

Long- term safety of Eruca sativa leaf ethanolic extract on Male Rats

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Research Article

Keywords: Eruca sativa, kidney function, liver function, apoptotic marker

Posted Date: March 9th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1370620/v1>

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Abstract

The present study was performed to evaluate the safe dose of ethanolic *Eruca sativa* leaves extract in healthy adult male rats. Twenty-five male Wister rats were divided into 5 groups: Group I (Control): received no treatment, groups 2, 3, 4, and 5 received 1, 3, 5, and 6 g/kg BW orally per day for 35 days. On the 36 day, rats were euthanized. Body weights were recorded. Serum biochemical analysis and the expression level of liver and kidney p53 and Bcl-2 were determined. Histopathology of liver and kidney, and TNF- α immunohistochemistry were examined. The ethanolic extract revealed the content of phenols, flavonoids, and fatty acids. Determination of biochemical parameters showed no significant difference in the estimated parameters between control and treated groups. While the treated groups did not show any significant changes expressions of p53 and Bcl-2 as observed by histopathological and immunohistochemical examination as compared to the control group, liver and kidney toxicity occurred for the rat groups administered 6g/kg. In conclusion, *Eruca sativa* is not toxic and there were no structural and functional changes in the liver and kidney when administered the doses of 1–5 g/Kg BW over a 35-day period through its potent antioxidant activity in rats and that toxicity that occurred in the 6g/kg-day group may be caused by a high concentration of glucosinolates.

Introduction

Non toxicity is one of the criteria set by the World Health Organization for the use of herbs as medicines. Medicinal plants have been considered a major source of therapeutic agents in traditional medicine for the treatment of human diseases because they are natural medicines are healthier than synthetic medicines (Cragg and Newman 2005).

Eruca sativa is an edible annual plant, commonly used as a salad and a spice. A wide range of phytochemicals have been recognized in the *Eruca sativa* such as fatty acids, phenolic acids, terpenes, alkaloid, glycosides, saponins, sterols, carbohydrates, vitamin A, and vitamin C. *Eruca sativa* is a rich source of minerals such as Calcium, Manganese, Potassium, Sodium, Iron, Copper, and Zinc, and secondary metabolites; glucosinolates (GSLs) which has anti-carcinogenic activity (Barlas et al. 2011; Chun et al. 2013; Kishore et al. 2017).

Eruca sativa has been shown to have many pharmacological activities against different diseases (El-Missiry et al. 2000; Alarcón et al. 2014; Shatta, 2014; Edrees et al. 2015, Mashi , 2017, Aabdul-jabbar, 2018).

Teratogenicity studies show that, *Eruca stavia* seed oil extract at doses of 0.25, 0.50 ml/kg administered to pregnant rats causes a mutagenic effect in bone marrow and cells of fetus. Low dose of *Eruca sativa* seed oil were not associated with teratogenic changes, while high dose cause some abnormalities (Moustafa and El-Makawy 2002).

Several studies have been shown that, glucosinolates were used in the treatment of cancerous diseases (Eisa et al. 2015; Sa'vio et al. 2015; Yang et al. 2016). But Akagi et al. (2003) and Sugiura et al. (2003)

showed that, the use of these glucosinolates (0.1% in the diet) alone as synthetic compounds resulted in toxicity of normal cells. Since *Eruca sativa* contains glucosinolates besides other component as phenols, polyphenols and fatty acids which acts as antioxidants and protect the cell from damage, but these compounds necessitate high doses in order to manifest such effects that may not always be achievable through the daily diet. So, this study aim to evaluate the safety effective dose of ethanolic extract of *Eruca sativa* leaves on adult male rats hepatorenal structure and functions.

Materials And Methods

Preparation of the ethanolic *Eruca sativa* leaves extract (ESLE)

Ethanolic extract was prepared according to the method of **Banso (2009)**. 2.2 kg powder of *Eruca sativa* shade dried leaves was extracted with 15 liter of 95% ethanol, the mixture was shaken by using a magnetic stirrer for 3hr/ daily and allowed to stand for 21 hr for three days, the mixture was filtered on Wattman filter paper # 45, hence they was re-extracted with 9 liter 95% ethanol and filtered. The soluble ethanol extract was concentrated to dryness under reduced pressure at 60°C. Solvent elimination of the extract was weighed and finally gave 270g. The percentage yield was calculated using this formula: (weight of extract/original weight × 100 giving 12.16% yields) of green fatty crude *Eruca sativa* leaf ethanol extract. The plant extract was stored in 4°C in the refrigerator. The extract was suspended in distilled water before administration.

Determination of total phenolic and flavonoid content

The total phenolic and flavonoid content was determined according to the method (**Zilic et al. 2012**).

Determination of total antioxidant activity by DPPH and ABTS

Free radical scavenging capacity was determined according to method (**Hwang and Do Thi 2014**).

Determination of fatty acids using GC-MS

Esterification of fatty acids were made according to (**Marchetti and Errazu 2008**). Fatty acids methyl esters were analyzed on Agilent Technologies 7890B GC equipped with Zebron ZB-FAME capillary column (60 m x 0.25 mm internal diameter x 0.25 μm film thickness; Agilent Technologies, Little falls, CA, USA) and a flame ionization detector. The injector and detector temperatures were set at 250 °C and 285 °C, respectively. The column temperature programmed; initial temp. 100 °C for 3 min; rising at 2.5 °C /min to 240 °C and held for 10 min. The flow rate of carrier gas (H₂) was 1.8 ml/min. A sample of 1.0 μl was injected, using split mode (split ratio, 1:50). Components of fatty acids were identified by comparing their retention times with their fatty acids methyl ester standard (GC-MS) spectra from a library (MassHunter GC/MS Acquisition B.07.03.2129).

Animals and dosing

Twenty five healthy adult male Wistar rats, weighing 90-120 g were obtained from the Institute of Ophthalmic Disease Research, Cairo, Egypt. Animals were housed in stainless steel cages in an artificially illuminated and thermally controlled room (22- 25°C and 12 h light / dark cycle). The animals were kept a week for acclimation. Experimental protocol approved by the Animal Ethics Committee of Mansoura University, Egypt.

Experimental protocol

Rats were randomly distributed five animals per cage. The first group was the control group without any treatment. The second, third, fourth, and fifth groups received 1, 3, 5, and 6 g/kg BW a daily dose of ESLE by gastric intubation for 35 days. The treated and control rats were weighed on day 7, day 14 and day 35, and the weight changes were calculated as percentages of body weight. On day 36, all lived rats were anaesthetized with halothane and sacrificed. Serum was separated after centrifugation at 860 xg for 20min. Liver and kidney were removed, washed with 0.9%NaCl solution and then wiped on a piece of filter paper. Tissues were washed with 50 mM (sodium phosphate buffer saline pH 7.4) in ice-containing 0.1 mM EDTA to remove any RBCs and clots. The liver and kidney weighed, each of them was divided into 3 portions, the first was fixed in 10% formaldehyde for sectioning and histopathological examination, the second was used for water free calculation, and the third portion were kept at -80°C for the determination of p53 and Bcl₂.

Estimated biochemical parameters:

The following parameters were determined using a serum biochemistry analyzer (AU400, Coulter, Beckman, USA): Total bilirubin (TBIL), Total protein (TP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Glucose (GLU), Cholesterol (CHOL), Triglycerides (TG), Blood urea nitrogen (BUN), Creatinine (CREA), Uric acid (UA), Sodium (Na), Potassium (K), Calcium (Ca), and Phosphorous (P).

