

# *Urtica dioica* L. Attenuate Methotrexate Induced Hepatotoxicity in Rats

Sheetal Rani<sup>1</sup> , Chandan Sharma<sup>1</sup> , Divya Jain<sup>2</sup> , Priya Chaudhary<sup>3</sup> , Deepika Bhatia<sup>1,\*</sup> 

<sup>1</sup> University Institute of Pharma Sciences, Chandigarh University, Gharuan, Mohali, Punjab, India; chandan.pharma@cumail.in (C.S.); sheetalshandilya94@gmail.com (S.R.); Deepika.pharma@cumail.in (D.B.);

<sup>2</sup> Department of Microbiology, School of Applied & Life Sciences, Uttaranchal University, Dehradun, Uttarakhand, India; divyajain31011996@gmail.com;

<sup>3</sup> Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India; priyachaudhary358@gmail.com;

\* Correspondence: Deepika.pharma@cumail.in;

Scopus Author ID 57218572491

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**Abstract:** The aim of the study was to investigate phytochemical content from the different extracts of *Urtica dioica* (*U. dioica*) using standardized protocols. The present study focuses on the hepatoprotective activity of the petroleum ether extract of *U. dioica* on MTX-induced hepatotoxicity in Wistar Albino Rats. The rats were treated with 20 mg/kg of MTX, 100 mg/kg, and 200 mg/kg of the extract of *U. dioica*. For hepatotoxicity, various parameters, including change in body and organ weight, serological parameters (SGOT, SGPT, and Bilirubin), oxidative stress parameters (SOD and GPx), and histopathological analysis were evaluated. Among all extracts, the petroleum ether extract of the leaf had the highest concentrations of phenols and flavonoids. Furthermore, this extract was tested for in vivo hepatoprotective efficacy against MTX-induced hepatotoxicity. The treated control group showed a significant drop in body weight as well as an increase in organ weight. The extract was demonstrated to reduce the levels of SGOT, SGPT, and bilirubin while increasing SOD and GPx levels. A histopathological study revealed that the MTX-induced hepatotoxicity recovered in the standard treated group and both test groups at minimum and maximum doses. Thus, the current analysis revealed the presence of active ingredients with hepatoprotective potential.

**Keywords:** *Urtica dioica*; hepatoprotective; anti-cancer; herbal drugs; methotrexate; phytochemicals.

**Abbreviations:** SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamate pyruvate transaminase; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; MTX: Methotrexate; FPGS: folypolyglutamate synthetase; ROS: reactive oxygen species; H&E: Hematoxylin & Eosin; IP: Intraperitoneal; PO: per OS (orally).

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## 1. Introduction

The liver is the body's primary organ, responsible for biochemical and physiological processes such as metabolism and detoxification of many chemicals that enter the liver. This organ is primarily responsible for oxidative stress response. Hepatotoxicity is the harm or damage caused to the liver by destructive or damaging agents such as industrial chemicals and medications, often known as hepatotoxins. Liver illness is a global issue [1]. Many medications are used to treat hepatotoxicity; however, they can have side effects. As a result, it is critical to seek alternatives to traditional treatment [2].

Nowadays, herbal medicinal plants are gaining popularity for the treatment of a variety of ailments. One such plant is *U. dioica*, whose petroleum ether extract was tested for MTX-

induced toxicity [3]. MTX was previously known as amethopterin. It works as an immune suppressant and chemotherapeutic agent. It inhibits cell division by inhibiting nucleotide synthesis. It prevents cell division by blocking nucleotide synthesis. MTX can cause liver damage, fibrosis, and cirrhosis if taken over an extended period of time. It can cause hepatotoxicity by increasing specific enzymes such as SGOT, SGPT, and bilirubin. The liver is the primary organ responsible for drug processing and managing various physiological functions. It ensures the body's functionality, efficiency, and equilibrium [4].

MTX can also have an effect on other systems, such as the respiratory, gastrointestinal, and blood. MTX enters the cell by active transport across the reduced folate carrier (RFC) and is effluxed by various ABC transporters, primarily ABCC1-5 and ABCG-2. It metabolizes through polyglutamation, which involves adding 2-7 Glutamic acid groups, and is catalyzed by FPGS. Because the polyglutamated form cannot permeate the cell membrane, its intracellular half-life is prolonged. So, *U. dioica* was found to have a variety of antioxidant phytochemicals, including phenols and flavonoids [5].

