.16       | 12.13±0.83           | 15.20±0.31 |
| CCl₄             | 1ml/kg     | 7.16±0.08       | 130±3.3         | 9.15±0.44            | 12.80±0.33 |
| CCl₄+Silym       | 1ml/kg+50mg/kg | 8.16±0.48     | 12.40±2.8       | 10.49±0.09           | 14.10±0.33 |
| CCl₄+En          | 1ml/kg+150mg/kg | 7.45±0.05     | 21.10±1.3       | 10.81±0.54           | 13.60±0.40 |
| CCl₄+En          | 1ml/kg+300mg/kg | 8.03±0.26     | 9.70±0.50       | 12.80±0.33           | 15.10±0.033 |
| En               | 150mg/kg   | 7.89±1.29       | 9.50±0.27       | 10.50±0.08           | 14.60±0.54 |
| En               | 300mg/Kg   | 8.12±0.25       | 8.50±0.37       | 11.00±0.05           | 13.0±0.93  |

Mean ± SD (n = 6), Means with superscript * in a column specify significance at p<0.05; En: *Euphorbia nivulia*, Silym: *Silymarin*, CCl₄: Carbon tetrachloride

3.3.5 Defensive role of En on hepatic enzymes activity (serum)

Liver enzymes activity, i.e. ALT, AST and ALP measured therapeutic effects of *En* extract on CCl₄-induced hepatotoxicity (Table 4, Fig. 3). CCl₄ administration in rats produced a major increase (p < 0.05) in the level of ALP = 378 ±2.58 u/L, AST = 98±1.15 u/L and ALT = 107 ±1.8 u/L as compared to the ALP = 144 ±1.29 u/L, AST = 43.0 ±2.08 u/L and ALT = 44 ±1.3 u/L in the control group. High and low doses of *En* extract (groups V-VI) markedly restored the CCl₄-produced raised levels of hepatic stress biomarkers. Strangely high level of serum markers was significantly decreased (p < 0.05)
by co-administration of En (150 mg/kg) which tends to regulate these elevated levels (p < 0.05) as compared to control group. Still, co-administration of the high dose of En (300 mg/kg) significantly decreased the level of these enzymes, and the levels acquired were as: ALP = 150 ±2.30 u/L, AST = 42 ±1.39 u/L and ALT = 48 ±2.70 u/L in serum. High and low doses of En alone did not induce any change (p > 0.05) in the serum level of ALP, AST and ALT.

3.3.6 Defensive role of En on hepatic biochemical parameters (serum)

Serum albumin and bilirubin level were examined. The albumin level reduced (p < 0.05) while bilirubin level increased in CCl₄ intoxicated rats in contrast to the control group (Table 4, Fig. 3). Results show that En possesses important hepatic-protecting role, and the altered level of albumin and bilirubin produced with CCl₄ improved after co-administration of En in a dose dependent manner. Protective effects of silymarin for albumin and bilirubin were comparable (p > 0.05) with the higher dose of En 300 mg/kg administered to rats. Dealing with En alone (group VII and VIII) did not change the normal biochemical profile in contrast to the control group (p > 0.05).

3.3.7 Effect of En on hepatic histoarchitecture

Histopathological investigations endorsed protective effects of En against CCl₄ induced hepatic damage (Fig. 2). Liver section of control group displayed smooth architecture and normal morphology with typical central vein, kupfer cells, hepatocytes and sinusoids as shown in Fig. 2a. Histopathological examination of CCl₄ treated animals (group III) caused noticeable elevation in fatty changes, inflammatory cells infiltrations (hepatic cytoplasm inflammation), cellular hypertrophy, centrlobular necrosis, vacuolization, ballooning and dilation of central vein as shown in Fig. 2b. Co-administration of high dose En (300 mg/kg) extract exhibited protective effects by restoring hepatic inflammation towards normal (Fig. 2d), which were comparable with shielding effects of Silymarin (group IV; Fig. 2c) with mild destruction of hepatocytes induced by CCl₄. Administration of low dose En (150 mg/kg) had lessened injuries of the hepatic necrotic cells. Administration of En alone depicted the normal histoarchitecture of the liver samples, showing the safe and non-toxic behavior of the extracts. Pathohistological changes are summarized in Table 5 using scoring method.

