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Original research

Evolution of protective effects of purslane on metformin induced hepatotoxicity on healthy rats fed on a low-fat diet

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Abstract:

One of the most often recommended drugs for type 2 diabetes is metformin (MT). Generally, metformin is considered safe and effective. However, there are some risks when it is used with a low-fat diet (LFD). Consequently, several attempts were carried out to find alternative natural drugs with minimum side effects rather than chemical drugs. Thus the current study is aimed to evaluate the hepatotoxicity effects of metformin on low-fat diet rats and the ameliorative effects of purslane (PE) extract against metformin toxicity. To achieve this objective, 32 male albino rats were divided into four equal groups; **(G1):** control group, **(G2):** LFD+PE, **(G3):** LFD+MT, and **(G4):** LFD+ MT+PE. Oxidative stress markers (NO and TBARS), antioxidant enzyme (SOD) activity, and inflammation cytokines (TNF-α and IL-1β) were detected as well as histopathological investigation. Our results revealed that metformin raised NO and TBARS levels, inhibited SOD activity, increased TNF-α and IL-1β levels and induced several histopathological alternations. On the contrary, PE administration-induced rats modulate the toxic effects induced by MT.

Keywords: Metformin; Purslane; Low-fat diet ; Hepatotoxicity

1- Introduction

Increased obesity prevalence in the modern world is linked to numerous obesity-related health issues **(Pi-Sunyer, 2002)**, such as nonalcoholic fatty liver disease (NAFLD), diabetes, cardiovascular disease, hypertension, and hyperlipidemia **(Boza et al., 2005**).

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By 2030, If secular trends continue, 38% and 20% of individuals globally are predicted to be overweight or obese (**Kelly et al., 2008**). According to (**Wolch et al., 2014**) humans have evolved amazing physiological defenses against weight loss, but these defenses are only insufficient against weight growth during periods of food plenty. Controlling overweight or obesity is a crucial part of managing diabetes. According to current international diabetes care guidelines, metformin should be the first medication recommended to all patients with type 2 diabetes. It is even recommended that non-overweight patients take it as a first line of treatment. Lifestyle changes should also be implemented alongside medication from the time of diagnosis and throughout treatment (**Nathan et al., 2006**).

Metformin (1.1-dimethyl biguanide hydrochloride) is a member of the biguanide class, including guanidine derivatives which are used for treating type 2 diabetes that cannot be controlled by a healthy diet, particularly in individuals who are overweight (**Halimi et al., 2008).** Although its mechanism of action is complex, metformin mainly improves insulin sensitivity, which is followed by a reduction in the liver's synthesis of glucose and an increase in the transport of glucose across the membrane of skeletal muscle (**Chait and Den Hartigh, 2020**). Even while being clinically successful, metformin has been associated with a number of adverse side effects. Prolonged use has been shown to decrease serum levels of vitamin B12 and folic acid, and they are primarily gastrointestinal in character (flatulence, bloating, vomiting, nausea, and diarrhea) **(Lu et al., 2021)**. Liver damage has also been linked to several oral antidiabetic medications (**Chitturi and George, 2002**). Hepatotoxicity in the context of metformin has very infrequently been documented **(Induri et al., 2022).** However, because metformin was given to overweight patients and resulted in notable improvements in cardiovascular outcomes when compared to diet-based treatment, national and international guidelines reviewed by **Consoli et al. (2002)** suggested that it can be used as a first-line pharmaceutical therapy for overweight patients, but not for non-overweight patients (**ElSayed et al., 2023**).

Numerous studies have been conducted to demonstrate how well certain herbs and their active metabolites work to cure metabolic illnesses, for example, purslane, or *Portulaca oleracea L.,* belongs to the global *Portulacaceae* family (**Iranshahy et al., 2017).** Numerous chemical substances, such as flavonoids, alkaloids, polysaccharides, and other substances including vitamins, minerals, sterols, and essential fatty acids, are found in purslane leaves (**Iranshahy et al., 2017**; **Hadi et al., 2019**). According to the results of several investigations, these substances have antioxidant (**Zhou et al., 2015**) and anti-inflammatory properties (**Gu et al., 2022**). Additionally, investigations on animals have revealed hepatoprotective (**Abd El-Azime et al., 2014**) and anti-diabetic (**Ahangarpour et al., 2018**) benefits of *Portulaca oleracea* extract. This study sets out to determine whether purslane ethanolic extract may cause metformin's harmful effects on the livers of healthy rats given a low-fat diet.