The predominant antioxidants were phenolic phytochemicals. These phenolic components were observed to limit ROS production [6]. The current study looks into how *U. dioica*'s petroleum ether extract protects against MTX-induced hepatotoxicity. For this reason, the petroleum ether extract of *U. dioica* was given to rats that were then treated with MTX. Histological assessments of the liver were evaluated with the light microscope, in addition to measuring SGOT, SGPT, and bilirubin [7, 8].

## 2. Materials and Methods

### 2.1. Chemicals used.

Silymarin was purchased from ANJ Biomedicals. Diagnostic kits were from Reckon Diagnostics Pvt. Ltd., Vadodara, India. The Chemicals like hydrochloric acid, Folin Ciocalteu reagent and diethyl Ether, formalin, and sodium chloride of analytical grade were obtained from Chandigarh University, Gharuan, Mohali, Punjab, and Central Drug House (P) Ltd, New Delhi, India. All other chemicals and reagents used in this study were of analytical grade.

### 2.2. Sample collection, preparation, and extraction.

*Urtica dioica* L. was purchased from Pujali, Banjar, Kullu, and Himachal Pradesh in August 2023. The plant was further identified and observed based on its morphological characters and was finally authenticated and certified by CSIR NISCAIR, Delhi, India. The washed, air-dried leaves of *U. dioica* were ground into a coarse powder using an electric grinder, 35 g of which was extracted with petroleum ether, methanol, and aqueous solvent at 300 ml. The soxhlet extractor was used for a sequential extraction process at 60-70°C for 32 hours, and the final dried extract obtained was kept in the refrigerator for further studies [9, 10].

### 2.3. Quantitative estimation of phytochemicals.

For the determination of total phenolic content (TPC) and total flavonoid content (TFC), three extracts were used, including petroleum ether, methanol, and aqueous extracts of the leaves of *U. dioica* [11].

### 2.3.1. Determination of total phenolic content (TPC).

For the determination of TPC, folin ciocalteu (FC) reagent was used. All three extracts (0.5 ml) were mixed with FC reagent (0.5 ml). The solutions were then maintained at 25°C for around 5-8 minutes before adding 2 ml sodium carbonate solution and increasing the volume to 8 ml with water. The absorbance was measured after 2 hours. Gallic acid was employed as a reference for curve calibrations. TPC was represented as Gallic acid (mg) equivalent as per weight of the extract (mg/g) [12].

### 2.3.2. Determination of total flavonoid content (TFC).

A colorimetric assay was performed to determine TFC. The extracts (100 µl) were added to 0.3 ml of 5% NaNO<sub>2</sub> solution. After five minutes, 0.3 ml of 10% AlCl<sub>3</sub> and 2 ml of 1 M NaOH were added to the mixture. Then, the mixture was diluted by adding distilled water (3.3 ml). Then, the mixture was mixed thoroughly. The absorbance was found to be 510 nm. Quercetin was used as a standard for curve calibrations. TFC was expressed as Quercetin (g) equivalent as per the weight of the extract (mg/g) [3].

### 2.4. Approval of experimental animals and their procurement.

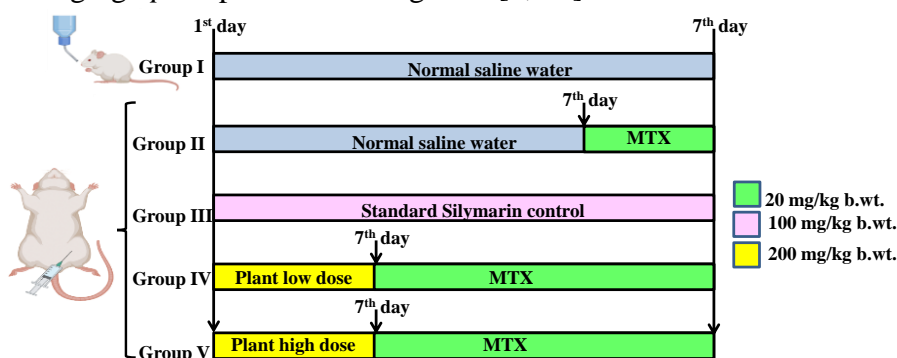
Wistar rats weighing 150-250 g were procured from Chandigarh University, Punjab, India. All the experiments were approved by the Institutional Animal Ethics Committee (IAEC) under protocol number CU/2021/IAEC/4/13. After the rats were procured, they were kept in conventional laboratory settings for approximately 15 days to acclimate in the animal house.

