The Natural Antioxidants Curcumin and Silymarin Afforded Hepatoprotective Effects against Deferasirox-Induced Hepatotoxicity

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Abstract

Deferasirox belongs to a new class of bis(hydroxyphenyl)triazoles iron chelator used for treatment of chronic iron overload. The use of Deferasirox is associated with hepatotoxicity. Silymarin is a benzopyrone flavonoid which affords hepatoprotection and preserves hepatocyte membranes by its antioxidant effect. Curcumin is a poly-phenol compound naturally concentrated in the herb *Curcuma longa*. The present study was conducted to evaluate and compare the hepatoprotective effects of both silymarin and curcumin against deferasirox-induced hepatotoxicity in rats and to explore the potential mechanisms account for their hepatoprotective effects. The present study was carried out using 32 male wistar randomly assigned into 4 groups (8 rats each) as follows; Group I: served as normal control group in which rats received distilled water (0.5ml per rat) by oral gavage. Group II (Deferasirox group) in which hepatotoxicity was induced by oral administration of deferasirox in a dose of 100 mg/kg once daily for 4 weeks dissolved in distilled water for a concentration of 35mg/ml. Group III (deferasirox+ curcumin) in which rats received curcumin in a dose of 100 mg/kg daily dose by oral gavage for 4 weeks suspended in distilled water for a concentration of 35 mg/ml one hour before administration of deferasirox. Group IV (deferasirox+ silymarin) in which rats received silymarin in dose of 7.56 mg/kg once daily by oral gavage for 4 weeks suspended in distilled water for a concentration of 2.7mg/ml one hour before administration of deferasirox. At the end of the treatment period, all rats were sacrificed by cervical dislocation, blood samples were collected for measurement of ALT, AST, ALP and total bilirubin. Liver samples were used for measurement of MDA, GSH and IL-6. Histopathological and immunohistochemistry were also done. The present data revealed that rats pretreated with curcumin or silymarin exhibited significant reduction in ALT, AST, ALP, total bilirubin, IL-6 and MDA levels with significant elevation of GSH level compared to deferasirox group. In-between group comparison revealed that there was a more significant reduction in ALT, AST, ALP, total bilirubin, IL-6 and MDA in group IV compared to group III. In conclusion, both curcumin and silymarin represent natural protective agents against Deferasirox-induced hepatotoxicity due to their antioxidant and anti-inflammatory effects.

Introduction

Hepatotoxicity refers to injury of the liver with impaired liver function. It is defined as chemically-induced liver damage due to exposure to drugs or other noninfectious agents. Many drugs, either in overdoses or within therapeutic ranges may result in liver injury [1]. Chemicals which cause liver injury are called hepatotoxins. These chemicals result in subclinical injury to liver manifested as abnormal liver function tests. Hepatotoxin-induced liver injury is responsible for 5% of hospital admissions and 50% of liver failures. About 75 % of idiosyncratic drug reactions may end by liver transplantation or death [2].

Liver injury results in an increase of the levels of serum alanine aminotransferase, conjugated bilirubin, aspartate aminotransferase, alkaline phosphatase and total bilirubin [3]. Liver injury may be hepatocellular with initial elevation of alanine aminotransferase level or cholestatic associated with elevated serum alkaline phosphatase level [4, 5]. Hepatotoxicity can be predictable or unpredictable.

Predictable reactions are dose-dependent while unpredictable hepatotoxic reactions are unrelated to dose [6].

Mechanisms of hepatotoxicity may include disruption of the cell membrane. Cell death also occurs due to covalent binding of the drug to cellular proteins associated with new adducts formation. Other mechanisms include abnormal bile flow due to disruption of subcellular actin filaments or interruption of transport pumps which results in cholestasis and jaundice. In addition, apoptosis, inhibition of mitochondrial function, accumulation of reactive oxygen species and lipid peroxidation are involved [7, 8].

