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Protective Activities of some Extracts from *Euphorbia retusa* Leaves towards CCl_4-Induced liver injuries in Rats

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Abstract Many studies have been conducted to identify natural compounds from plant materials for prevention of the development of cirrhosis or fibrosis of liver. The current study aimed at evaluating the protective effect of *Euphorbia retusa* Leaves butanol and methanol extracts towards the CCl_4-induced hepatotoxicity in male Wister rats. Evaluation was done through measuring certain hematological parameters, hepatic function markers, lipid and protein profiles in serum as well as the lipid peroxidation and endogenous antioxidants content in the liver were analyzed. CCl_4 diluted 1: 9 (v/v) in olive oil was injected intraperitoneally followed by butanol and methanol extracts (200 mg/kg body weight) were administered orally. The CCl_4-treated rats showed a significant decline in the studied hematological parameters, the serum levels of high-density lipoprotein (HDL), albumin (A) as well as the hepatic levels of glutathione (GSH) and activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR). This was accompanied by a significant elevation in the levels of total lipids (TL), triglycerides (TG), total cholesterol (TC), low-density lipoproteins (LDL), globulin (G), total bilirubin (TBil) and the activities of alanine and aspartate aminotransferase (ALAT and ASAT) and alkaline phosphatase (ALP) as well as the hepatic levels of malondialdehyde (MDA). In contrast, the administration of butanol and methanol extracts, notably improved all the studied parameters. This study disclosed that CCl_4 administration to Wister rats, at a high dose level, could induce a hepatic injury in addition to certain hematologic and metabolic alterations. The work was extended to examine tissue histopathology. Yet, the treatment with butanol and methanol extracts could ameliorate these alterations via their antioxidative effect. In conclusion, *Euphorbia retusa* Leaves butanol and methanol extracts, resulted in an attractive candidate for ameliorating of hepatotoxicity induced by CCl_4 through scavenging free radicals, improved liver functions, and normalizing the liver histopathological architecture. Further studies are required in order to identify the molecules responsible of the pharmacological activities.

Keywords *Euphorbia retusa* Leaves, butanol and methanol extracts, male Wister rats, liver injury, Carbon tetrachloride

Introduction

The liver is responsible for metabolism and detoxification of the most of components that enter the body [1]. Carbon tetrachloride (CCl_4) is a highly toxic chemical agent, the most famous drug used to induce liver damage experimentally. Histopathological sectioning of the liver tissues indicated that, CCl_4 induced fibrosis, cirrhosis and hepatocarcinoma [2]. The toxic effect of CCl_4 is attributed to trichloromethyl radical produced during oxidative

stress [3]. The number of infiltrated neutrophils, macrophages, Kupffer cells, lymphocytes and natural killer cells are significantly increased after liver injury induced by hepatotoxins such as CCl_4. It induced activation of liver resident macrophages and/or chemo attraction of extra hepatic cells (e.g. neutrophils and lymphocytes) [4]. The activated macrophages are released and contributed to liver fibrosis, inflammation and injury [5]. Once the liver became injured, its efficient treatment with famous chemical drugs is limited [6]. Therefore, interest concerned the use of alternative medicines for the treatment of hepatic disease has been arisen.

Many natural products plant extracts were reported for their hepatoprotective activity. These extracts are rich in phenolic metabolites [7-9]. Euphorbiaceae family is reported for providing plant extracts which are rich in phenolics [10]. *Euphorbia* genus belongs to the family Euphorbiaceae which comprises about 1000 species. *Euphorbia* species were reported to have antitumor activity, antimicrobial activity, inhibition of HIV-1viral infection, antihyperglycemic, hypolipidemic and hepatoprotective activities [11-14]. *Euphorbia retusa* is an annual herb found abundantly in the Mediterranean region. It is characterized by long alternate blue green leaves which contain toxic and skin-irritant milky latex. *E. retusa* has been used in traditional medicine for curing warts, trichiasis, and venomous bites [15]. *E. retusa* were reported to have antioxidant [16], inflammatory, and analgesic activities [15]. It was also reported to contain flavonol aglycones , their glycosides, ellagic acid and dimethoxy ellagic acid [17], in addition to carotenoids, fatty alcohol chains, diterpenes, triterpenes, sterols and essential fatty acids [18-19].

In the present study, we investigated the protective effects of *E. retusa* methanolic and butanolic extracts towards CCl_4-induced hepatotoxicity in rats by assaying liver functions, lipid profiles, histopathology of liver tissues, and its phytochemical composition.

Materials and Methods

Plant material and extraction

The aerial parts of *E. retusa* were collected from, Wadi Elnatron, Egypt in September 2018, and were identified by Prof. Ibrahim El-Garf, Prof. of Plant Taxonomy (Faculty of Science, Cairo University). Powdered plant material (500 g) was extracted three times at room temperature with 1 liter (7:3; MeOH: H_2O) to yield the obtained extracts were dried under vacuum then freeze dried to yield 60 g of the extract followed by suspension of 30 g in water and partitioned by DCM followed by ethyl acetate and butanol to yield 1.2 g DCM fraction, 2.5 g of ethyl acetate fraction and 12g of butanol fraction.

Animals

Male Wister albino rats (100 to 120 g) were selected for this study. They were obtained from the Animal House, National Research Center, Egypt. All animals were kept in controlled environment of air and temperature with access of water and diet ad libitum.

Ethics

Anesthetic procedures and handling with animals complied with the ethical guidelines of Medical Ethical Committee of the National Research Centre in Egypt.

- Doses of Administration. Administration regime was twice a week for six consecutive weeks. Five hundred microliters of CCl_4 diluted 1: 9 (v/v) in olive oil were injected intraperitoneally (0.1 ml). *E. retusa* extracts BT and ME (200 mg/kg body weight) were administered orally after intraperitoneal injection of CCl_4.

Experimental Design

24 male rats were used in this study. Animals were divided into 4 groups (6 rats each) as following:

Group 1 served as normal healthy control rats.

Group 2: Rats were intraperitoneally injected with CCl_4 alone.

Group 3: Rats were intraperitoneally injected with CCl_4 followed by oral administration of *E. retusa* extracts BT (200 mg/kg body weight)

Group 4: Rats were intraperitoneally injected with CCl_4 followed by oral administration of *E. retusa* extracts ME (200 mg/kg body weight)

Hematological and Biochemical studies

1- Sample Preparations

Blood was collected from each animal by puncture of sublingual vein. Blood samples were divided into two parts. The first part was collected on EDTA for hematological analyses. The second part was collected into dry test tubes and then centrifuged at 3000 rpm in order to separate serum. The sera were kept at -20 ºC for further biochemical analysis. In order to collect the hepatic tissues, rats were immediately dissected. The liver was homogenized with 10% w/v ratio in ice-cold 50 mM Tris HCl buffer at pH 7.4 and then centrifuged at 10,000 rpm for 20 min at 4EC. The supernatant was collected and kept in deepfreeze at -20 ºC for further analyses.

