



# Protective role of *Viola odorata* against hepatotoxicity induced by methotrexate in albino male mice

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## Abstract

Major organ of detoxification is Liver and first target exposure to side effect of drug, methotrexate(MTX) is one of different drugs that use as therapy for many diseases such as arthritis and cancer, besides that, show hepatotoxicity effect. For which strict laboratory and histological oversight has been launched. To estimate protective effect of *viola odorata* against hepatotoxicity induced by MTX this study has been designed.

Sixteenth male mice were divided into four groups (n=4 each), control negative group received normal saline while second group received MTX(positive control) at dose of (40 mg/kg). In addition to that, an interaction between *viola odorata* doses (200 mg/kg) and MTX were also made (third and fourth groups respectively in a pre- and post-treatment, positive role of *viola odorata* is assayed by determined total flavonoids ,liver function enzymes and histopathological examination of liver tissue in animals treated with MTX.

Results: Flavonoids content has been found approximately around  $(339 \pm 0.280) \mu\text{g/ml}$  , activity of AST and ALT were significantly increased to (125 and 65) Unit/L respectively after MTX-injection (control II). also has been observed highly increased in activity of ALP after MTX injection, while post-received *Viola odorata* methanolic extract had decreased level of AST,ALT and ALP to (101, 52 and 127) Unit/L respectively, while the results of pre-treatment interactions indicated that the plant extract able to decrease the level of all enzymes in comparison with MTX alone. besides that ,observed cloudy swelling of hepatocyte with marked aggregation of mononuclear cells particularly lymphocytes around central vein , proliferation and hypertrophy of kuffers cells , aggregation of mononuclear cells around bile duct and pyknotic nuclei inflammatory cells in congested sinusoids with marked fatty changes fibrosis around bile duct , large round clear fatty vacuolate in positive group,while third and fourth groups has mild mononuclear cells aggregation around central vein and in paranchyma finally, necrosis was disappeared and show normal structure of hepatocytes.

Conclusion: From our findings of this study , can conclude that flavonoid of *viola odorata* has been improved liver enzymes activity and reducing infiltration of inflammatory cells in various site of liver and disappeared of necrosis .

**Keywords:** Methotrexate , Hepatotoxicity , *viola odorata* .

## INTRODUCTION

Many medicinal plant are contain a great bioactive components that reported to preventive and therapeutic role on liver toxicity that carry out by chemicals (1) ,These components present effective as antioxidant, anti-necrotic, anti-inflammatory, anti-apoptotic activities and regulate pathways of lipids. (2), may be through activation antioxidant enzymes ,eliminated free radical or modulate protein / association genes expression. (3)

Liver is first location to detoxification and primary site of medications in the body. some drugs have been shown toxicity on different organ such as liver causes hepatic disorders by producing free radical , which were induced cellular injury in various steps or ways by affecting on biomolecules, such as lipids, proteins and DNA (4). although medications have a vital role in treatment of diseases ,but a common adverse effect of drugs leading to limiting uses of these drugs , (5 ). one of these drugs , methotrexate (MTX) which is the folic acid antagonist widely used in the therapy of various types of diseases. It is used in the treatment of psoriasis (6), psoriatic arthritis (7), rheumatoid arthritis in elderly and younger patients (8), acute lymphoblastic leukemia (9), ectopic pregnancy (10), inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (11), and chronic inflammatory demyelinating Polyradiculoneuropathy (12). Besides that, different chemicals can stimulated liver damage ,such as alcohol consumption , pollutants like heavy metals and organic toxicants. Furthermore, liver injuries can progress into fatty liver, fibrosis,liver failure, hepatitis and cirrhosis , even cancers. Thus, liver injuries have been looked as a critical health problem, raising trouble in toxicology fields and nutrition (13).

Many plants have been well-known as hepatoprotective like family violaceae that includes about 19 species of the genus *Viola* (14 ). *Viola odorata* L. known as banafsha or sweet violet is reiterated in Europe and Asia respectively (15) , *V. odorata* has antipyretic (16), anti-inflammatory (17), and antibacterial activity, flavonoids have been detected in *V. odorata* (18) and they showed

hepatoprotective against paracetamol induced hepatotoxicity (19) , due to role of flavonoids (isorhamnetin and luteolin) (20) .

## Aim of study

This work demonstrated hepatoprotective role of *Viola odorata* L.,against MTX through determined their bioactive compounds(flavonoids),assessments of liver function enzymes (L.F.E) and histopathological examination of liver.

