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Hepatoprotective Effect of EllagicAcid Rich Extract of *Moringa Oleifera Leaves* on the CCl_4 Intoxicated Rats

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Abstract: **This study was designed to evaluate the hepatoprotective effect of methanolic extract of *Moringa oleifera leaves* (MOLE) on the CCL_4 intoxicated rats. The antioxidative effect on the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), lipid peroxidation (MDA) and reduced glutathione (GSH) were investigated in carbon tetrachloride (CCl_4) intoxicated rats pretreated with MOLE or Silymarin and α-Tocopherol. HPLC analysis of MOLE revealed that Ellagic acid was the most abundant phenolic compound. Pretreatment with MOLE or Silymarin significantly ($P < 0.05$) reduced the serum levels of AST and ALT and MDA in the liver of CCl_4 treated rats. Pretreatment with MOLE increased the antioxidant activity of liver GSH. Histological analysis of liver tissues in groups pretreated with MOLE and Silymarin showed mild necrosis and inflammation of the hepatocytes compared to the CCl_4-intoxicated rats.**

Keywords: **Hepatoprotective, liver damage, *Moringa oleifera*, Silymarin.**

1. INTRODUCTION

Moringa serves as a medicinal plant, animal fodder, and a food source for humans [1]. The leaves, fruit, flowers and immature pods of Moringa oleifera are used as a highly nutritive vegetable in many countries, particularly in India, Pakistan, Philippines, Bangladesh, Afghanistan and many parts of Africa. Moringa oleifera leaves act as a good source of natural antioxidants such as ascorbic acid, flavonoids, and phenolics [2,3]. Physiologically, the purpose of antioxidants is to scavenge reactive oxygen species, most commonly hydrogen peroxide, superoxide radical and hydroxide radical that are produced within a cell due to oxidative stress. These reactive oxygen species (ROS) oxidize vital cell components such as amino acids, polyunsaturated fatty acids besides it damage the DNA and inactivate specific enzymes [4]. Oxidative stress is considered as the important cause of liver injury in several liver disorders [5]. Defense mechanisms have evolved within the body to limit the levels of ROS and the damage they induce. Superoxide dismutase converts superoxide to H2O2 and catalase removes H2O2. Glutathione peroxidase destroys organic peroxides [6]. Many compounds and extracts from plants have been evaluated for hepatoprotective and antioxidant effects against chemically-induced liver damage [7].

[8] Demonstrated the protective effect of Moringa oleifera leaves hydroethanol (80%) extract at oral doses of 200 and 800mg/kg against the hepatotoxicity of paracetamol in rats. The extract reduced hepatic lipid peroxidation while restoring levels of the enzymes glutathione S-transferase, glutathione reductase , and glutathione per- oxidase to normal. [9]studied the hepatoprotective activity of Curcumin and Ellagic acid in comparison to Silymarin in the CCL4 intoxicated mice. Phytochemicals treatments caused a normalized serum aminotransferases activities, decreased level of MDA and improved the antioxidant status. The present study investigated the protective effects of Moringa oleifera leaves extract

against CCl4- induced hepatotoxicity in rats by assaying liver functions, hepatic lipid peroxidation and histopathology of liver tissues.

2. MATERIALS AND METHODS

A. Plant materials:

Five kilograms of fresh leaves of Moringa oleifera were collected, identified and authenticated in the Egyptian Scientific Society of Moringa, Cairo, Egypt (2013) for its DNA against authenticated Moringa sample DNA. The fresh leaves were washed twice with distilled water and shade dried for 72 hours. The dried leaves were weighed and powdered using a domestic mixer grinder into a coarse powder, crouched and weighed.

B. Preparation of the extract:

Each 100 g dried leaves were extracted as mentioned by [10].The extract was stored in a refrigerator at 4 °C. The extract was analyzed for types and concentrations of polyphenolic compounds .the concentration of each phenolic compound was calculated as % of the leaves dray weight and also % of each compound was calculated as % of total identified phenolic compounds.