Deferasirox is one of a new class oral iron chelators called bishydroxyphenyltriazoles [9, 10]. The drug is used parentally for treatment of chronic iron overload occurs due to chronic blood transfusions used in treatment of B-thalassemia, sickle cell anemia and myelodysplastic syndrome [11]. The mechanisms of Deferasirox-induced hepatotoxicity may involve production of reactive intermediates by cytochrome P450, mitochondrial injury, and disruption of calcium homeostasis, stimulation of autoimmunity, canalicular injury and apoptosis [12].

Silymarin is one of the benzo gamma-pyrone flavonoids [13]. It is extracted from the seeds and fruit of *Silybum marianum*. It contains is a mixture of three components; silibinin, silydianine and silychristine [14]. Silymarin and silibinin are associated with cytoprotection and hepatoprotection. Silymarin is used for treatment of liver diseases associated with by degenerative necrosis and functional impairment. It also protects liver from injury caused by ischaemia, radiation, iron overload and viral hepatitis [15]. Silymarin preserved the functional and structural integrity of hepatocyte membranes by preventing alterations of their phospholipid structure and restoring alkaline phosphatase activity in liver cirrhosis induced in rats. Other studies investigated that treatment of rats with silymarin was associated with significant increase in GSH content in the liver, intestine and stomach [16, 17].

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]- 1,6 heptadiene- 3,5-dione) is a hydrophobic polyphenolic compound naturally found in the rhizome of *Curcuma longa*. Curcumin has several antioxidant, antimicrobial, anti-inflammatory, antiviral, anti-carcinogenic, anti-apoptotic and anti-hepatotoxic properties [18]. It is associated with decreased lipid peroxidation level and increased levels of reduced glutathione (GSH) and the enzymatic antioxidants catalase and superoxide dismutase [19].

The present study was conducted to evaluate and compare the hepatoprotective effects of both silymarin and curcumin against Deferasirox-induced hepatotoxicity in rats and to explore the potential mechanisms accounting for their hepatoprotective effects.

Material And Methods

Chemicals, kits and drugs

Deferasirox was purchased as Exjade® 500mg tablets from Novartis Company, Egypt. Curcumin was purchased as a yellow powder from Sigma chemical company, Egypt. Silymarin was purchased as

livamarin® 140mg sachets from European Egyptian pharma, Egypt. All other chemicals are of analytical gradient (Al Gomhoria pharmaceutical company, Egypt). Kits for serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum total bilirubin, malondialdehyde (MDA), reduced glutathione (GSH) and rat interleukin-6 (IL- 6) were all purchased from Biodiagnostic company, Egypt.

Experimental design

Animal groups

This experiment was conducted out using 32 male Wistar rats aged 7 weeks old, each weighing 150-200 gm. They were kept under similar housing conditions and had free access to food and water through the whole period of the work. All experimental procedures described in this study comply with the ethical principles and guidelines for care and the use of laboratory animals adopted by the “Research Ethics Committee” Faculty of Medicine, Tanta University (NIH publication NO.8023, revised 1978) to minimize animal suffering. The study was approved on 21/4/2020 and given an approval number 34876/4/20. The current study is in accordance with ARRIVE guidelines.

Rats were randomly divided into 4 groups (8 rats each) as follows; **Group I:** served as normal control group in which rats received distilled water (0.5ml per rat) by oral gavage. **Group II (Deferasirox group)** in which hepatotoxicity was induced by oral administration of Deferasirox in a dose of 100 mg/kg dissolved in distilled water for a concentration of 35mg/ml once daily for 4 weeks [20]. **Group III (Deferasirox+Curcumin)** in which rats received Curcumin in a dose of 100 mg/kg daily suspended in distilled water for a concentration of 35 mg/ml by oral gavage for 4 weeks one hour before administration of Deferasirox [21]. **Group IV (Deferasirox+ Silymarin)** in which rats received Silymarin in a dose of 7.56 mg/kg suspended in distilled water for a concentration of 2.7mg/ml once daily by oral gavage for 4 weeks one hour before administration of Deferasirox [22].

