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Histological changes induced by Piroxicam on the hepatic and renal tissues of mice with and without administration of Peppermint oil

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ABSTRACT

Piroxicam is a popular anti-inflammatory drug that displays palliative and antipyretic activity. Peppermint oil is a common flavoring used in foods and drinks. To investigate the defensive action of Peppermint oil against the hepatic and renal histological damage induced by Piroxicam in mice.

Forty healthy adult Swiss albino mice of both sexes were categorized into 4 groups (10 mice in each group): Control group (I); Treatment group (II) – injected with Piroxicam 0.3 mg/kg/rat/day via intraperitoneal route for 28 days; Treatment group (III) – oral Peppermint oil 0.2 ml/kg/day by oral gavage 24 hours preceding each injection of Piroxicam; Treatment group (IV) oral Peppermint oil alone. Blood samples were withdrawn to estimate the hepatic and renal functions. Immediately after death, specimens of liver and kidney from the four groups were isolated and put in 10% concentration buffered formalin for 24 hours then prepared for light microscopic examination.

There was a highly significant rise in the serum level of hepatic enzymes (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase and total serum bilirubin) in the group treated with Piroxicam, as compared to the control group. These returned to near normal level in the group treated with Piroxicam and Peppermint oil. Liver samples of the treated mice showed ballooning degeneration of hepatocytes, small apoptotic hepatocytes and inflammatory cellular infiltration, whereas kidney sections revealed cystic dilatation of Bowman's space, shrinkage of glomerular tuft and apoptosis of epithelial cells lining the tubules. In contrast, the addition of peppermint oil efficiently ameliorated the hepatic and renal tissue changes.

Piroxicam induces hepatorenal toxicity as exhibited by histological, histochemical and biochemical findings. Peppermint oil shows ameliorative properties against the hepatorenal toxic effects induced by Piroxicam.

INTRODUCTION

Pain is the novel cause of economic and social burden expressed by about 20% of the entire population. Narcotic analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) are optional for managing all types of pain, although NSAIDs are preferred above narcotics due to their good efficacy, fewer adverse effects and low drug abuse potential [1]. Piroxicam is a member of the oxicam family. These act by suppressing prostaglandins, prostacyclins and thromboxanes genesis via non-selective inhibition of the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [2]. Arbitrary clinical studies suggest the use of Piroxicam mesotherapy in patients diagnosed with osteoarthritis, rheumatoid arthritis, acute gout, even as an adjunct treatment of carcinoma [3]. Piroxicam (FELDENE®) sealed as 20 mg capsules has a long half-life (40 hrs). The therapeutic dose in human is 10-20 mg as a single or divided dose, and it is metabolized in the liver and excreted by the kidney [4]. Piroicam, unfortunately, induces several respiratory, gastrointestinal and cerebrovascular side effects. Oxidative stress mediates the Piroxicam

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toxicity via generation of free radicals, lipid peroxidation and degradation of glutathione [5]. Modified regimens have been suggested to minimize the predictable toxicity. Examples of this are the sulfonated Piroxicam derivatives [6]. Moreover, numerous natural antioxidants and herbal remedies might reverse the drug toxicity by neutralizing reactive oxygen species, strengthening the endogenous glutathione and restoring the optimal balance [7].

The natural aromatic herb, Peppermint (*Mentha piperita* L.), belongs to the Lamiaceae (Labiatae) family, and is commercially cultivated in the northern USA and Canada. Commercial preparations labeled 'Peppermint oil' also include a mixture of water mint (*Mentha aquatic* L.) and spearmint (*Mentha spicata* L.) [8]. The herb is widely consumed in the management of digestive, respiratory and nervous disorders and as a flavouring agent in foods or cosmetic products [9]. Many clinical studies have motivated the use of Peppermint oil in managing irritable bowel syndrome (IBS) owing to its worth as smooth muscle relaxant of the gastrointestinal tract [10]. Peppermint oil has a relatively high LD_{50} (Lethal $Dose_{50}$ is the amount of a substance that is needed to kill half of a test population of animals), and falls into the "safe" category, hence, it is widely used as a food additive [11]. A randomized trial in 2019 proposed that the antioxidant activity of Peppermint oil is mostly mediated by free radical scavenging and by inhibiting lipid peroxidation [12].

There are conflicting reports regarding the hepatorenal toxicity of Piroxicam and few studies have been issued to demonstrate the exact and most competent ameliorative remedy to the histological damage induced by Piroxicam on the tissues of liver and kidney. The target of the current study is thus to explore the histological changes induced by Piroxicam on the liver and kidney of mice and to investigate whether Peppermint oil has a defensive role against Piroxicam toxicity.