C. HPLC analysis:

Phenolic acids of the sample extract were determined using a Shimadzu HPLC instrument for detection of phenolic acid , at 320 nm using UV-VIS detector according to [11]. Identification was carried out by comparing the retention time with that of the external standard.

D. Experimental animals:

Forty eight adult male Sprague Dawley albino rats weighing 180-200g were purchased from the Egyptian Organization for Biological Products and Vaccines. The animals were housed individually in stainless steel cages and maintained on a 12 h light–dark cycle at 25 ± 2˚C and 60 % humidity. Animals were fed ad libitum stock basal diet [12]and water. Animals were allowed to acclimate to housing conditions in the Research Institute of Ophthalmology in Cairo, Egypt for 7 days prior to experimentation. Animals were maintained and handled according to [13].

E. Experimental design:

Plan of the experiment was conducted according to [14,15]for induction of hepatotoxicity with CCL4, and according to [16]for Silymarin treatment, [17,18] for Moringa oleifera extract treatment and [15]for α-Tocopherol treatment. The animals were grouped into six groups comprising 8 animals in each group as follows:

-Group 1 (G 1): (Negative control)

Rats were fed on a stock basal diet and received distilled water containing 0.3% sodium carboxy methyl cellulose (CMC-Na) (1 ml / Kg b.w. ,p.o.) daily for 10 days , and olive oil (1 ml / Kg b.w., s .c.) on days 2,4,6.

-Group 2 (G 2):

Rats were fed on a stock basal diet and received 0.3% (CMC-Na) (1 ml / Kg b.w., p. o.) daily for 10 days , and treated with a mixture of CCL4 and olive oil (1:1 , 2 ml / Kg b.w. ,s .c.) on days 2,4,6.

-Group 3 (G 3):

Rats were fed on a stock basal diet and treated with α-Tocopherol (50 mg / Kg b.w., p.o.) daily for 10 days , and received CCl4 – olive oil mixture (1:1, 2 ml /Kg b.w.,s.c.) on days 2,4,6 after 30 min of administration of vitamin E .

-Group 4 (G4):

Rats were fed on a stock basal diet and treated with standard drug Silymarin (150 mg / Kg b.w., p.o.) daily for 10 days, and received CCl4 – olive oil mixture (1:1 , 2 ml / Kg b.w., ,s. c.) on days 2,4,6 after 30 min of administration of Silymarin .

-Group 5 (G 5):

Rats were fed on a stock basal diet, and treated with MOLE containing (30 mg Ellagic acid /Kg b.w., p.o.) daily for 10 days , and received CCl4 – olive oil mixture (1: 1, 2 ml / Kg b. w., s.c.) on days 2,4,6 after 30 min of administration of MOLE .

-Group 6 (G 6):

Rats were fed on a stock basal diet, and treated with MOLE containing (50 mg Ellagic acid /Kg b.w., p.o.) daily for 10 days , and received CCl4 – olive oil mixture (1:1 , 2 ml /Kg b.w., s.c.) on days 2,4,6 after 30 min of administration of MOLE.

On day 11, blood samples were collected from retro-orbital plexus vein of all rats , and kept for about half an hour at room temperature before centrifugation at 3000 rpm for 10 min and the clear serum was separated and stored at -20˚C for analysis. Animals were then sacrificed by bleeding. Liver samples were dissected and immediately washed with ice-cold saline to remove blood, dried between filter papers and weighed. One gram of liver tissue was homogenized in 10 mL cold buffer (50 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA), using tissue homogenizer. The liver homogenate was centrifuged at 4000 rpm for 15 min at 4˚C. The supernatant was collected and stored at -80˚C for analysis.

