

AMELIORATIVE EFFECT OF HESPERIDIN ON CARBON TETRACHLORIDE INDUCED LIVER FIBROSIS IN RATS

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ABSTRACT

Objective: Exposure to carbon tetrachloride leads to serious liver injury and fibrosis. This study was aimed to evaluate the hepatoprotective effects of hesperidin against carbon tetrachloride (CCl₄)-induced liver fibrosis in rats compared with the reference drug silymarin.

Methods: Wistar albino rats were divided into five groups, each of eight rats. Animals were allocated into a control group, corn oil group and fibrosis control group. The remaining two groups received in addition to CCl₄, silymarin (100 mg/kg/d) as a reference treatment and hesperidin (200 mg/kg/d). At the end of experimental period, the biomarkers of specific fibrosis [hepatic transforming growth factor β₁ (TGF-β₁) and hydroxyproline (HYP)], liver function [serum alanine transaminase (ALT), aspartate transaminase (AST), albumin and total bilirubin], oxidative stress [hepatic malondialdehyde (MDA), glutathione (GSH) and catalase (CAT)], inflammatory [hepatic myeloperoxidase (MPO), serum tumor necrosis factor alpha (TNF-α)], relative liver weight, lipid profile [total cholesterol, serum triglycerides, high-density lipoprotein cholesterol (HDL-Ch) and low density lipoprotein cholesterol (LDL-Ch)] were evaluated, supported by liver histopathological study and immunohistochemistry of alpha-smooth muscle actin (α-SMA) in liver sections.

Results: Hesperidin significantly decreased hepatic transforming growth factor β₁, hydroxyproline, the serum liver function markers of ALT, AST and total bilirubin, the hepatic content of MDA and MPO activity, the serum pro-inflammatory cytokine TNF-α, relative liver weight, and the serum lipid profile markers cholesterol, triglycerides and LDL. On the other hand, Hesperidin significantly increased albumin, the hepatic content of GSH and CAT, and serum lipid profile of LDL. In addition, liver sections obtained from these groups showed marked histopathological and immunohistochemistry of α-SMA improvement.

Conclusion: Hesperidin may be promising protective agent against liver fibrosis through improvement of liver function, modulation of the fibrous scar formation, anti-inflammatory and antioxidant potentials.

Keywords: Liver fibrosis, CCl₄, Corn oil, Silymarin, Hesperidin, Rat

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INTRODUCTION

Hepatic fibrosis (HF) is a great health problem unless controlled it can progress to cirrhosis and primary liver cancer, which are responsible for most liver transplants and deaths [1]. HF is a healing respond of liver tissue damage. It is a reversible process and marked by over accumulation of extracellular matrix (ECM) protein [2]. The imbalance between enhanced ECM synthesis and decreased breakdown of connective tissue proteins will damage the normal architecture of the liver, which changes the normal function of the organ [3].

When the hepatic stellate cell (HSC) is activated, it loses its retinoid and starts expressing new receptors such as the platelet-derived growth factor (PDGF) receptor and transforming growth factor (TGFβ₁) receptor. It also expresses new proteins such as α-smooth muscle actin. The activated HSC proliferates and synthesises extracellular matrix proteins to produce the fibrous scar [4].

Free radical-initiated lipid peroxidation plays a role in hepatic fibrogenesis, which affected the cellular permeability of hepatocytes leading to increasing levels of liver enzymes [5]. Therefore, there is a possible role of antioxidants in the prevention and treatment of liver diseases [6]. The level of liver hydroxyproline (HYP) reflects the amount of hepatic collagen which makes it an important marker of liver fibrosis to be determined in liver tissue. It has been previously reported that CCl₄ intoxication leads to elevation of lipid biomarkers and accumulation of fat vacuoles, which may reflect impairment of liver function, particularly on lipid metabolism [7].

Until now, there is no standard treatment for liver fibrosis. In addition, the current therapies are often ineffective in treating the

attached causes of fibrosis and are associated with many side effects. Therefore, there is a great demand for new drug classes to be proved as potent and safe antifibrotic agents, aiming at least to prevent the progression to end-stage liver disease [6].

Hesperidin (3,5,7-trihydroxy flavanone-7-rhamnoglucoside) [8], a flavanone glycoside present abundantly in citrus fruits [9]. This flavonoid has potential therapeutic benefits including, antiviral, anti-allergic, antiplatelet, anti-inflammatory and antioxidant activities [10].

Silymarin is an extract from milk thistle *Silybum marianum*. It is used as hepatoprotective drug based on its free radical scavenging, anti-inflammatory and antifibrotic activities. Silymarin has clinical applications in liver fibrosis, liver cirrhosis and drug-induced liver diseases [11].

Based on this background, the present study aimed to evaluate the possible hepatoprotective effects of hesperidin, as compared to the reference drug silymarin, on experimentally-induced HF in adult male Wistar albino rats.

