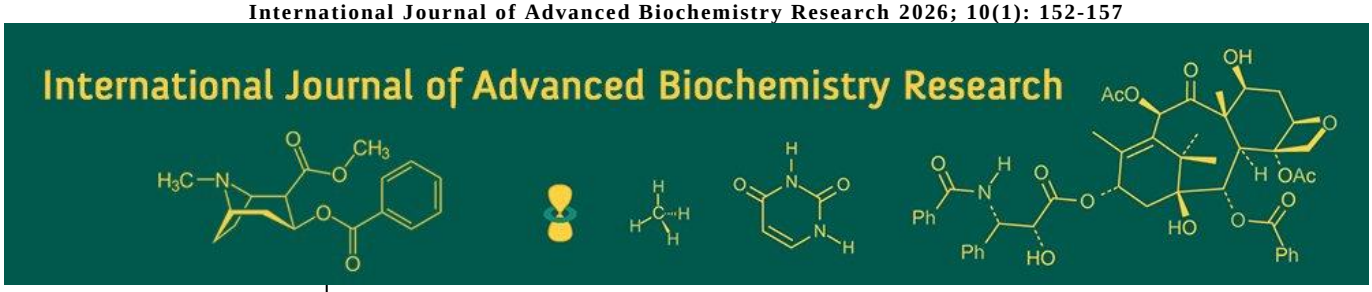

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Ritu J Patel
Department of Veterinary Pathology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat, India

Dinesh J Ghoadasara
Department of Veterinary Pathology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat, India

Bharat B Bhanderi
Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat, India

Anik M Mathakiya
Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat, India

Raish M Sherasiya
Department of Veterinary Medicine, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Junagadh, Gujarat, India

Corresponding Author:
Ritu J Patel
Department of Veterinary Pathology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand, Gujarat, India

Protective effect hesperidin on methotrexate induced toxicity in Wistar rats: A histopathological study

Ritu J Patel, Dinesh J Ghoadasara, Bharat B Bhanderi, Anik M Mathakiya and Raish M Sherasiya

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Abstract
This present research work was conducted on 40 Wistar rats to study the methotrexate (MTX) toxicity and ameliorative effects of hesperidin. Wistar rats were randomly divided into 4 different groups with 5 male and 5 female in each group. The group were numbered as group I to IV. Group I treated as a control and received 1% CMC (carboxymethyl cellulose) in distilled water thorough orally for 28 days. Group II treated with single dose of MTX at the dose rate of 20mg/kg/b.wt on 21st day of study via i.p. Whereas group III received hesperidin at the dose rate of 100mg/kg/b.wt orally for 28 days with single dose of MTX @20mg/kg on 21st day of study via i.p. and group IV 200mg/kg/b.wt orally for 28 days and single dose of MTX @20mg/kg on 21st day of study via i.p. All the surviving rats were sacrificed on by high dose of sodium pentothal 29th day of the experiment for necropsy. The appropriate tissue samples have been collected, fixed in 10% neutral buffered formalin, and histopathologically 85 examined in detail. Methotrexate group showed necrosis, congestion, degeneration of the renal tubules and infiltration of inflammatory cells in interstitial spaces of kidney. In liver congestion of central vein, dilatation of sinusoids, degenerative changes in hepatocytes and fatty changes were seen in methotrexate treated group. Methotrexate along with hesperidin treated female and male rats (group III & IV) showed improvement in histopathological lesion in liver and kidney. This study suggests hesperidin have a protective effect against MTX.

Keywords: Methotrexate toxicity Hesperidin, Wistar rats, Pathomorpholog0079

Introduction
Chemotherapy has been important component in human and animal cancer patients for last many years. Now a days in India cancer cases were rapidly increased in the animals. Cancer is an uncontrolled growth out of control and spread to other areas of your body. Chemotherapy drug has a cytotoxic effect on cancer cells as well as normal cells. Chemotherapy drug such as Methotrexate (MTX) has antimetabolite, immunomodulatory, anti-inflammatory, immunosuppressive and cytotoxic properties and used in a wide range of clinical practice (Peters *et al*., 2000) [17].

Methotrexate widely used as a first line of treatment in autoimmune disease, inflammatory disease such as a rheumatoid arthritis, crones' disease and psoriasis. Methotrexate also used in the treatment of lymphoma, breast tumours, Osteosarcoma, invasive urinary bladder tumours, autoimmune disease and in veterinary oncology. Numerous organs and tissue affected with MTX treatment including kidney, spleen, liver, small intestines, ovary, testicles and nerve cells (Li ex *et al*., 2016) [12].