### 2.5. Maintenance of animals.

They were maintained under conventional laboratory settings of 22 ± 2°C with a relative humidity of 50 ± 15% in an air-conditioned animal house during the period of acclimatization and experimentation. They were fed a pelleted diet and water *ad libitum*.

### 2.6. Experimental induction of hepatotoxicity in rats.

The rats were divided into five groups of six each. Group I served as Vehicle control. Group II treated control received (MTX 20 mg/kg *i.p.* on the 7<sup>th</sup> day). Group III standard control was treated with Silymarin 100 mg/kg *p.o.* once daily for 7 days. Both Group IV and Group V were treated with the extract of *U. dioica* at the minimum and maximum dose of 100 and 200 mg/kg body weight (b. wt.; *p. o.*), respectively, and were also treated with MTX on the 7<sup>th</sup> day at a dose of 20 mg/kg *i.p.* as presented in Figure 1 [4, 13].



**Figure 1.** Schematic representation of an experimental design. Number of animals in each group, n=6; Total number of animals = 30.

### 2.7. Collection of samples from the animals.

On the seventh day, three animals from each group were sacrificed after anesthetizing them with diethyl ether. Just before sacrifice, under mild anesthesia, the blood was collected from the rats by the retro-orbital route. After that, these were subjected to SGOT, SGPT, and Bilirubin estimations. The liver was stored in 10% neutral buffered formalin and saline for histopathological evaluations and oxidative parameter examination.

### 2.8. Preparation of tissues homogenate.

The liver (10% w/v) was excised and minced using a tissue homogenizer in an ice-cold 0.1 mol/L sodium phosphate buffer (pH 7.4).

### 2.9. Change in body weight and organ weight.

The body weight was determined as reported by Yadav *et al.* [14], and the following formula calculated the liver's absolute weight or relative weight ratio:

$$\text{Ratio} = \frac{\text{Liver weight (g)}}{\text{Body weight of mice on sacrificed day (g)}} \times 100 \quad (1)$$

### 2.10. Hepatic injury evaluation parameters.

#### 2.10.1. Serum biochemistry.

Biochemical analyses, including serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), and serum bilirubin, were carried out using a semi-automatic biochemical analyzer with commercial biochemical kits [15, 16].

#### 2.10.2. Antioxidant parameters.

Firstly, the liver was perfused with 0.86% cold saline to eliminate the red blood cells. The liver was then sliced into small pieces after hanging it in 10% (w/v) ice-cold 0.1 phosphate buffer (pH 7.4), and the needed number of liver pieces was homogenized using a Teflon homogenizer. The levels of SOD and GPx were measured and estimated by using the homogenate [17].

### 2.11. Histopathological evaluations.

Hepatic tissue was immersed in newly made 10% phosphate-buffered formalin (PBF) overnight to complete fixation, then trimmed into small pieces, re-fixed in PBF, and embedded in paraffin using a mechanical processing machine. Slices of 5  $\mu\text{m}$  thickness were stained with H & E for histopathology. By using a light microscope, magnified images of tissue structure were captured and micro-photographed [18].

### 2.12. Statistical analysis.

The observations were statistically analyzed with the help of GraphPad Prism 9. All results were presented as mean  $\pm$  SEM. The results were analyzed using One-Way Analysis of Variance (ANOVA), followed by Tukey's multiple comparison test, and  $p < 0.0001$  indicated statistical significance.

### 3. Results and Discussion

#### 3.1. Determination of quantitative phytochemical assays.

##### 3.1.1. Total phenolic content.

The average TPC range was found to be  $116.37 \pm 1.51$  to  $322.70 \pm 1.44$  mg GAE/g in all extracts. Following is the ascending order of phenol content at a significant level of ( $p < 0.05$ ) in different fractions: Petroleum ether extract of leaf < Aqueous extract of leaf < Methanol extract of leaf. The highest TPC was found to be  $322.70 \pm 1.44$  mg/g in petroleum ether extract (Table 1). This amount was compared with the gallic acid (standard) using a standard curve ( $y = 0.004x + 0.553$  and  $R^2 = 0.994$ ).