MATERIALS AND METHODS

Materials

Animals

This study was performed on healthy adult male Wistar albino rats, weighing 250±10 g. Animals were obtained from Animal House of Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt and they were housed in the air-conditioned pathogen-controlled animal room in the animal house. All experimental rats were kept under stable temperature (25±1 °C) and relative humidity and allowed free access to standard

forage and tap water *ad libitum*. All procedures performed in studies involving animals were in accordance with the ethical standards of the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Drugs, chemicals and reagent kits

CCl₄ and corn oil were purchased from El-Nasr Chemical Company (Abou-Zaabal, Cairo, Egypt). Silymarin was purchased from Sigma-Aldrich Chemical Company (St. Louis, USA). Hesperidin was purchased from Acros Organics Company (New Jersey, USA). Tissue enzyme-linked immune sorbent assay (ELISA) kits of transforming growth factor β1 (TGF-β1) was obtained from MyBioSource, Inc. (San Diego, USA). Serum ELISA tumour necrosis factor alpha (TNF-α) kit was supplied by RandD Systems (McKinley, Minneapolis, United States). Tissue colorimetric kit of myeloperoxidase (MPO) was purchased from Amsbio Company (Milton Park, Abingdon, UK). Tissue colorimetric antioxidant kits of malondialdehyde (MDA), glutathione (GSH) and catalase (CAT) were purchased from Bio-Diagnostic Company (Dokki, Giza, Egypt). Serum colorimetric kits of liver function serum alanine transaminase (ALT), aspartate transaminase (AST), albumin, total bilirubin and lipid profile [total cholesterol, triglycerides (TGs) and high-density lipoprotein cholesterol (HDL-Ch)] were purchased from BioMed company (Badr City, Industrial Area Piece, Egypt). All others chemicals, solvents and reagents used were of the highest analytical grade commercially available.

Experimental design

Rats were randomly allocated into 5 weight-matched groups, each of 8 rats. The first group was kept as a normal control group and received only saline 5 ml/kg/d, p. o. The second group was kept as a corn oil group and received only corn oil 1 ml/kg, i. p. two times per week for five weeks. The remaining three groups received intraperitoneal CCl₄ in corn oil (1:1) at a dose of 2 ml/kg, i. p. [12] one of them was left as fibrosis control group. The remaining 2 groups received the following treatments; silymarin 100 mg/kg/d, p. o. [13] as a reference treatment and hesperidin 200 mg/kg/d, p. o. [9], respectively. All the treatments were given daily for five consecutive weeks starting from the first day of induction. Doses of test agents were determined with pilot trials guided by the published literature. Blood and liver tissue samples were withdrawn 24 h after the last dose.

Methods

Induction of liver fibrosis

Induction of liver fibrosis was performed by i. p. injection of rats with CCl₄ in corn oil (1:1) at a dose of 2 ml/kg, twice weekly for five weeks according to Hui *et al.* [12].

Serum preparation

At the end of the experiment, rats were anaesthetized with light ether. Blood samples were withdrawn from the retro-orbital plexus in centrifuge tubes using heparinized micro capillary tubes. After collecting blood samples, the tubes were allowed to coagulate at room temperature. Then samples were centrifuged for 20 min at 4000 rpm using a cooling centrifuge (Sigma 3-30 k, USA). The clear serum layer was separated and stored at -80 °C in a deep freezer (Als Angelantoni Life Science, Italy) for the analysis of ALT, AST, albumin, bilirubin, cholesterol, triglycerides, HDL, LDL and TNF-α.

Calculation of relative liver weight

Firstly, the animals have been weighed just before killing. Then, they were rapidly killed by cervical dislocation soon after blood samples were collected. Abdominal cavities were opened, the whole liver tissues were carefully isolated and washed several times with 0.9% sterile ice-cooled saline to remove any blood from the tissues and then pressed between 2 filter papers to absorb the excess saline solution. Each liver was weighed.

CALCULATION

$$\text{Relative liver body weight} = \frac{\text{Weight of liver (gm)}}{\text{Body weight of rat (gm)}} \times 100$$

Preparation of tissue homogenate

Livers were cut into small portions and used for the preparation of liver homogenates and histopathology sections. A portion of the liver was homogenized with 5 volumes of isotonic ice-cold normal saline using a homogenizer (Ultra-Turrax T 25, made in Germany), to prepare 20% liver homogenate. Aliquots of liver homogenates (20%) were centrifuged at 4000 rpm for 15 min at 4 °C and the supernatant was collected, and then stored at -80 °C for analysis of HYP, TGFβ₁, MDA, GSH, CAT and MPO.

Assessment of serum biomarkers

Serum ALT and AST levels were assayed according to the method described by Reitman and Frankel [14]. Serum albumin and bilirubin were assessed as described by Doumas *et al.*, Malloy and Evelyn [15, 16], respectively. Serum TNF-α was assessed as described by Brouckaert *et al.* [17]. Serum total cholesterol, TGs and HDL were estimated by the method of Watson, Fossati and Prencipe, and Castelli *et al.* [18, 19, 20] and respectively, while LDL was assessed according to Friedewald *et al.* [21].