MATERIALS AND METHODS

Forty healthy adult Swiss albino mice of both sexes weighing from 25-30 g were obtained from the Animal House in the College of Veterinary Medicine, University of Mosul. The experiments were approved by the Medical Research Ethics Committee in the College of Medicine, University of Mosul at 4/6/2021. The work was done from May to November 2021.

The animals were accommodated in the laboratory room under controlled constant conditions of temperature and light with unrestricted access to laboratory commercial food and water ad libitum. They were adapted for one week prior to the experiments.

The mice were distributed into four groups, 10 mice in each. These consisted of: a control group (I) given only 0.9% normal saline intraperitoneally for 4 weeks; experimental group (II) treated with Piroxicam (0.3 mg/kg) as a toxic dose [13,14] by intraperitoneal injection for 4 weeks; experimental group (III) given Peppermint oil orally 0.2 ml/kg [15] by oral gavage 24 hours earlier to each intraperitoneal injection of Piroxicam (0.3 mg/kg) for 4 weeks; and experimental group (IV) given oral Peppermint oil 0.2 ml/kg day for 4 weeks. After the latest injection, the mice were sacrificed and their peritoneal cavities were explored, then specimens of liver and kidney of the four groups were removed and kept in buffered formalin of 10% concentration for 24 hours. Following fixation, the specimens were dehydrated and embedded into paraffin blocks. Subsequently, the blocks were sectioned, and the sections stained with Harris Hematoxylin and Eosin (H&E) and Periodic Acid Schiff's stains (PAS) so as to be examined under light microscope.

Biochemical analysis

Intracardiac blood samples were drawn by heart puncture, centrifuged, then serum samples were separated for estimation of hepatic function by measuring the level of marker enzymes serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) using commercial kits. Results were measured as units/uL. Evaluation of renal function was done by measuring the blood urea and the serum creatinine levels, using a specialized Urea Enzymatic Colorimetric Kit, and a Creatinine Colorimetric Kit, respectively.

Morphometric measurements

A morphometric study to compare some parameters among the groups was conducted by means of digital image camera (Scope Image 9.0 – Taiwan) accompanied by image processing software. Random non-overlapping fields were chosen from each section for capturing the images. The software of the camera was standardized to all lenses of an Olympus-CX31 microscope (Japan) by assistance of a 0.01 mm stage micrometer (ESM11/Japan).

Statistical analysis

Statistical analysis was expressed as mean±SD and analyzed using one-way Analysis of Variance (ANOVA) by means of the statistical package of SPSS-26 (2019, IBM Corp.®, USA) and Microsoft Excel 2019 (Microsoft ©, USA). The statistical significance was tested at a P-value ≤ 0.05 by applying Chi-square test and student t-test for analysis of the results.

RESULTS

During the period of experiment, no deaths occurred. According to daily monitoring of the activity of mice in the cages and the amount of food eaten by them, the animals of the control group and group IV were active with good appetite, group II mice became less active with less appetite, group III continued attentive with normal food intake.

Biochemical results

Serum ALT, AST and ALP and TSB in group II revealed a significant surge as in comparison with the control group. Significantly lower enzyme levels were noticed in group III following administration of Peppermint oil prior to Piroxicam, compared to the group II. Normal values of enzymes were observed in mice treated with Peppermint oil alone (group IV) (Table 1).

Table 1. Comparison of the level of the serum marker of hepatic and renal functions in the normal and experimental mice (Data expressed as mean±SD)

	Group I	Group II	Group III	Group IV	P-value
Liver					
ALT (IU/L)	18.5±0.5	28.5±0.7	22.5±0.8	17.5±0.6	0.001**
AST (IU/L)	50±0.8	65.5±0.7	53.4±0.6	54.6±0.7	0.001**
ALP (IU/L)	75±0.6	88.6±1.5	74.4±0.5	76.5±0.8	0.001**
TSB	0.45±0.13	0.45±0.17	0.45±0.14	0.45±0.1	0.15
Kidney					
Blood urea (mg/dl)	20.5±3.5	25±2.8	23.5±2.1	20.5±2.2	0.03
Serum Creatinine (mg/dl)	0.08±0.03	0.13±0.02	0.09±0.01	0.08±0.01	0.02

Non significance at P-value >0.05
* statistical significance at P-value <0.05
** high statistical significance at P-value <0.01

Histomorphometric results

Morphometric measurements of liver sections revealed that the number of Kupffer cells was not significantly different in the group treated with Piroxicam (group I) and in group IV, when compared with the control mice (P = 0.1), while the number is significantly more in group III as compared to the control (P = 0.01). A highly significant increase in the diameter of sinusoids in group II, in comparison with the control group (P = 0.001) was evident, while the diameter was not significantly different in group III and IV when compared to control mice (P-value >0.05) (Tab. 2), (Fig. 1 and 2).