F. Biochemical determinations:

Blood serum was used for the evaluation of alanine aminotransferase and aspartate aminotransferase (ALT and AST) activities, according to the method of [19]. The clear supernatant of liver homogenate was used for the determination of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH). The SOD activity was determined according to the method described by [20].Determination is based on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitro blue tetrazolium dye. Reduced GSH in liver tissues was determined according to the method described by [21]which measures the reduction of 5, 5`dithio-bis (2- nitrobenzoic acid (DTNB)) by SH groups to form a stable yellow color of 2-nitro-5-mercaptobenzoic acid which was measured colorimetrically at 412 nm using Jenway 6405 / vis Spectrophotometer . Lipid peroxidation content was determined as malondialdehyde (MDA) according to the method described by[22]. MDA reacts with thiobarbituric acid (TBA) in acidic medium at 95°C for 30 min giving a colored TBA complex that was measured calorimetrically at 520-535 nm using Unicaum Spectro-photometer. Biochemical determinations for ALT, AST were carried out using assay kits obtained from Bio diagnostic company, Cairo, Egypt according to the manufacturer's protocols.

Histopathological examination of liver sections:

A portion of the liver tissue was removed, fixed in buffered formalin and embedded in paraffin blocks. Tissue sections (5 µm) were prepared and stained with Hematoxylin and Eosin (H & E) stain for microscopic examination using magnification power × 400 of the light microscope according to [23].

3. STATISTICAL ANALYSIS

All experiments were done in triplicate and results were reported as mean ± standard deviation. The data were analyzed by one-way ANOVA; statistically significant effects were further analyzed and means were compared using Duncan's multiple range tests [24]. Statistical significance was determined at $p < 0.05$.

4. RESULTS AND DISCUSSION

Polyphenols composition of Moringa oleifera leaves methanolic extract HPLC analysis of Moringa oleifera leaves methanolic extract (MOLE) is illustrated in Table 1

<Table 1>

Results in Table 1 showed that 17 phenolic compounds were identified in the MOLE. Ellagic acid was the most abundant phenolic compound followed by benzoic acid and Pyrogallol and the those three phenolic compounds represent 80 % of the total identified compounds. Ellagic acid represents 52.7% of the total identified polyphenolic compounds (794.36mg / 100 g dry weight) of the investigated extract; benzoic acid represent 13.6 % and Pyrogllol represent 13.5 %of the total identified polyphenolic compounds. Other polyphenolic compounds such as epicatechin; chlorogenic acid; p-hydroxy benzoic acid and vanillic acid are present in low concentrations. These findings are in agreement with that reported by [25] for Ellagic acid, Gallic acid and Vanillic acid. Variations in the concentrations of other polyphenols could be due to differences in variety, location, genotype and the extraction method.

Biochemical examination:

Results in Table (2) indicate the effect of different doses of Moringa oleifera leaves methanolic extract (MOLE) (30 and 50 mg Ellagic acid /Kg b.w.), α-Tocopherol (50 mg / Kg b.w.) and Silymarin (150 mg / Kg b.w.) against CCL4.

<Table 2>

The effects of administration of Moringa oleifera leaves 100 % methanolic extract at two dose levels (30 and 50 mg Ellagic acid /kg b.w.), on relative liver weight, AST and ALT serum enzymes and GSH, SOD and MDA in the liver of CCl4-intoxicated rats are shown in Table 2. Results in Table 2 indicate that there were no significant differences of the relative liver weight between the experimental groups treated with CCl4. However, the rats in the CCl4-intoxicated groups exhibited significantly ($p< 0.05$) higher relative liver weight when compared to rats in the control group.