## MATERIALS AND METHODS:

### Collection of *Viola odorata*

The aireal parts of plant were collected from the local markets of Baghdad /Iraq during September (2017), which had been identified previously by National Herbarium of Iraq. After collection, tap water was used to eradicate any traces and grime then let's to dehydrate by air. Finally, fine particles were obtained.

### Preparation of Plant extraction:

The method of (21) was followed to prepare plant methanolic extract. Fifty grams powder was flooded in (300 ml) of 80% methanol in sonication apparatus for about one hour, then the muddle was then drinkable by a Buchner funnel after 24 hours under vacuum pressure, these progression repeated two times. Then, the extract dried up using a rotary evaporator under vacuum, and stored under sanitary conditions at -20°C until use.

### Assessments of flavoniods:

Stock Solution of plant Extract: about 100 mg of the extract which accurately weighed transferred to 10 ml flask and complete volume by methanol. After that,0.5ml of extract stock solution mixed with different material firstly (1.5) ml of methanol secondly (0.1) ml of aluminium chloride thirdly (0.1) ml of potassium acetate solution and finally (2.8) ml of distilled water were added and mixed well. also,Sample blank was prepared in similar way. absorbance step was done at 415 nm (22).The same procedure was followed up with rutin as standaed.

**Laboratory Animals**

The study is carried out on Albino male mice (*Mus musculus*) obtained from Pharmaceutical Control Department (Ministry of Health and Environment). Twelve mice weighing 23-27g, of 8-9 week age were housed in conventional conditions at animal house laboratory of Biotechnology Research Center/ Al-Nahrain University and fed on a standard pellet and libitium water is added.

**Experimental Design**

Sixteenth male mice were divided into four groups (n=4 each), control negative group received normal saline while second group received methotrexate drug (positive control) at dose of (40 mg/kg). In addition to that, an interaction between *viola odorata* doses (200 mg/kg) and methotrexate were also made (third and fourth groups respectively in a pre- and post treatment manner). Each mice administrated with 0.1 ml intraperetonilly of the tested materials for 7 days and then sacrificed at day 8 (from groups1-2) but for interactions groups, mice administrated methotrexate for day 1 and from day 2-7 received plant extract (post treatment) and for pre-treatment, the animals received plant from 1-6 and drug in day 7.

**Determinations of Aspartate Amino-Transferase (AST), Alanine Amino-Transferase (ALT), Alkaline Phosphatase (ALP)**

liver enzyme activity (AST, ALT and ALP ) were assayed in serum by using process of Reitman and Frankel (1957)(23)

**Tissue Preparation for Histology**

The liver of each mouse is prepared for histopathological study as described by Alwachi and Husain (2014)(24). Samples were fixed in 10% formaldehyde for 24h, processed by dehydration at gradual series of alcohol (30-100%) for (5)min each. Then the samples cleared in two changes of xylene before embedded in paraffin wax for sectioning. Cross sections of (5)µm thickness are prepared and stained with hematoxylin (Harison) and eosin according to standard method. Histopathological changes are performed under light microscope as compared to control group.

flavonoids content were determined in methanolic extract of *V. odorata* and its found approximately around 339 ± 0.280 µg/ml flavonoids as rutin standard.

**Hepatoprotective activity**

levels of AST , ALT in untreated group(negative control) was (30 and 28 ) Unit/L respectively, but their levels was substantially (P ≤ 0.05) elevated to (125 and 65) Unit/L respectively after MTX-injection (positive control), and ALP level was substantially (P ≤ 0.05) increased after MTX injection in same group contrast to negative control (153 vs. 40 )Unit/L. post-treatment with *Viola odorata* methanolic extract were also able to decreased the level of AST ,ALT and ALP as shown in Fig (1) to reach to (101, 52 and 127) Unit/L respectively, while the results of pre-treatment interactions indicated that the plant extract able to decrease the level of all enzymes in comparison with MTX alone as shown in fig(1).