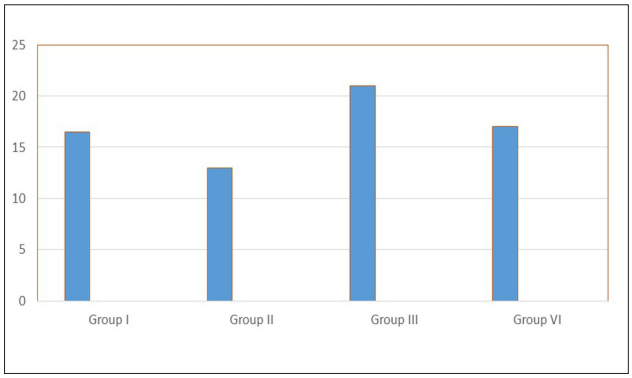

Figure 1. Morphometric comparison of number Kupffer cells among the groups (Data expressed as mean±SD)

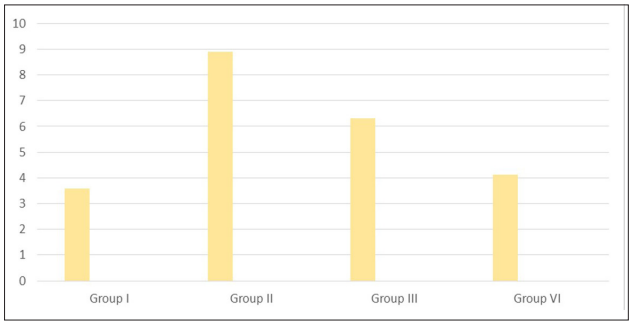

Figure 2. Morphometric comparison of diameter of sinusoids (μm) among the groups (Data expressed as mean±SD)

Table 2. Morphometric comparison of some parameters of the liver among the control and experimental groups (Data expressed as mean±SD)

	Group I	Group II	Group III	Group VI	Statistical significance	P-value
No. of Kupffer cells (46000 μm²/40 × field)	16.55 ±0.9	13.88 ±0.8	21.32 ±1.3	17.3 ±1.1	I vs. II I vs. III I vs. VI	0.1 0.01 0.1
Diameter of sinusoids /μm	3.57 ±0.2	8.9 ±0.5	6.34 ±0.4	4.11 ±0.1	I vs. II I vs. III I vs. VI	0.001** 0.1 0.2

non significant at P-value >0.05), *statistical significance at P-value> 0.05, **high statistical significance at P-value >0.01)

Morphometry of the kidney sections revealed a substantial decrease in the glomerular diameter in group II, compared to control and also when compared with all other groups. A highly significant increase in the diameter of Bowman's space in group III treated with Piroxicam in comparison with the control mice was noted. The diameter of lumen of both proximal and distal convoluted tubules showed an extremely significant rise in group II compared to the control group and the other groups, as well (Table 3).

Table 3. Morphometric comparison of some parameters of the kidney among the control and experimental groups (Data expressed as mean±SD)

	Group I	Group II	Group III	Group VI	P-value
Glomerular diameter/μm	112.25 ±6.3	87.91 ±3.5	104.275 ±7.4	109.76 ±5.1	0.001**
Bowman's space diameter/μm	6.3 ±0.2	15.7 ±0.9	8.2 ±0.8	5.1 ±0.1	0.001**
Proximal convoluted tubule lumen diameter/μm	10.12 ±1.2	20.33 ±1.7	9.34 ±0.7	11.56 ±0.8	0.001**
Distal convoluted tubule lumen diameter/μm	18.91 ±0.6	26.88 ±1.4	17.39 ±0.7	20.02 ±0.9	0.001**

(Data expressed as mean±SD,
non significant at P-value >0.05), *statistical significance at P-value >0.05, **high statistical significance at P-value >0.01)

Histological outcomes

The liver of the control mice seemed lobulated, soft and brown, the gross appearance was comparable in all the groups. The sections of hepatic tissue showed normal architecture (as plates or cords of polygonal hepatocytes), however, some were binucleated (Fig. 3).