##### 3.1.2. Total flavonoid content.

The average TFC range was found to be  $57.68 \pm 0.51$  to  $132 \pm 1.08$  mg QE/g in all extracts. Following is the ascending order of phenol content at a significant level of ( $p < 0.05$ ) in different fractions: Petroleum ether extract of leaf < Aqueous extract of leaf < Methanol extract of leaf. The highest TPC was found to be  $132 \pm 1.08$  mg/g in petroleum ether extract. This amount was compared with the quercetin (standard) using a standard curve ( $y = 0.153x + 0.345$  and  $R^2 = 0.997$ ). Among all three extracts, petroleum ether extract had a high content of phenols and flavonoids, as shown in Table 1. The quantification of phytochemicals revealed the presence of phenolics and flavonoids in *U. dioica* leaf extracts. These phytochemicals have already been shown to have antioxidant and hepatoprotective properties.

**Table 1.** Total phenolic and flavonoid content in different extracts of *U. dioica*.

Extract and standard	TPC (mg of GAE/g)	TFC (mg of QE/g)
Petroleum ether	$322.70 \pm 1.44^c$	$132.05 \pm 1.08^c$
Methanol	$116.37 \pm 1.51^a$	$57.68 \pm 0.51^a$
Aqueous	$196.33 \pm 1.52^b$	$116.3 \pm 1.37^b$
Gallic acid	$332.83 \pm 1.38$	-
Quercetin	-	$140.38 \pm 1.31$

The values represent the means of the triplicates  $\pm$  SD. Different letters show significance at  $p < 0.05$  as compared to standard.

#### 3.2. Change in body weight and organ weight.

Significant reduction in b. wt. was observed in MTX-treated mice compared to the non-toxic group (Group I). Mice treated with standard and low and high doses of petroleum ether extract of *U. dioica* showed a significant increase in body weight when compared with Group II (Table 2).

**Table 2.** Effect of MTX, silymarin, and *Urtica dioica* on body weight and organ weight of the experimental mice.

Parameters	Vehicle control group	Treated control group	Standard control group	Minimum dose group	Maximum dose group
Body weight (g)	$250 \pm 1.01$	$298 \pm 1.22^b$	$240 \pm 1.54^a$	$232 \pm 0.50^b$	$439 \pm 0.26^b$
Organ weight (g)	$2.51 \pm 0.09$	$3.11 \pm 0.11^a$	$2.65 \pm 0.04^a$	$2.95 \pm 1.22^a$	$2.71 \pm 1.13^a$

The values represent the means of the triplicates  $\pm$  SD. Different letters show significance at  $p < 0.05$  as compared to normal control.

The organ weight was significantly increased in Group II animals compared to the control, and the treated dosage of standard and extract alleviated this elevation. The animals in

the treated control showed significant loss of b. wt. which was caused by irregular intestinal clearance with diarrhea and increased urine excretion [19 20].

### 3.3. Effect of *Urtica dioica* Linn on biochemical parameters in MTX-induced hepatotoxicity.

#### 3.3.1. SGOT level.

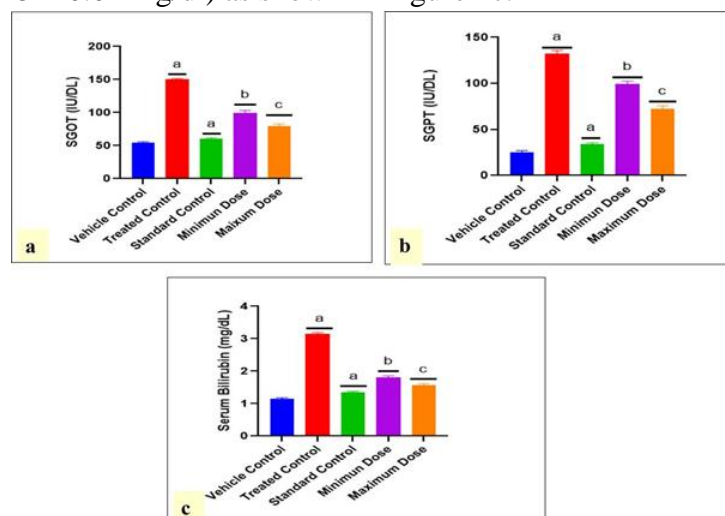
MTX significantly elevated the SGOT level from  $54.40 \pm 0.60$  (mg/dl) to  $150 \pm 0.37$  (mg/dl) compared with the Vehicle control group. In addition, an increase in the level of SGOT was significantly observed in the standard control group (Silymarin) from  $54.40 \pm 0.60$  (mg/dl) to  $60.6 \pm 0.60$  (mg/dl). On the other hand, the petroleum ether extract of *U. dioica* at 100 mg/kg and 200 mg/kg with MTX exhibited a significant reduction in the levels of SGOT from  $99.98 \pm 1.46$  (mg/dl) and  $79.53 \pm 1.36$  (mg/dl) when compared with treated control group  $150 \pm 0.37$  (mg/dl) as shown in Figure 2a.