**Histopathological Evaluations:**

Many histopathological changes are shown as the following , liver suffer from many changes, in first group given normal saline has normal appearance Fig (2), in second group administrated MTX observe cloudy swelling of hepatocyte with marked aggregation of mononuclear cells particularly lymphocytes around central vein , proliferation and hypertrophy of kupffers cells Fig (3) , aggregation of mononuclear cells around bile duct and pyknotic nuclei Fig (4), inflammatory cells in congested sinusoids with marked fatty changes Fig (5), fibrosis around bile duct Fig (6),congestion of blood vessel, mononuclear cells aggregation in dilated sinusoids Fig (7),and in addition to multiple granulomatous lesions were recorded in the liver parenchyma Fig (8), large round clear fatty vacuolate appear as cystic like structure in liver parenchyma Fig (9), fibrosis around multiple casease necrotic area in liver parenchyma Fig (10), the third group treated with 200 mg/kg of *V.odorata* has mild mononuclear cells aggregation around central vein and in paranchyma and necrosis was disappeared Fig(11), show normal structure of hepatocytes Fig(12), fourth group treated with 200 mg/kg of *V.odorata* posted treated with MTX also has shown mild mono inflammatory cells aggregation in parenchyma of liver and necrosis was disappeared(13)

**RESULTS:**

**Flavonoid Determination**

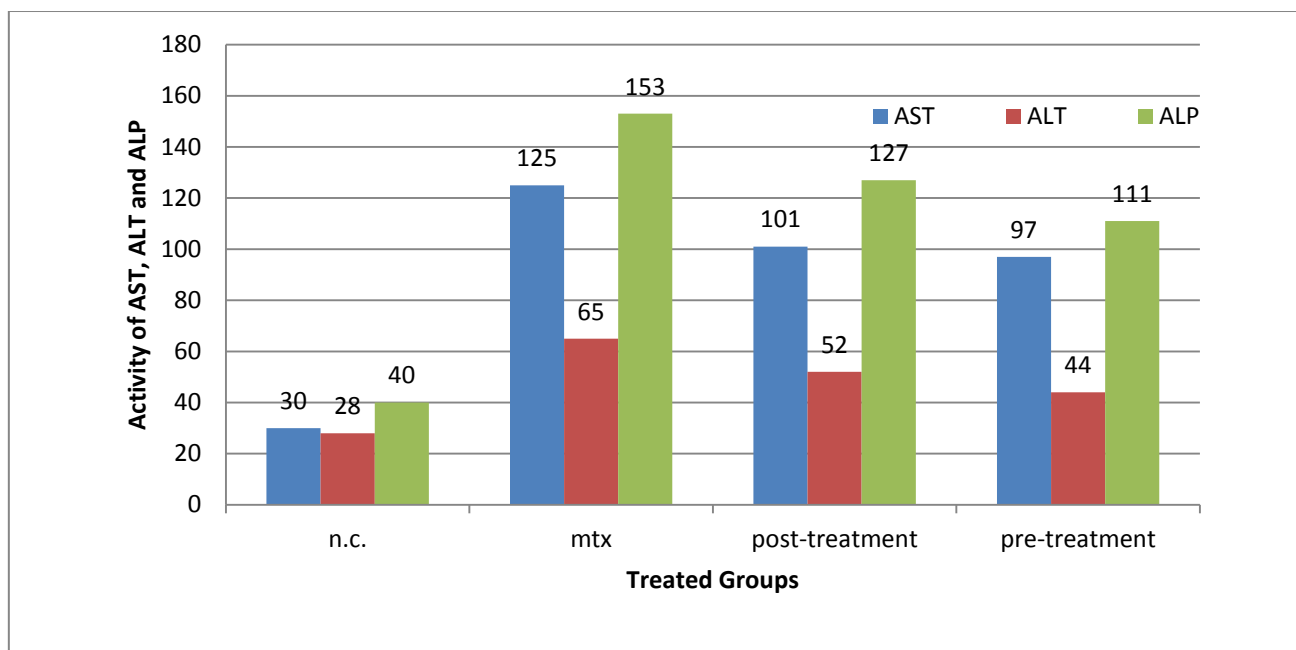


Fig 1.: Effect of *V.odorata* and methotrexate drug on liver function enzymes.