Estimation of hematological parameters

The hematological parameters including red blood cell (RBC) count, white blood cell (WBC) count, platelet (PLT) count, hemoglobin (Hb) content and packe cell volume (PCV) were analyzed using Medonic M-Series analyzer (Clinical Diagnostics solutions Inc, Florida, USA).

Estimation of serum biochemical parameters

In the serum of all the experimental groups, the levels of total lipids (TL), total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), total proteins (TP), albumin (A), globulin (G), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), total bilirubin (TBil) and direct bilirubin (DBil) were measured colorimetrically using Biodiagnostics kits (Dokki, Giza, Egypt). Lipid peroxide assay: The level of malondialdehyde (MDA) in the liver homogenate was assayed according to the technique described by Ohkawa *et al* [20]. The principle of this method depends on the reaction of the liberated MDA after lipid peroxidation (LPO) of the cell membranes with thiobarbituric acid in acidic medium.

Non-enzymatic and enzymatic antioxidant assay

The concentrations of non-enzymatic (glutathione, GSH) as well as enzymatic (catalase, CAT, superoxide dismutase, SOD, glutathione reductase, GR) antioxidants were estimated in the homogenate of the liver of control and treated rats. The method by which GSH content was measured was based on the reaction of 5,5'-Dithiobis-2-nitrobenzoic acid with GSH [21]. The CAT activity was estimated in accordance to the method described by Aebi [22]. The SOD activity assessment was based on the ability of SOD to inhibit the reduction reaction of nitrobluetetrazolium dye mediated by phenazine methosulphate [23]. The principle for measuring the GR activity was based on its ability to catalyze the reduction of glutathione (GSSG) as described by Goldberg and Spooner [24].

Determination of serum caspase-3 activity

Serum caspase-3 activity was measured by a quantitative ELISA technique using the kit provided by R and D systems (MN, USA) according to the manufacturer's instructions [25]. Quantitative reverse- transcription polymerase chain reaction (qRT-PCR) for analysis of serum Bax and Bcl2 mRNA levels:

Total RNA was extracted from serum using RNase mini kit (Qiagen, CA, USA).The isolated RNA was quantified using UV spectrophotometer (Beckmn, USA) and the purity of RNA was verified with 260/280nm ratio ranging from 1.9-2.1. The integrity of RNA was assessed by gel electrophoresis. The total RNA (0.5–2 µg) was used for cDNA conversion using high capacity cDNA reverse transcription kit (Fermentas, USA). Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The primer sequences shown in table 1 were provided by Shine Gene, China.

Table 1: The primer sequences of the studied genes.

Gene name	Primer sequence	Primer size (bp)
Refer-actin	Forward primer:5′-CAG GAT GGC GTG AGG GAG AGC-3′ Reverse primer:3′-AAG GTG TGA TGG TGG GAA TGG-5′	263
Bax	Forward primer: 5'-TTC CGA GTG GCA GCT GAG ATG TTT-3' Reverse primer: 3'-TGC TGG CAA AGT AGA AGA GGG CAA-5'	194
Bcl-2	Forward primer:5'-CAT GCC AAG AGG GAA ACA CCA GAA-3' Reverse primer:3'-GTG CTTTGCATT CTTGGA TGA GGG-5'	224

The PCR reactions included 10 min at 95^{o}C (activation), followed by 40 cycles at 94^{o}C for 15 sec (denaturation) and 60^{o}C for 1 min (annealing/extension) [26]. The expression level was calculated from the PCR cycle number (C_T) where the increased fluorescence curve passes across a threshold value. The relative expression of target genes was obtained using comparative C_T ($\Delta\Delta C_T$) method. The ΔC_T was calculated by subtracting β-actin C_T from that of the target gene whereas $\Delta\Delta C_T$ was obtained by subtracting the ΔC_T of the calibrator from that of the test sample. The relative expression was calculated from $2^{-\Delta\Delta CT}$ formula based on the method of Pfaffl [27].

Determination of percent of DNA damage by comet assay in liver tissues

Single cell gel electrophoresis assay (also known as comet assay) was performed as previously described by Singh et al. [28]. This test is a rapid, sensitive and simple method for detecting DNA damage. In this method, cellular DNA is detected by the migration of DNA fragments from the cell nucleus through an agarose gel using fluorescent dyes, under the influence of an electric field, resulting in a comet-like shape. With increasing number of breaks, DNA pieces migrate freely into the tail of the comet. The tail length and the percentage of total DNA in the tail reflect DNA damage, which is directly related to the frequency of breaks over a wide range of damage. All steps of the comet assay were conducted under dimmed light to prevent additional DNA damage. Image analysis was performed with a Leitz Orthoplane Pi fluorescence microscope (magnification 200) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The microscope was connected through a camera to a computer-based image analysis system (Comet Assay IV software, Perspective Instruments). One hundred randomly selected cells per slide were scored.

Statistical analysis

Data were statistically analyzed by the aid of Statistical Package of the Social Sciences, SPSS version 23 (copyrighted by IBM SPSS software, USA). Data were expressed as a mean ± standard error of mean (SEM).

Histopathological Study

Liver tissues were excised from sacrificed animals, individually weighed, and, from them, 5 μm thickness slices were cut, fixed in 10% paraformaldehyde, and embedded in paraffin wax blocks. Tissue sections of 5 μm thick were stained with hematoxylin and eosin (H&E).

Results and Discussion

Hepatoprotective activities

Effect on hematological parameters: The results of hematological parameters in Table (1) revealed that the type of treatment significantly affected all the studied blood parameters except for the PLT count that did not show any significant differences among all the studied groups. Rats of CCl_4-administered group showed a notable decline in the RBC and WBC counts, Hb content and PCV, as compared to the controls. As compared to the rats of CCl_4-treated group, the rats administered *E. retusa* extracts BT and ME after CCl_4 administration exhibited significant elevations in the RBC, WBC counts, Hb content and PCV. This data is in accordance with Meral and Kanter [29], who reported that rats treated with CCl_4 for 45 days significantly decreased the red blood cell count (RBC), white blood cell count (WBC), packed cell volume (PCV), and Hb levels while Nigella sativa treatment significantly increased the reduced RBC, WBC, PCV, and Hb levels.