Administration of CCl4 induced hepatic injury as shown by a significant elevation in serum levels of AST and ALT and MDA of liver by 3 to 7 folds and significant reduction in the activities of GSH and SOD of the liver of the investigated rats. These results are in agreement with those reported by [26,9]. Results in Table 2 reveal that administration of α-Tocopherol, Silymarin or MOLE lowered significantly the ALT and MDA levels that increased by the toxicity of CCl4. Silymarin (150 mg / Kg b.w.) was found to be significantly more effective than α-Tocopherol (50 mg / Kg b.w.) in reducing the levels of ALT and AST. Administration of MOLE (at 50 mg Ellagic acid /Kg b.w.) was found to be significantly close to the effect of Silymarin in reducing the AST level. The pretreatment with α-Tocopherol, Silymarin or MOLE (at 50 mg Ellagic acid /Kg b.w.) for 10 days, caused significant increase in the GSH and SOD liver levels that decreased by the toxicity of CCl4. Pretreatment with Silymarin (150 mg / Kg b.w.) significantly recovered these antioxidant enzymes levels to those of the control rats (Group1). On the other hand, pretreatment with α-Tocopherol (50 mg / Kg b.w.) significantly diminished lipid peroxidation. The 100 % methanolic extract used in this study showed a remarkable potentiality in restoring some physiological effect. This indicates a possible regeneration and recovery the damaged liver cell in animals treated with the extract. [27] suggested that the hepatoprotective effect against toxicity induced by CCl4 may be attributed to a decrease in the liver lipid peroxides and enhanced antioxidants levels.Ellagic acid, is a powerful bioactive compound with many potential pharmacological and industrial applications [28].

5. HISTOPATHOLOGICAL FINDINGS

The liver of control rats (Fig. 1) showed the normal histological structure of hepatocytes, hepatic cords, central vein and sinusoid. Liver of rats intoxicated with CCL_4 (Figs. 2 and 3) showed harmful effects on liver tissues the included , fatty changes of hepatocytes with the characteristic signet ring appearance (short arrow) and mono nuclear cell infiltration (tall arrow). Liver of rats intoxicated with CCL_4 and treated with standard drug α-Tocopherol (50 mg / Kg b.w., p.o.), (Fig. 4) showed fatty changes of hepatocytes (short arrow) with adjacent hepatocytes (tall arrow). Liver of rats intoxicated with CCL_4 and treated with standard drug Silymarin (150 mg / Kg b.w., p.o.) (Fig.5) maintained hepatic architecture with minimal fatty changes of hepatocytes. Liver of rats intoxicated with CCL_4 and treated with low dose of MOLE (containing 30 mg Ellagic acid/Kg b.w., p.o.) caused minor improvements in (Fig.6) and showed permanent fatty changes (tall arrow) and mono nuclear cell infiltration (short arrow) with adjacent necrosis of hepatocytes (double head arrow). Liver of rats intoxicated with CCL_4 and treated with high dose of MOLE (containing 50 mg Ellagic acid /Kg b.w., p.o.) showed obvious improvement in hepatocytes cords (astrisks) with few areas of fatty changes of hepatocytes (Fig. 7).

These results indicated that the use of high dose of MOLE as a natural source of hepatoprotective phenols could reduce the toxic effects of CCL_4 compared to some chemical drugs, which may cause side effects.

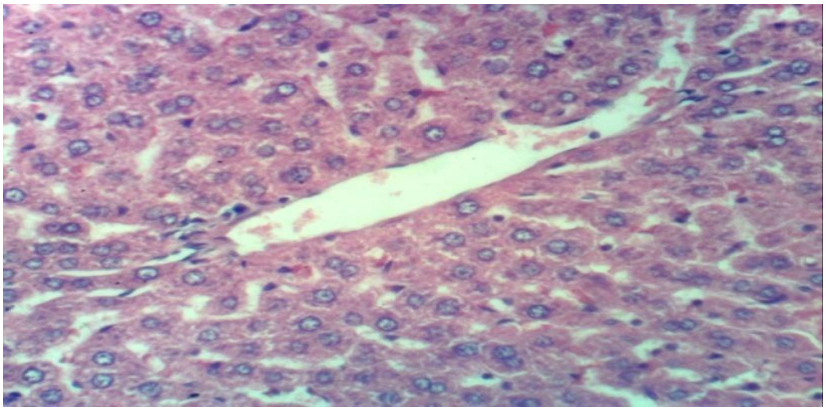

Fig. (1): Photomicrograph of liver of rat (Control group) showing the normal histological structure of hepatocytes, hepatic cords, central vein and sinusoids. (H & E X 400).