Formula: LDL = TC – HDL – (TG/5.0) mg/dl.

Assessment of liver homogenate biomarkers

Hepatic TGF-β1 was assessed by ELISA kit based on the principle previously described by Blanchette *et al.* [22]. Hepatic HYP was assessed as described by Patiyl and Katoch [23]. Hepatic MDA, GSH and CAT levels were measured colorimetrically according to Satoh, Beutler *et al.* and Aebi [24, 25, 26], respectively, while MPO was assessed as described by Weiss *et al.* [27].

Histopathological and immunohistochemical study

Samples were taken from the isolated livers of rats in different groups and immediately fixed in 10% formalin solution in normal saline and embedded in paraffin. Paraffin beeswax tissue blocks were prepared for sectioning at 4-5 microns. Sections of samples were collected on glass slides, deparaffinized, stained by hematoxylin and eosin stain for routine examination then examination was done through the light electric microscope.

Alpha-smooth muscle actin (α-SMA) was detected immunohistochemically as a marker of HSC activation. Paraffin-embedded tissue sections of 3 μm thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h. The sections were then immunostained with anti α-SMA antibody (Santa-Cruz Biotechnology, California, USA) at a concentration of 1 μg/ml containing 5% BSA in TBS and incubated overnight at 4 °C. After washing the slides with TBS, the sections were incubated with goat anti-rabbit secondary antibody. Sections were then washed with TBS and incubated for 5–10 min in a solution of 0.02% diaminobenzidine containing 0.01% H₂O₂. Counterstaining was performed using hematoxylin, and the slides were visualized under a light microscope. Sections of samples were investigated by the aid of two experienced pathologists blinded to the experiment.

Statistical analysis

All numerical data were expressed as means of 8 values ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) test followed by Tukey-Kramer multiple comparisons test by the aid of the statistical package for social sciences (SPSS; version 22.0) computer software program (SPSS Inc., Chicago, IL, USA), where the value of *p* < 0.05 was considered statistically significant.

RESULTS

Biochemical estimations

Effect of silymarin or hesperidin on serum level of liver function markers

CCl₄ significantly increased the serum level of ALT, AST and total bilirubin, while the serum level of albumin was significantly decreased as compared with control and corn oil groups.

Silymarin or hesperidin significantly reduced the serum level of ALT, AST and total bilirubin and significantly increased serum level of albumin as compared to the fibrosis control group.

Hesperidin treatment restored serum ALT and AST back to normal levels, while silymarin or hesperidin treatments restored serum bilirubin back to normal level (table 1).

Effect of silymarin or hesperidin on hepatic content of oxidative markers

Fibrosis control group showed a significant increase in the hepatic content of MDA and a significant decrease in hepatic GSH content and CAT activity as compared to normal control and corn oil rats.

Treatment of rats with silymarin or hesperidin significantly reduced the hepatic content of MDA and increased GSH and CAT as compared to the fibrosis control group (table 2).

Effect of silymarin or hesperidin on serum and tissue inflammatory markers

CCl₄ treated group showed a significant increase in serum level of TNF- α , the hepatic content of the inflammatory marker MPO and the relative liver weight as compared to the control and corn oil groups.

Treatment of rats with silymarin or hesperidin significantly reduced the serum level of TNF- α , the hepatic content of MPO and the relative liver weight as compared to the fibrosis control group.

Hesperidin showed significantly better improvements of MPO and TNF- α as compared to the reference silymarin and restored MPO back to normal levels (table 3).

Effect of silymarin or hesperidin on serum level lipid profile markers

CCl₄ showed a significant increase in serum total cholesterol, TGs and LDL levels, while a significant decrease in serum level of HDL as compared to normal control and corn oil groups.

Treatment of rats with silymarin or hesperidin significantly reduced serum total cholesterol, TGs and LDL levels and significantly increased serum level of HDL as compared to the fibrosis control group.

Hesperidin restored cholesterol back to normal levels, while silymarin or hesperidin restored TGs back to normal levels (table 4).

Effect of silymarin or hesperidin on hepatic content of specific fibrosis markers

Rats subjected to CCl₄ treatment showed a significant increase in the hepatic content of TGF- β 1 and HYP when compared with control and corn oil groups.

Silymarin or hesperidin significantly decreased the hepatic content of TGF- β 1 and HYP as compared with fibrosis control group.

Silymarin or hesperidin treatments were restored hepatic contents of hydroxyproline back to normal level (fig. 1 and 2).

