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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-nephronprotective 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 CCl_4 (1$mlkg^{-1}$BW). 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 CCl_4 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 CCl_4 treated rats. Histopathological cuts and damages were seen in hepatic cells and kidney of rats managed by CCl_4. But, co-administration of *En* extract, dose dependently, improved the CCl_4-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 CCl_4 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; ccl_4 induced toxicity.

ABBREVIATIONS

ALP : Alkaline Phosphatase
ALT : Alanine Transaminase
AST : Aspartate Transaminase
CCl_4 : 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, CCl_4 may be a reason for increasing the production of free radicals within liver including H_2O_2, which requires ferritin and hemoglobin for its conversion into $\dot{O}H$ ions. Decreased Hb level endorses excessive consumption of ferric ions for conversion of H_2O_2 into OH ions. Increased WBC count and ESR is a highly frequent finding in inflammation due to oxidative stress [8-9]. Systemically administered CCl_4 in rats is distributed at higher concentrations in the kidney than in the liver [10]. Kidney has high affinity for CCl_4 [11] and contains cytochrome P450 predominantly in the cortex [12-13], thus CCl_4 is extensively metabolized in the kidney generating more reactive species. CCl_4 exposure causes damage to the kidney due to enhanced production of reactive oxygen species. It has also been reported that chronic administration of CCl_4 (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 CCl_4 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, Glulam Sarwar, Departmentof 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^{0}C).Oven was used to get a thick & semisolid, dark brown gummy mass. The mass was weighed, marked and stored at 4^{0}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 IC_{50} calculations. The total inhibition percentage of DPPH radicals was calculated by:

$$\text{Inhibiton (\%)} = \left(\frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank}}\right) \times 100$$

Using EZ-Fit Enzyme Kinetics Software (Pirelli Scientific Inc. Amherst, (USA) IC_{50} 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% CCl_4 (in olive oil))
Group-IV: Drug control (CCl_4+ silymarin 50 mg/kg)
Group-V: CCl_4+ *En* (150 mg/Kg)
Group-VI: CCl_4+ *En* (300 mg/Kg)
Group-VII: *En* (150 mg/Kg)
Group-VIII: *En* (300 mg/Kg)

CCl_4 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 CCl_4. The treatments of CCl_4 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_{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 (*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_4 induced hepatic-nephrotoxicity at different doses (150 mg and 300 mg/kg BW).

3.3.4 Hematological parameters

Effects of *En* and *CCl_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_4 intoxicated group (III) as compared to the control group (I). Silymarin treated group (IV) restored toxic alteration induced via CCl_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_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 ($\times 10^6$)/µl RBCs, 12.80 ± 0.33 ($\times 10^5$)/µl platelet count and 9.70 ±0.50 ($\times 10^3$)/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.

Table 1. Phytochemical evaluation of *Euphorbia nivulia*

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_3 test		
Flavonoids	Dark yellow color	Flavonoids present
Test with Alkali solution		
Saponins	Froth formation	Saponins present
Froth test		
Phenolic Contents	Blackish color	Phenolic contents present
Fe Cl_3 test		

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

Sr#	Compounds	Retention time (min)	Area (mV.s)	Phenoliccontent(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