2, Material and methods

2,1, Chemicals and assay kits

Metformin was purchased from Merck Serono, Germany. Rat enzyme-linked immunosorbent assay (ELISA) kits of Rat TNF-α (Cat. No. ER1393), Rat IL-1β (Cat. No. ER1094), were purchased from Fine test Co., China. Sigma-Aldrich Co., USA, provided all additional chemicals and solvents of related biological reagents of excellent quality.

2,2, Preparation of Purslane Extract

Purslane extract was made with some adjustments to the guidelines provided by **Ezeabara et al. (2014)**. Using a sterile mortar and pestle, the plant parts (leaves and stems) were allowed to air dry at room temperature for four weeks. After being ground up and weighed at 433 g, the dry purslane stems and leaves were immersed in a 70% ethanol (1:4) combination. The rotary evaporator (Mod. 11100C101, Buchi, USA) was used to evaporate ethanol overnight. The remaining ethanol was then allowed to evaporate at room temperature. The semi-solid extracts were lyophilized using a freezing dryer (Mod. LyoConstellation S30, SP Scientific, USA), yielding 58g. After being lyophilized, the plant-extracted powder was kept at -20°C for further usage.

2,3, Experimental design and animal treatment

Thirty-two male albino rats weighing 125 ± 20 g were acquired from Alexandria University's Medical Technology Center's animal house in Egypt. For 15 days, the animals were kept in clean, well-ventilated polycarbonate cages with a 12-hour day-night cycle, 20±2.0 degrees Celsius, and 45–46% relative humidity. This made it possible for them to adapt to their new environment. There was a plenty of food and drink. With the approved number (IACUC #.79-2A-0121), the Ethics Committee of Pharmaceutical and Fermentation Industries Development Center (PFIDC), City of Scientific Research and Technological Application (SRTA-City), Borg Al Arab, Egypt . The rats were divided into four equal groups at random (each group consisting of eight rats) as follows:

Group I (Control group): the rats in this group fed on a commercial meal that was typical of balance. **Group II** (PE): the rats in this group were fed on a low-fat diet (11% fat, 15% carbohydrate, and 16% protein and treated with an oral dose of *purslane* ethanolic extract (100 mg/kg. b.w) for 4 weeks (**Ezeabara et al., 2014**). **Group III (**MT): the rats were fed on a low-fat diet (11% fat, 15% carbohydrate, and 16% protein **(Balbaa et al., 2016)** for four weeks. **Group IV** (MT+PE): the rats in this group were fed on a low-fat diet (11% fat, 15% carbohydrate, and 16% protein and treated with an oral dose of metformin (50 mg/kg b.w) and *purslane* ethanolic extract (50 mg/kg b.w) for 4 weeks.

2,4, Biochemical assays preparation

At the end of the trial, sodium pentobarbital (100 mg/kg i.p.) was used to put the rats to sleep. The liver tissue was removed as soon as possible, cleaned with a cold solution of 0.9% NaCl, frozen in liquid nitrogen, and kept at -80˚C. Following a 10-minute centrifugation at 10,000 rpm and 4˚C, the liver tissue was homogenized in a lysis solution containing a protease inhibitor (150 mM NaCl, 1% Triton X-100, and 10 mM Tris, pH 7.4). The purpose of collecting the supernatant was to measure oxidative stress, lipid peroxidation, antioxidant enzymes, and inflammatory cytokines.

2,5, Estimation of oxidative stress markers (NO and TBARS)

Nitric Oxide (NO) was detected using **Montgomery and Dymock's (1961)** methodology, which was based on the production of pink azo-dye by the interaction of nitrite with sulfanilamide in an acidic medium. Using spectrophotometry, the color's intensity was determined at 540 nm. **Zalkin and Tappel's (1960)** method for the Thiobarbituric Acid Reactive Substances (TBARS) assay. This technique was based on reacting to thiobarbituric acid as a reagent in an acidic, high-temperature environment, and measuring the absorbance of the colorful result at 532 nm.