Flow cytometric analysis of p53 and Bcl-2 proteins

Kidney and Liver tissue homogenate p53 and Bcl₂ protein were prepared with a phosphate buffer saline (PBS)/bovine serum albumin (BSA) buffer, the cells were incubated with mouse anti FITC p53 (Cat.No. 554218, BD Biosciences, Bicton, Dickinson, CA, USA) and with mouse anti FITC bcl-2 (Cat. No. 554221), mixed well and incubated for 30 min at RT. The cells were washed and resuspended in 0.5% paraformaldehyde in PBS/PSA and analyzed using a flow cytometry. Fluorescent histograms were derived from gated events with side and forward light-scattering characteristics of viable cells. Flow cytometry was performed on BD FACScanTM system (Becton Dickinson, SanJose, CA).

Histopathological examination

The liver and kidney tissues were fixed, embedded in paraffin wax, then sections 4µm thick were prepared and stained with Mayer's Haematoxylin and Eosin (H&E) stains for microscopic examination.

Immunohistochemical assay

For immunohistochemical assay, 4µm deparaffinized thick tissue section were used according to the method of **Magaki et al. (2019)**. The deparaffinized slices were rehydrated in descending grades of alcohol, incubated in 3% hydrogen peroxide, washed in PBS, and placed in 0.01mol/L citrate buffer (pH 6) in a microwave for 5 min., incubated in 1% BAS/PBS for 30 min. at 37° C, and incubated with the rabbit polyclonal anti-tumor necrosis factor alpha antibody (Anti-TNF α antibody (ab34674, 1:500, IHC-P, Abcam, Cambridge, Massachusetts) at 4°C overnight. Endogenous peroxidase activity was blocked by incubation in 0.075% hydrogen peroxide in PBS. For antibody PK-7800 (VECTOR Laboratories, CA) for 1h at RT, incubated with diaminobenzidine (DAB) tetrahydrochloride for 10 min. Sections were washed with distilled water and counterstained with hematoxylin, dehydrated with graded alcohols and xylene, and mounted on slides. The immunostaining intensity and cellular localization TNF- α were determined using light microscopy.

Statistical analysis

The obtained data were analyzed using statistical package for social science, version 23 (SPSS Software, SPSS Inc., Chicago, USA) and expressed as means ± standard error (Mean± SE). The significance were performed using analysis of variance (One Way ANOVA) and followed by Scheffe multiple-comparisons test. For data with Gaussian distribution with non-homogeneity of variances, statistical analysis were performed using analysis of variance (One Way ANOVA) followed by Dunnett's T3 test. For parameters with non-Gaussian distribution, Kruskal–Wallis test was employed followed by Mann-Whiney U test for multiple comparisons. Differences were considered significant at $p \leq 0.05$.

Results

Table 1 shows the total phenolic (TPC), total flavonoid (TFC) as well as antioxidant activity either by DPPH or ABTS. The results showed illustrated that phenolic content from *Eruca sativa* leaf extract revealed that, it has a good source of phenolic and flavonoids 12.522 mg GAE/g and 9.938 mg CE/g extract, respectively. Also, the antioxidant activity of ESLE displayed a decrease in the DPPH and ABTS with a 63.2% and 90.3% scavenging activity respectively.

Table 1: Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (AOA) by DPPH and ABTS of ELSE.

Total phenols (mg GAE/g)	Total flavonoids (mg CE/g)	AOA DPPH (mg TE/g) (%)	AOA ABTS (mg TE/g) (%)
12.522	9.938	2.71 (63.2)	5.548 (90.3)

(GAE) gallic acid equivalent, (CE) catechin equivalent , (TE) trolox equivalent

Fatty acids

Table 2 shows GLC analysis of the fatty acids methyl esters which represent the identification of 11 fatty acids in which lauric, palmitic, myristic, stearic, oleic, linoleic, linolenic, and arachidonic acids are the major component. Moreover, it was shown that the total unsaturated fatty acids represent the major constituents of the total mixture (46.1%) at which a monounsaturated fatty acids (14.25) whereas the total saturated fatty acids (36.55%).

Table 2: GLC-MS analysis of Fatty acids methyl esters of ELSE.

No	RT	Area	Name	C	Area Sum %
1	16.78	5483.5	Lauric acid	C12:0	8.45
2	20.74	11266	Non identified	—	17.35
3	22.74	2649.14	Myristic acid	C14:0	4.08
4	28.65	9246.8	Palmitic acid	C16:0	14.24
5	34.25	4251.95	Stearic acid	C18:0	6.55
6	35.09	9249.61	Oleic acid	C18:1	14.25
7	36.83	5306.79	Linoleic acid	C18:2	8.17
8	38.03	1340.42	Gamma-linolenic acid	C18:3	2.06
9	38.95	6100.62	Linolenic acid	C18:3	9.4
10	42.87	1339.42	Homo- γ -linolenic acid	C20:3	2.06
11	43.67	6594.98	Arachidonic acid	C20:4	10.16
12	49.06	2097.07	Lignoceric acid	C24:0	2.23

Body weight gain (BWG):

It is evident that there were no death was recorded in both control and treated rats up to 5g/kg, while (40%) of rats receiving high dose (6g/kg BW) died at 7 and 13 day of the beginning of the experiment, so rats of this group scarify at the day 14th. Evaluation of body weights for rats treated with 1, 3, and 5g/kg BW ESLE as compared to control, showed an increase in body weight of treated rats at 7th, 14th, and 35th day. These increases were significant ($p < 0.05$) at a dose of 5g at 7th, 14th day, while at the 35th day, the increase in the body weight was non significant ($p > 0.05$) at 5g/kg dose. Also, at the 35th day, the % of weight gain increased from dose of 1g/kg up to 5g (Table 3). The group received 6g /kg BW showed fluctuation, non significant decrease and increase at day 7,14 from the start of experiment.

Table 3: Body weights and % of bodyweight gains (g) from the control group and treated groups with 1, 3, 5, and 6g/kg body weight of ESLE.

Day	<i>Eruca sativa</i>				
	Control (n=5)	1g/kg (n=5)	3g/kg (n=5)	5g/kg (n=5)	6g/kg (n=3)
Day 0	119.50±1.12 ^b	113.25±1.65 ^{ab}	104.60±3.86 ^{ac}	122.00±1.61 ^b	93.00±3.61 ^c
Day 7	121.50±1.21 ^a	116.00±1.87 ^a	112.60±4.33 ^a	152.67±3.42 ^b	91.00±3.79 ^b
BWG [%]	+1.67±0.37 ^{ab}	+2.45±1.23 ^{ab}	+7.64±1.01 ^a	+25.21±3.18 ^c	-2.17±0.67 ^b
Day 14	133.00±3.11 ^a	143.00±4.85 ^a	145.00±4.65 ^a	170.22±4.50 ^b	100.67±1.45 ^b
BWG[%]	+11.34±2.85 ^b	+26.25±3.76 ^{ab}	+38.79±2.31 ^a	+39.61±4.06 ^a	+ 8.45±2.73 ^b
Day 35	154.20±6.45 ^a	188.80±4.13 ^b	177.80±6.16 ^b	175.20±3.93 ^{ab}	—
BWG [%]	+29.08±5.51 ^b	+66.76±3.42 ^a	+70.32±4.70 ^a	+43.70±3.68 ^b	

Each value represent the mean ± SE, values superscripts with different letters (a-c) were significantly different at $p \leq 0.05$

Statistical analysis of kidney and liver weights, and the relative weights of kidneys and liver for the control group and the groups treated with Eruca sativa-treated showed that there were no statistically significant differences ($p > 0.05$) between groups of ESLE- treated rats at the doses of 1, 3, 5 and 6g/kg BW and the control group or between the treated groups, except the weight of kidney of the ESLE- treated group of rats 6g/kg significantly ($P < 0.05$) reduced compared to control group (Table 4).

Table 4: Relative kidney and liver organ weights from the control group and treated groups with 1, 3, 5, and 6g/kg body weight of ELSE.