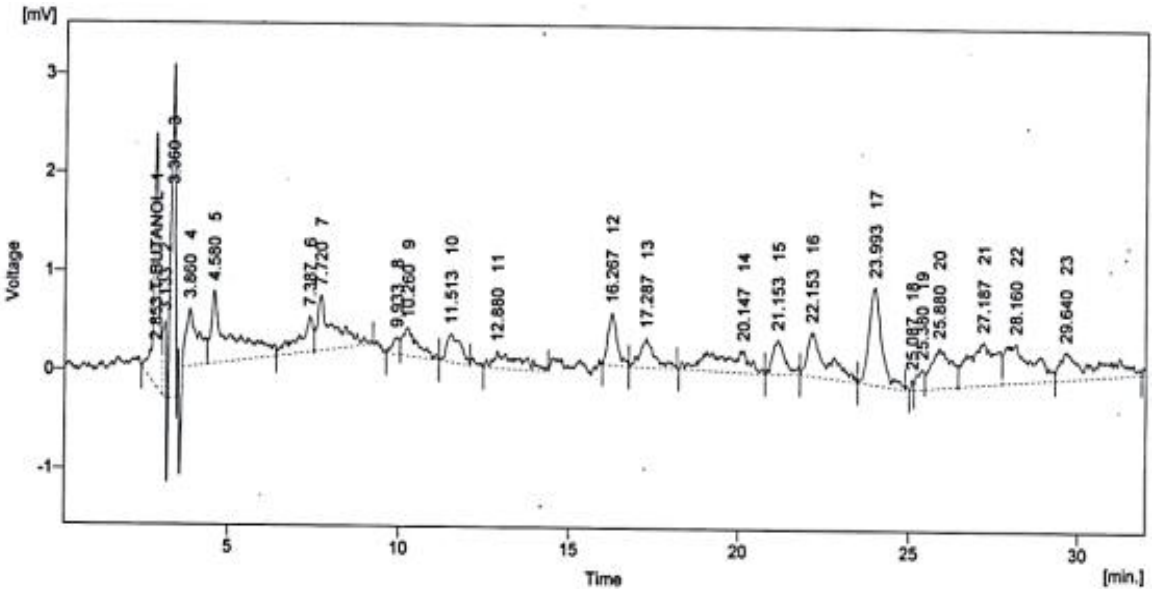


Fig. 1. HPLC chromatogram of *Euphorbia nivulia*

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

Groups	Dose	RBCs ($\times10^6$)/µl	WBCs ($\times10^3$) /µl	Platelets ($\times10^5$) /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±.09	8.70±0.16	12.13 ±0.83	15.20 ±0.31
CCl_4	1ml/kg	7.16±.0.08	130 ± 3.3	9.15 ±.04	12.80 ±0.33
CCl_4+ Silym	1ml/kg+50mg/kg	8.16±0.48	12.40±.2.8	10.49 ±.09	14.10±0.33
CCl_4+ *En*	1ml/kg+150mg/kg	7.45 ±.05	21.10 ± 1.3	10.81±o.54	13.60±.040
CCl_4+ *En*	1ml/kg+300mg/kg	8.03±0.26*	9.70 ±.050*	12.80±0.33*	15.10 ±.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±.05	13.0 ± .093

*Mean ± SD (n = 6), Means with superscript * in a column specify significance at p< 0.05; En: Euphorbia nivulia, Silym: Silymarin, CCl_4: 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 CCl_4induced hepatotoxicity (Table 4, Fig. 3). CCl_4 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 theCCl_4produced 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 CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 treated animals (group III) caused noticeable elevation in fatty changes, inflammatory cells infiltrations (hepatic cytoplasm inflammation), cellular hypertrophy, centrilobular 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 CCl_4. 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 CCl_4 to rats (Group III) significantly increases serum creatinine, serum urea and creratinine/ 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 CCl_4 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 CCl_4 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 CCl_4 group (Table 6; Fig. 4).

Table 4. Result of *Euphorbia nivulia* on biochemical indicators of liver in serum

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 0±.09
Vehicle control	1ml/kg	41±0.53	145±1.5	42±.90	4.21 ± .01	2.70±.09
CCl_4	1ml/kg	107 ± 1.8	378±2.58	98± 1.15	1.93 ±.012	5.79±.027
CCl_4+ Silym	1ml/kg+50mg/kg	54±2.3	159±2.60	31±1.73	4.1 ± .000	2.80±.0.0
CCl_4+ *En*	1ml/kg+150mg/kg	64±1.86	268±2.70	49±2.54	3.37±0.02	3.18±.02
CCl_4+ *En*	1ml/kg+300mg/kg	48 ± 2.7*	150 ± 2.30*	42± 1.39*	3.76±.02*	2.90 ± .02*
En	150mg/kg	44±2.3	142.8±.50	47±1.96	5.07± 1.3	2.90 ± .02
En	300mg/kg	43±1.5	132±0.65	43.15±3.83	4.10±0.33	2.93±.025