2,6, Estimation of antioxidant enzymes activity (SOD)

The approach developed by **Marklund and Marklund (1974)** was used to evaluate the activity of superoxide dismutase (SOD). Pyrogallol is inhibited in alkaline pH, which is the basis of this technique. Superoxide radicals are created when pyrogallol is autoxidized (O_2). Using spectrophotometry, the reduction in pyrogallol autoxidation rate caused by SOD is detected at 420 nm.

2,7, Estimation of inflammatory cytokines (TNF-α, IL-1β)

ELISA kits were used to measure liver tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1β). Sandwich enzyme-linked immune-sorbent assay technology served as the foundation for these kits. The well plate was previously coated with the appropriate anti-cytokine antibodies. The antibodies found were the appropriate biotin-conjugated anti-cytokine antibodies. A microplate reader was used to measure the optical density absorbance at 450 nm.

2,8, Histological examination

For microscopic preparations, liver tissues were extracted, washed with saline, and preserved in a 10% neutral buffered formalin solution (pH 7.0). The tissues were imbedded in molten paraffin wax at 58–62˚C after being dehydrated with increasing ethyl alcohol (50–99%). Hematoxylin and eosin was used to stain tissue sections that were 5 µm thick (**Harris, 1900**).

2,9, Statistical analysis

Minitab 12 software was used to perform Tuckey's test after one-way analysis of variance (ANOVA) was used to examine mean differences (Product Code: 0471360619, Minitab LLC, State College, Pennsylvania, U.S.A.). Every data point is expressed using the mean ±SD. The differences were considered statistically significant when $P < 0.05$.

3, Results and Discussion

3,1, *Oxidative stress markers (NO and TBARS), and Antioxidant enzyme activity (SOD)*

Figures 1 and 2 demonstrated that the NO (45.32±1.38) and TBARS (0.248±0.034) levels in the PE group were non-significant ($p > 0.05$) lower than those in the control group (46.39±1.23 and 0.257±0.26, respectively). In contrast to the control group, the MT-group's NO (78.32±1.33) and TBARS (0.468±0.012) levels were significantly ($p < 0.05$) higher. MT-treated rats' NO (57.88±0.62) and TBARS (0.338±0.015) levels significantly ($p < 0.05$) decreased when PE was administered.

Figure 3 provides details on how PE extract affected the amount of SOD in the liver tissues of the treated and control groups. Similar to the control group, the PE group's SOD activity (115.56±1.75) demonstrated non-significant ($p > 0.05$) variations. A significant ($p < 0.05$) decrease in SOD activity was seen in the MT group (84.33±1.99) as compared to the control group (96.547±0.852). On the other hand, PE treatment significantly ($p < 0.05$) increased SOD activity (107.94±3.26) in MT-treated rats.

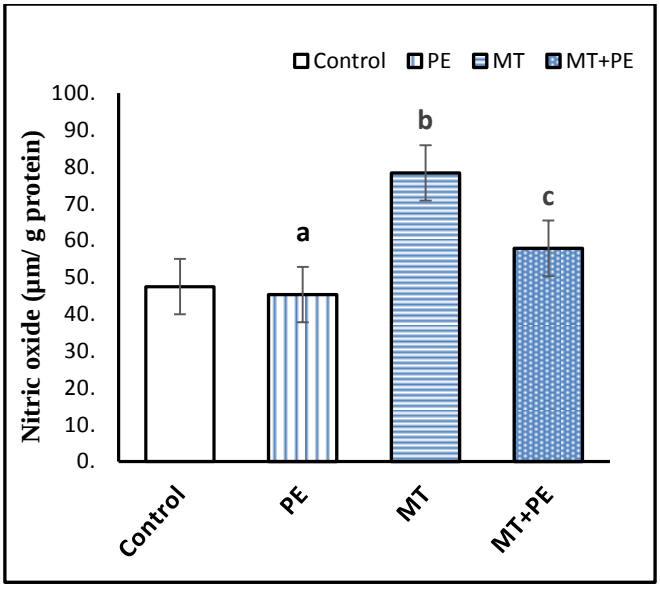

Fig (1): Mean ± SD of nitric oxide (NO) of the control and treated groups (n = 8 each). (a) Non-significant to the control group (P>0.05). (b) Significant to the control group (P<0.05). (c) Significant to the MT group (P<0.01).