Item	<i>Eruca sativa</i>				
	Control (n=5)	1g/kg (n=5)	3g/kg (n=5)	5g/kg (n=5)	6g/kg (n=3)
Kidney	1.06±0.08 ^a	1.13±0.03 ^a	1.12±0.05 ^a	1.23±0.03 ^a	0.75±0.01 ^b
RKW	0.69±0.05 ^a	0.60±0.03 ^a	0.63±0.03 ^a	0.70±0.01 ^a	0.74±0.02 ^a
Liver	5.07±0.24 ^{ab}	5.72±0.12 ^a	5.49±0.17 ^a	6.11±0.49 ^a	3.78±0.17 ^b
RLW	3.30±0.17 ^a	3.04±0.10 ^a	3.09±0.07 ^a	3.49±0.28 ^a	3.73±0.12 ^a

Each value represent the mean ± SE, values superscripts with different letters (a-b) were significantly different at $p \leq 0.05$. RKW: relative kidney weight, RLW: relative liver weight.

Table (5) shows that the liver and kidney weight water free for the control group and the *Eruca sativa*-treated groups. There were non statistically significant differences ($p>0.05$) between groups of rats treated with ESLE at doses of 1, 3, 5 and 6g/kg BW and the control group or between the treated groups.

Table 5: Kidney and liver weights water free (g) in the control group and treated groups with 1, 3, 5, and 6g/kg body weight of ELSE.

Item	<i>Eruca sativa</i>				
	Control (n=5)	1g/kg (n=5)	3g/kg (n=5)	5g/kg (n=5)	6g/kg (n=3)
KW	0.33±0.02 ^a	0.39±0.03 ^a	0.38±0.04 ^a	0.38±0.02 ^a	0.34±0.05 ^a
KWC (%)	68.31±2.83 ^a	65.38±1.62 ^a	66.11±2.29 ^a	68.89±1.11 ^a	54.76±6.29 ^a
LW	1.38±0.09 ^a	1.80±0.02 ^a	1.74±0.06 ^a	2.59±0.49 ^a	1.42±0.09 ^a
LWC (%)	71.02±0.65 ^a	68.62±0.86 ^a	68.15±0.95 ^a	54.65±14.09 ^a	62.16±4.16 ^a

Each value represent the mean ± SE, values superscripts with different letters were significantly different at $p\leq 0.05$. KW: kidney weight free of water, KWC: kidney water content (%), LW: liver weight free of water, LWC: liver water content (%).

Biochemical parameters

Table 6 shows biochemical parameters such as liver function, glucose, cholesterol, triglycerides, kidney function and electrolytes of ESLE-treated groups and control group animals. ESLE administration did not produce any significant ($p<0.05$) changes on biochemical parameters related to renal and hepatic function as compared to the control group. While, serum level of ca showed a significant ($p<0.05$) increase in the 5g and 6g groups compared to the control group.

Table 6: Serum biochemical parameters of the control group and treated groups with 1, 3, 5, and 6g/kg body weight of ELSE.

Estimated parameters	<i>Eruca sativa</i>				
	Control (n=5)	1g/kg (n=5)	3g/kg (n=5)	5g/kg (n=5)	6g/kg (n=3)
TBil (mg/dl)	0.12±0.02 ^a	0.12±0.02 ^a	0.12±0.02 ^a	0.11±0.01 ^a	0.13±0.03 ^a
TP (g/L)	7.14±0.35 ^a	7.42±0.21 ^a	7.72±0.24 ^a	6.96±0.11 ^a	7.30±0.60 ^a
ALB (mg/dl)	3.84±0.21 ^a	4.00±0.16 ^a	4.18±0.18 ^a	3.72±0.07 ^a	3.46±0.12 ^a
ALT (U/L)	38.00±4.81 ^a	40.27±3.56 ^a	51.50±4.62 ^a	53.00±4.09 ^a	54.67±5.04 ^a
AST (U/L)	339.80±32.84 ^a	313.00±32.43 ^a	402.40±24.14 ^a	289.00±20.02 ^a	349.00±87.47 ^a
ALP (U/L)	356.8±37.84 ^a	354.00±22.12 ^a	398.6±16.01 ^a	259.4±34.60 ^a	259.33±64.40 ^a
GLU (mg/dl)	110.80±23.80 ^a	150.90±7.79 ^a	117.20±11.94 ^a	154.40±6.42 ^a	122.33±3.18 ^a
CHOL (mg/dl)	57.40±8.07 ^a	62.25±5.43 ^a	81.60±6.18 ^a	68.20±5.11 ^a	60.67±4.10 ^a
TG (mg/dl)	57.50±10.78 ^a	73.51±7.60 ^a	77.12±5.05 ^a	67.33±10.37 ^a	93.16±13.33 ^a
BUN (mg/dl)	21.75±1.21 ^a	20.61±1.29 ^a	21.50±0.54 ^a	30.46±3.28 ^a	20.31±3.54 ^a
CREA (mg/dl)	0.28±0.02 ^{ab}	0.30±0.00 ^a	0.34±0.02 ^a	0.20±0.00 ^b	0.23±0.03 ^{ab}
UA (mg/dl)	2.72±0.47 ^a	2.60±0.47 ^a	2.62±0.13 ^a	3.32±0.32 ^a	3.53±0.53 ^a
Na (mg/dl)	145.80±1.59 ^a	147.00±1.14 ^a	146.2±1.07 ^a	139.0±1.79 ^a	142.66±0.88 ^a

K (mg/dl)	8.03±1.01 ^a	6.92±1.02 ^a	6.60±0.18 ^a	10.00±0.41 ^a	8.74±0.47 ^a
Ca (mg/dl)	10.22±0.36 ^a	10.85±0.16 ^{ab}	10.84±0.45 ^{ab}	11.50±0.18 ^b	11.63±0.12 ^b
PO ₄ ⁻ (mg/dl)	10.76±1.36 ^a	9.70±0.81 ^a	10.92±1.14 ^a	10.34±0.91 ^a	12.33±1.73 ^a

Each value represent the mean ± SE, values superscripts with different letters (a-c) were significantly different at p≤0.05.

Data presented in table 7 represent the effect of different concentrations of ESLE on the expression levels of p53 and Bcl-2 genes in the liver and kidneys of rats treated with 1, 3, 5 or 6 g/kg body weight. The expression levels of Bcl-2 and p53 genes for rats treated with ESLE at 1, 3, and 5g/kg BW were non-significant compared to control group. While, evaluation of p53 and Bcl-2 for rats treated with ESLE at 6g/kg BW as compared to control, showed a significant increase (p<0.05) in p53 and a decrease in Bcl-2 (Figure 2,3).

Table 7: Expression of p53 and Bcl-2 of liver and kidney of control group and rat treated group with 1, 3, 5, and 6 g/kg body weight of ESLE.

Group	Control (n=5)	1 g (n=5)	3 g (n=5)	5 g (n=5)	6g (n=3)
Liver p53	26.76± 1.46 ^a	30.08±1.17 ^a	29.02±1.45 ^a	24.46±1.72 ^a	52.20±1.49 ^b
Liver Bcl-2	6.46±0.39 ^a	7.00±0.26 ^a	7.02±0.13 ^a	6.92±0.28 ^a	2.83±0.12 ^b
Kidney p53	21.18±2.07 ^a	26.20±2.82 ^a	27.48±2.14 ^a	29.02±1.36 ^a	53.30±1.18 ^b
Kidney Bcl-2	16.22±1.96 ^a	16.29±1.39 ^a	17.50±1.66 ^a	18.86±1.65 ^a	6.77±0.29 ^b

Each value represent the mean ± SE, values superscripts with similar letters (a-b) were non significant different at p≤0.05.

Histopathology:

Photomicrograph 1 show the kidney sections of the control group and the treated groups with 1, 3 or 5g/kg body weights of ESLE that showed a normal architecture of glomeruli and tubules. On the other hand 6g/kg ESLE treatment shows severe degenerative alterations in the tubules were seen with

congestion of blood vessels and diffused inflammatory cell infiltration. Cloudy swelling in renal tubules, tubular hyaline casts, early coagulative necrosis of some renal tubules with signs of regeneration in some other renal tubules. Multiple foci of mononuclear cells aggregation were seen in interstitial tissue.