*Mean ± SD (n = 6), Means with superscript * in a column specify significance at $p < 0.05$. En: Euphorbia nivulia, Silym: silymarin, CCl_4: Carbon tetrachloride*

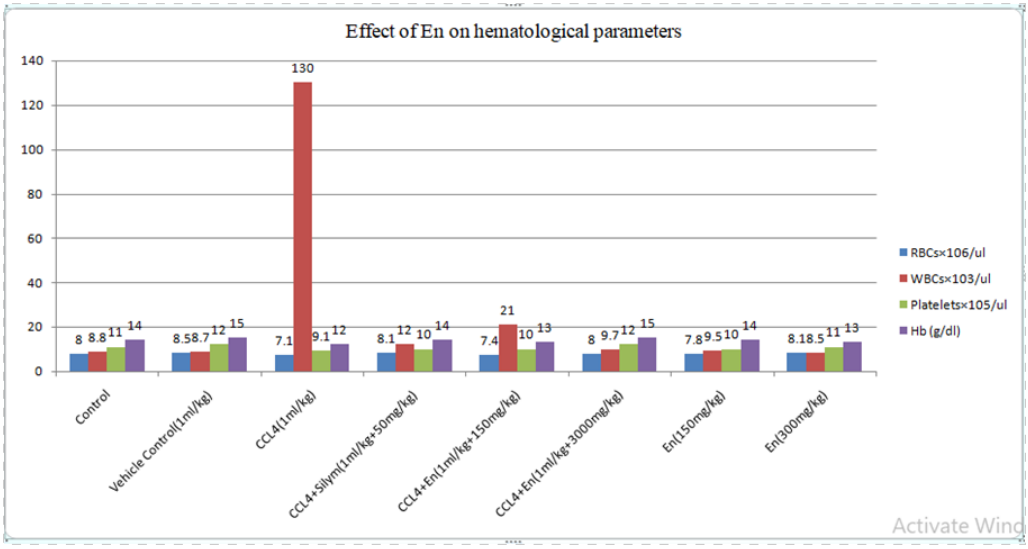


Fig. 2. Effect of *Euphorbia nivulia* on haematological parameters

Table 5. Grading showing histopathological changes induced by CCl_4 & *Euphorbia nivulia* in hepatic tissues (quantification scores)

Groups	Dose	Hepatic cytoplasm inflammation	Centrilobular necrosis	Cellular hypertrophy	Vacuolization	Steatosis
Control	-	–	–	–	–	–
Vehicle control	1ml/kg	–	–	–	–	–
CCl_4	1ml/kg	++	+++	+++	++	+++
CCl_4+ Silym	1ml/kg+50mg/kg	–	–	–	–	–
CCl_4+ *En*	1ml/kg+150mg/kg	+	+	+	+	+
CCl_4+ *En*	1ml/kg+300mg/kg	-	-	-	-	-

Histopathologic assessment was scored as follows: (−)—no meaningful histopathologic changes (+)—milddegree; (++)—moderate degree and (+++)—severe degree. En: Euphorbia nivulia, Silym: Silymarin, CCl_4: Carbon tetrachloride

Table 6. Effect of *Euphorbia nivulia* on serum biochemical markers of kidney in rats treated with CCl_4

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 ± .01	0.17±0.00
CCl_4	1ml/kg	50±0.82	2.12±0.03	1.93±0.03	1.09±0.03
CCl_4+Silym	1ml/kg+50mg/kg	23±1.97	0.79±0.06	4.10±0.22	0.19±0.03
CCl_4+ *En*E	1ml/kg+150mg/kg	26±1.86	0.99±0.00	3.37±0.09	0.26±0.01
CCl_4+ *En*E	1ml/kg+300mg/kg	17±2.21*	0.87±0.06*	3.76±0.06*	0.25±0.03*
*En*E	150mg/kg	15.30±0.67	0.91±0.04	3.84±0.03	0.23±0.03
*En*E	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, CCl_4: Carbon tetrachloride