At the end of the 4 weeks, all rats of the four studied group were sacrificed by cervical dislocation, blood samples were collected and immediately centrifuged at 3000 rpm for 15 minutes and the sera were separated for measurement of biological parameters such as serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP) and total bilirubin. The abdomens of the rats were opened and liver was dissected. One portion of the liver was washed with cold saline and stored at -80°C for measurement of tissue malondialdehyde, reduced glutathione and Interleukin 6. The other portion of the liver was fixed in 10% formalin (pH 7.4) for examination of histopathology and immunohistochemistry changes.

Determination of serum alanine aminotransferase (ALT) level (u/ml)

Serum ALT enzyme level was measured according to the method of [23]. The principle is based on colorimetric determination of ALT activities according to the following reactions:

Alanine + Alpha keto glutarate $\leftrightarrow$ Pyruvate + Glutamate

The keto acid pyruvate is measured in its derivatives form 2,4 – dinitrophenylhydrazine.

Determination of serum aspartate aminotransferase (AST) level (u/ml)

Serum aspartate aminotransferase enzyme level was measured according to the method of [23]. The principle is based on colorimetric determination of AST activities according to the following reactions:

Aspartate + Alpha keto glutarate $\leftrightarrow$ Oxaloacetate + Glutamate

The keto acid oxaloacetate is measured in its derivatives form 2,4 – dinitrophenylhydrazine.

Determination of serum alkaline phosphatase (ALP) level (IU/L)

Serum alkaline phosphatase enzyme level was measured according to the method of [24]. The principle is based on colorimetric determination of ALP activities according to the following reactions:

Phenyl phosphate $\rightarrow$ Phenol + Phosphate

This reaction occurs in presence of alkaline phosphatase enzyme in pH 10.0, the liberated phenol is measured colorimetrically in the presence of 4-aminophenazone and potassium ferricyanide where:

Determination of serum total bilirubin content (mg/dl)

Serum total bilirubin level was measured according to the method of [25]. The principle is based on the reaction between bilirubin and the diazonium salt of sulphanilic acid. Produced azobilirubin shows a maximum absorption at 535nm in an acid medium. In the presence of dimethylsulfoxide (DMSO), total bilirubin participates in the reaction and in the absence of (DMSO) only conjugated bilirubin reacts.

Determination of tissue malondialdehyde (MDA) content (nmol/g.tissue):

Malondialdehyde was determined according to the colorimetric method of [26]. The principle is that thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured at 534 nm. Mixing was done, and the absorbance of sample (A sample) was measured against blank and standard against distilled water at 534 nm.

Determination of tissue reduced glutathione (GSH) content (mg/g. tissue)

Reduced glutathione was determined according to the colorimetric method of [27]. The method is based on the reduction of 5,5` dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow

compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at wavelength of 405 nm.

Measurement of tissue interleukin-6 (IL- 6) content (pg/ml)

the assay is based on the method of [28]. The rat IL-6 ELISA kit is an enzyme-linked immunosorbent assay for the quantitative detection of rat IL-6. An anti-rat IL-6 coating antibody was adsorbed onto microwells. Rat IL-6 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin conjugated anti-rat IL-6 antibody was added and binds to rat IL-6 captured by the first antibody. Following incubation, unbound biotin-conjugated anti-rat IL-6 antibody was removed during a wash step. Streptavidin-HRP was added and binds to the biotin-conjugated anti rat IL-6 antibody. Following incubation, unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP was added to the wells. A coloured product is formed in proportion to the amount of rat IL-6 present in the sample or standard. The reaction was terminated by addition of acid and absorbance is measured at 450 nm. A standard curve was prepared from 7 rat IL-6 standard dilutions and rat IL-6 sample concentration determined.