Flavonoids are categorized as flavones (Apigenin, Tangeretin), flavanols (Quercetin, Rutin, Myricetin) flavanones (Hesperidin, Naringin, flavonols), isoflavones (Genistein, Daidzein), chalcone (Phlorectin, Arbutin) and anthocyanin (Cyanidin, Malvidin) according to structural variations (Panche *et al*., 2016) [16]. Hesperidin is one of the most commonly used and biologically active compounds in the flavonoid family **(**Turk *et al.*, 2019) [18]. Hesperidin also has antibacterial, antioxidant, lipid-lowering, anti-inflammatory, antiviral, anti-hypertensive, anti- carcinogenic, and antioedema effects. Hesperidin has some pharmacological effects like effects on anti-fertility, vascular system, action on enzyme, platelet and cell aggregation inhibition, ultra violet protecting activity and also some miscellaneous effect like anti-allergic, analgesic, antipyretic activity, effect on wound healing and anti-ulcer activity (Garg *et al*., 2001) [8].

Material and Methods

The study was conducted at small animal house and Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand. The study was approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, Gujarat, India. The animal facility of Cadila Pharmaceuticals Company Ltd. in Dholka, Gujarat, India provided a total of 40 Wistar rats (20 male and 20 female), which were used in experiments.

The procedures for animal care and management followed to those given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. All of the rats were kept in polypropylene cages at a laboratory animal house facility (College of Veterinary Science and Animal husbandry, Anand) in a climate-controlled room with a constant temperature of 22 ± 3 ºC and a humidity of 30-70%. There was a 12/12- hour cycle of light and dark. The rats were kept stress-free by using corncob as bedding and following all appropriate management protocols. Before experiments, all rats were acclimated for 15 days.

Rats were given free access to a regular pellet food and palatable water for the duration of the trial. The feed was provided by keval sales corporation, Vadodara, Gujarat. Rats were identified by having picric acid applied to their body coats to mark them. One cage had five rats, each of which had a ring at the base of its tail to represent the number of the animal (rats 1, 2, 3, 4, and 5 consecutively).

Methotrexate and hesperidin were purchased from cadila healthcare Ltd, Ahmedabad and Sigma Aldrich company respectively.

A total of 40 Wistar rats (20 males and 20 females) were divided into four groups at random, each including five female and five male rats. The groups give numbers ranging from I to IV. Group I (control): Rats treated with vehicle 1% carboxymethylcellulose (CMC) dissolved in distilled water through orally and single dose of normal saline via intraperitoneal route. Group II (MTX): Rats were received single dose of MTX at the dose of 20 mg/kg/bw via intraperitoneally. Group III (MTX and hesperidin 100 mg): Rats received MTX at the dose of 20 mg/kg through intraperitoneally at the 21st day and HSP @ 100 mg/kg/bw through orally for 28 days. Group IV (MTX and hesperidin 200 mg): Rats were given single dose of MTX @ 20 mg/kg i.p at 21st day and HSP at the dose of 200 mg/kg/bw for 28 days via oral route.

Every Wistar rats in the study was examined twice a day for morbidity and mortality. The clinical observations have been recorded once daily during the adaptation period. Clinical and behavioural observations were carried out at least twice on each day of dosing during the experimental period (before and after therapy).

All the surviving rats were sacrificed on by high dose of sodium pentothal 29th day of the experiment for necropsy. All rats were starved overnight before necropsy. The final body weight was recorded prior to necropsy.

For histopathology, kidney, liver, heart, lung, spleen, stomach, intestine, and brain tissues were collected and preserved in 10% neutral buffered formalin and testes have been stored in modified Davidson fluid. Postmortem examination were carried out of all the animal and gross lesion were recorded if any.

The tissue from sacrificed animals, including the kidney, liver, heart, brain, spleen, lungs, testes, stomach, and intestine, were collected. Any adherent tissue was subsequently removed if needed, and the tissue organ was preserved in 10% neutral buffer formalin for at least 48 hours. sTissue processing and staining: During necropsy, the tissue of the kidney, liver, heart, brain, spleen, lungs, testes, stomach, and intestine were collected in 10% neutral buffered formalin. The collected tissues were processed in an automatic tissue processor, and then paraffin blocks were prepared in an automatic tissue embanding station. 5-6 µm section were cut with an automatic microtome. Sections were stained with H&E (Haematoxylin & Eosin) stains (Luna, 1968) with standard protocol. Under a light microscope, the Haematoxylin and Eosin-stained slides were examined and microscopic lesion were recorded.