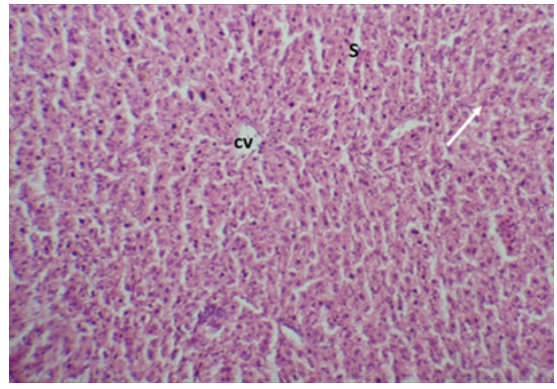

Figure 3. Micrograph of liver from control mice showing central vein (CV), hepatocytes (white arrows), hepatic sinusoids (S) (H&E × 100)

Liver sections of group II revealed focal areas of coagulative necrosis of hepatocytes (Fig. 4), as well as ballooning degeneration of hepatocytes observed around the central vein and in the whole hepatic lobules with cobweb-like cytoplasm and disintegrating central faint nuclei (Fig. 5). Dilatation and congestion of central vein, small apoptotic

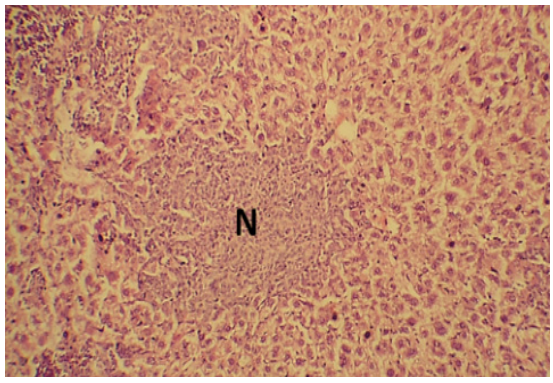

Figure 4. Micrograph of liver from group II showing focal area of coagulative necrosis of hepatocytes (N) (H&E × 100)

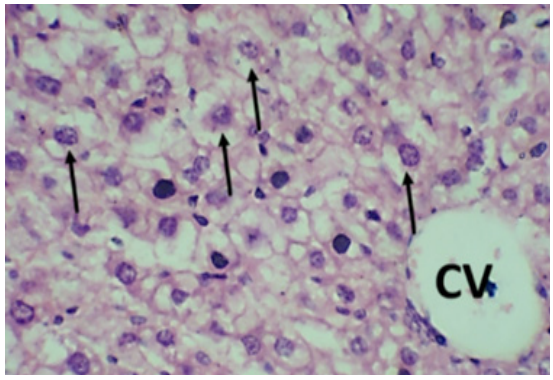

Figure 5. Micrograph of liver from group II showing ballooning degeneration of hepatocytes (black arrows) round the central vein (CV) and in the whole hepatic lobule (H&E × 600)

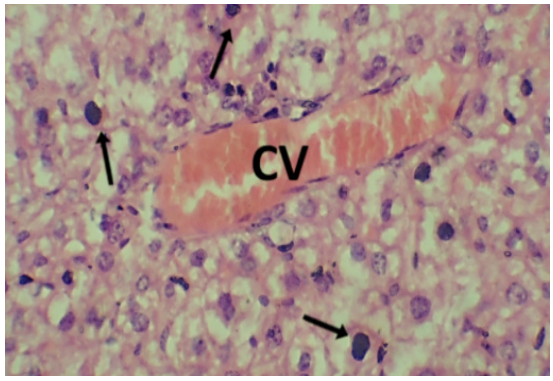

Figure 6. Micrograph of liver from group II showing dilatation and congestion of central vein (CV), apoptotic hepatocytes with eosinophilic cytoplasm and pyknotic nuclei (black arrows) (H&E × 600)

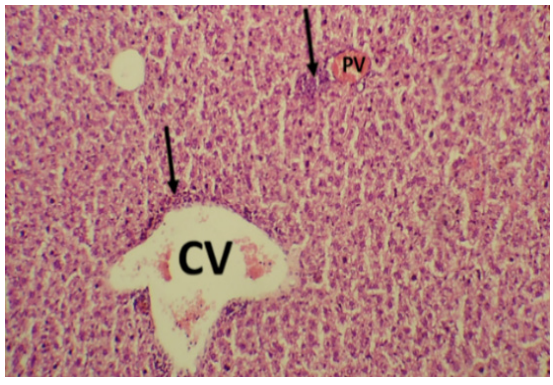

Figure 7. Micrograph of liver from group III showing reverse of normal architecture of liver with mild congestion of central vein (CV) and portal vein (PV)and few perivascular inflammatory cell infiltration (black arrows) (H&E × 100)

hepatocytes with deeply stained eosinophilic cytoplasm and condensed pyknotic nuclei (Fig. 6) was also noted. When Peppermint oil was administered prior to Piroxicam, the mice livers showed reversion to normal architecture of liver, with mild dilatation of central vein and portal vein and few perivascular infiltrations of inflammatory cells (Fig. 7). The livers of group IV mice showed the customary view of hepatocytes, apart from mild congestion of central vein and portal vein and dilated sinusoids (Fig. 8).