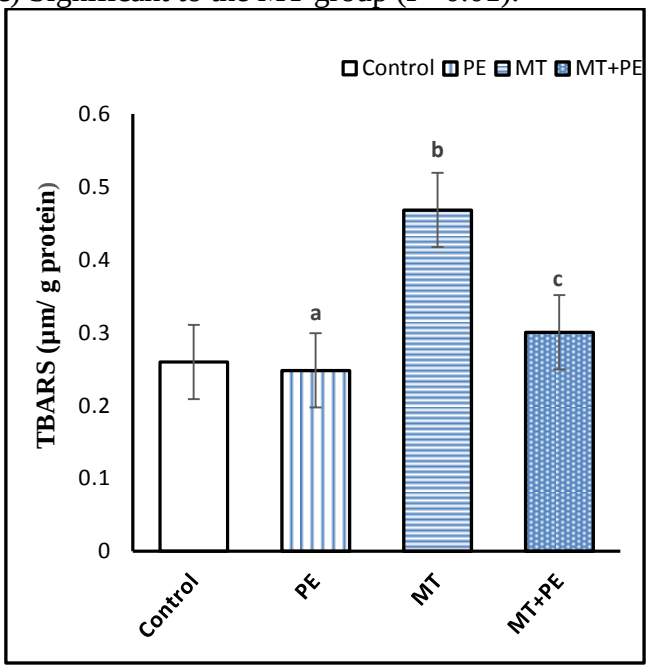

Fig (2): Mean ± SD of TBARS of the control and treated groups (n = 8 each). (a) Non-significant to the control group (P>0.05). (b) Significant to the control group (P<0.05). (c) Significant to the MT group (P<0.01).

According to **Bell and Chalasani (2009)**, drug-induced liver damage (DILI) accounts for nearly 13% of all cases of acute liver failure in the United States. Because there are currently no reliable tests or particular serum indicators to link liver damage to a medicine, diagnosing DILI is still challenging. This can be particularly difficult in cases where hepatotoxicity is brought on by a medication that is not considered intrinsically hepatotoxic such as metformin (**Mian et al., 2023**).

Metformin is thought to be safe; however it can infrequently result in severe hepatitis (**Mian et al., 2023**). According to the current study, metformin and the liver's oxidative stress and inflammatory response are closely related. The metformin group in this instance showed an excess of ROS levels associated with increased

TBARS levels and NO activity, and suppression of antioxidant enzyme activity, including SOD. A supporting study provided by **Anedda et al. (2008)** demonstrated how metformin affected the adipocyte cell line 3T3-L1, causing a significant rise in ROS levels that was mirrored in a decrease in aconitase activity. This is because high ROS levels activate the body's antioxidant defenses. The same article also pointed out that metformin lowers total cell mass, which could be a sign of stress-induced cell death. A supporting study by **Zheng (2016)** showed that metformin-induced liver damage has been infrequently documented.

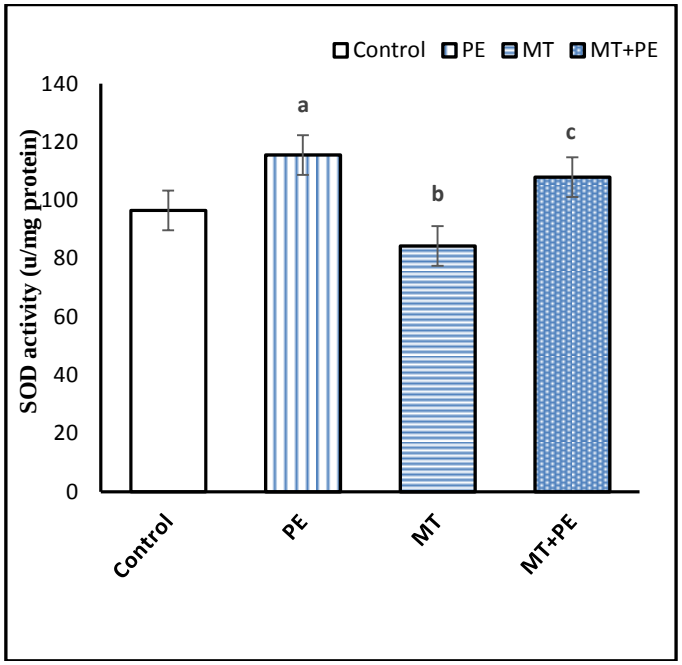

Fig (3): Mean ± SD of SOD of the control and treated groups (n = 8 each). (a) Non-significant to the control group (P>0.05). (b) Significant to the control group (P<0.05). (c) Significant to the MT group (P<0.01).