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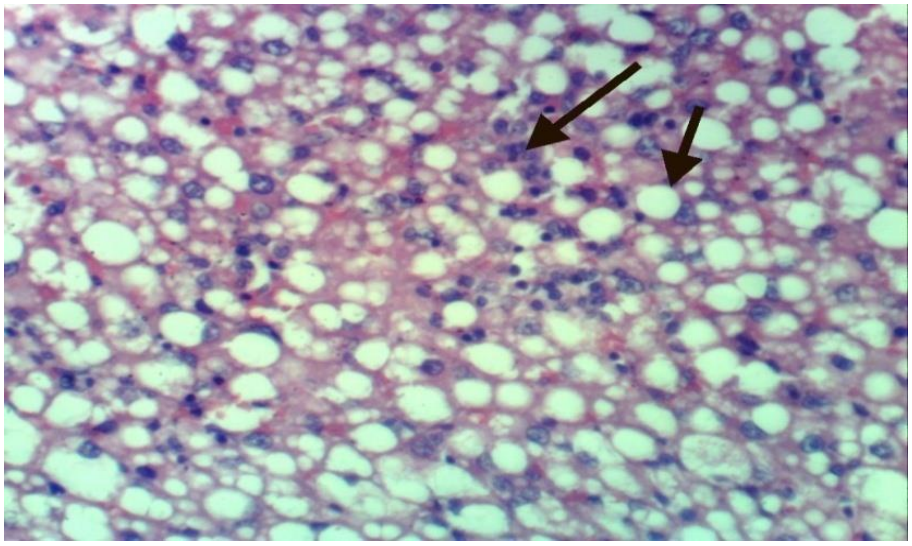

Fig. (2): Photomicrograph of liver of rat treated with CCL_4 and olive oil(1:1, 2 ml / Kg b.w., s.c.) on days 2,4,6. (H & E X 400).

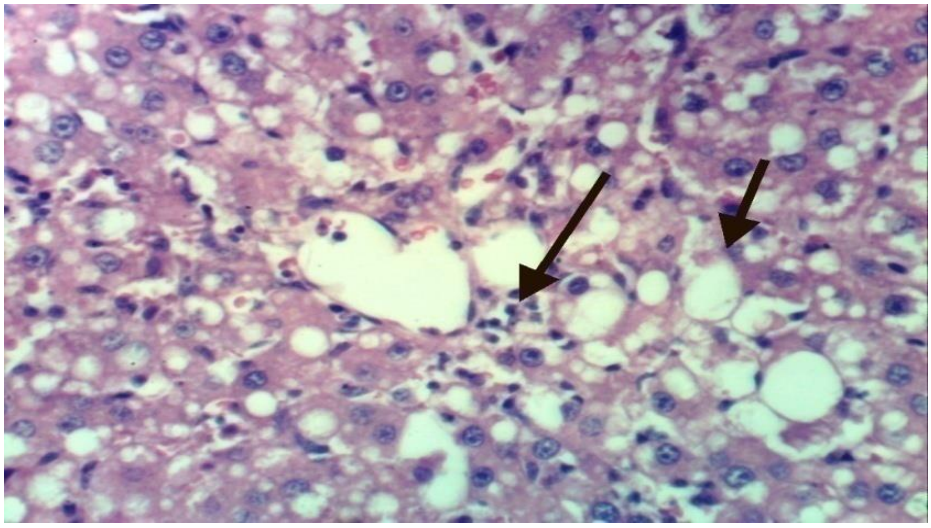

Fig. (3): Photomicrograph of liver of rat treated with CCL_4 and olive oil (1:1, 2 ml / Kg b.w., s.c.) on days 2,4,6. (H & E X 400).