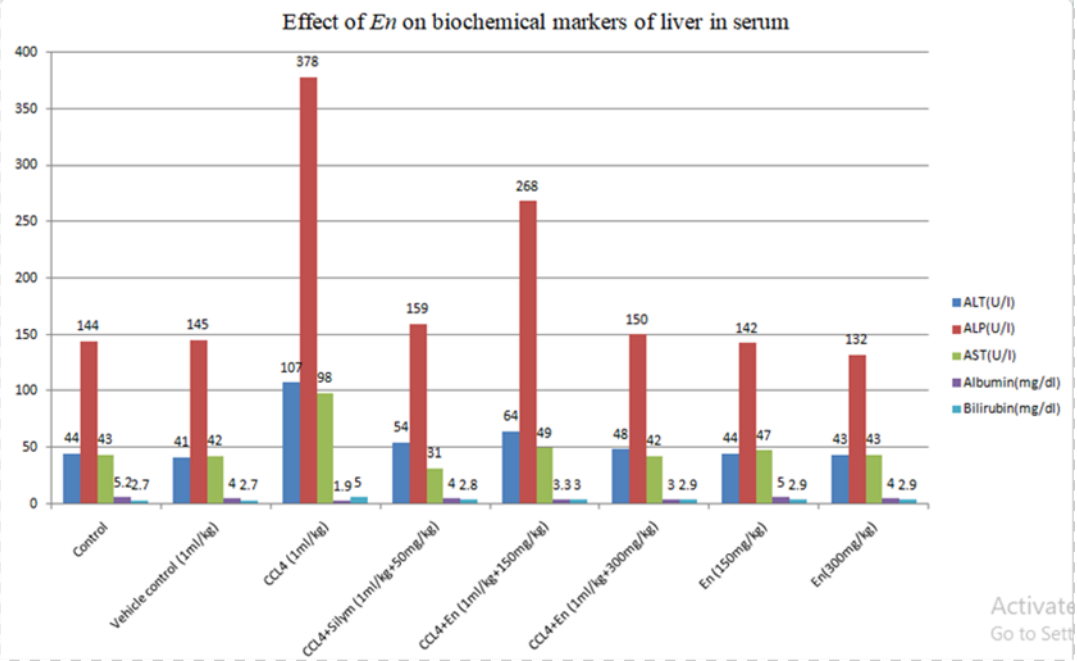


Fig. 3. Effect of *Euphorbia nivulia* on biochemical markers of liver

Table 7. Pathohistological changes induced by CCl_4 and *Euphorbia nivulia* in renal tissues (Quantification scores)

Parameter	Control	*CCl_4*	*CCl_4+ En*
Interstitial fibrosis	−	++	−
Interstitial cell infiltration	+++	−	−
Glomerular changes	−	++	−
Tubular dilatation	−	++	−
Vascular congestion	−	+++	+

(−) absent; (+) mild; (++) moderate; (+++) severe; (En) Euphorbia nivulia

3.3.9 Effect of *en* on renal histoarchitecture

Histopathological investigations endorsed protective effects of *En* against CCl_4 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 CCl_4 chronic administration caused significant renal morphological damage, especially in the renal cortex. However, CCl_4 effect on the medulla was limited. CCl_4-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 CCl_4 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. CCl_4- 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

shielding effects of silymarin (group IV; Fig. 3b). In the CCl_4+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 CCl_4 and *En* extract in renal tissues (Quantification scores) is summarized in Table 7.