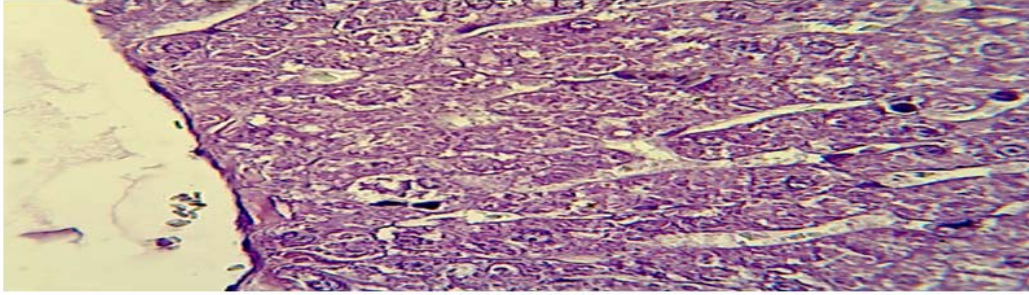


Fig: 2.H istopathological section in liver of control negative animal shows no clear lesions (H&E stain 40X)

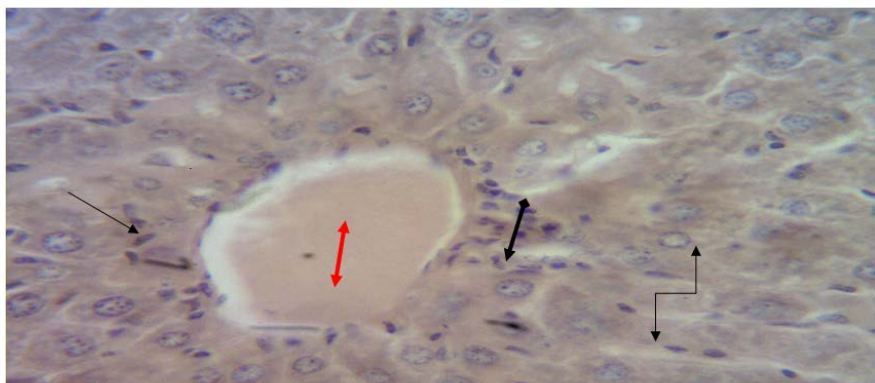


Figure3: Histopathological section of animals treated with drug showed congestion of blood vessels  $\leftrightarrow$  cloudy swelling of hepatocyte  $\uparrow$  with mononuclear cells aggregation around central vein  $\leftarrow$  and proliferation and hypertrophy of kupffers cells  $\rightarrow$  (H&E stain 40 X).

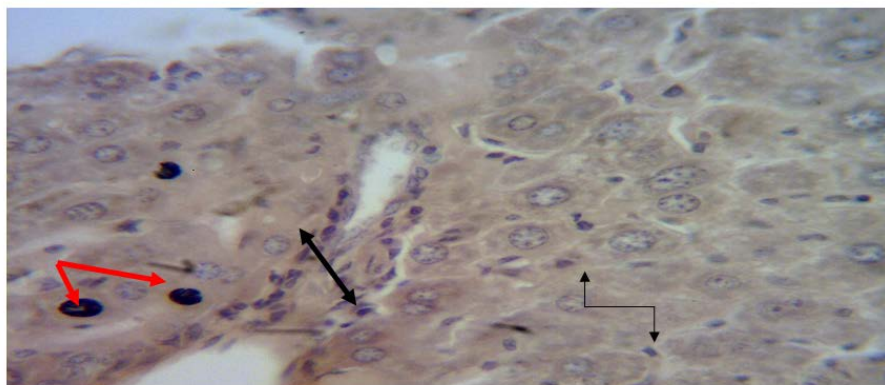


Figure4: Histopathological section of animals treated with drug showed congestion of blood vessels ,cloudy swelling of hepatocyte  $\uparrow$  with mononuclear cells aggregation around bile duct  $\leftarrow$  and few number of pyknotic nuclei  $\rightarrow$  (H&E stain 40 X).



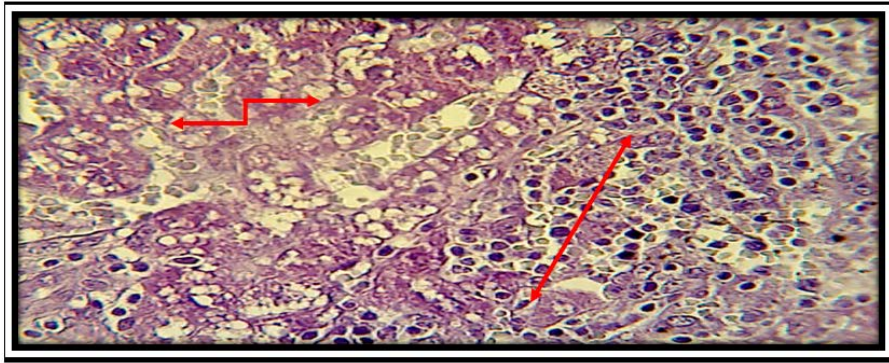


Fig5.:Histopathological section in liver of animal administration with MTX ,inflammatory cells in congested sinusoids  $\longleftrightarrow$  with marked fatty changes  $\longleftrightarrow$  (H&E stain 40X).