3.3.8 Effect of En on renal biochemical parameters (serum)

Administration of CCl₄ to rats (Group III) significantly increases serum creatinine, serum urea and creatinine/ albumin ratio and decrease serum albumin level (P<0.05, P<0.05, P<0.05 & P< 0.001 respectively) in contrast to control group (Group I) (p<0.001) (Table 6). A 4 weeks pre-treatment with En at 150 and 300 mg/kg dose (Groups V and VI) after CCl₄ intoxication showed renal safety in relations to serum urea, creatinine, and creatinine: albumin proportion levels in contrast to the toxic control group (Group III) (p<0.001). Associated administration of En to CCl₄ intoxicated rats caused a significant decrease in serum creatinine level and in creatinine:albumin ratio (P< 0.01) and significant increase in serum albumin level (P< 0.001) compared to CCl₄ group (Table 6; Fig. 4).

| Groups       | Dose          | ALT(U/l) | ALP(U/l) | AST(U/l) | Albumin (mg/dl) | Bilirubin (mg/dl) |
|--------------|---------------|----------|----------|----------|-----------------|------------------|
| Control      | -             | 44±1.3   | 144±1.29 | 43.0±2.08 | 5.2± .12        | 2.7± .09         |
| Vehicle control | 1ml/kg       | 41±0.53  | 145±1.5  | 42±90    | 4.21± .01       | 2.7± .09         |
| CCl₄         | 1ml/kg        | 107± 1.8 | 378±2.58 | 98±1.15  | 1.93±0.12       | 5.79±0.27        |
| CCl₄+ Silym  | 1ml/kg+50mg/kg| 54±2.3   | 159±2.60 | 31±1.73  | 4.1 ± .000      | 2.8±0.0          |
| CCl₄+ En     | 1ml/kg+150mg/kg | 64±1.86  | 268±2.70 | 49±2.54  | 3.37±0.02       | 3.18±0.02        |
| CCl₄+ En     | 1ml/kg+300mg/kg | 48±2.7*  | 150±2.30*| 42±1.39* | 3.76±0.02*      | 2.90±0.02*       |
| En           | 150mg/kg      | 44±2.3   | 142±5.0  | 47±1.96  | 5.07±1.3        | 2.90±0.02        |
| En           | 300mg/kg      | 43±1.5   | 132±6.05 | 43.15±3.83| 4.10±0.33       | 2.93±0.25        |

Mean ± SD (n = 6); Means with superscript * in a column specify significance at p < 0.05. En: Euphorbia nivulia, Silym: silymarin, CCl₄: Carbon tetrachloride
Table 5. Grading showing histopathological changes induced by CCl₄ & *Euphorbia nivulia* in hepatic tissues (quantification scores)

| Groups          | Dose          | Hepatic cytoplasm inflammation | Centrilobular necrosis | Cellular hypertrophy | Vacuolization | Steatosis |
|-----------------|---------------|--------------------------------|------------------------|----------------------|---------------|----------|
| Control         | -             | -                              | -                      | -                    | -             | -        |
| Vehicle control | 1ml/kg        | -                              | -                      | -                    | -             | -        |
| CCl₄            | 1ml/kg        | ++                             | +++                    | +++                  | ++           | +++      |
| CCl₄ + Silym     | 1ml/kg + 50mg/kg | -                              | -                      | -                    | -             | -        |
| CCl₄ + En       | 1ml/kg + 150mg/kg | +                              | +                      | +                    | +             | +        |
| CCl₄ + En       | 1ml/kg + 300mg/kg | -                              | -                      | -                    | -             | -        |

Histopathologic assessment was scored as follows: (−)—no meaningful histopathologic changes (+)—mild degree; (++)—moderate degree and (+++)—severe degree. En: *Euphorbia nivulia*, Silym: Silymarin, CCl₄: Carbon tetrachloride

Table 6. Effect of *Euphorbia nivulia* on serum biochemical markers of kidney in rats treated with CCl₄

| Groups          | Dose          | Urea (mg/dl) | Creatinine (mg/dl) | Albumin (mg/dl) | Creatinine/albumin |
|-----------------|---------------|--------------|--------------------|----------------|--------------------|
| Control         | -             | 16.50±2.13   | 0.69±0.03          | 4.58±0.15      | 0.15±0.01          |
| Vehicle control | 1ml/kg        | 13±1.73      | 0.73±0.02          | 4.21±0.01      | 0.17±0.00          |
| CCl₄            | 1ml/kg        | 50±0.82      | 2.12±0.03          | 1.93±0.03      | 1.09±0.03          |
| CCl₄ + Silym     | 1ml/kg + 50mg/kg | 23±1.97    | 0.79±0.06          | 4.10±0.22      | 0.19±0.03          |
| CCl₄ + En       | 1ml/kg + 150mg/kg | 26±1.86    | 0.99±0.00          | 3.37±0.09      | 0.26±0.01          |
| CCl₄ + En       | 1ml/kg + 300mg/kg | 17±2.21*   | 0.87±0.06*         | 3.76±0.06*     | 0.25±0.03*         |
| EnE             | 150mg/kg      | 15.30±0.67   | 0.91±0.04          | 3.84±0.03      | 0.23±0.03          |
| EnE             | 300mg/Kg      | 17.10±0.53   | 0.82±0.06          | 3.42±0.06      | 0.23±0.03          |

Mean ± SD (n = 6), Means with superscript * in a column specify significance at p < 0.05.