Results

Pathomorphology Kidney

In male rats, kidney section showed normal histoarchitecture details in control group

(I) rats (Fig. 1.1). The kidney section of methotrexate group (II) revealed severe degeneration of tubules, decreased lumen size of tubules (Fig. 1.2), infiltration of inflammatory cells, congestion in interstitial spaces along with necrosis of glomeruli (Fig. 1.3, Fig. 1.4). The kidney section of hesperidin 100mg+methotrexate group (III) showed mild degeneration of tubular epithelial cells and presence of red blood cell cast (Fig. 1.5). The kidney section of hesperidin 200mg+methotrexate group (IV) showed mild tubular degeneration (Fig. 1.6).

In female rats, control group revealed normal histoarchitecture details in the kidney section (Fig. 1.7). Methotrexate treated group (II) rats showed severe necrosis and tubular degeneration, presence of tubular cast along with infiltration of inflammatory cells and detachment of glomeruli from basement membrane (Fig. 1.8, Fig. 1.9). The kidney section of hesperidin 100mg+methotrexate group (III) showed mild degeneration, infiltration of inflammatory cells and capillary congestion in cortex (Fig. 1.10). The kidney section of hesperidin 200mg+methotrexate group (IV) revealed mild congestion in interstitial spaces of kidney (Fig. 1.11).

Liver

Histopathological lesion in the liver from male rats in control group revealed normal histoarchitecture details (Fig.1.12). The liver of rats treated with methotrexate group (II) showed severe degeneration of hepatocytes, dilation of sinusoids near portal triad, fatty changes in hepatocyte (Fig. 1.13, Fig. 1.15), congestion of central vein (Fig. 1.14) and focal infiltration of inflammatory cells (Fig. 1.13). The liver section of hesperidin 100mg+methotrexate group (III) rat revealed mild to moderate dilatation of sinusoids and fatty changes of hepatocyte (Fig. 1.16). The liver of rats treated with hesperidin 200mg+methotrexate group revealed only mild dilation of sinusoids (Fig. 1.17).

In female rats, liver section showed normal histoarchitecture details in control group (I) (Fig. 1.18). The liver of rat treated with methotrexate group (II) revealed diffuse degeneration and fatty changes of hepatocyte (Fig. 1.19). The liver section of hesperidin 100mg+methotrexate group (III) rats observed fatty changes in hepatocyte (Fig. 1.20). The liver of rats treated with hesperidin

200mg+methotrexate group revealed mild congestion in central veins (Fig.1.21).

Discussion

Similarly, to present findings, Jahovic *et al.*, bserved extensive degeneration of glomeruli, swelling of proximal tubule in methotrexate treated rats. Vardi *et al.* (2013) [1+], Morsy *et al.* (2013) [14] and Erboga *et al.* (2015) noticed tubular dilation, tubular degeneration and glomerular congestion in methotrexate treated rats. Cakir *et al*., noticed tubular necrosis, tubular epithelial degeneration, narrowing of Bowman's space, congestion and interstitial inflammation in methotrexate treated rats. Ahmed and Abdulmajeed (2017) [3] observed focal degeneration of renal tubular epithelial cells, infiltration of inflammatory cells in interstitial spaces and necrosis in methotrexate treated rats.

Moustafa *et al.* (2022) [15] noticed aluminum phosphide treated rats showed vacuolar degeneration, cloudy swelling, degeneration of renal tubular epithelial cells while co-administration of hesperidin and aluminium phosphide treated rats revealed restoring of the normal glomerular architecture with reappearance of a number of normal capillary loops and the proximal convoluted tubule in hesperidin treated rats. Kasem *et al.* (2022) [11] observed Bisphenol treated rats revealed infiltration of inflammatory cells in interstitial tissue and glomerular destruction while co administration of bisphenol and hesperidin treated rats revealed infiltration of few inflammatory cells with no congestion.