Light microscopic and Histopathological examination

A piece of liver of each animal was taken and immediately fixed in 10% formal saline solution for 24 hours, then dehydrated in ascending grades of alcohol followed by clearing with xylol. Impregnation was carried out in pure soft paraffin for 2 hours at 50°C followed by embedding in hard paraffin. Sections of 3–5 microns in thickness were cut by the microtome, then stained with hematoxylin and eosin stain to study the general histological structure and Masson`s trichrome stain to demonstrate the collagen fibers [29].

Immunohistochemical detection of caspase-3 in liver tissue

Immunohistochemical assessment of the reactivity of the liver tissue to the antibody of caspase-3 was performed. The method is based on that Caspase normally exists in the cytoplasm as an inactive proenzymes that can be activated once apoptosis starts and it causes proteolysis of various cellular components and so contributes to the pathogenesis of cell death. Caspase-3 immunoreactivity appears as brownish cytoplasmic discoloration using the avidinbiotin-peroxidase complex technique. Liver tissues were Deparaffinated and rehydrated. Sections (5 um) were cut from Bouin's-fixed paraffin embedded blocks on positive charged slides (Super frost plus Biogenic). They were dewaxed in fresh xylene bath. For antigen retrieval, Slides were put in a slide rack and placed in a coplan jar containing 0.01 M citrate buffer (pH 6). Coplan jar is placed in a water bath to keep a humid atmosphere inside the microwave oven. Slides were then washed in distilled water several times then placed in PBS (pH 7.4). For blocking endogenous peroxidase, sections were immersed in 3% hydrogen peroxide in methanol for 30 minutes, and then slides were washed by PBS. For blocking nonspecific staining; two drops of non-specific blocking reagent (normal goat serum) were placed on each slide. Primary rabbit polyclonal caspase-3 antibody (CPP32 Ab-4, RB-1197-PCS, Lab Vision Corporation, United Kingdom), were placed on each slide. The polyclonal antibody was used at a 1:50 dilution. Then secondary biotinylated antibody was

added and drops of streptavidin enzyme label were applied. Diamino-Benzidine (DAB). In Preparation of working color reagent, chromogenic was prepared by addition of one drop of DAB chromogenic per 1ml of buffered substrate. Several drops of the working color reagent were placed on each slide. Slides were washed by Tap water. Counter-stain with hematoxyline then the slide was washed in distilled water. Sections were dehydrated in alcohol, put in xylene then mounted in Distrene-plasticizer-xylene. Positive cells were stained brownish while negative remained blue [30].

Morphometric analysis

The image analysis software program (ImageJ 1.46 version) was used to examine sections from liver in all groups. Mean Area percentage of Masson's trichrome and optical density of caspase-3 immuno-expression in hepatocytes were measured in five randomly different microscopic fields for each slide for each specimen. The data obtained were statistically analyzed using GraphPad InStat Software and then compared by one-way analysis of variance (ANOVA) test followed by Tukey's test to compare different groups with the control group. The results were expressed as mean $\pm$ standard deviation (SD). The differences were considered statistically significant if probability value $P < 0.05$, highly significant if $P < 0.001$ and non-significant if $P > 0.05$ [31].

Statistical analysis

All values were presented as mean $\pm$ S.D. Data were analyzed by One-Way analysis of variance (ANOVA). All statistical analyses were processed using statistical package for the social sciences (SPSS) for windows, version 20. $p \leq 0.05$ value was set for statistical significance.

Results

As shown in Figure 1, group II (Deferasirox group) exhibited a significant increase in serum ALT, a significant increase in serum AST (Figure 2) and a significant increase in ALP (Figure 3), a significant increase in serum total bilirubin (Figure 4) compared to control group. There was also a significant increase in tissue IL-6 (Figure 5), a significant increase in tissue MDA content (Figure 6), a significant decrease in tissue GSH content in compared to control group (Figure 7).