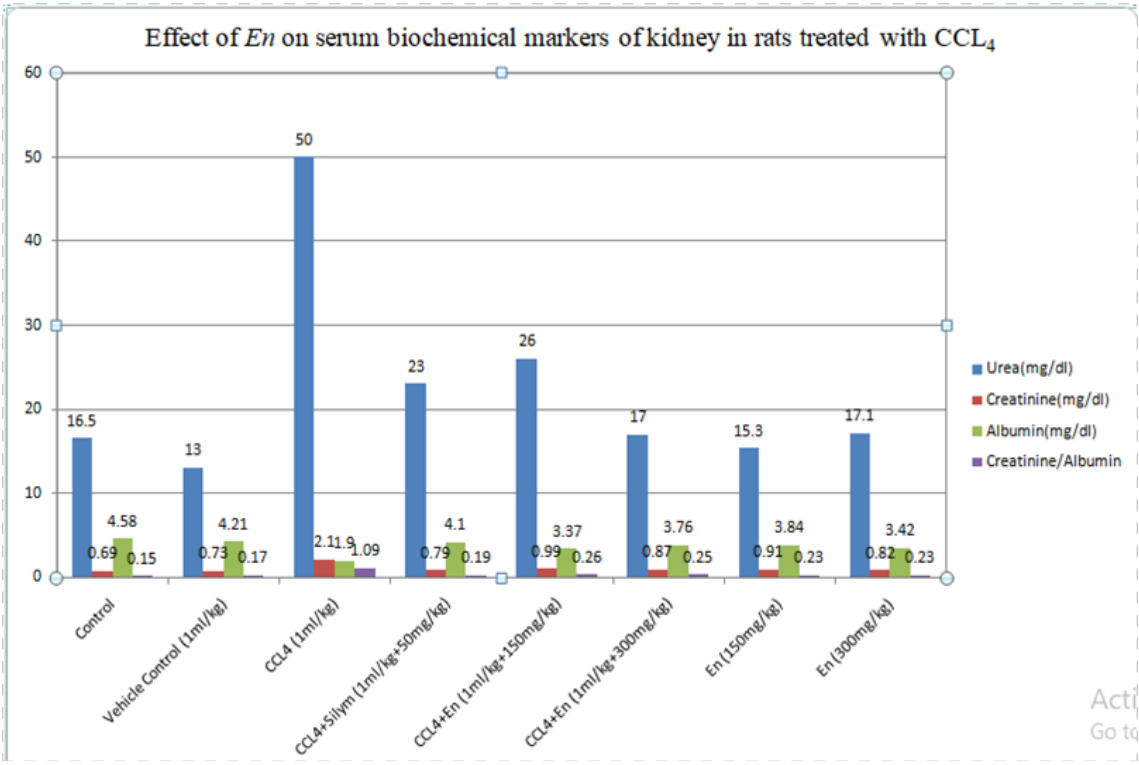
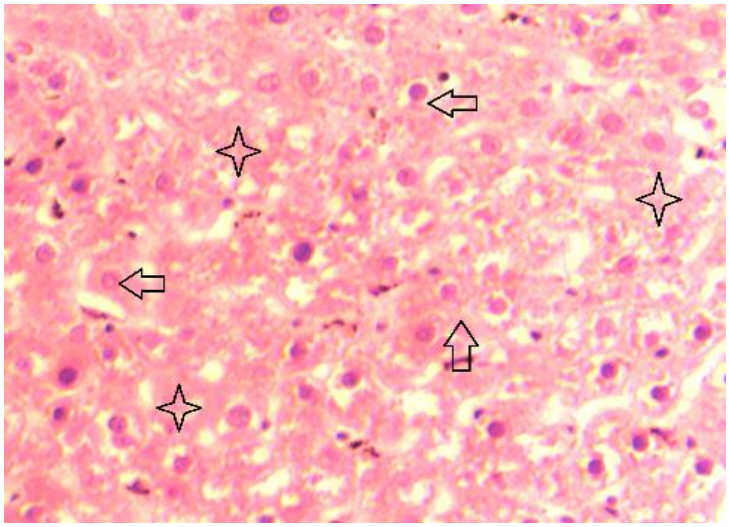
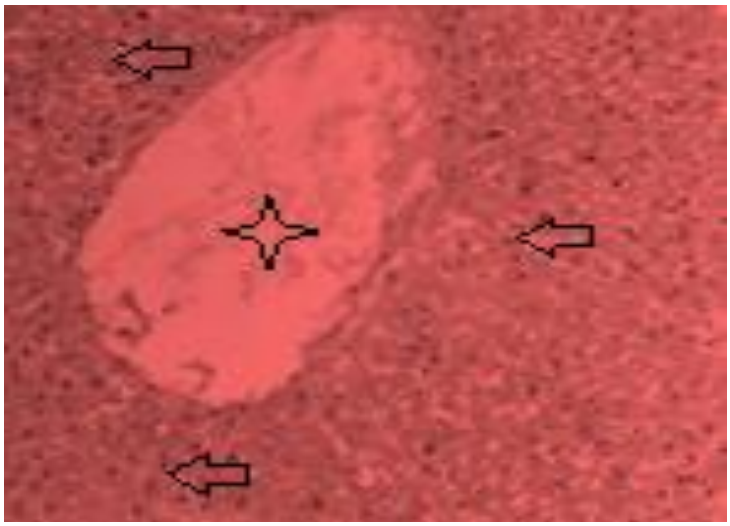