Effect on serum biochemical parameters: The lipid profile of the experimental animals as affected by the administration of CCl_4 alone, *E. retusa* extracts BT or ME plus CCL4 are shown in Table (2). The serum levels of TL, TC, TG, LDL-C and HDL-C of the rats were markedly influenced by the type of treatment. In comparison to control group, all the studied lipid profile parameters of CCL4-treated group were significantly elevated except the levels of HDL-C that were notably reduced. On the other hand, rats treated E. retusa extracts BT or ME plus CCL4 exhibited a marked reduction in the levels of TL, TC, TG and LDL-C, as compared with the CCL_4-treated group. The results of the present study have also established that CCl_4 treatment could have affected the lipid metabolism of liver (triglyceride and cholesterol levels). This is evidenced from the present observations in which CCl_4 caused a significant ($p < 0.05$) increase in the levels of lipid parameters. In this connection, Muller *et al* [30] stated that CCl_4 intoxication is similar to hepatitis in case of the triglycerides catabolism. This situation could be also attributed to the reduction of lipase activity, which could lead to decrease in triglyceride hydrolysis [31]. On the other hand, it can be assumed that hypercholesterolemia in CCl_4 intoxicated rats was resulted from damage of hepatic parenchymal cells that lead to disturbance of lipid metabolism in liver [32]. However, rats treated with *E. retusa* extracts ME showed a significant ($p < 0.05$) decline in triacylglycerol and cholesterol values compared to CCl_4-intoxicated rats. The mechanism of lipid lowering effects of *E. retusa* extract might be attributed to an inhibitory activity on microsomal acyl coenzyme A: cholesterol acyltransferease *in vitro*. This enzyme is responsible for acylation of cholesterol to cholesterol esters in liver [33].

Serum protein profile of different groups of rats in Table (3) was noticeably affected by the type of treatment as rats administered CCl_4 alone exhibited marked reductions in the levels of albumin simultaneous with a significant increase in the levels of globulin, as compared to the controls. Thus, the A/G ratio of this group was remarkably reduced. On the other hand, the rats of *E. retusa* extract plus CCl_4 -treated groups displayed a marked increase in the levels of albumin and A/G ratio but a marked decrease in the levels of globulin, as compared to the CCl_4-treated group.

In this study the significant ($p < 0.05$) decrease in serum albumin of rats treated with CCl_4 as compared to control may indicates poor liver functions or impaired synthesis, either primary as in liver cells damage or secondary to diminished protein intake and reduced absorption of amino acids caused by a malabsorption syndromes or malnutrition, or loss protein in urine, due to nephritic syndrome and chronic glomerulonephritis [34]. On the other hand, a significant ($p < 0.05$) increase in concentration of serum albumin was observed in rats received *E. retusa* extracts plus CCl_4 in comparison to rats received CCl_4 alone. The increase of albumin concentration after treatment with *E. retusa* extract may be attributed to the decrease in lipid peroxidation processes and increase in the activities of plasma protein thiols as a result of the treatment [34].

Liver function markers, as influenced by the administration of CCl_4 - *E. retusa* extract alone and mixed, were presented in Table (4). The activities of ASAT, ALAT and ALP and TBil, in serum of rats were significantly affected by the type of treatment, whereas the serum levels of DBil were not affected by any of the studied factors. In comparison to the controls, the CCl_4-treated rats showed significant elevations in the activities of ASAT and

ALAT and ALP as well as the levels of TBil. On the contrary, the activities of ALP, ASAT and ALAT as well as the levels of TBil and DBil of *E. retusa* extract plus CCL_4 -treated rats were not significantly different from those of the control group.

In the present study serum hepatic biomarkers, AST and ALT activities were greatly increased ($p < 0.05$) in rats treated with the CCl_4 compare to control. As in the present investigation, previous studies have shown that $CC1_4$ increased significantly serum ALP levels, and total protein and albumin levels [35-36]. The increased serum levels of hepatic markers have been attributed to the liver injury, because these enzymes are found in cytoplasmic area of the cell and they are released into circulation in case of cellular damage [37]. On the other hand, treatment with *E. retusa* extract plus CCL_4 was found to suppress ($p < 0.05$) the increase of serum AST and ALT activities. In accordance with the present results, many other plant extracts were reported to have considerable therapeutic effects on liver injury induced by chemical agents, for example, administration of poly phenolic extracts from chicory (Cichorium intybus) resulted in wholly normalization of the serum AST and ALT levels in mice exposed to thioacetamide, a hepatotoxic organosulfur compound [38]. Rafiei *et al* [39] have also reported similar effects from barberry extract upon administration to CCl_4 induced hepatotoxic animals. These finding implies that challenge to protect liver tissue from CCl_4 injury.

Effect on the hepatic lipid peroxidation and endogenous antioxidants

The effects of CCl_4 alone or with *E. retusa* extracts BT or ME administrations on the levels of hepatic MDA and GSH and the activities of endogenous antioxidant enzymes were shown in Table (5). The hepatic levels of MDA and GSH as well as the activities of CAT, SOD and GR were significantly influenced by the type of treatment. In the liver of rats administered CCl_4 alone, there was a meaningful elevation in the levels of MDA accompanied by a marked reduction in the GSH content, SOD and GR activities as compared to those of controls. In the rats of *E. retusa* extracts BT or ME plus CCl_4 -treated groups, the mean values of hepatic MDA concentration were significantly lower than those of CCl_4-treated rats and were not significantly different from those of the controls. On the other hand, the mean values of hepatic GSH content of *E. retusa* extract plus CCl_4-treated rats were significantly higher than those of CCl_4-treated group. As compared to the CCl_4-treated group, the rats administered *E. retusa* extract plus CCl_4 showed a marked elevation in the activities of CAT and SOD and GR, that did not significantly differ from those of the controls.

Data of the present study is in accordance with the findings of other workers such as Park *et al* [40] who reported that hepatotoxic effects by CCl_4 are lipid peroxidation origin, and are largely due to its active metabolite CCl_3 (This metabolite can abstract hydrogen from fatty acids, initiating the lipid peroxidation), lead to cell injury, and finally liver damage. Moreover, Palanivel *et al* [41], stated that the efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. In this connection, the present study revealed that E. retusa extracts BT or ME decreased ($p < 0.05$) CCl_4 induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells. As previously noted and similar to the results achieved for other plants in the literature [42-43], our observations and findings can be attributed to the antioxidant ingredients of *E. retusa* extract that probably inhibit lipid peroxidation and consequently inhibition of oxidative stress. Therefore, the cell membranes remain intact and as a result cells are prevented to enter the necrosis step.