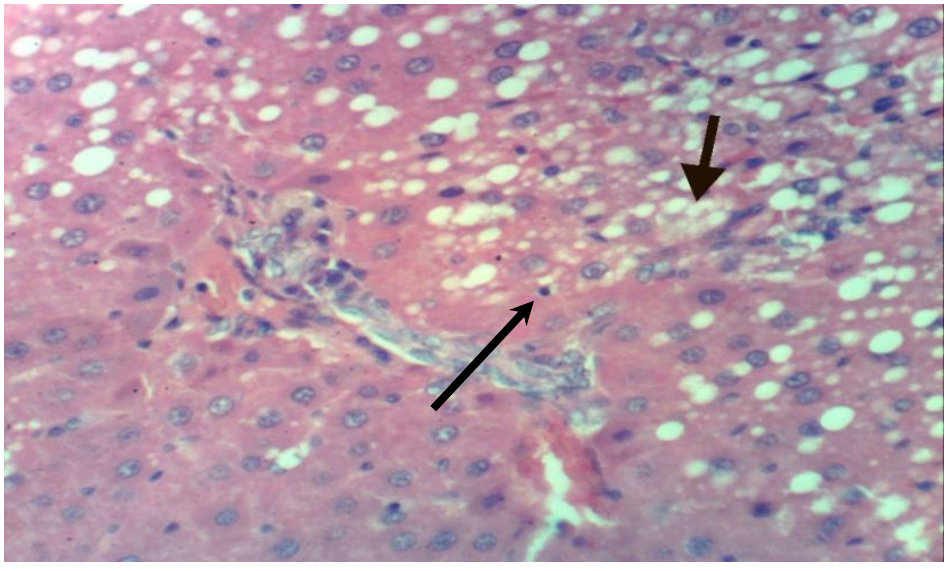

Fig. (4): Photomicrograph of liver of rat treated with α-Tocopherol (50 mg / Kg b.w., p.o.) daily for 10 days and received CCl_4 - olive oil mixture (1:1, 2 ml / Kg b.w., s. c.) on days 2,4,6. (H & E X 400).

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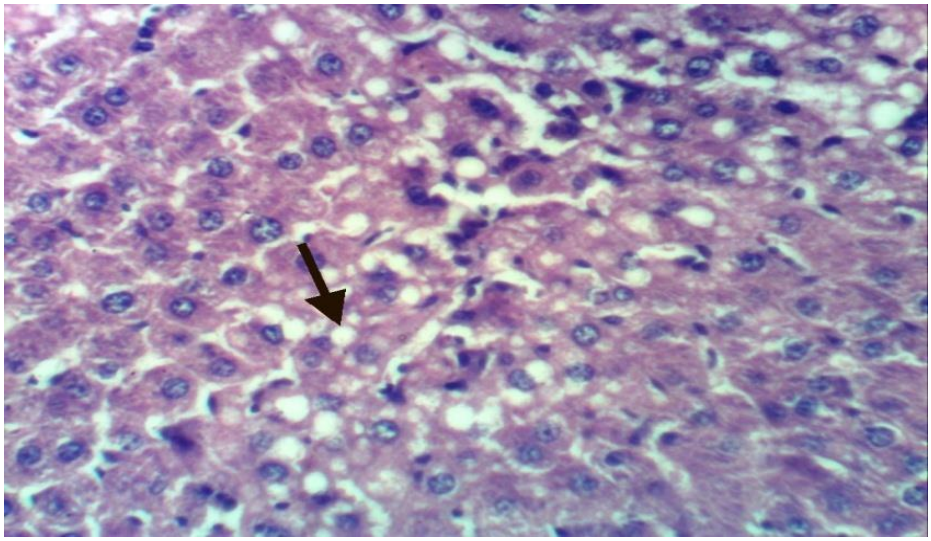

Fig. (5): Photomicrograph of Liver of rat treated with standard drug Silymarin (150 mg / Kg b.w., p.o.) daily for 10 days , and received CCl_4 - olive oil mixture (1:1, 2 ml / Kg b.w., $_{S.C.}$) on days 2,4,6. (H & E X 400).