Fig. 4. Effect of *Euphorbia nivulia* on serum biochemical markers of kidney



(a)



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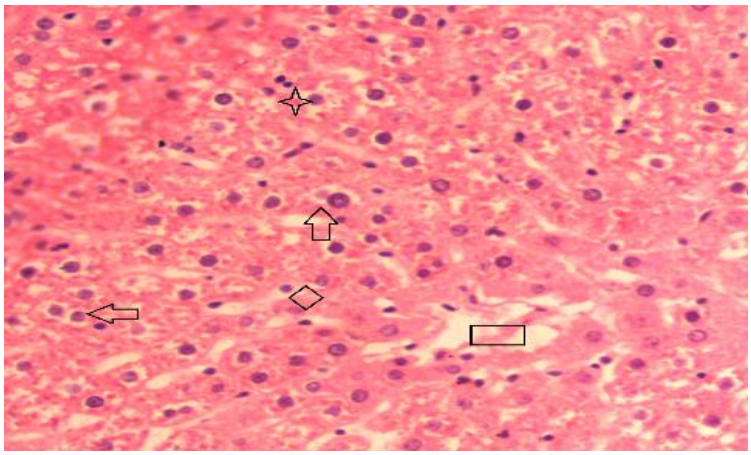

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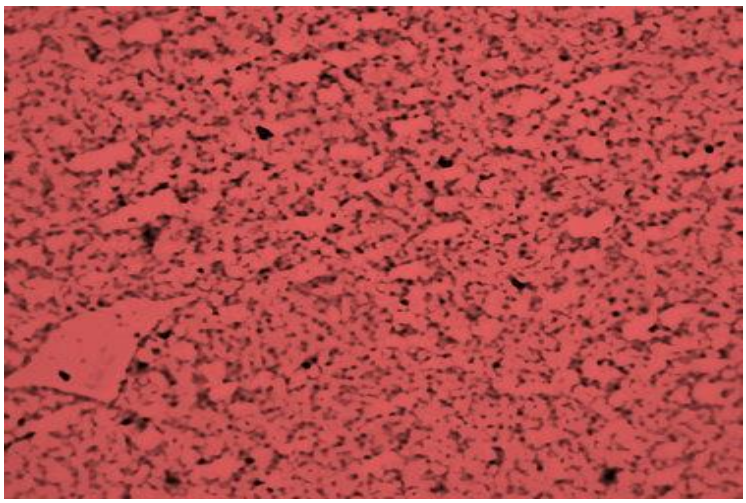

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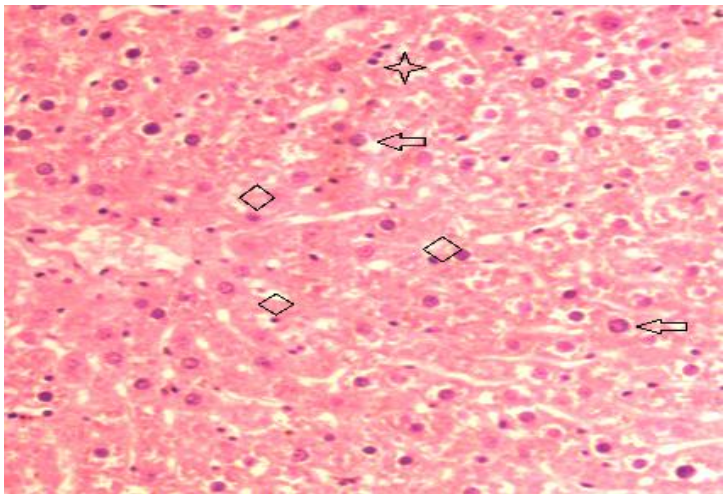