Determination of serum caspase-3 activity

The data in Table (6) revealed that CCl_4 intoxication produced a significant elevation in serum caspase-3 activity reaching 324% of the control group. On the other hand, administration of *E. retusa* extract significantly reduced serum caspase-3 activity as compared to CCl_4-intoxicated group reaching 215 and 182% of the control group, respectively.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for analysis of serum Bax and Bcl2 mRNA levels

Table (7) showed that CCl_4 intoxication produced a marked up-regulation of Bax mRNA serum level along with an obvious down-regulation of Bcl2 mRNA level by almost 11.22 and 0.19 folds of the control group, respectively. Administration of either *E. retusa* extracts BT or ME reduced the serum level of Bax mRNA, but elevated that of Bcl2 mRNA as compared to CCl_4-intoxicated group reaching 4.31 and 0.55 folds of the control group, respectively for E. retusa BT extract -treated group and 6.08 and 0.65 folds of the control group, respectively for E. retusa ME extract -treated group.

Our results are in coincidence with those of other workers who reported that specific activator of peroxisome proliferator-activated receptor-α (PPARα) [44-45]. Such Activation causes an upregulation of mRNA and protein levels of a number of peroxisomal and non-peroxisome-associated enzymes and structural proteins [46].

In addition, the current results at the molecular level, showed a substantially high expression of the pro-apoptotic protein (Bax) in the liver tissues of CCl_4-intoxicated rats, while the anti-apoptotic protein; Bcl-2 was significantly down-regulated. Together, this implies that the Bax/Bcl-2 ratio, a main index for apoptosis, might be significantly elevated; indicating CCl_4–enhanced apoptosis in the livers of intoxicated rats. Also, the present study showed a significant inhibition of serum caspase-3 activity in CCl_4-intoxicated group. This may be attributed to the over production of ROS which induced a significant elevation of caspase-3 activity leading to apoptotic condition [47].

Our findings are supported by previous reports that certain chemical agents induced apoptosis in the rat kidney and liver directly through the alteration of apoptotic gene expression [48]. Accumulating evidences have indicated that these agents could induce cellular apoptosis by targeting the mitochondrial apoptotic pathway. This may be through the activation of cytochrome c release from the mitochondria, down-regulation of Bcl-2 expression, up-regulation of Bax expression, translocation of Bax into the mitochondrial membrane, and activation of caspase-3 [49].

Our study revealed that treatment of CCl_4-intoxicated rats with the *E. retusa* extracts BT or ME down-regulated the expression of Bax and up-regulated Bcl-2 expression. The present results also declared significant inhibition of caspase-3activity in groups treated with *E. retusa* extracts BT or ME.

Determination of percent of DNA damage by comet assay in liver tissues

The data in table (8) and figure (1) revealed that CCl_4 liver intoxication produced a significant elevation in tail moment compared to control group of rats. On the other hand, administration of either *E. retusa* extracts BT or ME plus CCl_4 significantly reduced tail moment and consequently significant reduction in the percent of DNA damage as compared to CCl_4-intoxicated group in comparison to the control group.

Table 1: Effect of oral administration of CCl_4 alone or with different *E. retusa* extracts, on certain hematological parameters of male albino rats

Parameters	**Experimental groups**			
	Control	**CCl_4**	**E. retusa BT extract + CCl_4**	**E. retusa ME extract + CCl_4**
Red blood cell count ($\times 1012\ L^{-1}$)	5.99 ± 0.31	6.71 ± 0.62	6.91 ± 0.51	6.06 ± 0.4
White blood cell count ($\times 109\ L^{-1}$)	5.9 ± 0.82	15.5 ± 1.91	14.7 ± 0.87	14.4 ± 0.74
Platelet count ($\times 109\ L^{-1}$)	471 ± 43.3	783.5 ± 41.6	483.3 ± 33.7	435.6 ± 34.84
Hemoglobin content ($g\ d\ L^{-1}$)	12.6 ± 0.38	9.3 ± 0.32	12.36 ± 0.85	11.6 ± 0.92
Packed cell volume (%)	34.2 ± 1.23	39.1 ± 1.91	36.7 ± 1.78	36.1 ± 2.46

Data are represented as mean ± standard error.

Table 2: Effect of oral administration of CCL4 alone or with different *E. retusa* extracts, on the concentrations of serum total lipid (TL), total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) of male albino rats

Parameters	Experimental groups			
	Control	**CCl_4**	**E. retusa BT extract + CCl_4**	**E. retusa ME extract + CCl_4**
TL ($mgdL^{-1}$)	512.04 ± 43.06	658.8 ± 50.38	488.40 ± 38.07	440.80 ± 31.76
TC ($mgdL^{-1}$)	118.20 ± 2.97	228.8 ± 20.31	122.40 ± 13.68	*103.80 ± 4.54*
TG ($mgdL^{-1}$)	104.40 ± 7.34	164.80 ± 14.59	106.00 ± 9.39	*101.40 ± 8.33*
LDL-C ($mgdL^{-1}$)	61.20 ± 9.87	159.02 ± 16.76	55.60 ± 8.03	*43.80 ± 4.49*
HDL-C ($mgdL^{-1}$)	36.60 ± 6.40	27.06 ± 3.95	40.00 ± 5.52	*39.80 ± 4.73*

Data are represented as mean ± standard error

Table 3: Effect of oral administration of CCl_4 alone or with different E. retusa extracts, on the concentrations of serum total protein (TP), albumin (A), globulin (G) and A/G ratio of male albino rats

Parameters	Experimental groups			
	Control	**CCl_4**	**E. retusa BT extract + CCl_4**	**E. retusa ME extract + CCl_4**
TP ($g\ d\ L^{-1}$)	6.68 ± 0.22	6.52 ± 0.30	6.24 ± 0.05	6.19 ± 0.08
A ($g\ d\ L^{-1}$)	4.42 ± 0.13	3.42 ± 0.15	4.12 ± 0.09	4.36 ± 0.07
G ($g\ d\ L^{-1}$)	2.46 ± 0.24	3.70 ± 0.18	2.62 ± 0.19	2.59 ± 0.11
A/G ratio	1.72 ± 0.16	0.85 ± 0.09	1.38 ± 0.16	1.36 ± 0.13

Data are represented as mean ± standard error.