The present findings were in accordance with by Tunali-Akbay *et al*. They noticed degeneration of hepatocytes, infiltration of inflammatory cells around portal vein and central vein in methotrexate treated rats. Abdul *et al.* (2012) [1] revealed scattered dysplastic hepatocytic changes, periportal inflammatory cell infiltration and sinusoidal dilation in methotrexate treated rats. Yucel *et al.* (2017), Ekinci-akdemir *et al.* (2018) [7], Kalantari *et al.* (2018) [10], Hoshyar *et al.* (2020) [9], Azadnasab *et al.* (2021) and Abdel-Raheim *et al.* (2022) [15] also observed infiltration of inflammatory cells, fatty changes, congestion of central vein, necrosis and dilation of sinusoids in methotrexate treated rats.

The hepato protective effect of hesperidin were also observed by Ansar *et al*. (2018) [4]. They observed expansion of portal tract, hyalinization of hepatocytes, infiltration of inflammatory cells, increased number of Kupffer cell and increased accumulation of cells around the central vein in zinc oxide treated rats. Co administration of hesperidin and zinc oxide in rats revealed mild degeneration of hepatocyte and dilation of sinusoids. Mesallam and Atef (2020) [13] noticed diazinon treated rats showed congestion of the sinusoids and central vein, hepatocytes had pyknotic nuclei and intracytoplasmic vacuolation, dilation of bile ducts, hyalinization and thickening of the arterial wall and infiltration of inflammatory cells. Co administration of hesperidin with diazinon-treated rats revealed improvement indicated by congestion and dilation of the central vein and sinusoids and the majority of the hepatocytes were polyhedral with acidophilic cytoplasm and rounded vesicular nuclei. Aboraya *et al*. (2022) [2] observed Cisplatin treated rats showed local extensive necrosis of hepatocytes, congestion of the sinusoids while hesperidin + cisplatin treated rats showed mild focal necrosis of the hepatocytes.

Conclusion

Methotrexate group showed necrosis, congestion, degeneration of the renal tubules and infiltration of inflammatory cells in interstitial spaces of kidney. In liver congestion of central vein, dilatation of sinusoids, degenerative changes in hepatocytes and fatty changes were seen in methotrexate treated group. Methotrexate along with hesperidin treated female and male rats (group III & IV) showed improvement in histopathological lesion in liver and kidney.

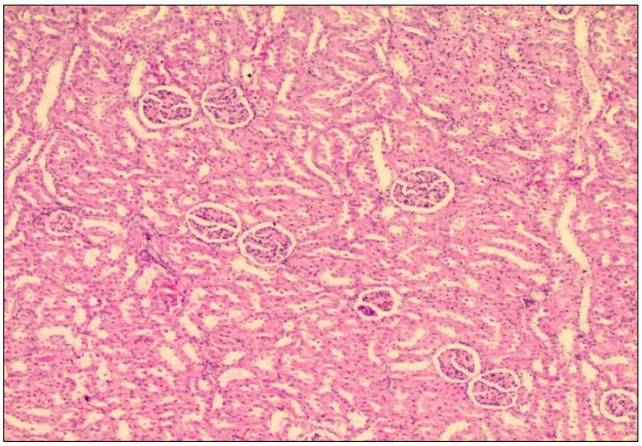

Fig 1.1: Section of Kidney from control group (I) male rats showing normal histological architecture. (H &E stain, 100X)

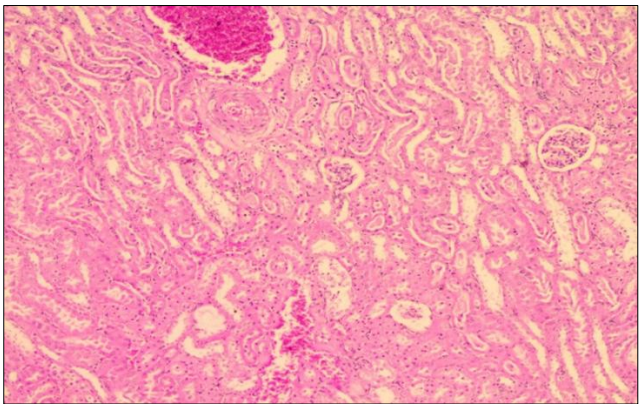

Fig 1.2: Section of kidney from methotrexate group (II) male rats showing severe degeneration of tubules, decreased lumen size of tubules and congestion in interstitium. (H & E stain, 100X)