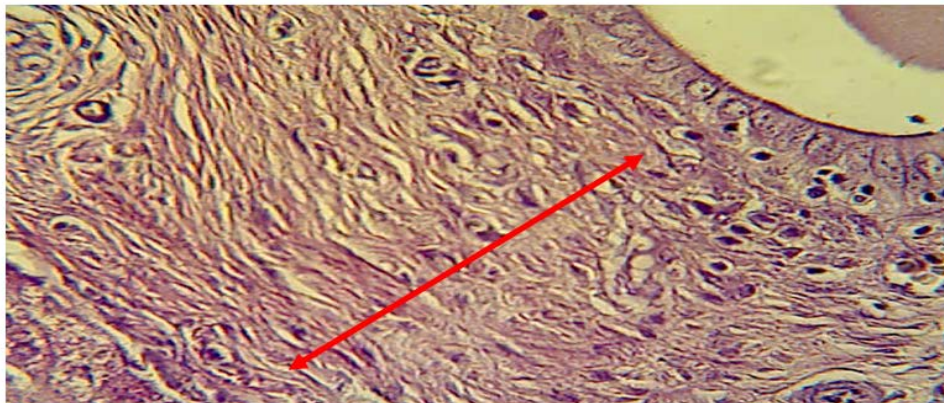


Fig:6.Histopathological section in liver of animal administration with MTX shows marked fibrosis around bile duct  $\longleftrightarrow$  (H&E stain 40X)

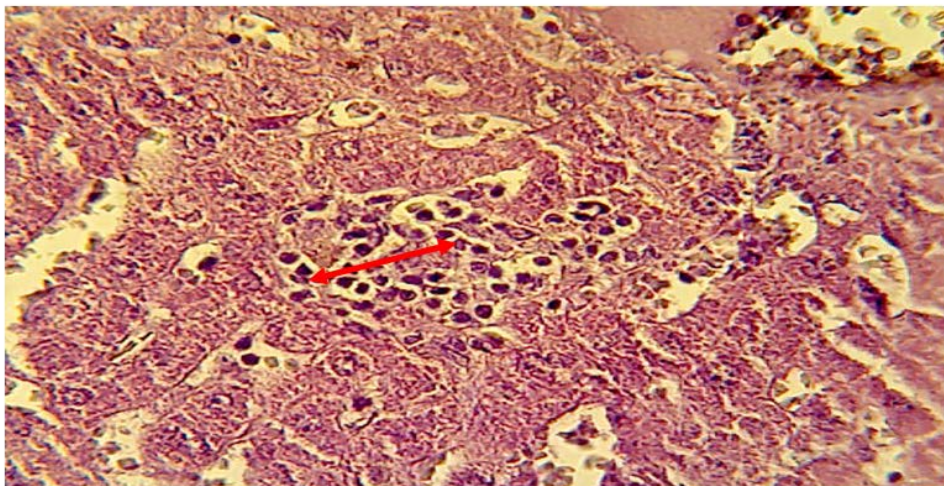
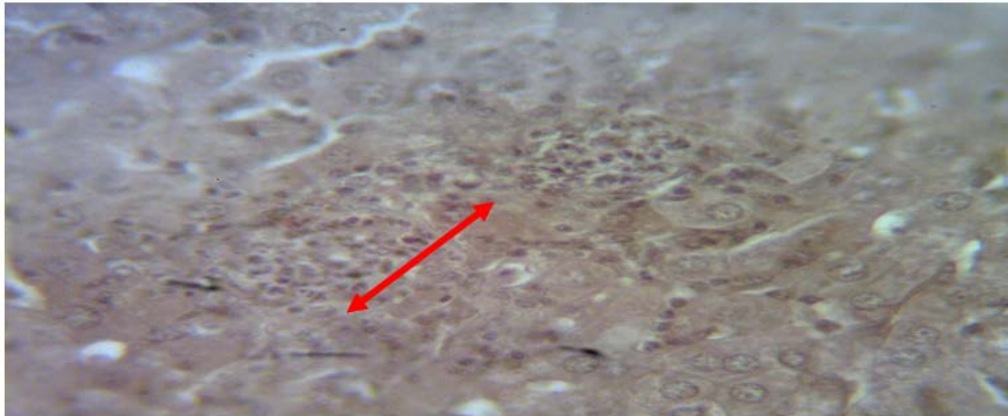
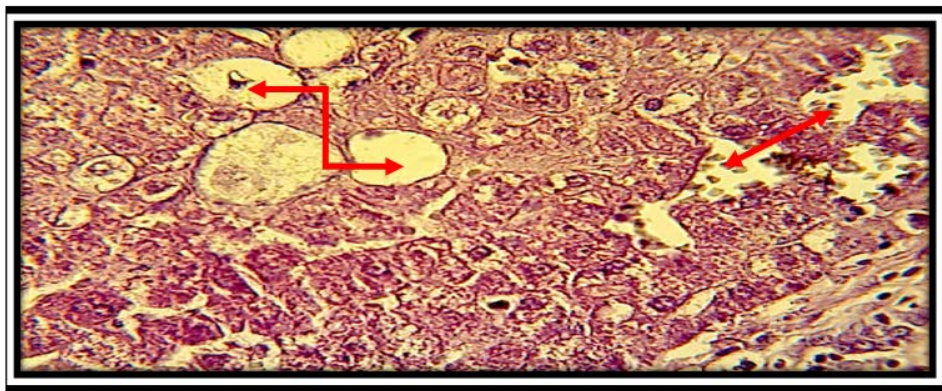