EnE: *Euphorbia nivulia* extract, Silym: Silymarin, CCl₄: Carbon tetrachloride
3.3.9 Effect of en on renal histoarchitecture

Histopathological investigations endorsed protective effects of En against CCl₄ induced renal damage (Fig. 3). Kidney section of the control group displayed smooth architecture and normal morphology with normal structural and architectural integrity. The control group kidney showed normal proximal and distal tubules and normal and intact glomeruli (Fig. 3a). Four week CCl₄ chronic administration caused significant renal morphological damage, especially in the renal cortex. However, CCl₄ effect on the medulla was limited. CCl₄-treated kidneys exhibited different forms of degeneration in affected glomeruli. Some showed mild dilatation of Bowman’s space along with glomerular atrophy, while others exhibited congestion in the capillary loops. Proximal and distal tubules showed histological changes with inflammation, dilation and degeneration with preliminary signs of acute tubular necrosis. Due to sporadic congestion there was sporadic haemorrhage that results in renal tubular degeneration and detachment of tubules, and vacuolation of epithelial cells (Fig. 3b). Vacuolations or fatty changes in the renal cortex were clearly apparent after CCl₄ treatment. As an indicator of fibrosis and considerable congestion in the blood vessels, there was an evident increase in the connective tissue cells in these regions of infiltration. Rats treated with En alone (150 and 300 mg/kg) depicted the normal histoarchitecture of the kidney samples having no histological changes in kidney tissues, thus showing the safety and non-toxic behavior of the extract. CCl₄ induced abnormal histopathological changes significantly reduced in the En-treated groups. High dose En (300 mg/kg) co-administration exhibited protective effects towards normal by decreasing renal inflammation (Fig. 3e), these effects were comparable with
In the CCl₄+ENE groups (V and VI), ENE markedly prevented congestion in glomeruli and vessels and other alterations, and the glomeruli and tubules were normal in their histology. Any increase in the connective tissue cells was not observed as well (Fig. 5e). Low dose En (150 mg/kg) administration lessened injuries of renal necrotic cells. Histopathological changes induced by CCl₄ and En extract in renal tissues (Quantification scores) is summarized in Table 7.

Fig. 4. Effect of *Euphorbia nivulia* on serum biochemical markers of kidney
Fig. 5. Microphotographs representing the effect of CCl₄, Silymarin, various doses of *Euphorbia nivulia* on liver in rat (40X Hematoxylin-eosin stain)

a. Vehicle (negative control): liver section of the control group displayed smooth architecture and usual morphology along with central vein, liver cells and sinusoids vacuolation and normal sinusoids are evident (star and short arrow).

b. CCl₄ treated (disease control): showed high dilatation of portal vein due to engorgement of blood (star) and pyknotic hepatocytes (arrow). The liver tissue showed acute toxicity leads to cell injury accompanied by lesion of early necrosis.

c. CCl₄+ Silymarin (50mg/Kg) (Positive control): reduced inflammation in tissue, few monocytes cells (star) abundant normal hepatocytes (arrow) normal central vein (rectangle) and few psychotic hepatocytes (rhombus). The photograph revealed recovery stage and normal histological architectural detail of liver tissue

d. CCl₄+ En (150mg/Kg): mild to moderate eduction in inflammation and degenerative changes

e. CCl₄+ En (300mg/Kg): reduced inflammation in tissue, few monocytes cells (star) abundant normal hepatocytes (arrow) no dilatation and few psychotic hepatocytes (rhombus). The photograph reflected recovery and normal histological architectural detail of liver tissue.

4. DISCUSSION

Traditional medicinal practices designed the base of clinical, pharmacological and chemical studies [29]. Plants have been the essential component of human culture due to their medicinal uses since time immemorial. In early drug discovery, plants as initial source of medicines have been used in ethno-pharmacological stuff [30-31]. Herbs as a source of medicine are popular in almost every culture of the world because of their easy availability, effectiveness, economy and compatibility to human physiology.