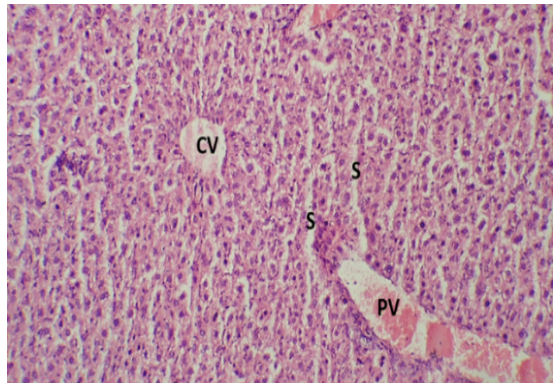

Figure 8. Micrograph of liver from group IV normal view of hepatocytes apart from mild congestion of central vein (CV) and portal vein (PV) and dilated sinusoids (H&E × 100)

The sections of kidney obtained from the control mice showed normal proximal convoluted tubules (PCT), distal convoluted tubules (DCT) and renal corpuscles, each comprised of glomerular tuft surrounded by Bowman›s capsule (Fig. 9). The mice kidneys of group II revealed shrinkage of glomerular tuft with widening of Bowman's space (Fig. 10). In addition, we saw cystic dilatation of Bowman's space, with focal inflammatory cell infiltration (Fig. 11), as well as apoptosis of epithelial cells lining the renal tubules with eosinophilic cytoplasm and pyknotic nuclei (Fig. 12). The mice kidneys of group III displayed reversal to normal architecture of kidney, apart from moderate dilatation of Bowman's space (Fig. 13). The mice kidneys of group IV showed normal appearance of renal tissue, apart from interstitial hemorrhage within the renal parenchyma (Fig. 14).

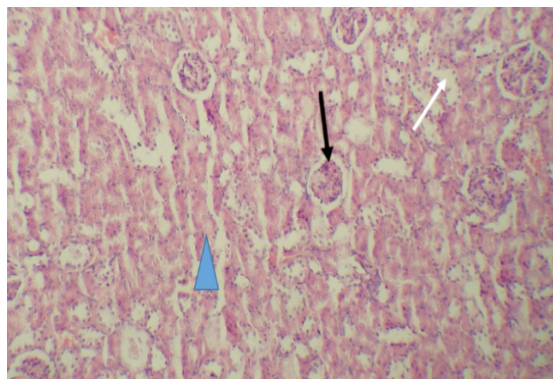

Figure 9. Micrograph of kidney from group I revealed renal corpuscles (black arrow), proximal convoluted tubule (arrow head), distal convoluted tubule (white arrow) (H&E × 100)

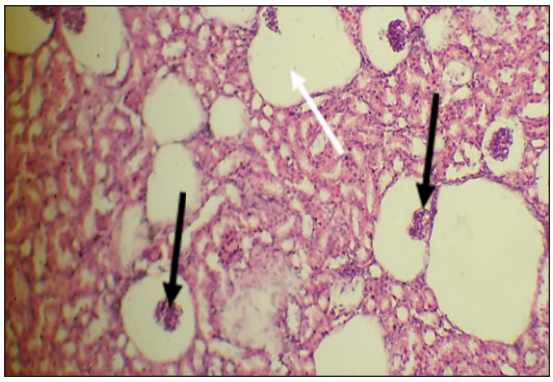

Figure 10. Micrograph of kidney from group II revealed shrinkage of glomerular tuft (black arrow) with widening of bowman's space (white arrow) (H&E × 100)

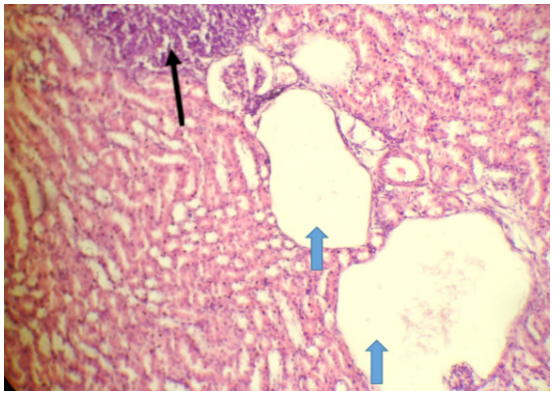

Figure 11. Micrograph of kidney from group II revealed cystic dilatation of bowman's space (blue arrows) with focal inflammatory cell infiltration (black arrow) (H&E × 100)