(e)

Fig. 5. Microphotographs representing the effect of CCl_4, 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. CCl_4 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. CCl_4+ 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. CCl_4+ En (150mg/Kg): mild to moderate eduction in inflammation and degenerative changes

e. CCl_4+ *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

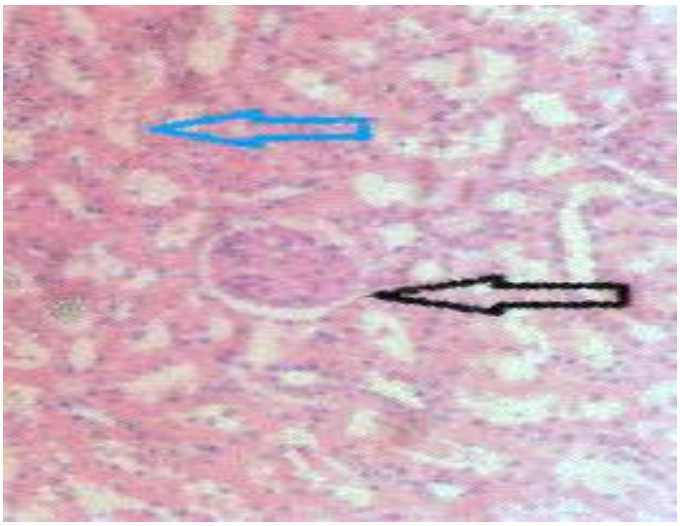
Traditional medicinal practices designed the base of clinical, pharmacological and chemical studies [29]. Plants have been the essential component of human culture due to their medicinal uses since time immemorial. In early drug discovery, plants as initial source of medicines have been used in ethno-pharmacological 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 $DPPH_2$ 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 CCl_4 induced toxicity.

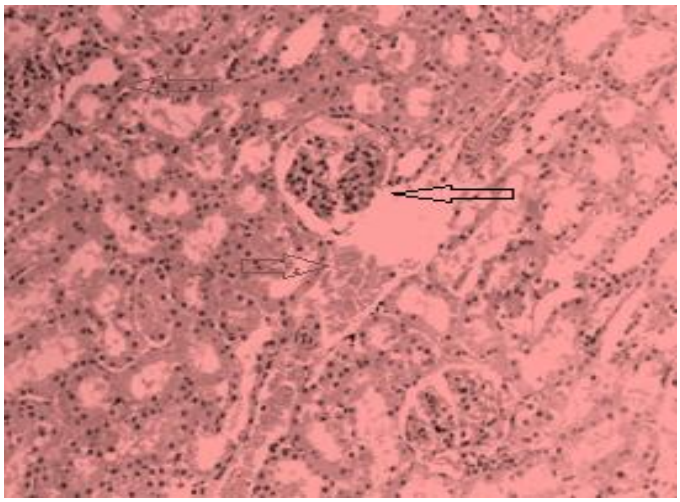
Determination of serum AST, ALT and ALP enzyme activity is an important indicator for hepatic membrane functional integrity. Elevated ALP, AST and ALT serum levels are measured as hepatotoxicity markers, these enzymes ooze out into the plasma when hepatic injury disturbs cellular membrane probity. In the current

investigation, increased levels of hepatic serum markers (AST, ALT and ALP) might reverse hepatic damage due to CCl_4 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 concommitant administration with CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 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).