Fig:7. Histopathological section in liver of animal administration with MTX shows mononuclear cells aggregation in dilated sinusoids  $\longleftrightarrow$  (H&E stain 40X).

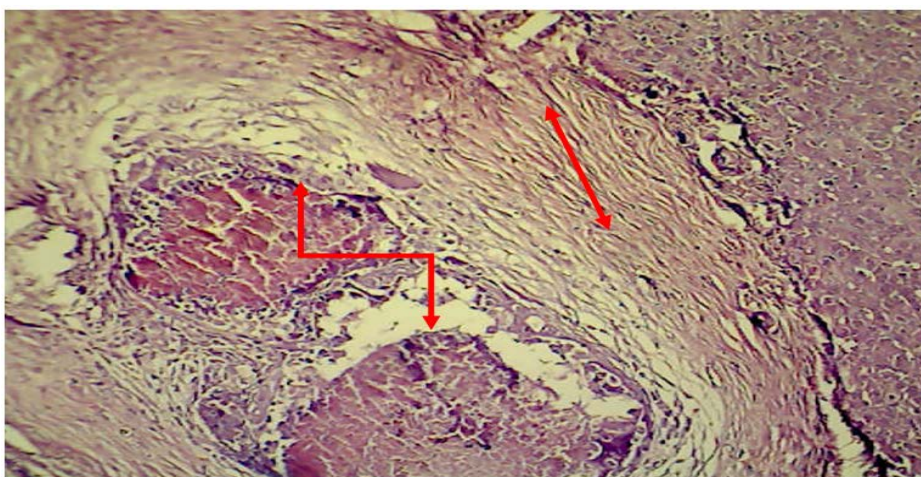




**Fig8:** Histopathological section in the liver of animal post-treated with drug showed mononuclear cells aggregation in liver parenchyma  $\longleftrightarrow$  (H&E stain 40X).



**Fig:9.** Histopathological section in liver of animal MTX large round clear fatty vacuolate in liver parenchyma  $\hookrightarrow$  and congested dilated sinusoids  $\longleftrightarrow$  (H&E stain 40X)



**Fig:10.** Histopathological section in liver of animal administration with MTX shows marked fibrosis  $\longleftrightarrow$  around multiple casease necrotic area  $\hookrightarrow$  in liver parenchyma , (H&E stain 40X)