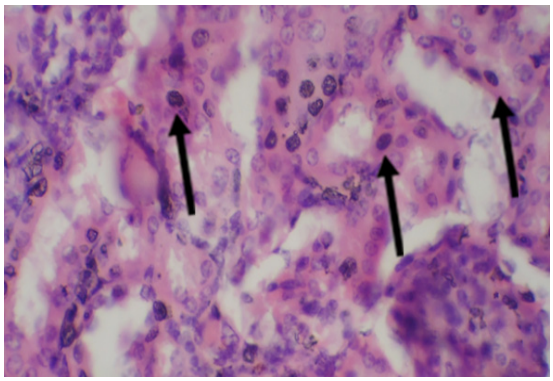

Figure 12. Micrograph of kidney from group II revealed apoptosis of epithelial cells lining the renal tubules with eosinophilic cytoplasm and pyknotic nuclei (black arrows) (H&E × 400)

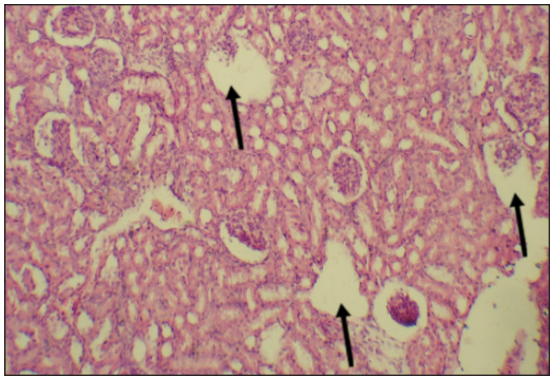

Figure 13. Micrograph of kidney from group III revealed reverse of normal architecture of kidney apart from mild dilatation of bowman's space (black arrows) (H&E × 400)

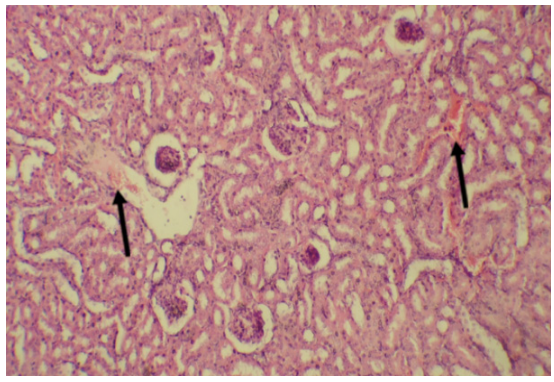

Figure 14. Micrograph of kidney from group IV revealed normal appearance of renal tissue apart from interstitial hemorrhage within the renal parenchyma (black arrow) (H&E × 100)

A strong positive magenda color of glycogen in the cytoplasm of the hepatocytes stained with PAS stain was observed in group I (Fig. 15). In contrast, a weak positive reaction to PAS in the liver sections of group II was evident as compared to the control mice (Fig. 16) with non-homogenous distribution of the glycogen contents. We saw a strong positive reaction in group III that was nearly similar to the control mice (Fig. 17), as well as a strong positive reaction in group VI (Fig. 18).

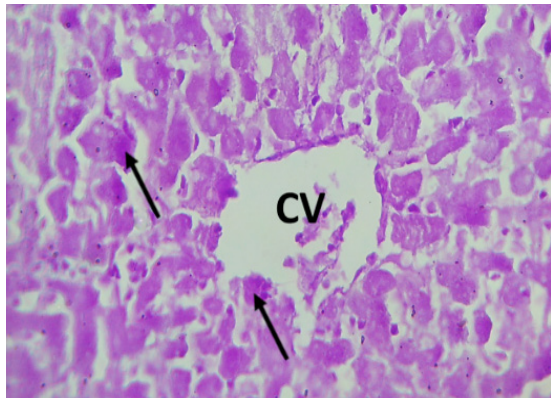

Figure 15. Micrograph of liver from control group illustrating a strong positive magenta color of PAS stain in the hepatocytes (black arrows) around the central vein (CV) (PAS × 600)