DPPH analysis is well known to measure the scavenging potential of antioxidants appearing deep purple in color. In this analysis there is reduction of DPPH to DPPH₂ wherein the scavenger antioxidant molecule donates the proton. Reagent color modified from purple to yellow at 515 nm [32]. In short, antioxidant ability may be characteristics to phenolic as well as flavonoids contents present in *En*. Acute toxicity investigation confirmed the non-toxic behavior of the extract where during two weeks no death was noticed. In vivo studies the initial safety of the plant was analyzed. So the powerful antioxidant ability and non-toxic nature of the extract was acceptable reason for further assessment of its hepato-nephron protective potential against CCl₄ induced toxicity.

Determination of serum AST, ALT and ALP enzyme activity is an important indicator for hepatic membrane functional integrity. Elevated ALP, AST and ALT serum levels are measured as hepatotoxicity markers, these enzymes ooze out into the plasma when hepatic injury disturbs cellular membrane probity. In the current
investigation, increased levels of hepatic serum markers (AST, ALT and ALP) might reverse hepatic damage due to CCl₄ intoxication. Increased ROS generation in hepatocytes leads to hepatic damaging action and cellular death because of protein oxidation, lipid peroxidation, and DNA damage [33]. Similarly, increased bilirubin serum level clearly indicates blockage in bile elimination due to hepatic cells’ damage. Hepatocellular injury can be determined by assessment of serum bilirubin and hepatic biomarkers [34-35]. In case of decreased albumin level inflammation occurs due to oxidative stress [8]. In the current investigation, polyphenol enriched En (Table 2 and Fig. 1) concomitant administration with CCl₄ treatment reversed the toxic effects by conserving structural integrity of hepatic cellular membrane. The extract protective potential may be attributed to the antioxidant phytoconstituents present [36]. The serum transaminase level becomes normal with recovery of parenchyma and hepatic cells. Silymarin co-administration as well as CCl₄ exhibited the protective capability of En. AST, ALT and ALP values were found immediate values of control groups in experimentation. En (70% aq. ethanol) extract showed protective aptitude in a dose dependent manner against CCl₄ induced hepatotoxicity. 150 mg/kg En, i.e. lower dose utilizes protective tendency and lowers the levels of AST, ALT and ALP to a reasonable extent. Higher dose En (300 mg/kg) exhibited potent toxic-suppressive effects and the level of liver bio markers was found near to the level of control animals in experimentation. Liver enzymes restoration towards control levels indicates hepatoprotective potentials of polyphenolics of the plant. Thus, from Fig. 2 it is observed that co-administration of low and high dose En as well as silymarin ameliorated and decreased the CCl₄ induced toxic effects and damages. It is suggested that En Protective effects may be due to the phytoconstituents like polyphenols, sterols, tannins, terpenoids and flavonoids as suggested by Nabavi et al. [37].

En may be a strong hepatoprotective agent with free radical scavenging ability in hepatotoxicity. These investigations suggest a direct means of assessing the hepatic-protective effects against CCl₄ induced damages in liver Fig. 2. Liver section of the control group displayed smooth architecture and normal morphology with normal structural and architectural integrity Fig. 2a. Histopathological examination of CCl₄ treated animals (group III) show noticeable abnormal changes, like elevation in fats, hepatic cytoplasm inflammation, cellular hypertrophy, necrosis, ballooning, vacuolization, and central vein dilatation as shown in Fig. 2b. High dose En (300 mg/kg) co-administration showed protective effects towards normal by decreasing hepatic cells inflammation Fig. 2d, these effects were similar with protective effects of silymarin (group IV: Fig. 2c). Administration of low dose En (150 mg/kg) reduced the injuries of necrotic cells of the liver. Administration of En alone depicted the normal histoarchitecture of the liver, proving the safety and non-toxic behavior of the extract. Pathohistological changes are concised in Table 6 (scoring method was used).
Fig. 6. Microphotographs of renin histology of different groups representing the effect of CCl4, Silymarin, various doses of *Euphorbia nivulia* on kidney in rat (40X Hematoxylin-eosin stain)

a. Vehicle (negative control): No change in Bowmans capsule (black arrow) & epithelial cells (blue arrow); kidney with normal structure and architecture

b. CCl4 treated (disease control): showing extensive vacuolar degeneration, congestion & hemorrhage; disruption of Bowman’s capsule is evident (black arrow) whereas coagulate necrosis is also exhibited (blue arrow)

c. CCl4+ Silymarin (50mg/Kg) (positive control): Marked improvement in disruption is produced in the Bowman’s capsule on exposure to silymarin; reduced inflammation and degenerative changes (black arrow)

d. CCl4+ En (150mg/Kg): Improvement in the disruption of Bowman’s Capsule (black arrow) comparable to disease control; showing moderate inflammation and degenerative changes

e. CCl4+ En (300mg/Kg): Improvement in the disruption of Bowman’s Capsule (black arrow) comparable to disease control, although no change in coagulate necrosis (blue arrow); reduced inflammation and degenerative change sufficiently.