Values of chemical parameters obtained from group III (Curcumin+ Deferasirox) revealed significant reduction in ALT, AST, ALP, total bilirubin, IL-6 and MDA contents with significant elevation in GSH content in comparison to Deferasirox group.

In comparison to Deferasirox group, rats pretreated with Silymarin (group IV) exhibited significant reduction in ALT, AST, ALP, total bilirubin, IL-6 and MDA contents with significant elevation of GSH content.

In-between group comparison revealed more significant reduction in ALT, AST, ALP, total bilirubin, IL-6 and MDA contents in Silymarin group compared to Curcumin group.

Results for Histopathological examination of liver tissue by H&E staining

Microscopic examination of group I (control group) showed normal histological structure of the liver with classic hepatic lobular architecture consisting of cords of hepatocytes radiating around central veins and portal areas at the corners (Fig. 8a). Hepatocytes revealed acidophilic cytoplasm, vesicular nuclei and some of them appeared binucleated. Blood sinusoids separating hepatocyte cords were lined by flat endothelial cells (Fig. 8b).

Meanwhile, examination of group II (deferasirox-treated group) showed disturbed hepatic lobular architecture with inflammatory cellular infiltration in between hepatocytes, around central vein and portal area (Fig. 8c,d). Bile duct proliferation was also observed in some sections (Fig. 8e). Regarding hepatocytes, they appeared swollen with highly vacuolated cytoplasm and shrunken deeply stained pyknotic nuclei (Fig. 8f). In group III (curcumin-treated group) partial improvement in hepatic architecture was detected (Fig. 8g). Some hepatocytes appeared acidophilic with less vacuolated cytoplasm and vesicular nuclei while others showed more vacuolated cytoplasm (Fig. 8h). Some inflammatory cellular infiltration within the hepatic lobule and around the portal area was still observed (Fig. 8i). Regarding group IV (silymarin-treated group), there was marked improvement in the histological structure (Fig. 8j). Classic hepatic lobular architecture with hepatocytes radiating around central vein was observed. Hepatocytes appeared with acidophilic less vacuolated cytoplasm and vesicular nuclei. Few inflammatory cellular infiltrations around the portal area were still observed (Fig. 8k).

Results for Histopathological examination of liver tissue using Masson's Trichrome

Examination of sections obtained from the liver of group I (control group) revealed minimal deposition of collagen fibers around the central vein and in the portal area (Fig. 9a). While group II (deferasirox-treated group) showed excessive deposition of collagen fibers around central veins and in the portal area (Fig. 9b-c). Group III (deferasirox+curcumin treated group) exhibited deposition of relatively moderate amount of collagen fibers in the portal area (Fig. 9d). Moreover, group IV (deferasirox+ silymarin treated group) showed deposition of relatively small amount of collagen fibers in the portal area (Fig. 9e).

Immunohistochemical expression of Caspase-3:

Immunohistochemically stained sections of group I (control group) showed few hepatocytes with faint cytoplasmic & nuclear immunoreactivity (Fig. 10a). While group II (deferasirox-treated group) revealed many hepatocytes with intense nuclear & cytoplasmic immunoreaction (Fig. 10b). Group III (deferasirox+curcumin treated group) showed some hepatocytes with moderate immunoreactivity mainly nuclear (Fig. 10c). In group IV (deferasirox+silymarin treated group) there was faint nuclear reaction in some hepatocytes (Fig. 10d).

In addition, there was a highly significant increase in the mean optical density of caspase-3 positive cells in group II (deferasirox-treated group) as compared to control group ($p < 0.001$). While group III (deferasirox+curcumin treated group) revealed a significant increase in the mean optical density of caspase-3 positive cells as compared to control group ($p < 0.05$). Meanwhile, there was non significant increase in the mean optical density of caspase-3 positive cells in group IV (deferasirox + silymarin treated group) as compared to control group (Table 1 and Figure 11)

Table 1
Comparison between studied groups for the mean $\pm$ SD of the optical density.