Photomicrograph 2 show a kidney immunohistochemical stained with TNF- α , for the control group and the treated groups with 1, 3, 5 or 6g/kg body weights of ESLE negative stain of both glomeruli and tubules are noticed.

Photomicrograph 3 show the liver sections of the control group and the treated groups with 1, 3, 5, and 6g/kg body weights of ESLE (A-E) which showed a normal architecture, characterized by polyhedral hepatocytes shaped and cytoplasm granules with uniform small nuclei, normal radial arrangements around the central vein were well observed. While, treatment with 6g/kg body weights of ESLE which showed fatty changes, and extensive vacuolization with degenerated nuclei (E).

Photomicrograph 4 show a liver immunohistochemical stained with TNF- α for the control group and the treated groups with 1, 3, 5, or 6g/kg body weights of ELSE, negative stain of liver sections are noticed.

Discussion

This work was designed to highlights the safety of chronic administration of ESLE in male Wistar rats fed standard shew diet contain 1, 3, 5, and 6 g/kg for 35 days to explore whether it had a toxic effect within this dose. Obtained results did not record any mortality from the experimental animal through the study from 1 up to 5g/kg body weight indicating its primary safety, these results agree with previous study of Saleh et al. (2015) who noticed that acute administration of *Eruca sativa* leaf extract did not manifest any significant toxicity at any of the biochemical examined parameters and histological observations up to 5 g/kg. While the rats treated with 6g/kg of BW show an early death at 14th day, and the percentage change in body weight for the other rats was significantly lower than other groups, which may reflect the toxicity at this dose. This result may be due to the cells being exposed to high levels of thiocynates resulting in glutathione reductase inhibitor which lead to an unbalance in cellular GSH/GR system causing depletion of cellular GSH, induce oxidative stress and cell death (Li et al. 2020).

Fats are essential for nutrition and energy source and must be obtained from diet. The obtained result of *Eruca sativa* leaf analysis showed the identification of twelve fatty acids. These observations agreed with Hetta et al. (2017). These F.A. may enhances the secretion of digestive enzymes which result in improving the digestion rate (Jang et al. 2004). Furthermore, ESLE stimulate the activity and secretion of pancreatic enzymes which stimulate the digestion of proteins, fat and cellulose (Jamroz and Kmel 2002; Botsoglou et al. 2002). In addition the chemical components and mineral content of ESLE which stimulates the metabolic process which keep animal healthy (Khalil et al. 2015; Tassi et al. 2018). On the other hand, the decrease in the % body weight gain at 6g /kg may be attributed to high level of glucosinolates which lead to antinutritional effect, reduce protein digestibility and food consumption (Bjerg et al. 1989) and/or decrease thyroid hormone production which lead to loss of weight (Felker et al. 2016; Li et al. 2020).

The results of the present study show that administration of ESLE from 1 to up to 5g/kg and also at 6g/kg were not correlated with any significant change in the weight of liver and kidney. These results were supported by the observation of histological and immunohistochemical examination of both kidney and liver tissue at which no changes related with the different doses of ESLE. These results was in accordance to the findings of **Saleh et al. (2015)** who noticed non significant change in the weights of liver and kidney fed with ESLE as compared with control groups, These results rats may be related to the presence of phenolic and flavonoid content which protect cells and tissues against oxidative free radicals through increasing or maintaining the levels of antioxidant molecules and antioxidant enzymes, preventing cell damage.

The organ-body weight ratio is usually used to evaluate the effects of a test compound on organ weights (**Nirogi et al. 2014**). The results of the present study show that, administration of ESLE from 1 up to 6g/kg was not associated with an increase in relative kidney or liver weight due to the effective doses of this extract on the whole body and internal organ weight, an explanation which coincide with that of **Saleh et al. (2015)**.

The increase in the activity of ALT and AST is usually associated with increased plasma membrane permeability, resulting from the damage of hepatocytes (**Bloom 1993**). The results of the present study show that, administration of ESLE from 1 up to 6g/kg was not associated with any abnormal changes in liver function, indicated its safety on liver structure and function. The non significant alteration in lipid profile can also be correlated to the liver health, since the liver is the site for cholesterol degradation and glucose storage (**Bechmann et al. 2012**). Similarly, the non significant changes in blood urea nitrogen, creatinine, uric, sodium and potassium indicates the safety of the extract on renal functions. These finding were in accordance with **Alam et al. (2007)** and may be due to the fatty acids composition of the extract and to its powerful antioxidant to inhibit free radical that protect cells from oxidative damage by maintaining or rising the antioxidant molecules content and antioxidant enzymes (**Maia et al. 2015**). The effect of the ESLE may be not only attributable to the content of phenolic and flavonoid, suggesting a synergism between the phenolics and the other nonphenolic compounds that work synergistically to contribute to the antioxidant activity (**Llorach et al. 2004**). On the other hand, the increase in content blood calcium at rat receive 5g and 6g ESLE groups compared to control group are in agreement with (**Mashi and Dheyab 2018**). This might be due to leaves contain high content of oxalates and phytate (**Keyata et al. 2020**) which prevent absorption of calcium leading to elevation of parathyroid hormone cause release of calcium from bones into blood stream (**Kim et al. 2020**).

The results of the current study show that, administration of ESLE from 1 up to 5g/kg was not associated with any significant changes in p53 and Bcl-2 of both liver and kidney homogenate, these results are in agreement with **Abdel-Rahman et al. (2016)**, and may be attributed to the anti-apoptotic properties of ESLE extract (**Abd El-salam et al. 2021**) which indicates that the ESLE at these doses keep normal renal or hepatic cells without any noticed abnormalities. Whereas, the obtained results of high expression of p53 and low Bcl-2 at rat giving 6g/kg of treated rats may be related to the high concentrations of isothiocyanates (glucosinolates) that induce p53 elevation and reduce Bcl-2 expression, these results are in

agreement with **Lenzi et al. (2017)** who showed a significant increase in the percentage of apoptotic cell in a dose dependent manner with an increase in the necrotic cell fraction at 128 μ M (26.5mg/ml) of isothiocyanates.

The microscopic examination of liver in control and the treated groups from 1 up to 5g/kg BW showed normal hepatocytes and cytoplasm granules. Concerning the kidney tissue of the control and the treated groups from 1 up to 5g/kg BW there are, normal architecture of glomeruli and tubules as recorded by **Alqasoumi (2010); Meligi and Hassan (2017)**. These observations may be attributed to antioxidant compounds in the extract which keep the hepatocyte and glomerulus in normal state (**Jin et al. 2009**).

In immunohistochemical stain of both kidney and liver tissue of the control and treated groups from 1 up to 5g/kg body weights showed a negative stain were noticed in kidney glomeruli and tubules or negative stain in liver tissue. Thus, the present study demonstrated that the normal renal or hepatic cells are kept without any abnormalities after the treatments of these doses of *Eruca sativa* extract.

Conclusion

The results of the present study indicate that the ethanolic ESLE is not toxic and there were no changes in the structure and functions of the liver and kidneys when administered to rats orally at doses of 1–5 g/Kg BW through the experimental period (35 day) where the extract containing antioxidant that protect the cell from damage, but the group given 6g/kg showed hepatorenal abnormalities which may be due to the presence of high concentration of glucosinolates. Hence, we understand the factors involved in identifying the other contributing factors that synergistically protect cell from damage

Declarations

Ethical Approval

All the experiments were conducted in agreement with the 'Institutional Animal Ethics Committee at Mansoura University, Mansoura, Egypt' regulation, which is in accordance with the 'Handbook for the Care and Use of Laboratory Animals' issued by the 'National Academy of Sciences.