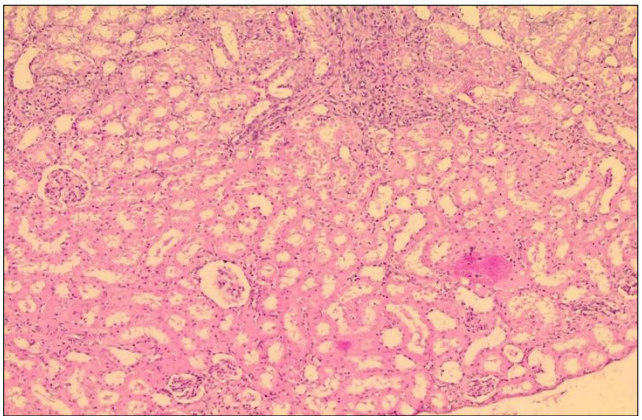

Fig 1.3: Section of kidney from methotrexate group (II) male rats showing severe degeneration of tubular epithelial cells, infiltration of the inflammatory cells and necrosis of the glomeruli. (H & E stain, 100X)

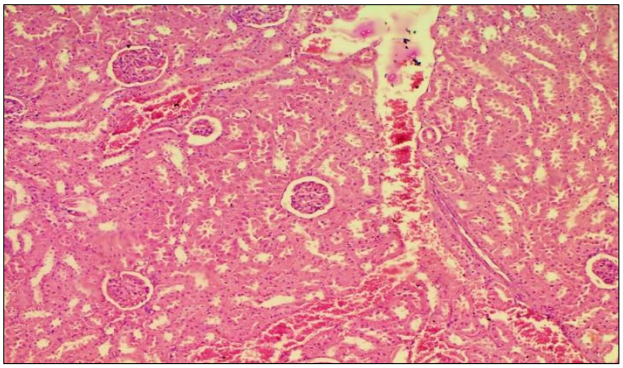

Fig 1.4: Section of kidney from methotrexate group (II) male rats showing severe degeneration of tubular epithelial cells, severe necrosis of glomeruli and congestion in the interstitial spaces. (H & E stain, 100X)

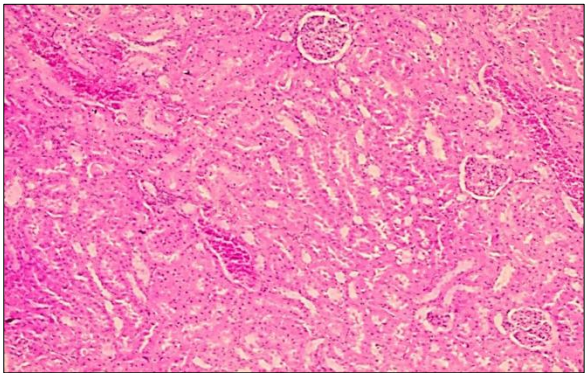

Fig 1.5: Section of kidney from hesperidin 100mg + methotrexate group (III) male rats showing moderate degeneration of tubular epithelial cells and congestion in the interstitium of the kidney. (H & E stain, 100X)

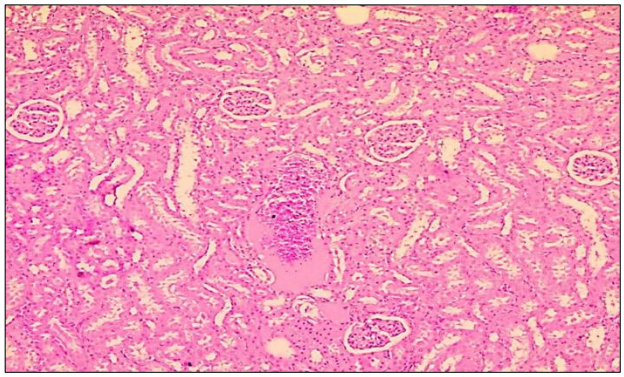

Fig 1.6: Section of kidney from hesperidin 200mg + methotrexate group (IV) male rats showing mild degeneration of tubular epithelial cells. (H & E stain, 100X)

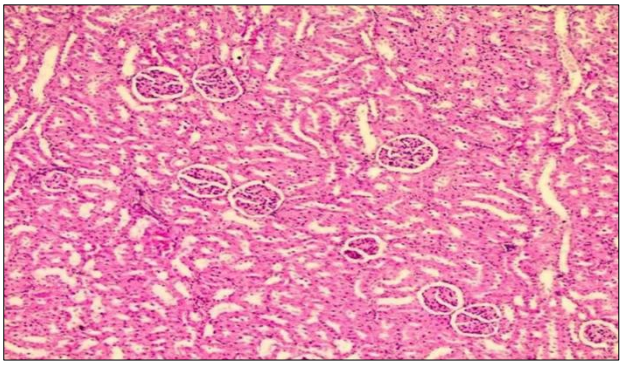

Fig 1.7: Section of Kidney from control group (I) female rats showing normal histological architecture. (H & E stain, 100X)