(a)



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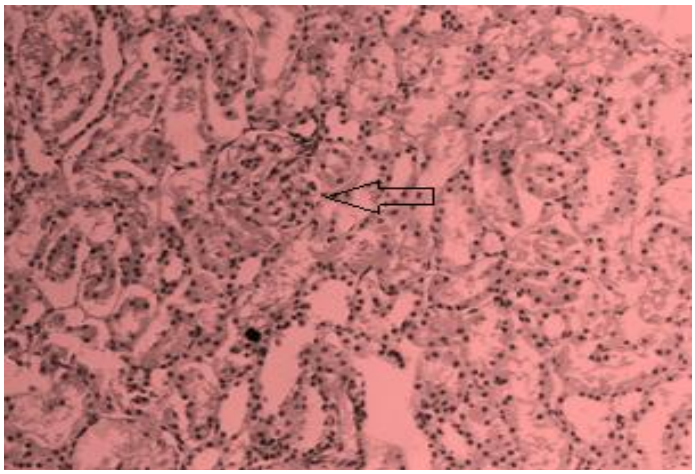

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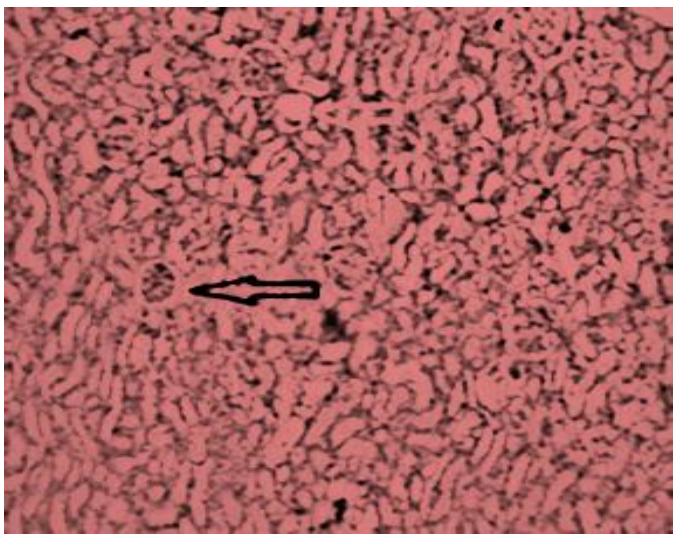

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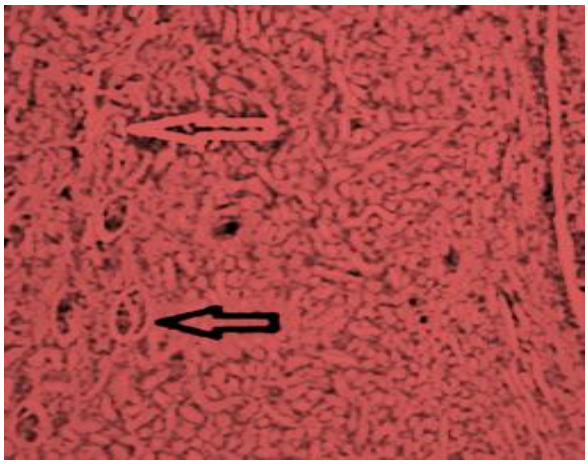

(e)

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. CCl_4 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. CCl_4+ 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. CCl_4+ *En* (150mg/Kg): Improvement in the disruption of Bowman's Capsule (black arrow) comparable to disease control; showing moderate inflammation and degenerative changes

e. **CCl_4+ *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 CCl_4 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 CCl_4 chronic administration caused significant renal morphological damage, especially in the renal cortex. However, CCl_4 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 CCl_4 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. CCl_4- 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 CCl_4+ *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 CCl_4 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 CCl_4 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 CCl_4 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 CCl_4-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 CCl_4 intoxication in rats. Hematological investigation is used for assessment of hepatic disorders like inflammatory diseases. CCl_4 may be a reason for enhancing the production of free radicals including H_2O_2, 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, CCl_4 intoxicated rats exhibited increased WBC levels and decreased Hb, RBCs and platelets levels. The extract significantly restored CCl_4 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 CCl_4 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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