Furthermore, **Hashmi (2011)** proposed that there were underreported cases of metformin-induced hepatotoxicity in published cases, most likely as a result of inconsistent nomenclature. These findings are in harmony with our previously published data by **Bahr et al. (2022)** which showed that the liver enzymes increased in the groups that were administered with metformin which confirmed the hepatotoxicity of metformin in healthy liver. Though, hepatocytes may be impacted by ROS as these cells infiltrate the liver parenchyma. Despite, the plasma membrane prevents superoxide from diffusing freely, SOD within the membrane can internalize into hepatocytes and convert O2- to H_2O_2 **(Higgs et al., 2014)**. Interestingly, given hepatocarcinogenesis is closely associated with elevated oxidative stress via chronic inflammation and lipids, reducing oxidative stress should be beneficial in preventing liver disease. Remarkably, the current findings indicated that metformin groups treated with purslane ethanolic extract presented a significant reduction in oxidative stress, inflammatory markers, and enhanced liver antioxidant levels.

Our results are in line with studies by **Abdel-Moneim et al. (2011) and Zakizadeh et al. (2015)**, which demonstrated the antioxidant qualities of purslane by showing that higher glutathione levels in rats are associated with lower levels of malondialdehyde (MDA) and NO as well as higher levels of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), catalase (CAT), and SOD. According to **Aladaileh et al. (2019)**, purslane treatment may also offer

protection against damage brought on by STZ, most likely via reducing oxygen-free radicals.

3,2, *Inflammatory cytokines (TNF-α, IL-1β)*

There is a non-significant (P > 0.05) drop in TNF-α and IL-1β (16.56±0.74 and 94.45±1.53, respectively) in the liver of the PE group compared to the control group (20.21±0.829 and 131.64±1.424, respectively), according to the levels of inflammatory cytokines (**Figs. 4 and 5 respectively**). However, when MT was administered, TNF-α and IL-1β levels were significantly (P < 0.05) higher than in the control group (27.08±0.56 and 145.66±1.50, respectively). However, when compared to the MT group, the PE+MT group exhibited a significant (P < 0.05) drop in TNF-α (20.93±0.84) and IL-1β (126.18±1.32).

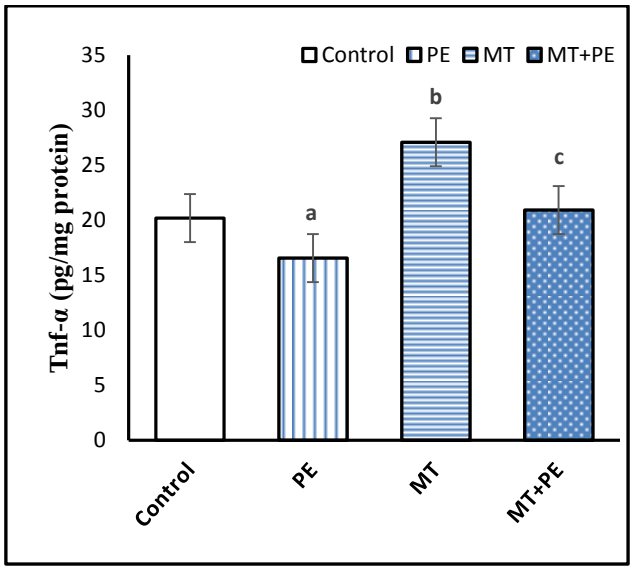

Fig (4): Mean ± SD of TNFα of the control and treated groups (n = 8 each). (a) Non-significant to the control group (*P*>0.05). (b) Significant to the control group (*P*<0.05). (c) Significant to the MT group (*P*<0.01).