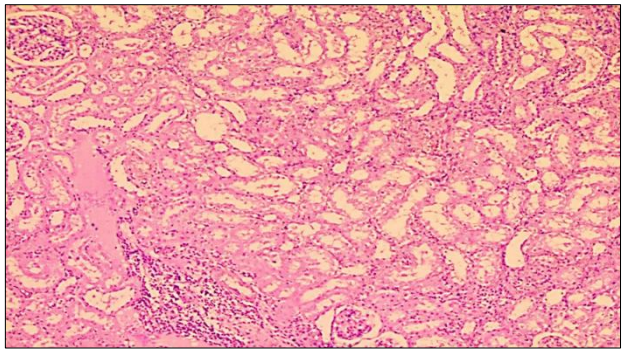

Fig 1.8: Section of kidney from methotrexate group (II) female rats showing severe tubular degeneration, presence of tubular cast along with infiltration of inflammatory cells and detachment of glomeruli from basement membrane. (H & E stain, 100X)

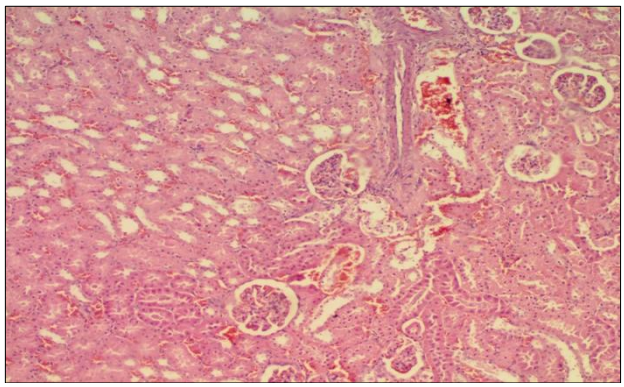

Fig 1.9: Section of kidney from methotrexate group (II) male rats showing severe necrosis and degeneration of tubular epithelial cells and congestion in the interstitium of the kidney. (H & E stain, 100X)

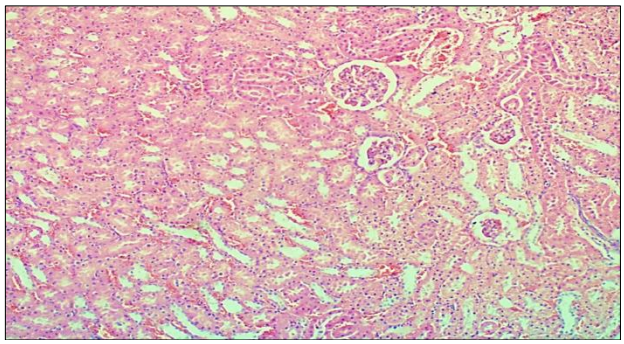

Fig 1.10: Section of kidney from hesperidin 100mg + methotrexate group (III) female rats showing mild degeneration of renal tubules, infiltration of inflammatory cells and capillary congestion in cortex. (H & E stain, 100X)

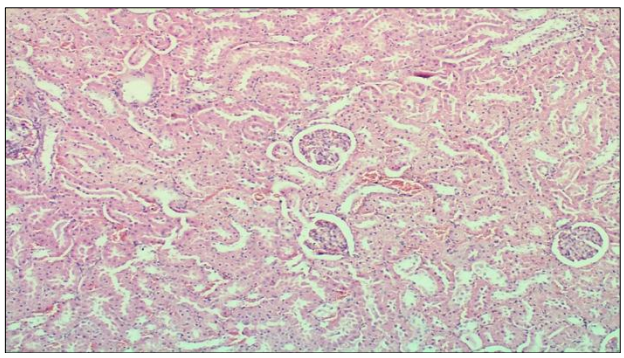

Fig 1.11: Section of kidney from hesperidin 200mg + methotrexate group (IV) female rats showing congestion in interstitial spaces of kidney. (H & E stain, 100X)