#### 3.3.2. SGPT level.

The MTX produced a significant elevation in the levels of SGPT from  $25.4 \pm 0.74$  (mg/dl) to  $132.6 \pm 1.36$  (mg/dl) when compared with the vehicle control group. In addition, the level of SGPT significantly increased from  $25.4 \pm 0.74$  to  $34 \pm 0.70$  (mg/dl). On the other hand, the petroleum ether extract of *U. dioica* at doses of 100 mg/kg and 200 mg/kg with MTX showed a significant reduction of SGPT levels  $99.49 \pm 1.31$  and  $72.40 \pm 1.34$  (mg/dl) level when compared with treated control group  $132.6 \pm 1.36$  (mg/dl) as shown in Figure 2b.

#### 3.3.3. Bilirubin level.

MTX significantly elevated serum bilirubin levels from  $1.14 \pm 0.01$  (mg/dl) to  $3.15 \pm 0.01$  (mg/dl) when compared with the vehicle control group. In addition, the bilirubin level was observed to increase from  $1.14 \pm 0.01$  (mg/dl) to  $1.34 \pm 0.17$  (mg/dl) in the standard group compared to the vehicle control group. On the other hand, the petroleum ether extract of *U. dioica* at doses of 100 mg/kg and 200 mg/kg with MTX showed a significant reduction in the levels of serum bilirubin  $1.81 \pm 0.01$  and  $1.57 \pm 0.10$  (mg/dl) level when it was compared with treated control ( $3.15 \pm 0.01$  mg/dl) as shown in Figure 2c.



**Figure 2.** Effects of *Urtica dioica* Linn on (a) SGOT; (b) SGPT; (c) Serum bilirubin levels in MTX-induced hepatotoxicity. The values are expressed as mean  $\pm$  SEM. a =  $p < 0.0001$  vs. vehicle control; a vs. treated control (a =  $p < 0.0001$ ); a vs. b = ( $p < 0.0001$ ) minimum dose of *Urtica dioica*; c =  $p < 0.0001$  vs. maximum dose of *Urtica dioica*.

MTX in different doses is used for treating various diseases such as cancer, acute leukemia, or severe psoriasis. Still, in high doses and continuous use, it can cause organ toxicity, such as cirrhosis, progressive hepatic fibrosis, and hepatotoxicity. The mechanism involved in MTX-induced toxicity is the conversion of MTX into MTX-Glu by different enzymes present in the liver, which increases the mean residence time of MTX in the organ and leads to hepatotoxicity by induction of inflammatory agents and oxidative stress. Oxidative stress is the main causal factor involved in hepatotoxicity, so antioxidant parameters were also evaluated in this plant extract [21].

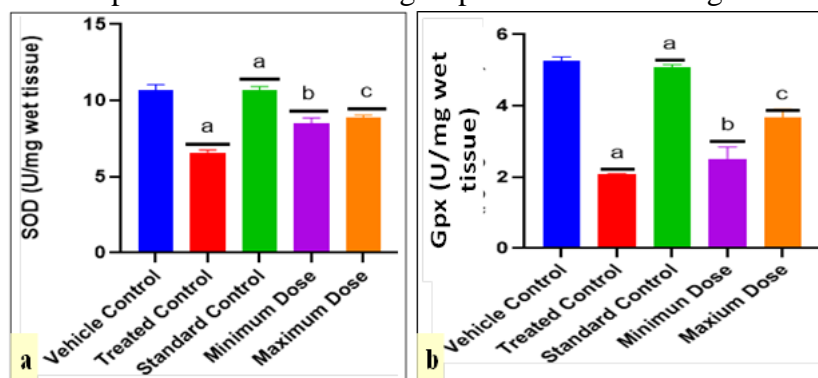
### 3.4. Level of oxidative stress biomarkers in response to *Urtica dioica* treated MTX-induced hepatotoxicity.