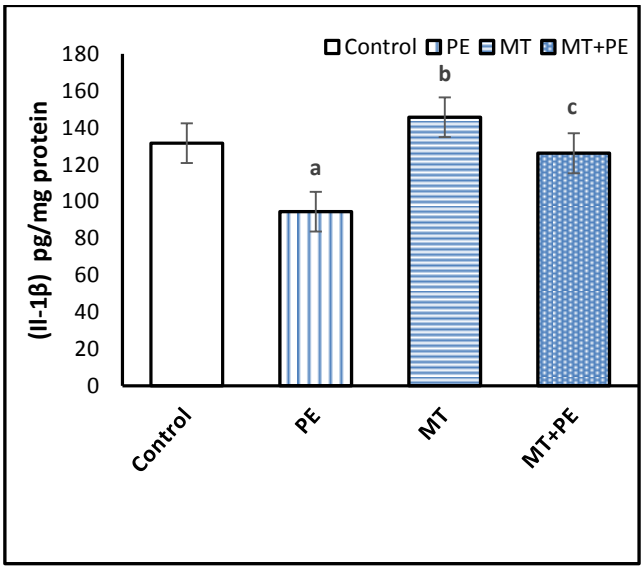

Fig (5): Mean ± SD of IL-1β of the control and treated groups (n = 8 each). (a) Non-significant to the control group (*P*>0.05). (b) Significant to the control group (*P*<0.05). (c) Significant to the MT group (*P*<0.01).

ROS can cause the synthesis of inflammatory mediators, which in turn causes liver damage, according to a study by **Zhao et al. (2017)**. It is noteworthy that our data showed a significant increase in the pro-inflammatory markers TNF-α and IL-1β in rats administered metformin. Similar results were reported by **de Souza Teixeira et al. (2018)**, who demonstrated that metformin increased hepatocyte production of cytokines, which in turn led to a pro-inflammatory profile in the liver and elevated levels of specific inflammatory cytokines. Furthermore, the same author discovered that, in comparison to their respective controls, metformin also raised the levels of adiponectin and interleukin-4 (IL-4) in both diets. Additionally, after exposure to numerous hazardous substances, target organs exhibit increased levels of chemokines, reactive oxygen and nitrogen species, and inflammatory mediators such TNFα (**Luster et al., 2001**).

According to additional supporting research by **Ramadan et al. (2017)**, In line with our findings, a purslane polysaccharide extract was shown by **Bai and colleagues (2016)** to dramatically lower levels of TNF-α and IL-6. These results could be explained by **Meng et al. (2016)**, who noted that several alkaloids produced from purslane have been demonstrated to have anti-inflammatory qualities. Purslane's anti-inflammatory, regenerative, and antioxidant properties are often credited with its hepatoprotective effects, which may help shield the liver from harm.

3,3, *Histological examination*

Compared to the control group, the microscopic inspection of liver sections from rats fed a low-fat diet and treated with purslane ethanolic extract revealed no pathological changes compared to the control group. The hepatic lobules maintained their typical structure, similar to that of the control group, consisting of radially arranged hepatocyte cords separated by normal blood sinusoids. Each hepatocyte was polygonal, with eosinophilic cytoplasm and one or two centrally located rounded nuclei (**Fig. 6B).** Contrary, the histological liver sections from rats on a low-fat diet and treated with metformin showed histopathological alteration including acidophilic cytoplasm, pyknotic nuclei, nuclear fragmentation, karyomegaly, and karyolysis (**Fig. 6C)**. Interestingly, the liver tissue from rats given a low-fat diet along with a combination of purslane extract and metformin showed no histological alterations. The hepatic lobules displayed a similar structure, consisting of normally appearing hepatocytes organized into radially extending cords. These cords were separated by blood sinusoids, resembling the normal architecture observed in the control group (**Fig. 6D).**

Metformin can cause liver damage, including hepatotoxicity. Mt can cause acute liver injury, leading to jaundice, hepatitis, or liver failure (**Mian et al., 2023**). Interestingly, a study by **Eidi et al. (2015)** showed that purslane extract treatment helped maintain the histological integrity of liver cells in rats and significantly reversed the elevated hepatic enzyme levels caused by carbon tetrachloride (CCl_4). The same author reported that the hepatoprotective effects of purslane may be attributed to its antioxidant, anti-inflammatory, and cell-regenerative properties, which may help treat or prevent liver damage; this elucidation is consistent with our findings. In addition, **Al-Howiriny et al. (2004)** found that both ethanolic and aqueous extracts of purslane possess antioxidant properties, protecting tissues from lipid peroxidation. The protective effects of the extract strongly suggest that it can help prevent or reduce enzyme leakage into the bloodstream, promote hepatocyte regeneration, maintain plasma membrane integrity, and ultimately enhance enzyme levels.