Histopathological investigations recognized protective effects of *En* against CCl4 induced renal damage Fig. 3. Kidney section of the control group displayed smooth architecture and normal morphology with normal structural and architectural integrity. Moreover, the control group kidney showed normal proximal and distal tubules and normal, and intact glomeruli Fig. 3a. The CCl4 chronic administration caused significant renal morphological damage, especially in the renal cortex. However, CCl4 effect on the medulla was found limited. Due to sporadic congestion there was sporadic hemorrhage that results in renal tubular degeneration and detachment of tubules, and vacuolation of epithelial cells Fig. 3b. Vacuolations or fatty changes in the renal cortex, fibrosis and considerable profusion in the blood vessels were clearly apparent after CCl4 treatment Fig. 3.

Rats treated with *En* alone (150 and 300 mg/Kg) depicted the normal histoarchitecture of the kidney samples having no histological changes in kidney tissues, thus showing the safety and non-toxic behavior of the extract. CCl4 induced abnormal histopathological changes significantly reduced in the *En*-treated groups. High dose *En* (300 mg/kg) co-administration exhibited protective effects towards normal by decreasing renal inflammation Fig. 3e, these effects were analogous with protecting effects of silymarin (group IV; Fig. 3). In the CCl4+ *En* groups (V and VI), *En* obviously prevented profusion in glomeruli and vessels and other changes, and histologically glomeruli and tubules were normal. Any increase in the connective tissue cells was not observed as well Fig. 3. Low dose *En* (150 mg/kg) administration lessened injuries of renal necrotic cells. Pathohistological changes are
summarized in Table 4 (scoring method was used).

Results are in agreement with Moneim and El-Debi, 2012, who demonstrated that CCl₄ made a significant increase in kidney weight and comparative kidney weight due to a significant decrease in body weight and kidney swelling of rats [38]. Abnormal increased level of urea, and serum creatinine are possible gages of kidney injuries induced through CCl₄ treatment [39], also accompanied by histological changes such as severe proximal renal tubular necrosis followed by renal failure [40-41]. Serum creatinine level does not rise until at least half of the kidney nephrons are destroyed or damaged. After CCl₄ intoxication, rats developed urea elevation and chronic renal injury [42]. Level of proteinuria is high in renal injuries. Moreover, there is reduction in serum albumin in CCl₄-treated rats due to glomeruli and tubules, which resulted in remarkable leakage. Results of the current study reveals that En significantly recovered renal injuries induced through CCl₄ intoxication in rats. Hematological investigation is used for assessment of hepatic disorders like inflammatory diseases. CCl₄ may be a reason for enhancing the production of free radicals including H₂O₂, which needs ferritin and hemoglobin for OH ions conversion. Lower Hb level, and increased WBC count is a highly frequent indicator in oxidative stress inflammation [8-9]. In this investigation, CCl₄ intoxicated rats exhibited increased WBC levels and decreased Hb, RBCs and platelets levels. The extract significantly restored CCl₄ mediated hematological abnormalities towards normal level. This restorative and protective property may be due to the protective potential of the polyphenols present in extract.

5. CONCLUSION

The current study recommends that Euphorbia nivulia Buch.–Ham (70% hydro alcoholic) extract possesses the possibility to improve the histopathological injuries triggered by CCl₄ and has the ability for restoration of normal hepatic and renal histoarchitecture. All this justifies its protective and shielding capacity to revoke the hepatic and renal damage caused by oxidative stress due to free radicals/ROS. Protective properties of the extract might probably be due to its various phytochemical constituents and antioxidant potential. Hepato and nephron-protective role of Euphorbia nivulia extract was comparable with silymarin which may be attributed to the presence of polyphenols and flavonoids. En allows us to conclude that extract may further be subjected to bio-assay guided isolation to identify the active component(s). The extract may be a good candidate as antioxidant and hepato-nephron-protective agent for developing new formulation(s).

ETHICAL APPROVAL

Strategies of National Institute of Health, Islamabad were firmly monitored to conduct the trial. The designed protocol was approved (Bch#0265) by the Ethical Committee of Quaid-i-Azam University, Islamabad, Pakistan.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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