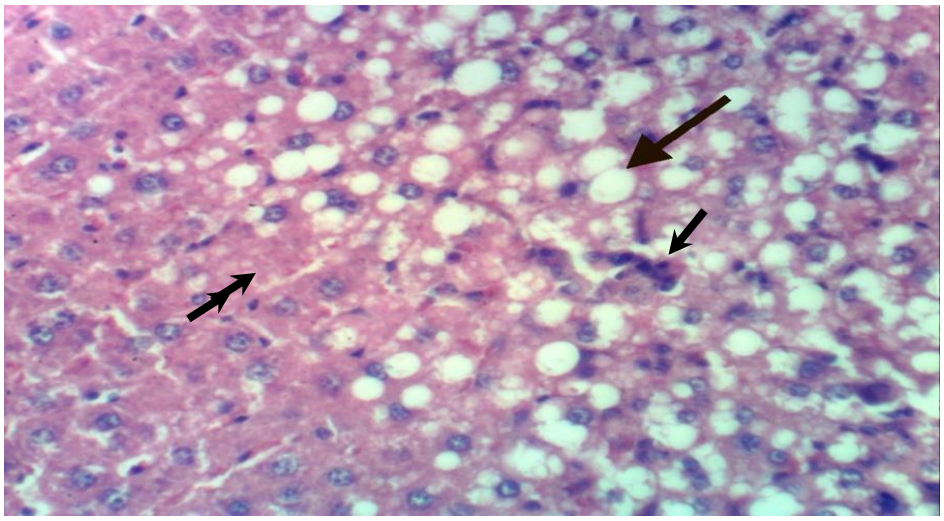

Fig. (6): Photomicrograph of liver of rat treated with MOLE (containing 30 mg Ellagic acid /Kg b.w., p.o.) daily for 10 days , and received CCl_4 – olive oil mixture (1:1 ,2 ml /Kg b.w., s. c.) on days 2,4,6. (H & E X 400).

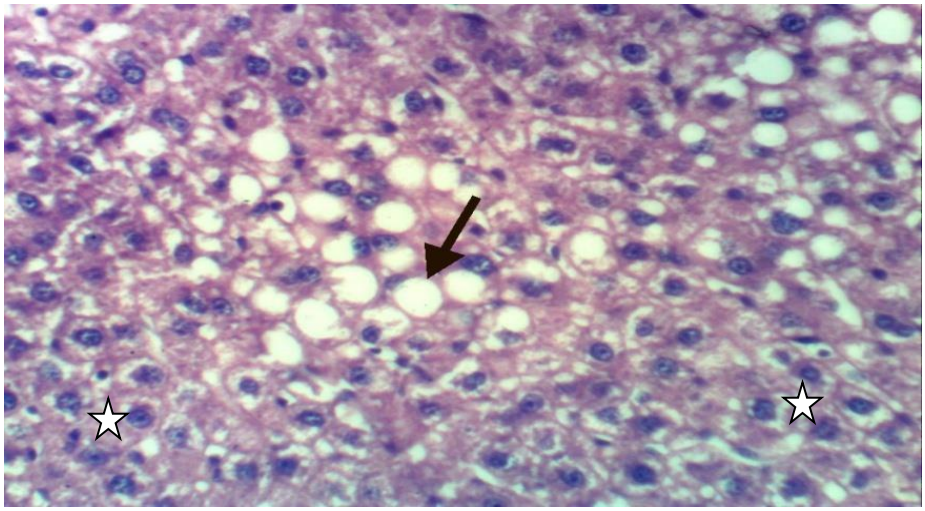

Fig. (7): Photomicrograph of liver of rat treated with MOLE extract (containing 50 mg Ellagic acid /Kg b.w., p.o.) daily for 10 days and received CCl_4 – olive oil mixture (1:1 , 2 ml /Kg b.w., s. c.) on days 2,4,6. (H & E X 400).