Table 1: Effect of silymarin or hesperidin on serum levels of ALT, AST, albumin or total bilirubin in rats with experimentally-induced liver fibrosis

Parameters \Rightarrow Groups \Downarrow	ALT (u/l)	AST (u/l)	Albumin (gm/dl)	Bilirubin (mg/dl)
Control (5 ml saline/kg/p. o.)	18.0 \pm 1.2	13.1 \pm 1.1	4.7 \pm 0.4	1.0 \pm 0.1
Corn oil (1 ml/kg/i. p.)	14.6 \pm 1.4	15.0 \pm 0.8	5.0 \pm 0.2	0.9 \pm 0.1
Fibrosis control CCl ₄ with corn oil (1:1) (2 ml/kg/i. p.)	72.5 \pm 7.3 ^{a,b}	54.3 \pm 2.8 ^{a,b}	1.4 \pm 0.1 ^{a,b}	1.9 \pm 0.2 ^{a,b}
Silymarin (100 mg/kg/p. o.)	42.5 \pm 3.6 ^{a,b,c}	26.8 \pm 2.2 ^{a,b,c}	2.9 \pm 0.1 ^{a,b,c}	1.1 \pm 0.1 ^c
Hesperidin (200 mg/kg/p. o.)	24.5 \pm 1.7 ^{c,d}	20.2 \pm 2.0 ^c	3.4 \pm 0.3 ^{a,b,c}	1.0 \pm 0.1 ^c

Each value represents the mean of 8 animals \pm standard error of the mean (SEM), ^aSignificantly different from control group value at $p < 0.05$, ^bSignificantly different from corn oil group value at $p < 0.05$, ^cSignificantly different from fibrosis control group value at $p < 0.05$ and ^dSignificantly different from silymarin reference treated group value at $p < 0.05$

Table 2: Effect of silymarin or hesperidin on liver contents of MDA, GSH and liver CAT activity in rats with experimentally-induced liver fibrosis

Parameters \Rightarrow Groups \Downarrow	MDA (nmol/mg ptn)	GSH (u/mg ptn)	CAT (mmol/mg ptn)
Control (5 ml saline/kg/p. o.)	1.2 \pm 0.1	63.8 \pm 1.6	117.5 \pm 2.3
Corn oil (1 ml/kg/i. p.)	1.3 \pm 0.1	61.1 \pm 3.3	119.8 \pm 3.9
Fibrosis control CCl ₄ with corn oil (1:1) (2 ml/kg/i. p.)	19.9 \pm 1.6 ^{a,b}	18.9 \pm 1.6 ^{a,b}	47.0 \pm 3.5 ^{a,b}
Silymarin (100 mg/kg/p. o.)	13.1 \pm 0.6 ^{a,b,c}	35.7 \pm 4.0 ^{a,b,c}	102.8 \pm 3.4 ^c
Hesperidin (200 mg/kg/p. o.)	10.1 \pm 0.7 ^{a,b,c}	37.5 \pm 2.7 ^{a,b,c}	104.1 \pm 7.5 ^c

Each value represents the mean of 8 animals \pm standard error of the mean (SEM), ^aSignificantly different from control group value at $p < 0.05$, ^bSignificantly different from corn oil group value at $p < 0.05$, ^cSignificantly different from fibrosis control group value at $p < 0.05$ and ^dSignificantly different from silymarin reference treated group value at $p < 0.05$

Table 3: Effect of silymarin or hesperidin on serum level of TNF- α , liver MPO activity and relative liver weight in rats with experimentally-induced liver fibrosis

Parameters \Rightarrow Groups \Downarrow	MPO (u/mg ptn)	TNF α (pg/ml)	Relative liver/Body weight
Control (5 ml saline/kg/p. o.)	2.9 \pm 0.2	31.1 \pm 1.8	3.1 \pm 0.2
Corn oil (1 ml/kg/i. p.)	2.7 \pm 0.1	31.1 \pm 1.1	3.5 \pm 0.1
Fibrosis control CCl ₄ with corn oil (1:1) (2 ml/kg/i. p.)	16.7 \pm 1.4 ^{a,b}	130.4 \pm 5.6 ^{a,b}	4.9 \pm 0.2 ^{a,b}
Silymarin (100 mg/kg/p. o.)	8.5 \pm 0.9 ^{a,b,c}	92.7 \pm 2.5 ^{a,b,c}	3.7 \pm 0.2 ^c
Hesperidin (200 mg/kg/p. o.)	4.0 \pm 0.7 ^{c,d}	73.4 \pm 2.8 ^{a,b,c,d}	3.9 \pm 0.1 ^{a,c}

Each value represents the mean of 8 animals \pm standard error of the mean (SEM), ^aSignificantly different from control group value at $p < 0.05$, ^bSignificantly different from corn oil group value at $p < 0.05$, ^cSignificantly different from fibrosis control group value at $p < 0.05$ and ^dSignificantly different from silymarin reference treated group value at $p < 0.05$

Table 4: Effect of silymarin or hesperidin on serum levels of Cholesterol, TGs, HDL and LDL in rats with experimentally-induced liver fibrosis