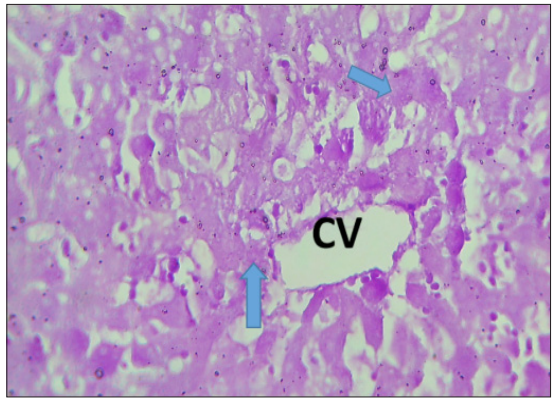

Figure 16. Micrograph of liver from group II illustrating a weak positive reaction to PAS stain in hepatocytes (arrows) (PAS × 600)

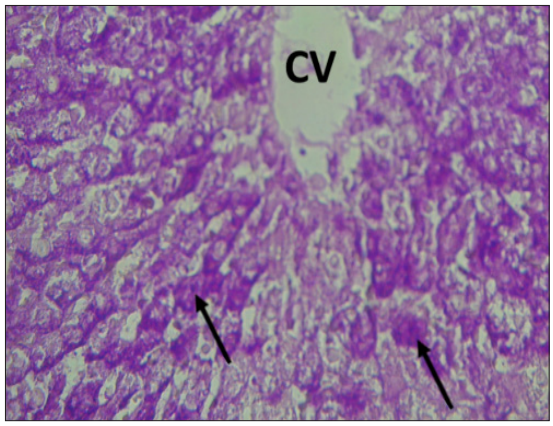

Figure 17. Micrograph of liver from group III illustrating a strong positive reaction to PAS stain in hepatocytes (PAS × 600)

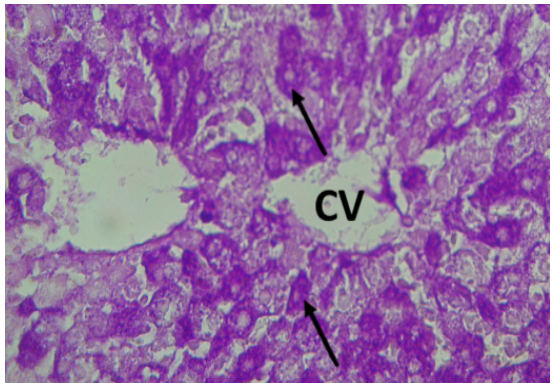

Figure 18. Micrograph of liver from group IV illustrating a strong positive reaction to PAS stain in hepatocytes (PAS × 600)

DISCUSSION

Piroxicam is a conventional NSAID that belongs to the oxicam category. A substantial amount of literature suggests that the damaging effects of PM may be attributed to oxidative stress, in addition to its nonselective-inhibitory effect on COX enzymes [17]. Oxidative damage is initiated due to exhaustion of endogenous antioxidant molecules, followed by generation of reactive oxygen species (hydrogen peroxide, hydroxyl radical, and superoxide anion. Subsequently, tissue damage is elicited by DNA damage, mitochondrial perturbation and lipid peroxidation and apoptotic cell death [18]. In the present study, the elevated serum AST, ALT, and ALP enzymes could be explained due to the oxidative hepatic cells damage and loss of membrane integrity (which contributes to leaking out of these enzymes). Such findings are similar to previous reports [19].

In the current study, prominent alterations in the histological architecture of the liver in group II could be due to intermediate substances which mediate the inflammatory process secreted by the infiltrated inflammatory cells following administration of Piroxicam in a dose higher than the therapeutic dose. This caused oxidative hepatic tissue damage via vasodilatation with excessive blood loss. These substances include proinflammatory cytokines, vasoactive amines eicosanoids and acute-phase proteins [20]. Previous studies state that mitochondrial damages can be provoked by enhanced generation of reactive oxygen species induced by Piroxicam resulting in altered gene expression and disturbances of liver function. Consequently, the impaired liver function may reduce blood osmotic pressure, thus the dilated sinusoids become more permeable to erythrocytes with subsequent interstitial hemorrhage and clot formation [21]. Moreover, as suggested by Omar R, ballooning degeneration of hepatocytes might be a result of preferential inhibition of COX-I and oxidative damage induced by Piroxicam causing fluid retention into the hepatocytes [22]. In addition, vacuolar degeneration of hepatocytes might be a consequence of substantial disturbance in the lipid metabolism, since that the suppressed synthesis of prostaglandins caused by Piroxicam leads to a reduced blood flow and hepatic tissue ischemia with subsequent mitochondrial dysfunction. This is considered to be the principal mode of a cascade of events including DNA damage and lipid peroxidation [23].