Groups	Mean $\pm$ SD
Group I	1.11 $\pm$ 0.022
Group II	1.7** $\pm$ 0.16
Group III	1.31* $\pm$ 0.124
Group IV	1.24 $\pm$ 0.039
Note: Data are expressed as mean $\pm$ SD= the standard deviation.	
** Highly significant from control group ($p \leq 0.001$). * Significant from control group.	

Results for area percentage of collagen fibers

There was a highly significant increase in the mean area percentage of collagen fibers in group II (deferasirox- treated group) as compared with the control group ($p < 0.001$) while group III (curcumin- treated group) revealed a significant increase in the mean area percentage as compared to control group ($p < 0.05$). Meanwhile, there was nonsignificant increase in the mean area percentage of collagen fibers in group IV (silymarin- treated group) as compared to control group (Table 2 and Figure 12).

Table 2
Comparison between studied groups for the mean $\pm$ SD of the area percentage of collagen fibers.

Groups	Mean $\pm$ SD
Group I	0.66 $\pm$ 0.39
Group II	5.76** $\pm$ 2.05
Group III	3.39* $\pm$ 0.83
Group IV	2.4 $\pm$ 0.86
Note: Data are expressed as mean $\pm$ SD= the standard deviation	
** Highly significant from control group ($p \leq 0.001$). * significant from control group.	

Discussion

Drug-induced hepatotoxicity is a common clinical problem caused by a large number of drugs with different mechanisms and variable outcomes. Deferasirox (DSX) was approved by the FDA in 2005 as an iron chelator for treatment of chronic iron overload in patients requiring multiple blood transfusions. However, the use of the drug was associated with post-market reports of hepatic injury with unknown mechanism of liver injury [32]. In the present study, Deferasirox induced a significant increase in serum AST, ALT, ALP and total bilirubin. This is in agreement with pre-marketing studies that demonstrated marked elevation of liver transaminases in about one third of patients taking Deferasirox. These data indicate drug-induced hepatitis and liver failure. The rationale of the present findings is that elevated transaminases level is associated with the occurrence of hepatic injury while increased bilirubin level is an indication of the severity of the liver injury. This means that Deferasirox-induced liver injury was substantially enough to impair liver function.

Another study has found that Deferasirox caused death of a patient with sickle cell anemia due to hepatitis. The patient possesses polymorphism in the *Abcc2* gene. This gene encodes MRP2 protein, thus genetic polymorphism in this gene is associated with increased susceptibility to Deferasirox-induced hepatotoxicity [33].

Also, it was found that Deferasirox (10–20 mg/kg) sickle cell anemia patients ≥ 40 years old caused severe changes in renal function or hepatotoxicity that resulted in discontinuation of the treatment. It was suggested that hepatotoxicity is related to the Haplotype MRP2 and the UGT1A1*6 genotype resulted in elevation of creatinine. Glucuronidation by UDP-glucuronosyltransferase 1A (UGT1A) subfamily which encodes UDP-glucuronosyltransferase. is the main metabolic pathway of Deferasirox. In bile, Deferasirox is then excreted through the multidrug resistance protein (MRP2) which is a transporter anion expressed in the hepatocytes membranes. MRP2 is encoded by the *Abcc2* gene [34].

In contrast to the present findings, Miura et al., 2010 found that three cases of transfusion-mediated iron-overloaded patients with hematological diseases had elevated ALT levels. Treatment with DSX decreased and subsequently normalized elevated ALT levels. This occurred also with the abnormal AST level [35]. Other parameters related to impaired liver function including ALP, γ -GTP, T-Bil, and Alb were not changed. However, we cannot rely on these findings that DSX may improve liver damage in those patients because of the small number of patients and lack of supportive histopathological findings of liver tissue examination.