#### 3.4.1. SOD level.

MTX produced a significant reduction in the SOD level from  $10.70 \pm 0.15$  to  $6.57 \pm 0.07$  U/mg when compared with the vehicle control group. In addition, the level of SOD significantly decreased from  $10.70 \pm 0.15$  to  $10.70 \pm 0.14$  in the standard group when it was compared to the vehicle control group because of the opening of the mitochondrial permeability transition pore. On the other hand, the petroleum ether extract of *Urtica dioica* at doses of 100 mg/kg and 200 mg/kg with MTX showed significant elevation in serum SOD levels  $8.47 \pm 0.17$  and  $8.87 \pm 0.07$  U/mg when compared with treated control  $6.57 \pm 0.07$  U/mg as shown Figure 3a.

#### 3.4.2. GPx level.

MTX significantly reduced the levels of GPx from  $5.26 \pm 0.47$  to  $2.07 \pm 0.009$  U/mg when compared with the vehicle control group. In addition, the level of GPx was significantly decreased from  $5.26 \pm 0.47$  to  $5.09 \pm 0.02$  U/mg in the standard group compared to the vehicle control group. On the other hand, the extract of *Urtica dioica* at 100 mg/kg and 200 mg/kg with MTX showed significant elevation in the levels of serum GPx  $2.51 \pm 0.14$  and  $3.67 \pm 0.10$  U/mg when it was compared with the control group  $2.07 \pm 0.009$  U/mg as shown in Figure 3b.



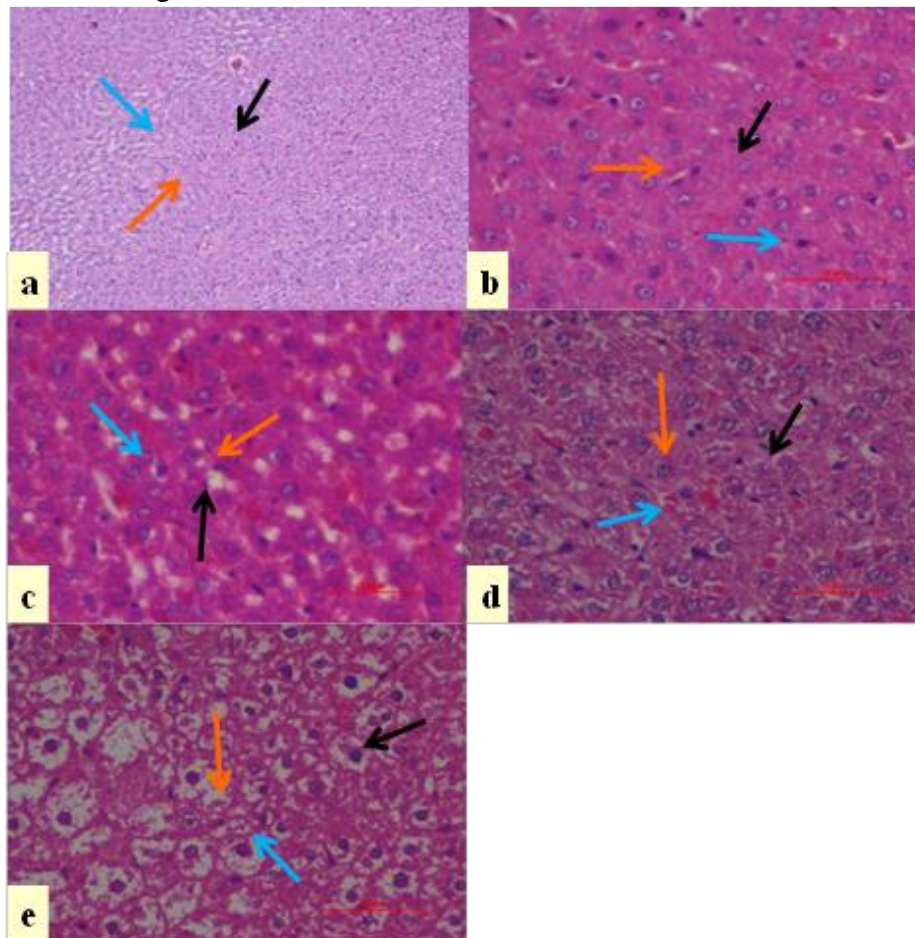
**Figure 3.** Effects of *Urtica dioica* Linn on (a) SOD level; (b) glutathione peroxidase level in MTX-induced liver toxicity. The values represent the means of the triplicates  $\pm$  SEM. a =  $p < 0.0001$  vs. vehicle control; a vs. treated control (a =  $p < 0.0001$ ); a vs. b ( $p < 0.0001$ ) minimum dose of *Urtica dioica*; vs. c =  $p < 0.0001$  vs. Maximum Dose of *Urtica dioica*.