Table 4: Effect of oral administration of CCL4 alone or with different *E. retusa* extracts, on the activities of serum aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and alkaline phosphatase (ALP) and the levels of total bilirubin (TBil) and direct bilirubin (DBil) of male albino rats

Parameters	Experimental groups			
	Control	**CCl_4**	**E. retusa BT extract + CCl_4**	**E. retusa ME extract + CCl_4**
ASAT ($U\ L^{-1}$)	33.02 ± 1.30	118.7 ± 24.49	48.20 ± 8.01	52.7 ±11.2
ALAT ($U\ L^{-1}$)	25.60 ± 1.50	75.60 ± 2.77	39.02 ± 5.52	38.9 ± 7.63
ALP ($U\ L^{-1}$)	55.30 ± 3.84	70.02 ± 8.08	53.22 ± 5.72	56.14 ± 7.61
TBil ($mg\ d\ L^{-1}$)	0.66±0.02	0.89 ± 0.03	0.73 ± 0.05	0.77 ± 0.03
DBil ($mg\ d\ L^{-1}$)	0.11 ± 0.005	0.14 ± 0.006	0.10 ± 0.008	0.10 ± 0.004

Data are represented as mean ± standard error.

Table 5: Effect of oral administration of CCl_4 alone or with different *E. retusa* extracts, on the levels of hepatic malondialdehyde (MDA) and glutathione (GSH) and the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) of male albino rats

Parameters	Experimental groups			
	Control	**CCL4**	**E. retusa BT extract + CCl_4**	**E. retusa ME extract + CCl_4**
MDA ($nmol\ g^{-1}$ liver)	4.48 ± 0.11	9.18 ± 0.26	4.15 ± 0.22	4.78 ± 0.34
GSH ($mg\ g^{-1}$ liver)	40.04 ± 5.10	19.72 ± 0.98	37.34 ± 2.84	38.91 ± 2.31
CAT ($U\ g^{-1}$ liver)	104.3 ± 17.1	39.40 ± 8.27	99.03 ± 13.38	101.56 ± 14.74
SOD ($U\ g^{-1}$ liver)	9.56 ± 0.17	4.36 ± 0.19	9.41 ± 0.16	10.23 ± .35
GR ($U\ g^{-1}$ liver)	73.20 ± 2.71	27.80 ± 1.28	68.40 ± 3.48	69.76 ± 3.93

Data are represented as mean ± standard error.

Table 6: Effect of E. retusa extracts BT or ME on serum caspase-3 activity following CCl_4 intoxicated rats

Parameters **Groups**	**Serum Caspase-3** ***(ng/ml)***
Control	1.86±0.18
CCl_4 alone	5.37±0.17
CCl_4+ E. retusa ME extract	4.03±0.39
CCl_4+ E. retusa BT extract	3.16±0.14

Values are expressed as mean ± S.E.M. (n=10).

Table 7: Effect of E. retusa extracts BT or ME on the serum mRNA level of Bax and Bcl2 in CCl_4 intoxicated rats

Parameters Groups	Serum Bax mRNA	Serum Bcl2 mRNA
Control	1.02±0.009 fold	1.0125± 0.007 fold
CCl_4 alone	11.098 ± 1.290 fold	0.187 ± 0.017 fold
CCl_4+ E. retusa ME extract	4.300± 0.193 fold	0.565 ±0.044 fold
CCl_4+ E. retusa BT extract	5.837± 0.337 fold	0.646±0.010 fold

Values are expressed as mean ± S.E.M. (n=10).

Table 8: Effect of E. retusa extracts BT or ME on the percentage of DNA damage in the liver tissue of CCl_4–intoxicated rats

Groups	Tailed cell (%)	Untailed (%)	Tail Length (µm)	DNA Tail (%)	Tail Moment units
Control	4.8±1.31	95.2±1.31	1.74±0.52	1.72±0.24	3.22±0.98
CCl_4 alone	18±0.57	82±0.57	3.59±0.04	3.63±0.10	13.06±0.53
CCl_4+ E. retusa ME extract	14.2±0.73	85.8±0.73	3.22±0.08	3.15±0.08	10.17±0.56
CCl_4+ E. retusa BT extract	12±0.44	88±0.44	2.81±0.03	2.76±0.01	7.83±0.11

Values are expressed as mean ± S.E.M. (n=10).

Class

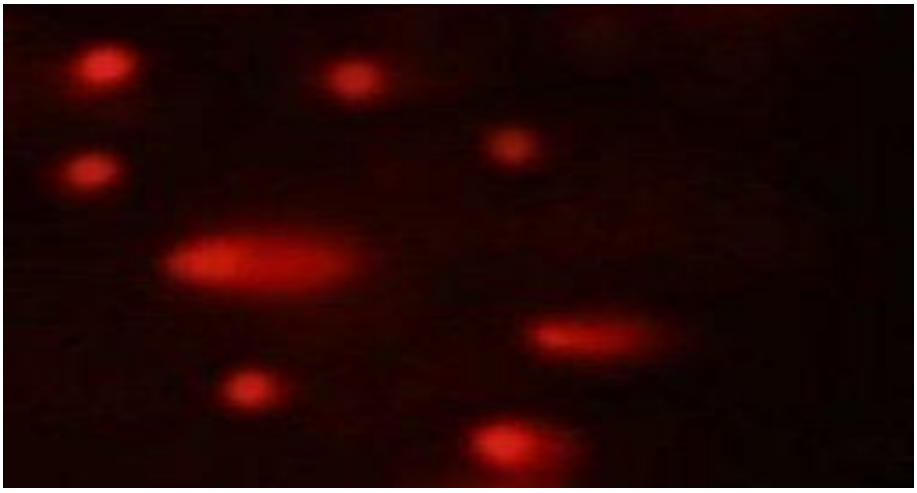

Figure 1: Visual score of DNA damage (classes 0, 1, 2 and 3) using comet assay in liver tissues of treated rats

Histopathological Results

Brain

Microscopic investigation of control brain sections of rats show highly active neurons which having huge pale-stained nuclei, nuclear chromatin and prominent nucleoli disappeared. The glial cells surrounded the neurons and support it. These cells have small densely stained nuclei with condensed chromatin and no visible nucleoli. Neuropil or background substances are shown in the cortex (Figure 2). Examination of sections of brain cortex of rats administered with CCl_4 alone showed dark neurons with irregular shape and glial cells that appeared inside white vacuoles. Neurofibrillary tangles stained with magenta color and looking like flames were founded. The tangle appears as long pink filaments in the cytoplasm. The neuropil is appeared vacuolated (Figure 3). Photomicrograph of section in brain cortex of rat administered with CCl_4 and *E. retusa* BT extract showing the structure of neurons appeared more or less like normal and regular shape (Figure 4). Photomicrograph of section in brain cortex of rat administered with CCl_4 and E. retusa ME extract showing dark neurons with irregular shape and surrounded by pericellular halos (blue arrows). No extracellular vacuoles are found in the neuropil (Figure 5).