The present work clarified that Piroxicam administration induces small apoptotic hepatocytes with condensed pyknotic nuclei. Such finding is similar to widespread apoptosis in the mice liver following administration of Indomethacin, and can be attenuated by L-cystein [24], Furthermore, apoptosis might switch to focal areas of coagulative necrosis when large numbers of hepatocytes are undergoing apoptotic changes [25]. Inflammatory cells infiltration around the blood vessels is a noticeable response to any detrimental impacts. However, the pathogenesis of NSAID toxicity has been suggested to be related to the adherence of the accumulated inflammatory cells to the vascular endothelium [26].

Hepatic tissue damage caused by free radicals is similar to the hepatic ischemia associated with hypoxia, and the cytokine storm syndrome was noticed in COVID-19 patients, particularly in elderly and liver transplant recipients [27]. Similarly, elevated liver enzymes levels were previously observed in patients regularly treated with chemotherapeutic agents such as 5- fluorouracil, and infliximab, which induces oxidative stress and Kupffer cell activation [28].

In the current study, shrinkage of the glomerular tuft with widening of Bowman's space might be attributed to vasoconstriction of the glomerular capillaries and retraction of the mesangial cell processes that may be stimulated by the presence of angiotensin II in their cytoplasm. Such findings might agree with the interesting study of Abdeen *et al.* [29], who investigated the role of Coenzyme Q10 in ameliorating the oxidative injury induced by Piroxicam in the stomach, liver and kidney.

The tubular cell damage detected in this study was accompanied by inflammatory cells infiltration that invaded into the intertubular tissues to minimize the injury. Similar acute kidney injury has been known to be induced by Diclofenac [30]. Other studies suggest that toxicity of Piroxicam comes about because it acts as a potent chelator of zinc, copper, selenium and manganese – which are the cofactors of the endogenous antioxidant, and because it has a notable negative impact on their scavenging activity [31].

The reversibility of disturbed hepatic and renal histological structure in the mice treated with Peppermint oil might be attributed to its antioxidant properties that are demonstrated by its scavenging of free radical and suppressing of lipid peroxidation and its increasing of the tissue contents of glutathione and peroxidase enzymes [32]. However, other studies indicate that the antioxidant activity of *Mentha*

piperita essential oil might be primarily explained due to the synergistic interaction between its active component (menthol and iso-menthone) creating a satisfactory defense against oxidative damage [33]. The results of the current study agreed greatly with that of Bellassoued *et al*. [34], who suggested that the *Mentha piperita* leaf essential oil has protective effects against oxidative damage induced by CCl4 in rats. Similarly, Mariani *et al*. [35] declared that the hepatic damage occurred after immobilization stress in mice could be reversed to normal following administration of Peppermint oil.

Reductions in proteins and polysaccharides contents in the liver are reflected as weak positive reactions to PAS staining. Such alteration in the carbohydrates metabolism have been attributed to the oxidative stress, insulin resistance hepatic steatosis and improper hepatic energy homeostasis [36]. Other investigators state that the reduced protein contents in the liver could be due to the distraction of lysosomal membranes provoked by several toxicants, with subsequent liberation of their hydrolytic enzymes. This effect promotes marked disbanding and lysis of the cells – as occurs in the liver of mice treated with high-fat Diet induced liver steatosis [37]. The defensive effects of Peppermint oil against the oxidative damage induced by Piroxicam might explain the strong positive reaction to PAS staining in the liver of group III. Such findings have been previously confirmed by Mostapha *et al*. [38].

CONCLUSION

The current study concluded that Piroxicam via oral or parenteral routes might induce drastic adverse effects on the liver and kidney, including degenerative changes, vascular congestion and apoptosis, and may affect the functions of these organs by reducing serum marker enzymes. Thus, Piroxicam should be used under stringent medical control. Oral Peppermint oil has been revealed to have defensive effects against Piroxicam-induced hepatic and renal toxicity by combating oxidative stress, scavenging free radical and suppressing lipid peroxidation. Further investigations are essential to expose the precise mechanism of the active constituents of *Mentha piperita* leaf essential oil against the hepatotoxicity and nephrotoxicity caused by Piroxicam.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Al-Hamdany MZ. Injection of the animals and histological preparation: Al-Hamdany MZ and Al-Tai, Reading of pathological changes and analysis of the results: Ismail H.K. Writing of the manuscript: Al-Hamdany MZ. Jointly developed the structure and arguments for the paper. All authors reviewed and approved of the final manuscript.

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