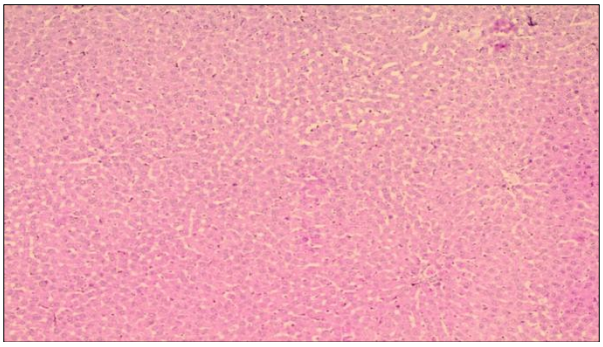

Fig 1.12: Section of liver from control group (I) male rats showing normal histological architecture. (H & E stain, 100X)

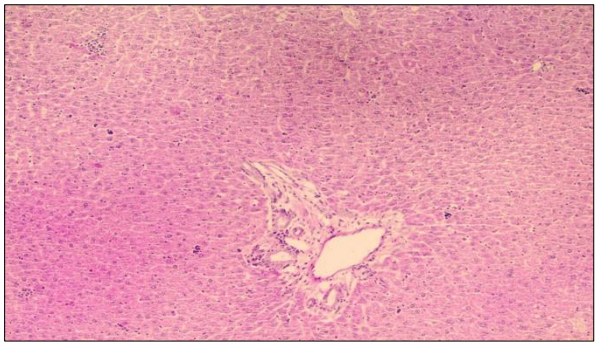

Fig 1.13: Section of liver from methotrexate group (II) male rats showing severe degeneration of hepatocyte, mild dilation of sinusoids near portal triad and focal infiltration of inflammatory cells. (H & E stain, 100X)

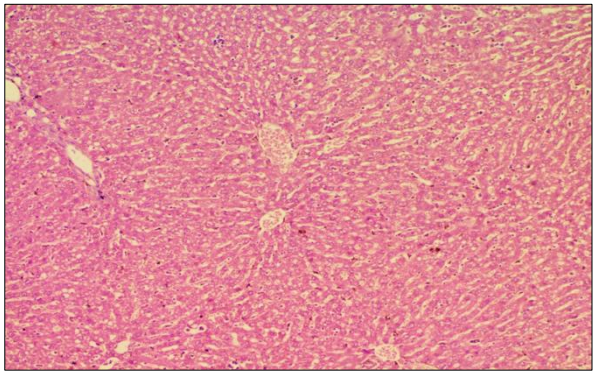

Fig 1.14: Section of liver from methotrexate group (II) male rats showing severe degeneration of hepatocyte, dilation of sinusoids, congestion of central vein and fatty changes. (H & E stain, 100X)

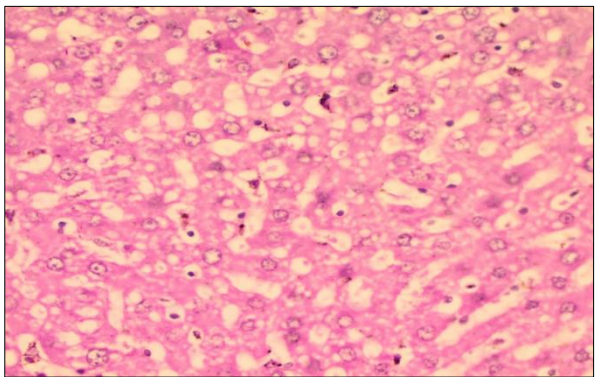

Fig 1.15: Section of liver from methotrexate group (II) male rats showing dilation of sinusoids, and fatty changes. (H & E stain, 400X)

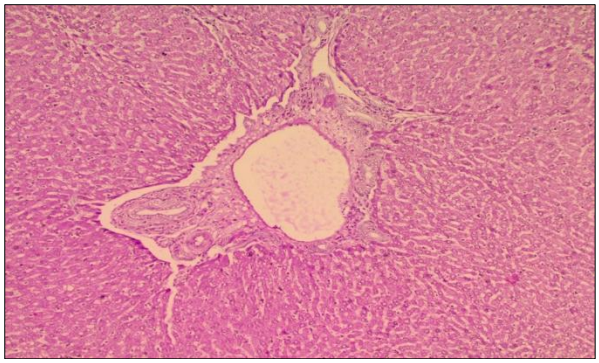

Fig 1.16: Section of liver from hesperidin 100mg + methotrexate group (III) male rats showing mild to moderate dilation of sinusoids and fatty changes of hepatocytes around portal triad. (H & E stain 100X)