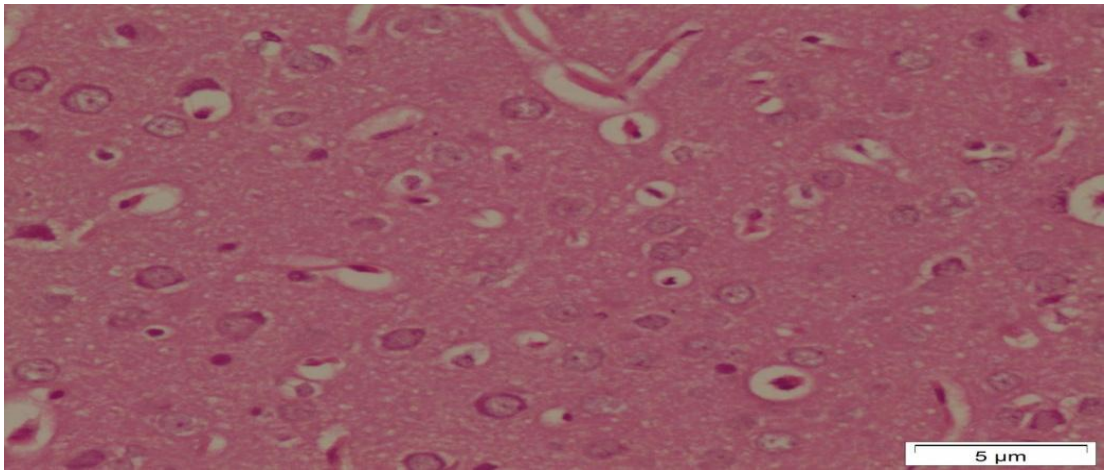

Figure 2: Photomicrograph of section in brain cortex of control rat shows the nerve cells (neurons) (blue arrow) that having pale-stained huge nuclei, disappeared nuclear chromatin and prominent nuclei, surrounding support cells (glial cells) (blue arrow head) having small nuclei with densely stained, condensed chromatin with no visible nucleoli, background substance (neuropil) (asterisk) and perivascular space are shown in the cortex (blue arrow) (H and E, Scale bar 5 µm).

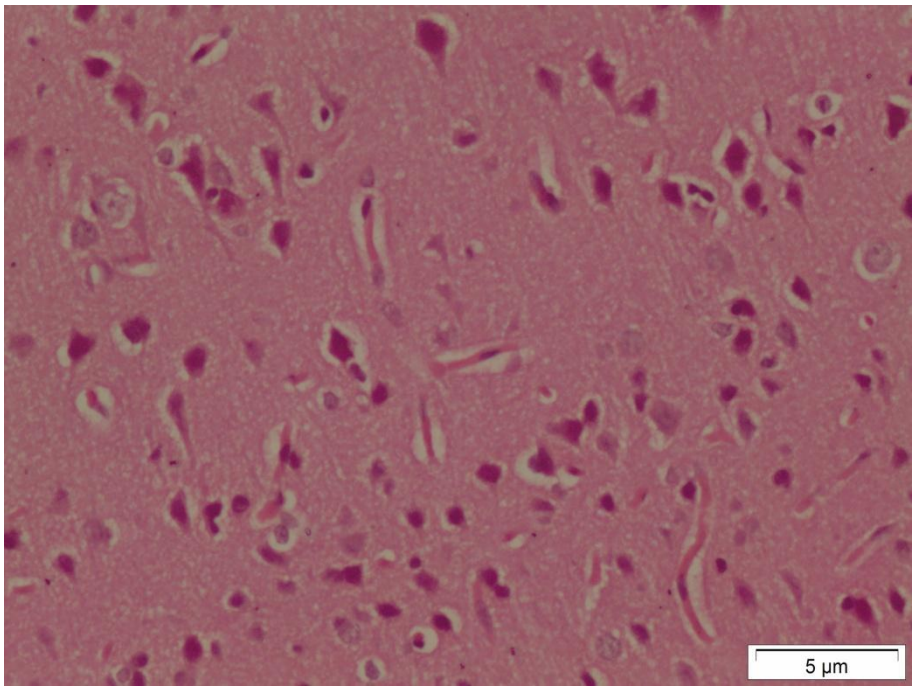

Figure 3: Photomicrograph of section in brain cortex of rat administered with CCl_4 alone showing dark neuron with irregular shape (arrows) and glial cells that appeared inside white vacuoles (arrowheads). Neurofibrillary tangles stained with magenta color and looking like flames were founded (red arrows). The tangle appears as long pink filaments in the cytoplasm (red arrows). The neuropil is appeared vacuolated (asterisk) (H and E, Scale bar 5 µm).

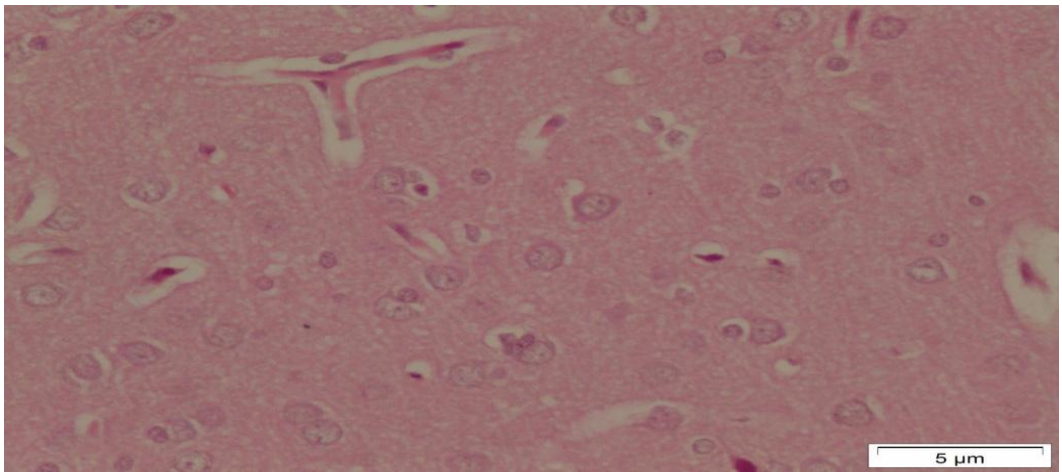

Figure 4: Photomicrograph of section in brain cortex of rat administered with CCl_4 and E. retusa extracts BT or ME showing the structure of neurons appeared more or less like normal and regular shape (blue arrows) (H and E, Scale bar 5 µm).