Parameters⇒ Groups⇩	Cholesterol (mg/dl)	TGs (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control (5 ml saline/kg/p. o.)	145.11±5.86	89.09±6.06	61.30±1.00	65.99±5.76
Corn oil (1 ml/kg/i. p.)	144.01±6.30	82.80±3.36	63.08±1.46	64.37±6.32
Fibrosis control CCl ₄ with corn oil (1:1) (2 ml/kg/i. p.)	236.80±13.40 ^{a,b}	133.40±3.37 ^{a,b}	26.44±1.68 ^{a,b}	183.68±14.15 ^{a,b}
Silymarin (100 mg/kg/p. o.)	191.31±3.41 ^{a,b,c}	99.78±3.33 ^c	38.80±1.34 ^{a,b,c}	122.53±4.07 ^{a,b,c}
Hesperidin (200 mg/kg/p. o.)	179.28±7.09 ^c	89.34±3.16 ^c	41.08±1.66 ^{a,b,c}	113.08±5.58 ^{a,b,c}

Each value represents the mean of 8 animals±standard error of the mean (SEM), ^aSignificantly different from control group value at $p<0.05$, ^bSignificantly different from corn oil group value at $p<0.05$, ^cSignificantly different from fibrosis control group value at $p<0.05$ and ^dSignificantly different from silymarin reference treated group value at $p<0.05$

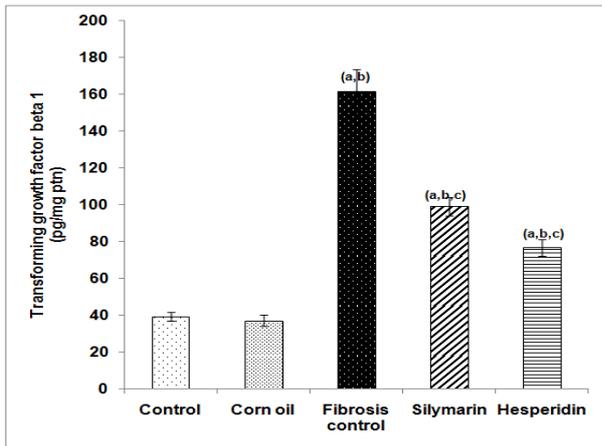


Fig. 1: Effect of silymarin or hesperidin on hepatic content of TGF-β₁ in rats with experimentally induced liver fibrosis

Each value represents the mean of 8 animals±standard error of the mean (SEM)

^aSignificantly different from control group value at $p<0.05$, ^bSignificantly different from corn oil group value at $p<0.05$, ^cSignificantly different from fibrosis control group value at $p<0.05$ and ^dSignificantly different from silymarin reference treated group value at $p<0.05$

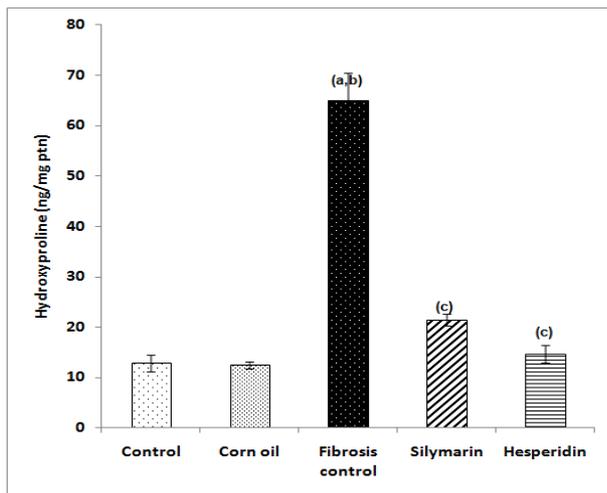


Fig. 2: Effect of silymarin or hesperidin on hepatic content of HYP in rats with experimentally-induced liver fibrosis

Each value represents the mean of 8 animals±standard error of the mean (SEM).

^aSignificantly different from control group value at $p<0.05$, ^bSignificantly different from corn oil group value at $p<0.05$, ^cSignificantly different from fibrosis control group value at $p<0.05$ and ^dSignificantly different from

silymarin reference treated group value at $p<0.05$

Histopathological study

Liver sections obtained from the control and corn oil groups showed normal hepatic architecture with central vein and radiating cords of normal hepatocytes with central rounded vesicular nuclei and prominent nucleoli. Hepatic cords are separated by blood sinusoids lined with endothelium and Von-Kupffer cells (fig. 3a, b). CCl₄ group showed increased fibrous tissue with dilated blood vessels. In addition to dilated congested blood sinusoids and activated Von Kupffer cells (fig. 3c, d). However, Silymarin treated group showed normal acidophilic hepatocytes with vesicular nuclei. Somewhat dilated central vein and von kupffer cells can also be noticed (fig. 3e). Hesperidin treated group showed normal central vein with slightly dilated congested blood sinusoids. Most hepatocytes are normal with acidophilic cytoplasm and vesicular nuclei, activation of Von Kupffer cells can be also observed (fig. 3f).