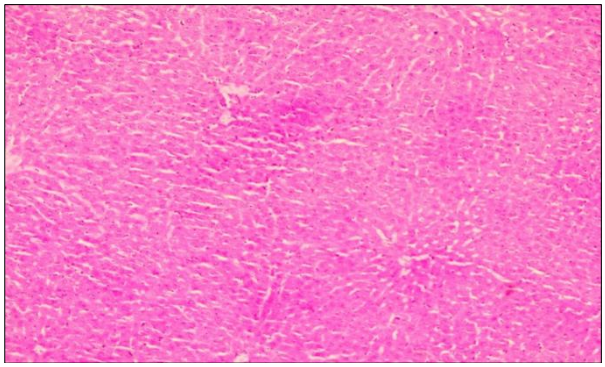

Fig 1.17: Section of liver from hesperidin 200mg + methotrexate group (IV) male rats showing mild dilation of sinusoids. (H & E stain, 100X)

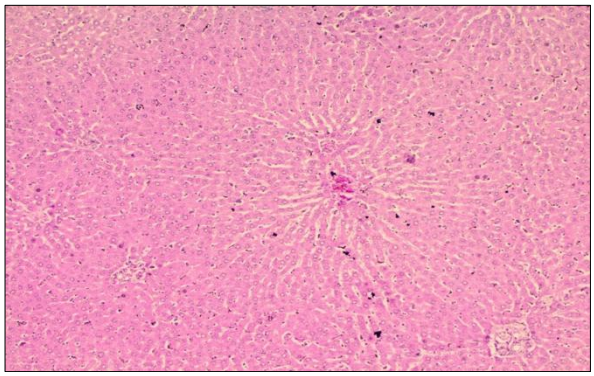

Fig 1.18: Section of liver from control group (I) female rats showing normal histological architecture. (H & E stain, 100X)

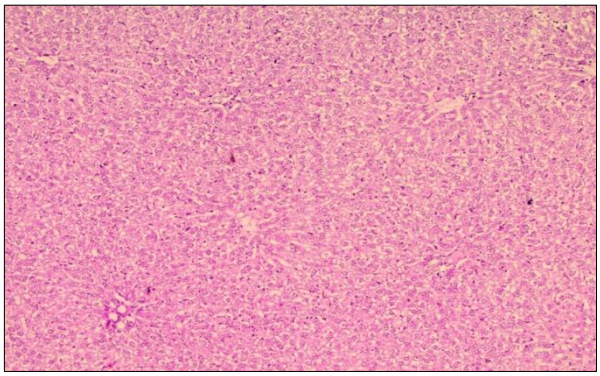

Fig 1.19: Section of liver from methotrexate group (II) female rats showing diffuse degeneration and fatty changes

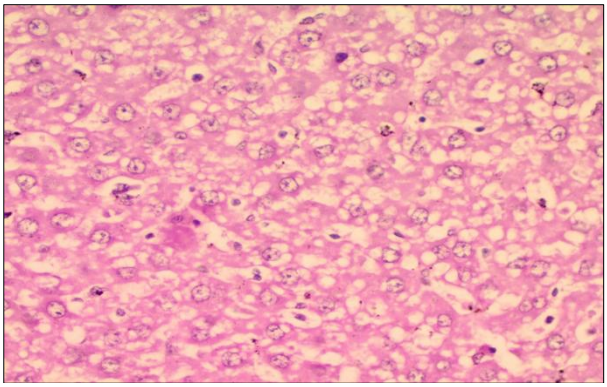

Fig 1.20: Section of liver from hesperidin 100mg+methotrexate group (III) female rats showing degeneration of hepatocyte along with fatty changes

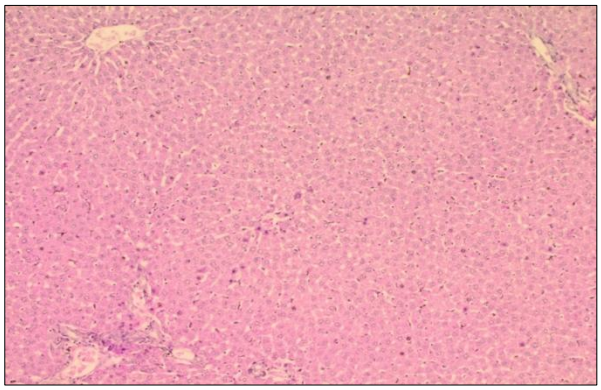

Fig 1.21: Section of liver from hesperidin 200mg+methotrexate group (IV) female rats showing mid congestion in central vein. (H & E stain, 100x)

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