Consent to participate: Not applicable

Consent for publication: Not applicable

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hussam Ahmed El-Gayar, Safaa Derbala, Sahar Hamed, Reda Soliman El-Demerdash, Wael Mohamady Afifi, Sameh Ibrahim Ahmed El Emam, and Gamal Mohamed Edrees. The first draft of the manuscript was written by Hussam Ahmed El-Gayar and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests

The authors declare no financial or commercial conflict of interest.

Data Availability: Data available upon request.

Code availability: Not applicable

References

1. Abdel-Rahman S, Shaban N, Haggag A, Awad D, Bassiouny A, Talaat I (2016) Combination between taxol-encapsulated liposomes and *Eruca sativa* seed extract suppresses mammary tumors in female rats induced by 7,12 dimethylbenz(a)anthracene. *Asian Pac J Cancer Prev* 17:117-123. <https://doi.org/10.7314/apjcp.2016.17.1.117>
2. Abd-Elsalam RM, El Badawy SA, Ogaly HA, Ibrahim FM, Farag OM, Ahmed KA (2021) *Eruca sativa* seed extract modulates oxidative stress and apoptosis and up-regulates the expression of Bcl-2 and Bax genes in acrylamide-induced testicular dysfunction in rats. *Environ Sci Pollut Res Int* 28 (38): 53249-53266. <https://doi.org/10.1007/s11356-021-14532-y>
3. **Aabdul-jabbar** RS (2018) Polyphenol and Flavonoid Contents and Antioxidant Activity in Freshly Consumed Rocket (*Eruca sativa*). *IOP Conf. Series: Materials Science and Engineering* 454 012158. <https://doi.org/10.1088/1757-899X/454/1/012158>
4. Akagi K, Sano M, Ogawa K, Hirose M, Goshima H, Shirai T (2003) Involvement of toxicity as an early event in urinary bladder carcinogenesis induced by phenethyl isothiocyanate, benzyl isothiocyanate, and analogues in F344 rats. *Toxicol Pathol* 31(4):388-396. <https://doi.org/10.1080/01926230390202326>
5. Alam MS, Kaur G, Jabbar Z, Javed K, Athar M (2007) *Eruca sativa* seed possess antioxidant activity and exert a protective effect induced on mercuric chloride induced renal toxicity. *Food and Chem Toxicol* 45(6): 910-920. <https://doi.org/10.1016/j.fct.2006.11.013>
6. Alarcón M, Fuentes M, Carrasco G, Palomo I (2014) A novel role of *Eruca sativa* mill. (Rocket) extract: Antiplatelet (NF-κB Inhibition) and antithrombotic activities *Nutrients* 6:5839-5852. <https://doi.org/10.3390/nu6125839>
- 7.
8. Alqasoumi S (2010) Carbon tetrachloride-induced hepatotoxicity: Protective effect of 'Rocket' *Eruca sativa* L. in rats. *Am J Chin Med* 38(1):75-88. <https://doi.org/10.1142/S0192415X10007671>
9. Bansa A (2009) Phytochemical and antibacterial investigation of bark extracts of *Acacia nilotica*. *J. Med. Plants Res.* 3(2): 082-085. <https://doi.org/10.5897/JMPR.9000985>

10. Barlas NT, Irget ME, Tepecik MM (2011) Mineral content of the rocket plant (*Eruca sativa*). *AJB* 10(64):14080-14082. <https://doi.org/10.5897/AJB11.2171>
11. Bechmann LP, Hannivoort RA, Gerken G, Hotamisligil GS, Trauner M, Canbay A (2012) The interaction of hepatic lipid and glucose metabolism in liver diseases. *J. Hepatol.* 56(4): 952–964. <https://doi.org/10.1016/j.jhep.2011.08.025>
12. Bjerg B, Eggum B.O, Jacobsen I, Otte J, Sørensen H (1989) Antinutritional and toxic effects in rats of individual glucosinolates (+/- myrosinase) added to a standard diet (2). *J Animal Physiol a Anim Nutr* 61(1-5): 227-244. <https://doi.org/10.1111/j.1439-0396.1989.tb00105.x>
13. Bloom JC (1993) Principles of hematotoxicology: laboratory assessment and interpretation of data. *Toxicol Pathol* 21(2):130–134. doi: 10.1177/019262339302100203
14. Botsoglou NA, Florou-Paneri P, Fletouris DJ, Spais AB (2002) Effect of dietary oregano essential oil on performance of chickens and on iron-induced lipid oxidation of breast, thigh and abdominal fat tissues. *Br Poult Sci* 43(2): 223-230. <https://doi.org/10.1080/00071660120121436>
15. Chung FL, Morse MA, Eklind KI, Lewis J (1992) Quatitation of human uptake of the anticarcinogen phenethyl isothiocyanate after a watercress meal. *Cancer Epidemiol Biomarks Prev* 1(5): 383–388. PMID: **1305471**
16. Cragg GM, Newman DJ (2005) Plants as a source of anti-cancer agents. *J Ethnopharmacol* 100(1-2): 72–79. <https://doi.org/10.1016/j.jep.2005.05.011>
17. Edrees GM, El-Gayar HA, Habibi EME, Salem ET (2015) Protective effect of *Petroselinum crispum* or *Eruca sativa* extract on testes against dioxin intoxication in rats. *European Journal of Biomedical and Pharmaceutical Sciences* 2(7): 57-66
18. Eisa NH, ElSherbiny NM, Shebl AM, Eissa LA, El-Shishtawy MM (2015) Phenethyl isothiocyanate potentiates anti-tumour effect of doxorubicin through Akt-dependent pathway. *Cell Biochem Funct* 33(8):541-551. <https://doi.org/10.1002/cbf.3153>
19. El-Missiry MA, El Gindy AM (2000) Amelioration of alloxan induced diabetes mellitus and oxidative stress in rats by oil of *Eruca sativa* seeds. *Ann Nutr Metab* 44(3):97-100. <https://doi.org/10.1159/000012829>
20. Khalil FF, Mehrim AI, Refaey MM (2015) Impact of Dietary Rocket (*Eruca sativa*) Leaves or Seeds on Growth Performance, Feed Utilization, Biochemical and Physiological Responses of *Oreochromis niloticus*, Fingerlings. *Asian Journal of Animal Sciences* 9 (4):134-147. <https://doi.org/10.3923/ajas.2015.134.147>
21. Felker P, Bunch R, Leung AM (2016) Concentrations of thiocyanate and goitrin in human plasma, their precursor concentrations in brassica vegetables, and associated potential risk for hypothyroidism. *Nutr Rev* 74(4):248-258. <https://doi.org/10.1093/nutrit/nuv110>
22. Hetta MH, Owis AI, Haddad PS, Eid HM (2017) The fatty acid-rich fraction of *Eruca sativa* (rocket salad) leaf extract exerts antidiabetic effects in cultured skeletal muscle, adipocytes and liver cells. *Pharm Biol* 55(1):810-818. <https://doi.org/10.1080/13880209.2017.1280687>