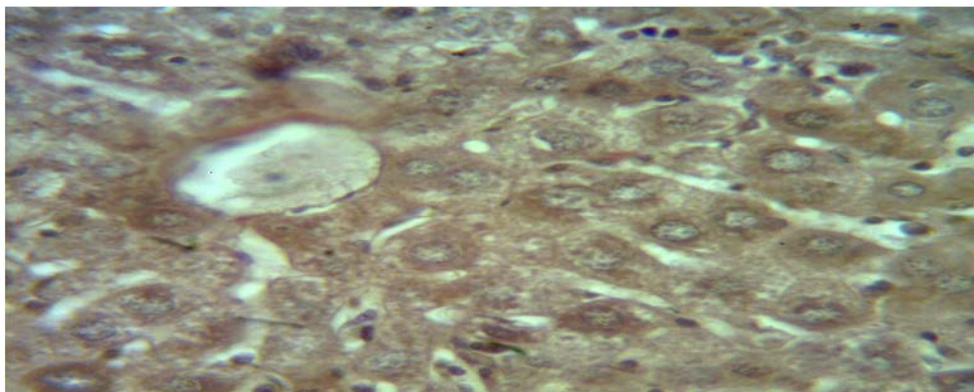


Fig 11: Histopathological section of animals treated with plant 200mg/kg after treated with MTX no clear lesion (H&E stain 40X)

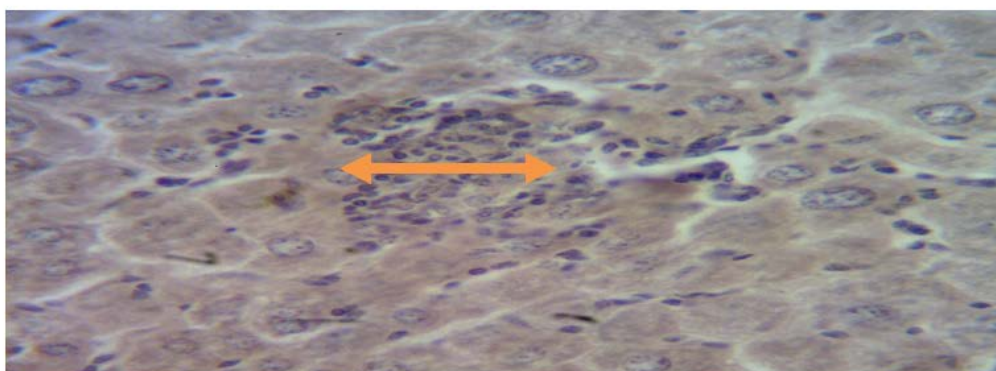



Fig12: Histopathological section in the liver of animal treated with plant 200mg/kg after treated with MTX showed mononuclear cells aggregation  in liver parenchyma (H&E stain 40X).

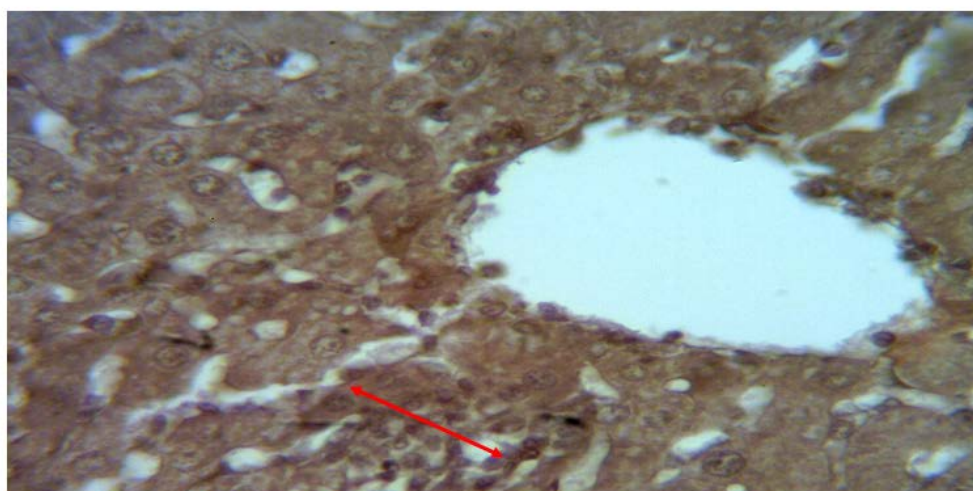



Fig13: Histopathological section in the liver of animal treated with plant 200mg/kg before treated with MTX, showed mononuclear cells aggregation  in liver parenchyma (H&E stain 40X)

**DISCUSSION:**

Liver has been more exposed to toxic and side effect of medications resulting in formation of free radical that are responsible partially of liver degeneration, Clinically MTX has approved therapeutic effective in many chronic inflammation, rheumatoid arthritis and cancer that are weakly respond to other medications.( 25).