The hepatotoxicity directed by MTX elevates the liver marker enzymes and releases them into the bloodstream. Treatment with petroleum ether extract of *Urtica dioica* reduced serum levels of SGOT, SGPT, and bilirubin to normal levels, indicating plasma membrane stabilization and healing of toxicant-induced hepatic tissue damage. MTX also has an effect on

the liver's antioxidant system, causing antioxidant levels to drop considerably. The *Urtica dioica* extract greatly increased the levels of SOD and GPx. These results are consistent with those of Joshi *et al.* [4]. SOD is the initial line of defense against the harmful effects of free radicals, whereas GPx oxidized the hydrogen peroxide-free radicals using reduced glutathione [2, 22].

### 3.5. Effects of *Urtica dioica* Linn on histological changes in MTX-induced hepatotoxicity.

The histological changes in the liver were observed in the experimental protocol shown in Figure 4. The hepatic cells in vehicle control were found to be normal, with proper hepatocytes around the central vein and a clear architecture of the nucleus and membrane of the cell, as shown in Figure 4a.



**Figure 4.** Effects of various treatments on histology of rat's liver. (a) vehicle control [normal liver architecture with mild congested central vein (red arrow), normal hepatocyte (blue arrow), and normal sinusoids (black arrow)]; (b). treated control [extensive inflammatory cells reaction (black arrow) with dilated and congested central vein (red arrow) and multifocal marked hepatocyte degenerative changes and cytoplasmic vacuolation (blue arrow)]; (c). standard control [significant reduction in the histopathological lesions in all hepatic lobular zones congested central vein (red arrow), normal hepatocyte (blue arrow), and normal sinusoids (black arrow)]; (d) *Urtica dioica* extract (minimum dose) + MTX [showed severe hepatocyte degenerative changes (blue arrow) with mild inflammatory cells reaction (black arrow) and mildly dilated and congested central vein (red arrow)]; (e) *Urtica dioica* extract (maximum dose) + MTX [showed mild degenerative changes (blue arrow) with mild perivascular inflammatory cells (black arrow) reaction and mild congestion in the central vein (red arrow)].

In the present study, the treated control group showed severe hepatic damage regarding degenerative alterations such as vacuolation of the cytoplasm of cells and fatty changes in scattered cells. Moreover, the central vein's hepatocytes showed extensive necrosis with

nuclear psychosis and vascular cytoplasmic degeneration in MTX-treated rats (Figure 4b). Standard control (Silymarin) showed a significant hepato-protective effect (Figure 4c). The petroleum ether extract of *Urtica dioica* attenuated hepatocyte necrosis and restored very normal morphological characteristics at doses of 100 mg/kg (Figure 3d) and 200 mg/kg (Figure 4e). The histopathological findings indicated that the extract normalized the microscopic pathological alterations. Thus, the plant extract was found to be more effective in treating liver dysfunction at a dose of 200 mg/kg than 100 mg/kg. This finding is ascribed to the phenolic compounds found in the leaves of *U. dioica*, which have possible hepatoprotective activity.

#### 4. Conclusions

The current study aims to evaluate the therapeutic effects of *U. dioica* on MTX-induced liver injury in rats, as well as the role of oxidative stress and serum markers in liver injury. Rat was administered MTX (20 mg/kg) over a 7-day period to produce hepatotoxicity. The index of liver damage was determined by monitoring the levels of SGOT, SGPT, and bilirubin. Furthermore, the liver's oxidative stress was measured. The current investigation found that *U. dioica*, both in preventative and curative, greatly reduced liver dysfunction and oxidative stress caused by MTX. In the present study, MTX administration resulted in severe fatty change vacuolization, cellular degeneration, an extension of the cellular vein, and necrosis with nuclear pycnosis in hepatic cells. However, *U. dioica* Linn's higher dose effectively restored the normal architecture of the liver and ameliorated MTX-induced histopathological changes. The current finding may indicate that MTX produces severe liver damage, which is improved by the therapy of herbal medication *U. dioica*.

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#### Conflict of interests

The authors have declared that no conflict of interest exists.

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