23. Hwang ES, Do Thi, N (2014) Effects of Extraction and Processing Methods on Antioxidant Compound Contents and Radical Scavenging Activities of Laver (*Porphyra tenera*). *Prev Nutr Food Sci* 19(1): 40-48. <https://doi.org/10.3746/pnf.2014.19.1.040>
24. Jamroz D, Kamel C (2002) Plant extracts enhance boiler performance. In non-ruminant nutrition: antimicrobial agents and plant extracts on immunity, health and performance. *Journal of Animal Science* 80(1):41-46
25. Jang IS, Ko YH, Yang , HY et al (2004) Influence of essential oil components on growth performance and the functional activity of the pancreas and small intestine in broiler chickens. *Asian-Australasian Journal of Animal Sciences* (17) 3: 394-400. <https://doi.org/10.5713/ajas.2004.394>
26. Jin J, Koroleva OA, Gibson T, Swanston J, Magan J, Zhang Y, Rowland IR, Wagstaff C (2009) Analysis of phytochemical composition and chemoprotective capacity of rocket (*Eruca sativa* and *Diplotaxis tenuifolia*) leafy salad following cultivation in different environments. *J Agric Food Chem* 24; 57 (12):5227-5234. <https://doi.org/10.1021/jf9002973>
27. Keyata EO, Tola YB, Geremew B, Forsido SF (2020) Proximate, mineral, and anti-nutrient compositions of underutilized plants of Ethiopia: Figl (*Raphanus sativus* L.), Girgir (***Eruca sativa*** L) and Karkade (*Hibiscus sabdariffa*): Implications for in-vitro mineral bioavailability. *Food Res Int* 137:109724. <https://doi.org/10.1016/j.foodres.2020.109724>
28. Kishore L, Kaur N, Kajal A, Singh R (2017) Extraction, characterization and evaluation of *Eruca sativa* against Streptozotocin-induced diabetic nephropathy in rat. *Bangladesh J Pharm* 12 (2):216-227. <https://doi.org/10.3329/bjp.v12i2.32065>
29. Lenzi M, Cocchi V, Malaguti M, barbalace MC, Marchionni S, Herlia S, Herlia P (2017) 6-(Methylsulfonyl) hexyl isothiocyanate as potential chemopreventive agent: molecular and cellular profile in leukaemia cell lines. *Oncotarget* 4;8 (67): 111697-111714. <https://doi.org/10.18632/oncotarget.22902>
30. Li X, Ni M, Xu X, Chen W (2020) Characterisation of naturally occurring isothiocyanates as glutathione reductase inhibitors. *J Enzyme Inhib Med Chem* 35(1):1773-1780. <https://doi.org/10.1080/14756366.2020.1822828>
31. Llorach R, Tomas-Barberan FA, Ferreres F (2004) Lettuce and chicory byproducts as a source of antioxidant phenolic extracts. *J Agric Food Chem* 52(16): 5109–5116. <https://doi.org/10.1021/jf040055a>
32. Magaki S, Hojat SA, Wei B, So A, Yong WH (2019) An introduction to the performance of immunohistochemistry. *Methods Mol Biol* 1897:289-298. https://doi.org/10.1007/978-1-4939-8935-5_25
33. Maia ML, Correia-Sá L, Coelho A, Barroso MF, Domingues VF, Delerue-Matos C (2015) *Eruca sativa*: Benefits as antioxidants source versus risks of already banned pesticides. *J Environ Sci Health B* 50(5):338-345. <https://doi.org/10.1080/03601234.2015.1000178>
34. Marchetti JM, Frazu AF (2008) Esterification of free fatty acids using sulfuric acid as catalyst in the presence of triglycerides. *Biomass and Bioenergy* 32(9): 892-895.

<https://doi.org/10.1016/j.biombioe.2008.01.001>

35. Mashi SK (2017) Effect of *Eruca sativa* leaves extract on liver enzymes and lipid profile in phosphoric acid induced liver damage in male rabbits. *J Entomol Zool Stud* 5(6):1011-1015
36. Mashi SK, Dheyab DS (2018) Effect of *Eruca sativa* leaves extract on osteoporosis induced by phosphoric acid in adult male rabbits. *Indian Journal of Natural Sciences*, 8(46): 13098-13106
37. Meligi NM, Hassan HF (2017) Protective effects of *Eruca sativa* (rocket) on abamectin insecticide toxicity in male albino rats. *Environ Sci Pollut Res Int* 24(10):9702-9712.
<https://doi.org/10.1007/s11356-017-8671-8>
38. Moustafa NA, EL- Makawy A (2002) Effect of *Eruca sativa* seeds oil on the non pregnant and pregnant female rats. *Sc. J. Az. Med. Fac. (Girls)*. 23(2):521-543
39. Nirogi R, Goyal VK, Jana S, Pandey SK, Gothi A (2014) What suits best for organ weight analysis: review of relationship between organ weight and body / brain weight for rodent toxicity studies. *International Journal of Pharmaceutical Sciences and Research* 5(4): 1525-1532
40. Kim O-H, Booth CJ, Choi HS, Lee J et al (2020) High phytate/ low-calcium diet is a risk factor for crystal nephropathies, renal phosphate wasting, and bone loss. *Elife* 9: e52709.
<https://doi.org/10.7554/eLife.52709>
41. Saleh MM, Qader SW, Thaker AA (2015) Acute toxicity of alcoholic leaf extract of *Eruca sativa* in albino rats *Rattus norvegicus*. *Al-Anbar J Vet Sci* 8 (2):1-4
42. Sa'vio AL, da Silva GN, Salvadori DM (2014) Inhibition of bladder cancer cell proliferation by allyl isothiocyanate (mustard essential oil). *Mutat Res* 771:29-35.
<https://doi.org/10.1016/j.mrfmmm.2014.11.004>
43. Shatta GF. A review article on hyperlipidemia: Types, treatments and new drug targets. *BPJ* 7:399-409.
DOI : <https://dx.doi.org/10.13005/bpj/504>
44. Sugiura S, Ogawa K, Hirose M, Takeshita F, Asamoto M, Shirai T (2003) Reversibility of proliferative lesions and induction of non-papillary tumors in rat urinary bladder treated with phenylethyl isothiocyanate. *Carcinogenesis* 24(3):547-553
45. Tassi EMM, Duarte RMT, Amaya-Farfan J (2018) Partial nutrient characterization of arugula (rocket - *Eruca sativa* L.) and the effect of heat treatment on its lipoxidase activity. *Braz J Food Technol* 21, e2017024. <https://doi.org/10.1590/1981-6723.02417>
46. Yang M, Wang H, Zhou M, Liu W, Kuang P, Liang H, Yuan Q (2016) The natural compound sulforaphene, as a novel anticancer reagent, targeting PI3K-AKT signaling pathway in lung cancer. *Oncotarget* 7:76656-76666. <https://doi.org/10.18632/oncotarget.12307>
47. Zilic S, Serpen A, Akillioglu G, Jankovic M, Gokmen V (2012) Distributions of phenolic compounds, yellow pigments and oxidative enzymes in wheat grains and their relation to antioxidant capacity of bran and debranned flour. *J Cereal Sci* 56 (3): 652-658. <https://doi.org/10.1016/j.jcs.2012.07.014>

Figures

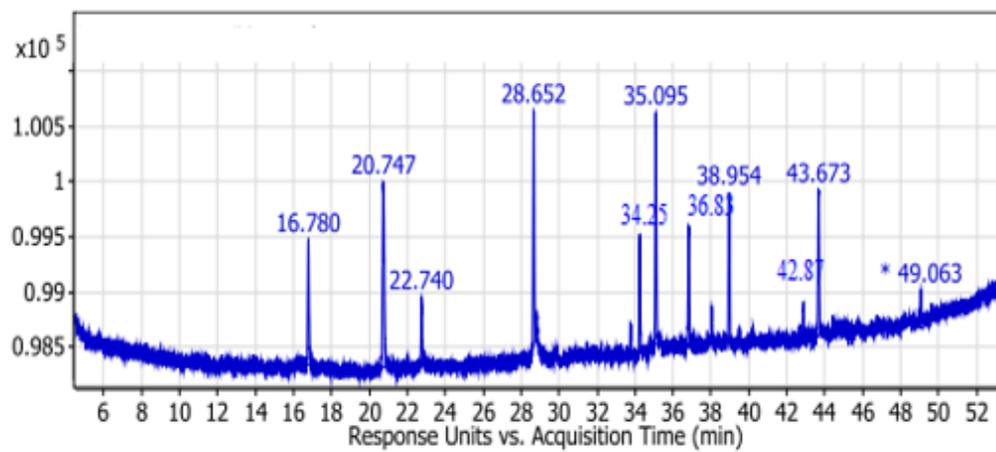


Figure 1

Gas chromatography-mass spectrometry chromatogram of the ELSE.