In our work determined methanolic extract of *Viola odorata* have contain flavonoid, this resulting is in a well agreement with a study done by karioti *et al.*, 2011(26) whose found that *V.odorata* contains flavonoid, glycoside, alkaloid, saponins, vitamin C, all these compound represent anti-oxidant action and flavonoid had hepatoprotective role.(27) it was clear that treating mice with MTX for 7 days caused highly increased in activities of the following enzymes (ALT),(AST)and(ALP), These findings are concur with other report .( 28 and 29) .also increase free radical, hydrogen peroxides and lipid peroxidation were responsible of toxicity of MTX (30) and hepatotoxicity (31) and (32), by damage of antioxidant mechanisms (66-68) and stopping processing of folic to folic acid, supposed these changes drive to damage of plasma membranes of hepatic parenchymal(33), leading to liberation of AST and ALT from cell to serum. so the presence of AST and ALT into the blood flow refer to deterioration of liver structure and cell death(34). other reported confirmed presence of these enzymes in serum is due to cellular damage and loss of integrity of cell membranes(35) and previous report has been viewed that necrosis in the liver lead to increase release these of enzymes into the blood stream(36).

Today, people trend to use medical plants and herbs that have antioxidant activity(37) and anti-inflammatory (19), and hepatoprotective (38) to avoid or few side effect of these drug. The current study determined flavonoid contents, and hepatoprotective role of *Viola odorata*. level of ALP, ALT and AST was elevated significantly by MTX given (Fig 1) while administration of *V.odorata* lead to reduction in level of ALT, ALP and AST, significantly in compare to the MTX treated group ( $p < 0.05$ ), these finding are agreement other study has been done by (39) this research finding, substantially elevation of serum levels of liver function biomarker enzymes (ALT, AST, ALP) in MTX administration to group of albino mice with correspond to control group While administration of MTX and *V.Odorata* in group III and VI caused insignificant changes in serum levels of liver enzyme as compared to the first group (negative control). *V.Odorata* represent best choice to use it as antioxidant because contain flavonoid which represent as hydrogen donor that reducing oxidative stress(40). but, not found mechanisms explain how *V.Odorata* control liver enzyme activity. in current histological finding, The main lesion characterized by cloudy swelling of hepatocyte with marked aggregation of mononuclear cells particularly lymphocytes around central vein and proliferation and hypertrophy of kupffers cells Fig.( 2 ) that as well mentioned by Patel *et al*(41). These finding are in accordance with other researches that cleared Methotrexate (MTX)-induced cloudy swelling, hydropic degeneration and fatty change(42) also observe congestion of blood vessels with mononuclear cells aggregation around bile duct and few number of pyknotic nuclei cells Fig.( 3 ) that agree with another report done by (43). Furthermore, in another section, small granulomatous lesion consisting from aggregation of lymphocytes and macrophages also this result observe previous in patient suffering liver injury.( 44)

MTX induced of hepatic fibrosis that stated in our work and by( O'Rourke and Eckert, 1964) (45). As well as, Ros *et al.*, (2002)(46) and Hytioglou *et al.*, (2004)(47) reported that fibrosis in peri-sinusoidal caused by toxic effects of MTX. Moreover, we observe in another section, inflammatory cells in congested sinusoids with marked fatty changes( Fig5 ), fibrosis

around bile duct( Fig6) and mononuclear cells aggregation in dilated sinusoids( Fig7).

On the other hand, Histopathological section of animals treated with plant 200mg/kg post treated with MTX showed moderated mononuclear cells aggregation in the liver parenchyma that shown (Fig12) while in another animals show no clear lesion. In last animal group treated with MTX then treated with 200mg/kg showed moderated mononuclear cells aggregation in the liver parenchyma that shown (Fig13) from our result and another studies agree that *V.odorata* are shown as a hepatoprotective (48), and in treatment of liver injury with *V.odorata* extract that showing development and reduce in score of necrosis - inflammation and restore of normal structure of hepatocytes(19). while other work explain hepatoprotective of *v.odorata* because capable of its maintain membrane stabilization and free radical scavenging ability (49)

Present histological examination illustrated that *V.Odorata* has used as hepatoprotective, because it moderate or diminished deterioration of hepatocytes, that may be due to membrane stabilization, their free radical scavenger properties because *V.Odorata* contain flavonoid that bind to free radical and remove it and reduce lipid peroxidation.

In conclusion, we demonstrated here in a strikingly *V.Odorata* could be reduced side effect of methotrexate and recover of liver architecture.

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