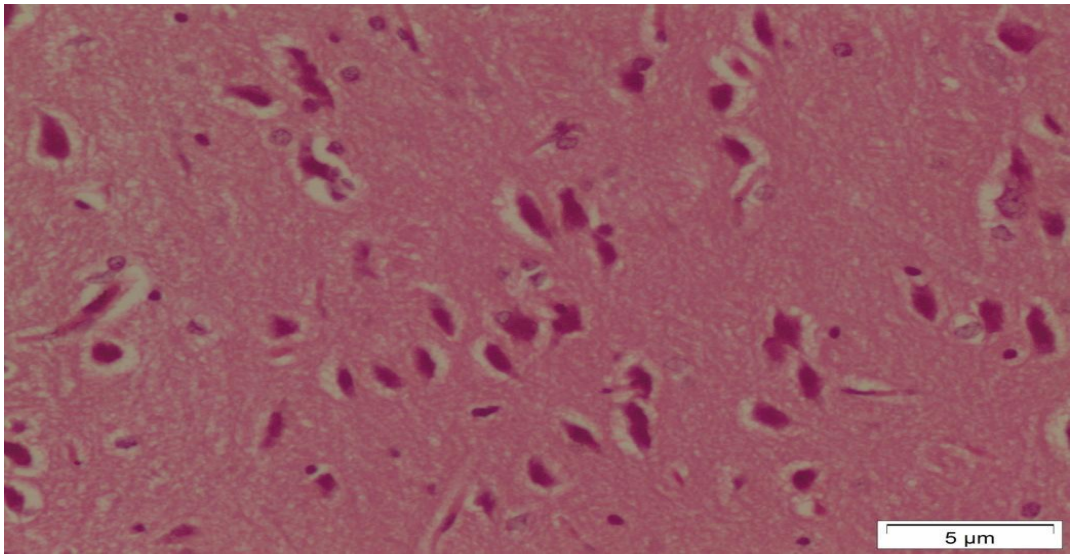

Figure 5: Photomicrograph of section in brain cortex of rat administered with CCl_4 and E. retusa extracts BT or ME showing dark neurons with irregular shape and surrounded by pericellular halos (blue arrows). No extracellular vacuoles are found in the neuropil (H and E, Scale bar 5 µm).

Liver

Microscopic examinations of sections of liver from normal control rats show the normal architecture of hepatic lobules. The central veins lies at the center of the lobules surrounded by cords of hepatocytes. Between the strands of hepatocytes, the hepatic sinusoids are seen (Figure 6). Histopathological investigation of liver from rats administered with CCl_4 alone showing disruption of the liver tissue with loss of lobular arrangement, bridging fibrosis with collagenous septa formation expanded portal tract to central vein with mononuclear cells, vacuolar degeneration and necrosis of hepatocytes (Figure 7 *).* Liver sections of rats administered with CCl_4 and E. retusa BT extract showing mild inflammatory cells infiltrations around central vein, vacuolar degeneration, and necrosis of hepatocytes. Binucleated and activated Kupffer cells were noticed (Figure 8). In case of rats administered with CCl_4 and E. retusa ME extract it was observed that liver section maintained hepatic architecture, with only few

inflammatory cells infiltrations around central vein, and centrilobular hepatic necrosis with mild vacuolar degeneration of hepatocytes (Figure 9).

In the present investigation, the biochemical findings were also confirmed by histpathological observations. The changes mostly include hepatocellular necrosis or apoptosis, fatty accumulation, inflammatory cells infiltration and other histological manifestations which were also consistent with the findings of other authors [36].

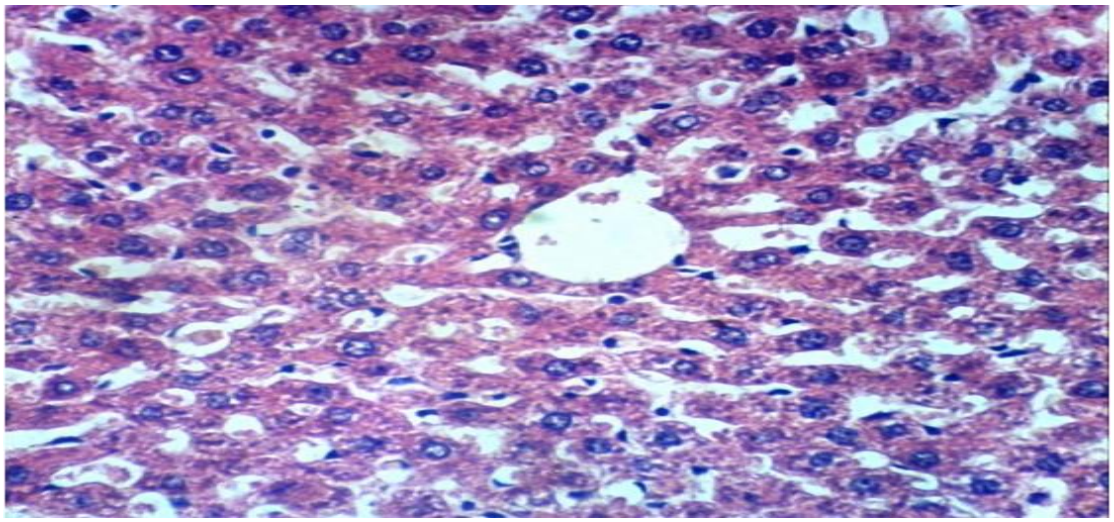

Figure 6: Photomicrograph of section in liver of control rat shows normal histological structure of hepatic lobules central vein, hepatocytes, blood sinusoids, and nuclei (H&E, ×400)