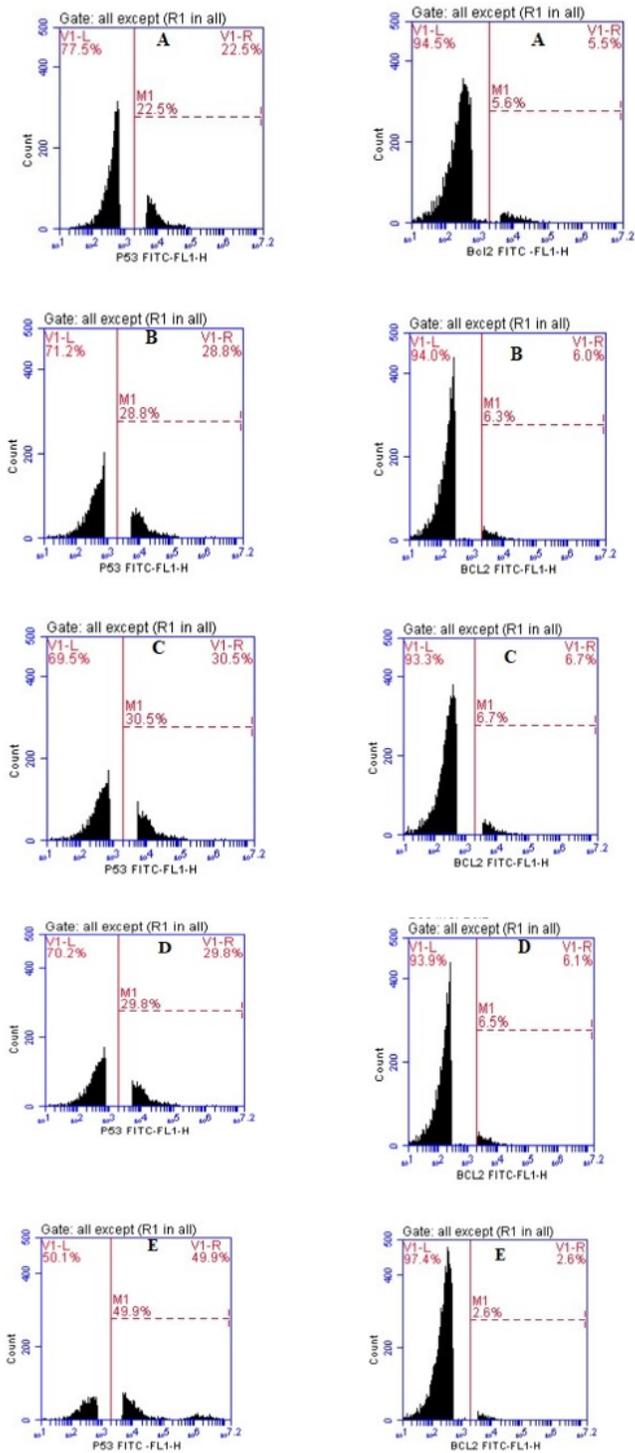


Figure 2

Flow cytometric analysis of Bcl-2 and p53 in liver of control and different treated rat groups. A: control; B: 1g/kg C: 3g/kg, D: 5g/kg, and E: 6g/kg ELSE.

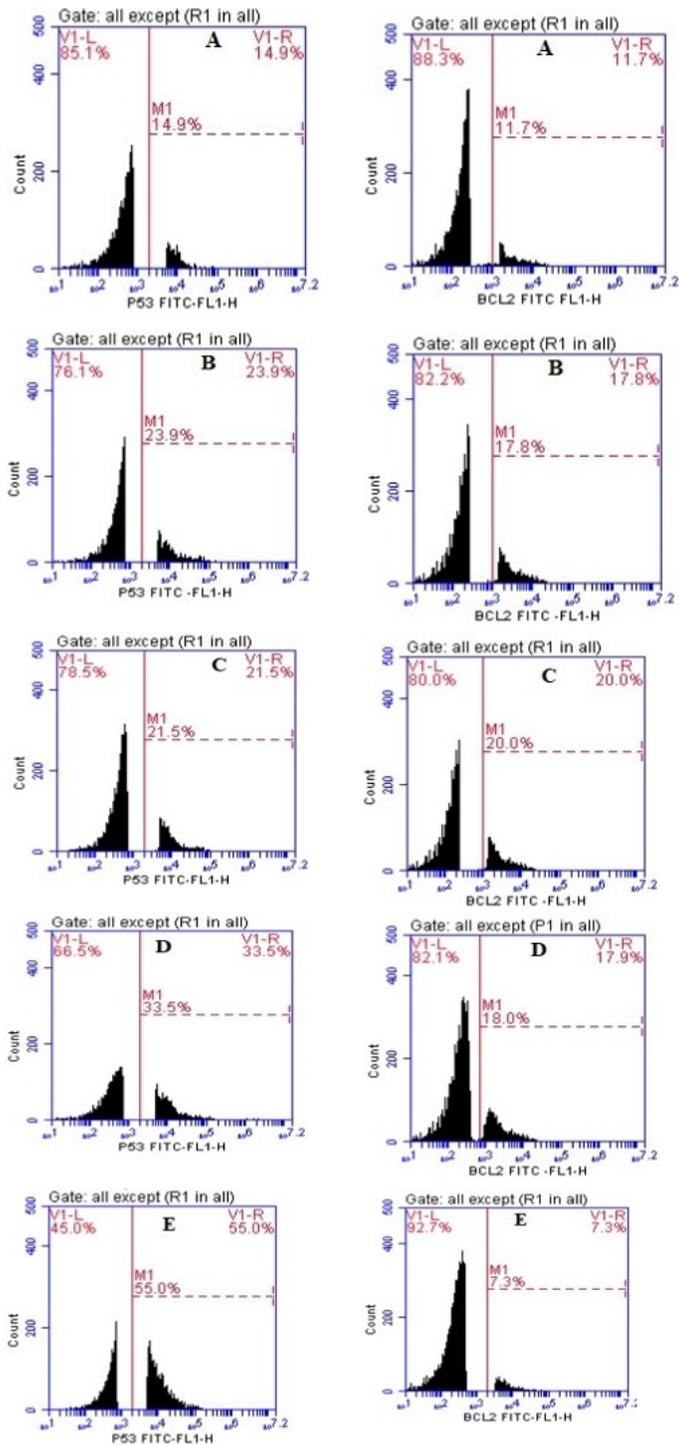


Figure 3

Flow cytometric analysis of Bcl-2 and p53 in kidney of control and different treated rat groups. A: control; B: 1g/kg C: 3g/kg, D: 5g/kg, and E: 6g/kg ELSE.