Immunohistochemical study

Immunostaining for [complete name] (α-SMA) antigen in the liver showing minimal expression of α-SMA in the portal area of control and corn oil groups (fig. 4a, b) respectively, whereas HSCs strongly positive of α-SMA in the portal areas and along the fibrous septa around the hepatic lobule were observed in group of CCl₄-intoxicated group (fig. 4c, d).

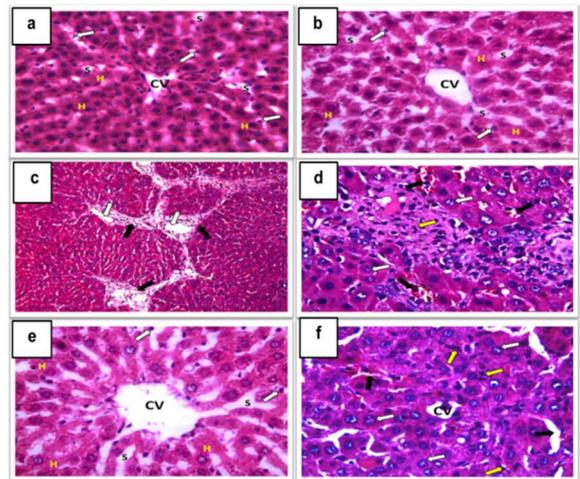


Fig. 3: Histopathological examination of liver sections stained with H and E (×400) in normal and treated groups represented as (a) normal control group (CV: central vein, H: hepatocytes, S: sinusoids, white arrow: von-kupffer cells), (b) corn oil group (CV: central vein, H: hepatocytes, S: sinusoids, white arrow: von-kupffer cells), (c) fibrosis control group CCl₄ in corn oil HandE (×100) (black arrow: fibrous tissue, white arrow: dilated blood vessels), (d) fibrosis control group (black arrow: dilated congested blood sinusoids and activated von-kupffer cells, yellow arrow: fibrous tissue), (e) CCl₄-intoxicated rat co-treated with silymarin group (CV: central vein, H: hepatocytes, S: sinusoids, white arrow: von-kupffer cells) and (f) CCl₄-intoxicated rat co-treated with hesperidin group (CV: central vein, black arrow: blood sinusoids, white arrow: hepatocytes with vesicular nuclei, yellow arrow: von kupffer cells).

Section of a liver obtained from CCl₄-intoxicated group co-treated with silymarin and hesperidin showing moderate positive expression of α -SMA in the portal areas and along the incomplete fibrous septa around the hepatic lobules (fig. 4e, f) respectively. Compared with CCl₄ intoxicated group, liver of rats treated concomitantly with silymarin and hesperidin showed markedly reduced α -SMA positive HSCs.

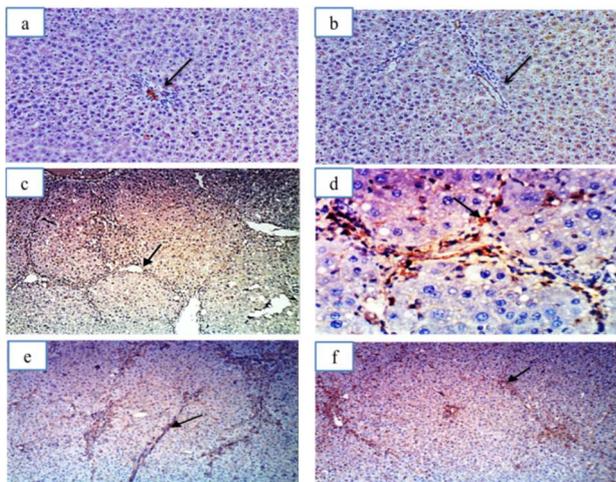


Fig. 4: Examination of alpha-smooth muscle actin (α -SMA) antigen by immunohistochemical staining (magnification \times 100) in liver sections represented as (a) normal control group (b) corn oil group (c) fibrosis control group CCl₄ in corn oil (d) fibrosis control group (magnification \times 400) (e) CCl₄-intoxicated rat co-treated with silymarin group and (f) CCl₄-intoxicated rat co-treated with hesperidin group

DISCUSSION

In the present study, hesperidin was evaluated regarding its possible beneficial effect on CCl₄-induced liver fibrosis in adult male Wistar albino rats as compared to the reference drug silymarin.

Carbon tetrachloride is an industrial solvent and one of the most commonly experimental models used in the induction of liver fibrosis and for the screening of hepatoprotective agents [28]. It is considered as a toxic chemical that induces hepatotoxicity including fibrosis, fatty degeneration, inflammation, carcinogenicity and hepatocellular death [29].