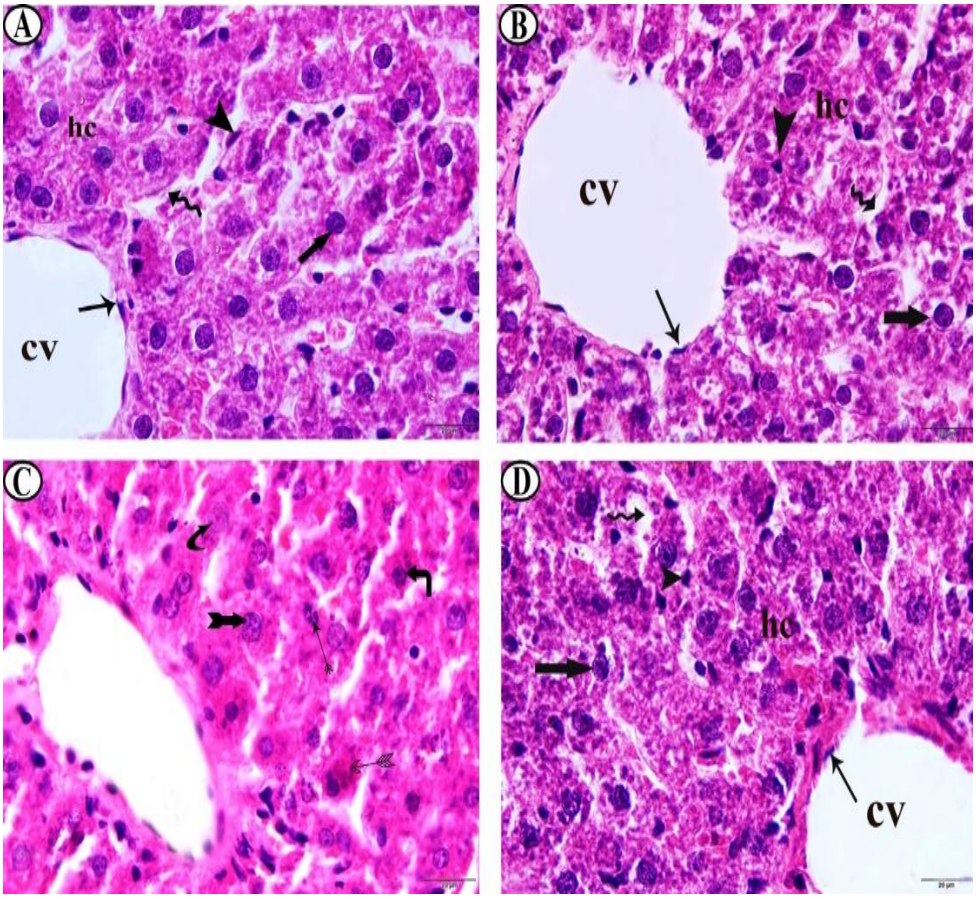

Fig (6): Photomicrograph of liver section stained with H&E. (A) control group, (B) PE group, (C) MT group, (D) MT+PE group. Hepatic cords (hc), blood sinusoids ( ), hepatocytes (thick arrow), Kupffer cells (arrowhead), central vein (CV), endothelial lining cells (thin arrow), karyocytosis ( ), pyknotic nuclei ( ), nuclear fragmentation ( ), acidophlic cytoplasm ( ), and karyomegaly ( ).

4, Conclusion

Metformin is considered one of the first-line anti-diabetic drugs, which is also used to treat obesity and overweight. According to the current study, metformin directly affects male liver-healthy rats. Our study's findings indicated that metformin might impair inflammatory and oxidative stress biomarker function. Furthermore, our study demonstrated the therapeutic and preventive efficacy of purslane extract against metformin toxicity generally, and it recommended that people receiving metformin medication be closely monitored.

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