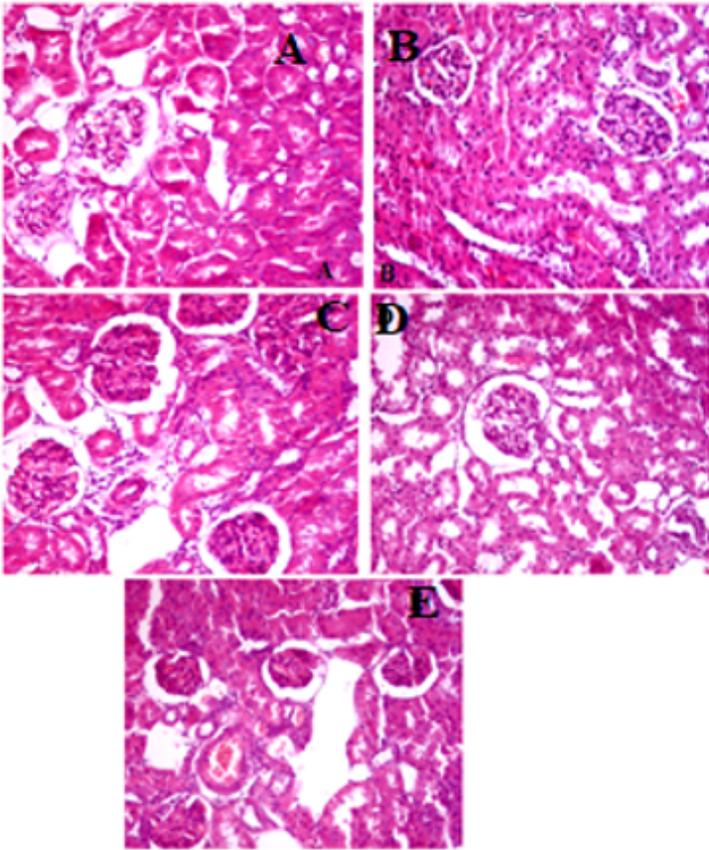


Figure 4

Photomicrograph 1: Histological sections of kidney from different groups show normal architecture of glomeruli and tubules. A) Control, B) 1g ESLE, C) 3g ESLE, D) 5g ESLE, and E) 6g ESLE. ($\times 200$).

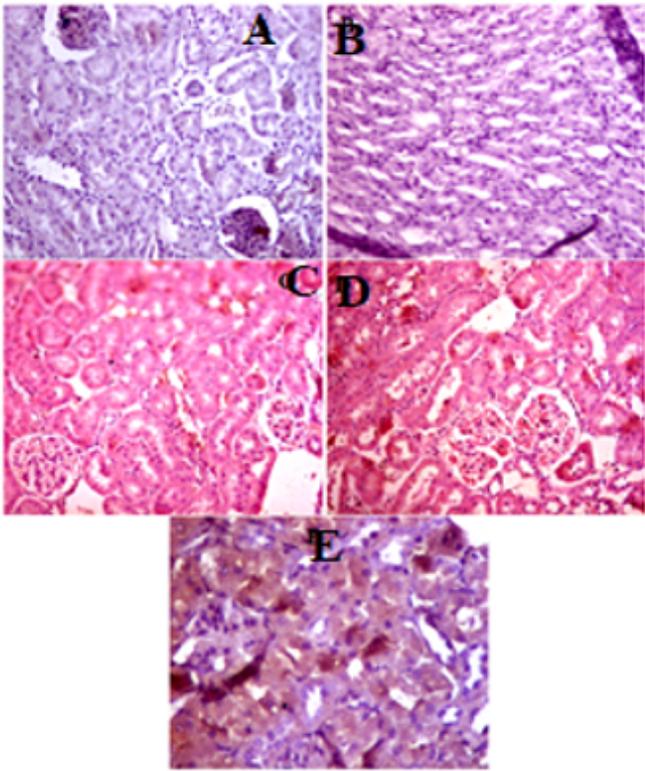


Figure 5

Photomicrograph 2: Representative immunohistochemical photomicrographs of TNF- α immunoreactivity from the kidney (A-E) of rat treated with saline, ESLE (1-6mg/kg), A: control rat treated with saline, B: rat treated with 1g/kg, C: rat treated with 3g/kg, D: rat treated with 5g/kg, E: rat treated with 6g/kg show positive stain of both glomeruli and tubules ($\times 100$).

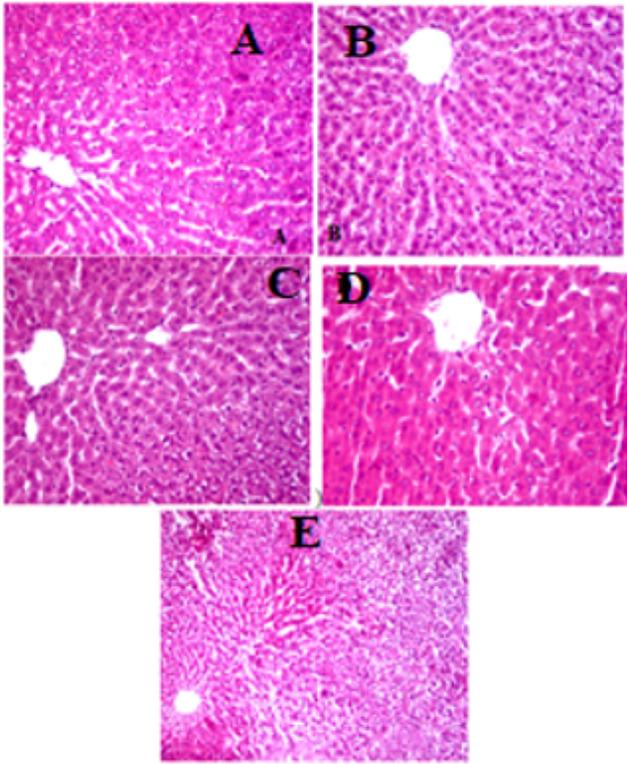


Figure 6

Photomicrograph 3: Histological sections of liver from different groups A) control, B) 1g ESLE, C) 3g ESLE, D) 5g ESLE, and E) 6 g ESLE. (A-D) show normal architecture, characterized by polyhedral shaped hepatocytes and cytoplasm granulated with small uniform nuclei, the normal radial arrangements around central vein is well observed. (E) show mild micro- and macrovesicular steatosis with inflammatory changes ($\times 100$).

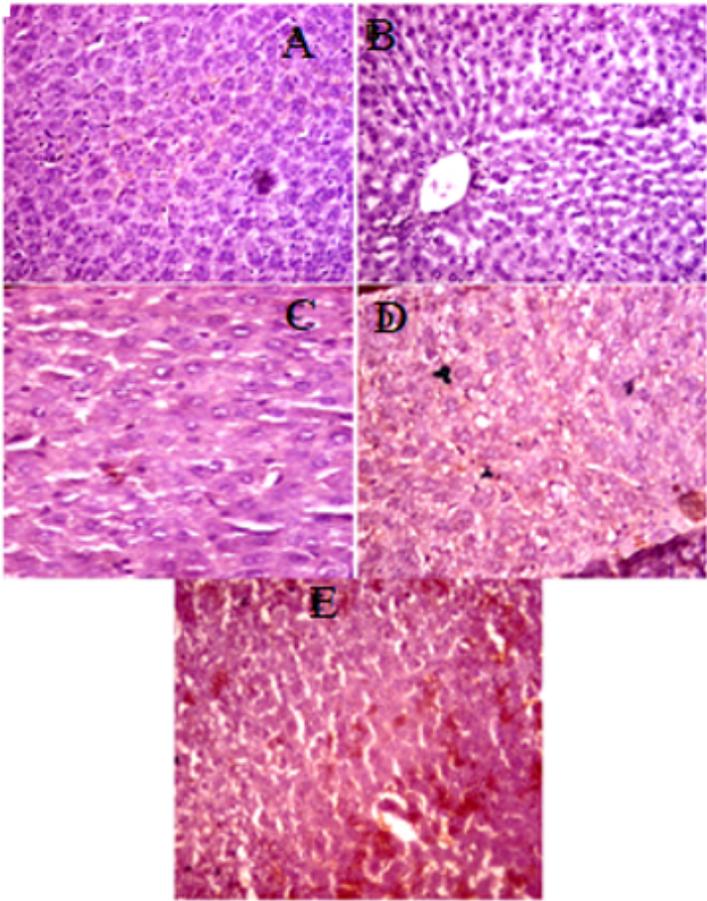


Figure 7

Photomicrograph 4: Representative immunohistochemical photomicrographs of TNF- α immunoreactivity from the liver (A-D) of rat treated with saline, ESLE (1-5mg/kg), A: control rat treated with saline, B: rat treated with 1g/kg, C: rat treated with 3g/kg, D: rat treated with 5g/kg, E: rat treated with 6g/kg, it shows positive stain of liver section ($\times 100$).