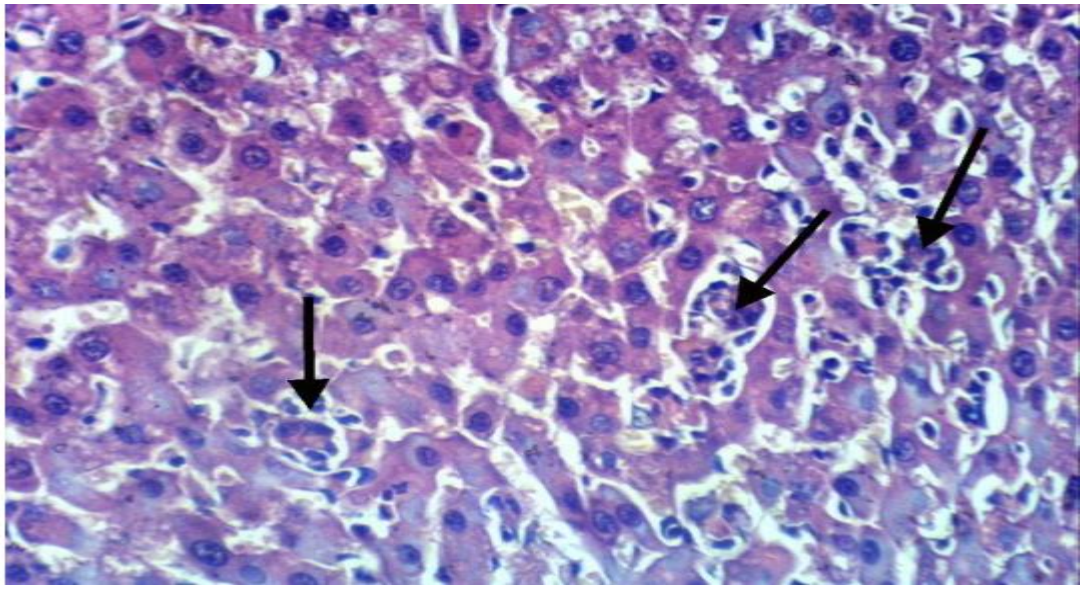

Figure 7: Photomicrograph of section in liver of rat administered with CCl_4 alone showing disruption of the liver tissue with loss of lobular arrangement, bridging fibrosis with collagenous septa formation expanded portal tract to central vein (arrow) with mononuclear cells, vacuolar degeneration and necrosis of hepatocytes (star). Dilated and congested central vein was observed (arrowhead) and pyknotic nuclei (H&E, ×400).

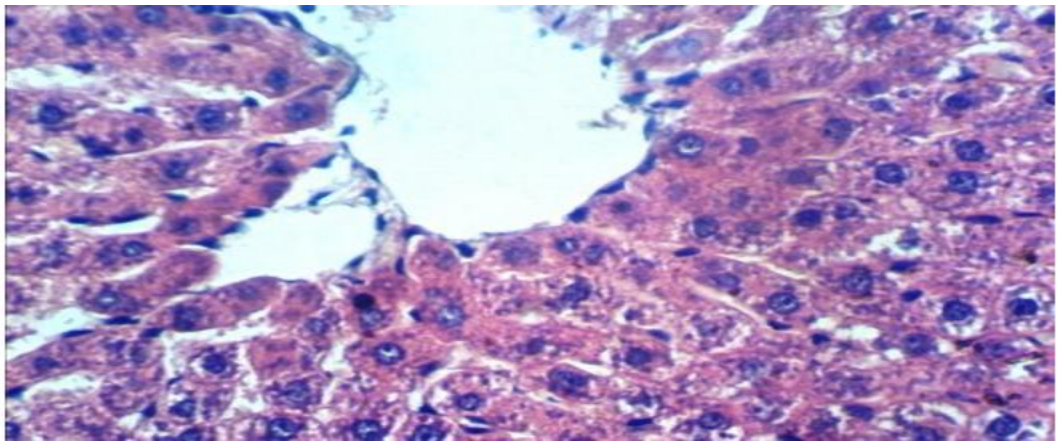

Figure 8: Photomicrograph of section in liver of rat administered with CCl_4 and E. retusa extracts BT showing showing mild inflammatory cells infiltrations around central vein (arrow), vacuolar degeneration, and necrosis of hepatocytes (star). Binucleated and activated Kupffer cells were noticed (H&E, ×400).

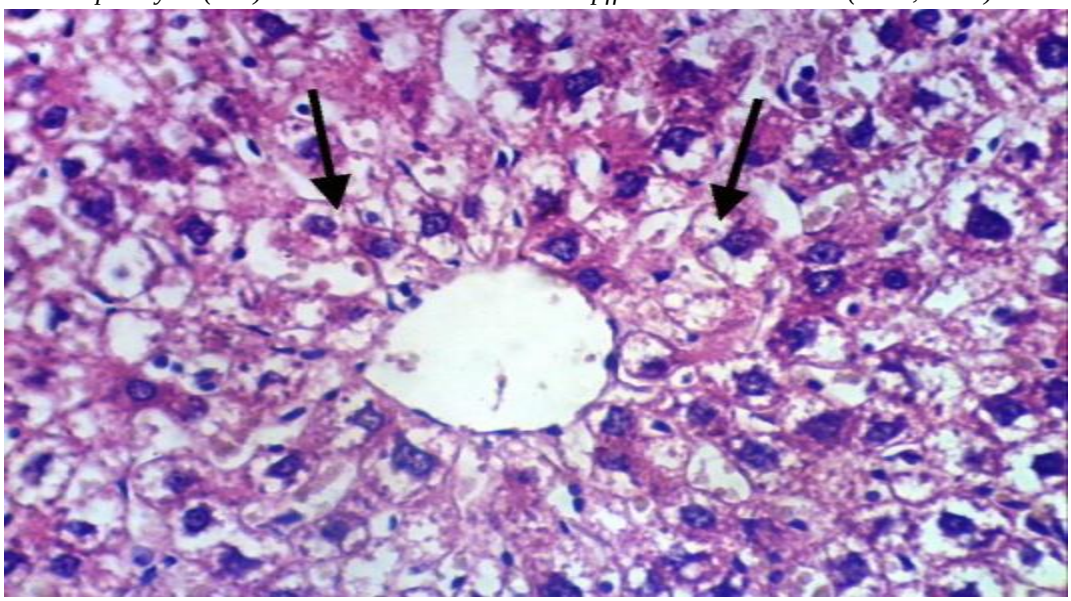

Figure 9: Photomicrograph of section in liver of rat administered with CCl_4 and E. retusa extracts ME showing maintained hepatic architecture, with only few inflammatory cells infiltrations around central vein (arrow), and centrilobular hepatic necrosis with mild vacuolar degeneration of hepatocytes (star). Dilated and congested central vein. Binucleiated and activated Kupffer cells were noticed (H&E, ×400).

Conclusion

Hepatoprotective effects of E. retusa extracts BT or ME on CCl_4-induced hepatic damage in male Wister rats were observed in the present study. Probably, antioxidative properties of the extract helped hepatic cells to obviate CCl_4-induced necrosis and inflammation which can be also observed in histopathological findings. The results obtained here and the reports from previous studies suggest that *Euphorbia retusa* extracts BT or ME may function as a good candidate for the treatment or prevention of liver failure. However, further investigations are required to unveil the molecular identification of the active ingredients and elucidation of the mechanisms involved in the effect.

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