In the current study, CCl₄ intoxication induced a significant increase in serum levels of AST, ALT and total bilirubin which are the most sensitive biomarkers used in the diagnosis of liver injury and hepatic necrosis. These results are in full agreement with Li *et al.* [30]. During the hepatocellular damage, these enzymes are released into the blood flow from the cytoplasm after the rupture of the hepatic plasma membrane. In addition, CCl₄ causes a destruction of hepatic cells and blocking of bile ducts which lead to an increase in serum total bilirubin levels [13]. In addition, CCl₄ produced a significant decrease in serum level of albumin, the most important protein synthesised in the liver. This is in accordance with [30] who considered this as an indication of hepatocyte damage and loss of functional integrity.

The protective potential of hesperidin against liver fibrosis was evidenced in this study from its ability to significantly suppress serum levels of liver function markers (ALT, AST and total bilirubin) and to significantly increase serum level of albumin, revealing its hepatoprotective nature against CCl₄ hepatotoxicity. This comes in agreement with the results of Elshazly and Mahmoud [9], who reported that hesperidin evoked a hepatoprotective effect against dimethylnitrosamine-induced fibrosis in rats, which is likely attributed to its antioxidant and antiapoptotic effects. These effects would reduce HSCs activation and the progression of fibrosis.

According to the findings of this study, CCl₄ intoxication led to a

significant decrease in the activity of the antioxidant enzyme catalase, depletion of hepatic GSH content and a significant increase in the hepatic content of MDA. These results are in accordance with those of Tirkey *et al.* [31] who reported that CCl₄ induces a marked oxidative stress in rat liver. Our observations obviously suggested that the oxidative damage might explain at least in part the CCl₄-induced liver fibrosis.

The main sources of oxidants in the liver are phagocytes and inflammatory state mediators which are present in the tissues of patients with liver diseases and could generate oxidants upon activation. Oxygen radical production increased lipid peroxidation, a process of oxidative conversion of polyunsaturated fatty acids to products known as MDA or lipid peroxides. Malondialdehyde has high cytotoxicity and inhibitory action on protective enzymes causing it to act as a tumour promoter and co-carcinogenic agent [32].

Regarding the oxidative stress biomarkers, results of the present investigation declared that hesperidin restored the normal values of GSH and MDA contents and CAT activity in the liver confirming its antioxidant potential. These results are in harmony with those of Pari *et al.* [8] who reported that hesperidin may play a protective role in reducing the toxic effects of iron-induced oxidative damage in liver and kidney, which could be due to its antioxidant potential by scavenging the free radicals.

In this investigation, the ability of hesperidin to improve these oxidative stress biomarkers reflects its antioxidant properties, its ability to suppress lipid peroxidation, and its good free radical scavenging properties.

Data of the current study showed that CCl₄ increases the hepatic MPO activity, TNF α and the relative liver weight to all rat body weight. According to the results obtained from our research, we realised that hesperidin was able to significantly suppress the hepatic MPO activity, which is a good indicator for neutrophil infiltration and tissue inflammation and to significantly suppress the serum level of the pro-inflammatory cytokine TNF α , which is one of the most important cytokines released during liver fibrosis. In agreement with our results, [33] Fouad *et al.* inferred the anti-inflammatory activity of hesperidin from its ability to reduce the release of inflammatory cytokines like TNF α and cyclooxygenase enzymes, which catalyse a key step in the conversion of arachidonate to prostaglandin (PGs) that plays a critical role in inflammation. Therefore, it could be concluded that inhibition of COX₂ will suppress the production of inflammatory prostaglandins.

Myeloperoxidase was found to catalyse the reaction between chloride and hydrogen peroxide to generate hypochlorous acid and other reactive oxidants. The production of these oxidants beside the cytokines will damage the hepatocytes and results in fibrosis [34]. Therefore, the antifibrotic action of hesperidin may in part be explained by its anti-inflammatory potential.

Results of the current study suggested that hesperidin reduced the amount of fibrous tissue in the liver as presented by its ability to reduce the relative liver weight as compared to the fibrosis control group. Therefore, it may be suggested that hesperidin could alleviate hepatic injury caused by CCl₄ partly through suppression of the inflammatory response.

It has been previously reported that high cholesterol diet leads to rapid deposition of lipid droplets in the liver. Hypercholesterolemia should be considered as a risk factor for hepatic damage as well as atherosclerosis and coronary artery disease [35].

Results of the current investigation revealed that CCl₄ increased serum levels of cholesterol, triglycerides and LDL-Ch, while it produced a significant decrease in serum level of HDL-Ch. Our observations add further evidence for the previous reports of [36] who reported that CCl₄ increases lipid profile through cellular oxidative stress, one of the main causes of hepatic lesions produced by CCl₄ mediated by the free radicals derived from this toxic substance. The intensification of the oxidative stress enhances the influence of non-essential fatty acids which, in turn, increase the serum and tissue levels of cholesterol and triglycerides.