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Table (1): Polyphenolic compounds of *Moringa oleifera* leaves (mg/100g dry weight) and % of each compound compared to the polyphenolic compounds Identify .

Identified polyphenolic compounds	Concentrations (mg/ 100 g dry weight)	% of polyphenolic compounds Identified
Ellagic Acid	418.5	52.7
Benzoic	108.16	13.6
Pyrogllol	107.60	13.5
Epicatechen	32.40	4.07
Chlorogenic acid	27.36	3.44
P.OH.benzoic	21.21	2.67
Vanillic	20.18	2.5
Catechol	12.02	1.5
Protocatechuic	10.7	1.3
Coumarin	8.72	1.09
Gallic acid	8.61	1.08
Feruillic	7.97	1.0
Cinnamic	4.19	0.52
Catechein	3.21	0.4
Caffeic acid	2.15	0.27
4-Aminobenzoic	0.98	0.12
Salicylic	0.40	0.05
Total Identified polyphenolic compounds	794.36	100.00 %

Table (2): Changes in relative weight of liver, levels of serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) , liver glutathione(GSH), liver superoxide dismutase (SOD) and liver malondialdehyde (MDA) in normal and experimental rats.

Group / Parameters	Vehicle (control)	Vehicle + CCl_4	Tocopherol + CCl_4	Silymarin + CCl_4	MOLE (30 mg Ellagic acid) + CCl_4	MOLE (50 mg Ellagic acid) + CCl_4
Relative weight of liver	c 2.78 ± 0.16	ab 3.44 ± 0.31	a 3.67 ± 0.36	ab 3.26 ± 0.26	ab 3.50 ± 0.25	b 3.26 ± 0.22
ALT (U\L)	d 19.29 ±4.03	a 148.57 ±16.10	b 120.42 ±19.05	c 61.29 ±15.46	b 118.29±25.84	b 102.0 ±7.30
AST (U\L)	c 54.14±8.38	a 173.43 ±12.22	a 143.14 ± 15.12	b 128.57 ±2.15	a 137.43 ±11.56	b 122.0 ±9.52
GSH (mg / g liver)	a 1.49 ± 0.28	d 0.76 ±0.172	bc 1.28 ± 0.163	ab 1.37 ± 0.107	d 0.88 ± 0.130	c 1.14 ±0.136
SOD (U/mg liver)	a 39.75± 3.016	d 19.11 ± 3.279	bc 33.88± 3.913	ab 35.18 ±5.120	d 21.58 ± 5.997	c 29.98±2.743
MDA (n mol/g liver)	d 24.58 ±7.62	a 77.67± 9.032	cd 31.91 ± 8.076	c 39.43 ±12.15	b 59.10 ± 0.066	b 54.64±4.267

Means with the same letter in the same row are not significantly different at 0.05 level of significance.
Data are expressed as mean ± SD. Each group consisted of 8 rats.
Relative weight of liver = Liver weight / Final body weight of rat.

6. CONCLUSION

In this study revealed that Ellagic acid (EA) was mast abundant phenolic compound in Methanolic extract of Moringa oleifera leaves (MOLE) we have attempted to evaluate the hepatoprotective activity of MOLE in comparison to Vit E (α-Tocopherol) and standard drugs (Silymarin) in carbon tetrachloride (CCL4) in deuced acute liver toxicity in rats . Administration of MOLE (containing 50 mg EA / Kg. b.w.) showed a remarkable potentiality in restoring some physiological effect. This indicates a possible regeneration and recovery the damage liver cell in animal treated with MOLE.

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