Results of the present study showed that hesperidin significantly reduced serum levels of lipid biomarkers such as total cholesterol, triglycerides and LDL cholesterol, while it produced a significant elevation in serum level of HDL cholesterol, probably through the antioxidant mechanism exerted by the flavones in hesperidin. It has been shown that antioxidants and flavonoids can act as inhibitors of lipid peroxidation by neutralising the radicals of polyunsaturated fatty acids and by interrupting the chain reactions, suppress the influence of non-essential fatty acids which, in turn, decrease the serum and tissue levels of cholesterol and triglycerides [36].

Similar results were also shown by Wang *et al.* [37] who stated that hesperidin effectively alleviated the steatosis of fatty liver, adipose tissue, liver weights and serum total cholesterol concentrations in rats fed with high cholesterol diet and play an important role in reducing the risk of cardiovascular disease.

Hepatic fibrosis is initiated by a damage of hepatocytes that lead to a formation of inflammatory cells and activation of kupffer cells, which subsequently lead to enhanced production of profibrotic cytokines such as TNF α and TGF- β . Activated TGF- β activates expression of many ECM proteins and decreases their degradation by matrix metalloproteinases through increasing the level of tissue inhibitor of metalloproteinases [38]. When the overexpression of TGF- β is inhibited, the result is a marked improvement of hepatic fibrosis, and for this reason, several inhibitors of TGF- β are investigated as potential drug candidates [39].

Data of the current study declared that CCl₄ significantly increased the hepatic content of TGF- β ₁ and HYP as compared to the normal control group. Our observations add further evidence for the previous reports of Aldaba-Muruato *et al.* [39] who mentioned that prolonged CCl₄ treatment was associated with progressive fibrogenesis, even after stopping administration of CCl₄.

Results of the current investigation showed that hesperidin significantly suppressed the hepatic content of TGF- β ₁ which is a major indicator for liver fibrosis and HYP which serves as a biochemical indicator of collagen production. This suggests that hesperidin might have a protective potential against liver fibrosis. This comes in agreement also with the results of Pérez-Vargas *et al.* [40] who reported that hesperidin prevents fibrosis through its ability to modulate profibrotic signals.

Importantly for further molecular and clear interpretation, the expression of α -SMA through the immunohistochemical study was examined. It was found that a dramatic increase in the expression of α -SMA in the CCl₄-intoxicated group. This was considered as an evidence of severe liver fibrosis induced by CCl₄. It is a good marker for HSCs activation and establishment of liver fibrosis model.

These findings were in line with the previous reports of Domitrović *et al.* [41] who stated that liver fibrosis was characterised by activated HSC with accelerated proliferation and enhanced production of ECM components. Hepatic stellate cell activation involves the trans-differentiation from a quiescent state into myofibroblast-like cells with the appearance of α -SMA and loss of cellular vitamin A storage.

On the other hand, it was noticed that hesperidin decreases the expression of α -SMA as compared to the CCl₄-treated group. In the agreement, previous investigations [9] showed similar results, they reported that hesperidin suppressed the progression of HSCs activation, which is manifested as a significant reducer of α -SMA expression.

In this study, deposition of collagen in the damaged hepatic areas, associated with increased α -SMA, indicates that activated HSCs are responsible for the fibrosis seen in CCl₄-intoxicated rat.

The aforementioned biochemical results were supported by histopathological improvement observed in the group treated with hesperidin. Hesperidin modulated the severity of hepatic damage caused by CCl₄ leading to a further confirmation of its anti-fibrotic effect.

Silymarin has been recorded as a hepatoprotective agent against different toxicants as alcohol, CCl₄ or in the case of long uses of many drugs such as acetaminophen [42]. In this study, silymarin showed

generally hepatoprotective effects. It attenuated the effect of CCl₄ toxicity in all the measured biochemical parameters, which is also supported by histopathological examination. Interestingly, hesperidin showed generally superior hepatoprotective effects compared to silymarin. These results suggest that hesperidin might be more therapeutically beneficial in ameliorating the progression of hepatic fibrosis than silymarin and sheds light on its potential value for treating various liver injuries.

CONCLUSION

In conclusion, results of the present study indicate that hesperidin significantly ameliorated CCl₄-induced liver fibrosis in rats based on its antioxidant, anti-inflammatory, antilipidemic and anti-fibrotic activities. These effects would reduce HSCs activation and the progression of fibrosis. The effects of hesperidin were not only comparable to but also even better than silymarin, making it a potential therapeutic option from natural sources to prevent liver fibrosis induced by CCl₄.

AUTHORS CONTRIBUTION

ASMAA RAMADAN ABD EL-STTAR: Design of the work, data collection, and writing the manuscript.

MARWA MAHMOUD KHALAF: Data analysis and interpretation, drafting the article.

AMIRA M. ABOYOUSSEF: Critical revision of the article.

ALI AHMED ABOSAIF: Final approval of the version to be published.

CONFLICT OF INTERESTS

Declared none

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