Another study found that DFX at a dose of 100 mg/kg was the most effective in preventing elevation of ALT, AST enzymes and histopathologic changes induced by concanavalin A [36].

Several mechanisms may account for Deferasirox –induced hepatotoxicity such as production of reactive intermediates by cytochrome P450, stimulation of autoimmunity, apoptosis, and disruption of calcium homeostasis, canalicular injury and mitochondrial injury [37]. This was supported by the findings

that treatment of rats with Deferasirox induced a significant elevation of hepatic tissue MDA content with a significant reduction in hepatic tissue GSH content.

The most frequent mechanism for drug-induced liver injury is metabolism by cytochrome P450 that involves the formation of high energy reactive intermediates which then undergo conjugation process. Subsequent covalent bonding of these reactive intermediates to cellular components, such as proteins or nucleic acids produces cellular dysfunction [38]. However, Deferasirox undergoes minimal metabolism by CYP-450 system which makes this mechanism unlikely for Deferasirox.

Immune-mediated liver toxicity may also account for Deferasirox-induced hepatotoxicity. The drug may bind to an enzyme and becomes immunologically active which then activates cellular and humoral immune responses leading to cellular death. This may account for the significant elevation of hepatic tissue IL-6 content in Deferasirox-treated group observed in the present study.

Cholestatic injury may be considered as another possible mechanism for Deferasirox-induced liver injury [39]. The drug may block bile salt transport proteins in the canalicular membranes responsible for the generation of bile. This in turn leads to cholestasis and jaundice and accounts for the observation that total bilirubin is significantly elevated in Deferasirox group.

Another possible mechanism that may account for Deferasirox-induced liver injury is mitochondrial injury. The main characteristics of this injury are macrovesicular lesions, focal necrosis, fibrosis and cholestasis. Mitochondrial injury may result from inhibition of fatty acid β -oxidation.

In the present work, H&E-stained liver sections of Deferasirox group revealed disturbed hepatic lobular architecture, swollen hepatocytes with highly vacuolated cytoplasm and shrunken deeply stained pyknotic nuclei, extensive inflammatory cellular infiltration and proliferation of bile ducts. Similar results were obtained by other authors [39, 40] studying the damaging effect of zinc oxide nanoparticles and carbon tetrachloride on rat's liver, respectively.

Swelling of hepatocytes with vacuolation of their cytoplasm could be explained by ballooning degeneration associated with dilatation of endoplasmic reticulum. This dilatation results from mitochondrial dysfunction and ATP depletion, leading to loss of ion homeostasis and plasma membrane integrity resulting in fluid influx. The cytoplasm is partially rarefied along the cellular periphery and the cytoplasmic cytoskeleton clumps around the nucleus [41].

In cases of drug-induced liver injury, damaged hepatocytes release molecules called damage-associated molecular patterns. This results in activation of liver immune cells, including Kupffer cells, natural killer lymphocytes and dendritic cells thus which triggers an inflammatory cascade involving recruitment of neutrophils and monocyte from blood [42]. This explanation coincides with the detection of extensive inflammatory cellular infiltration in the present study.

Activation of hepatic stem cells. These stem cells are activated in response to liver injury and differentiated into progenitor cells for both hepatocytes and cholangiocytes. This ultimately leads to

proliferation of bile ducts [43, 44].

In the current study, excessive deposition of collagen fibers in the portal area and around central veins was detected in Masson's trichrome-stained sections of Deferasirox group. This finding was confirmed by morphometric and statistical studies. There was a highly significant increase in the mean area percentage of collagen fibers in this group as compared to control group.

The process of liver fibrogenesis is initiated by damage of hepatocytes which results in recruitment of inflammatory cells and activation of Kupffer cells, with subsequent release of cytokines and growth factors [45, 46]. These factors stimulate differentiation of hepatic stellate cells into myofibroblasts-like cells that have the ability of synthesis and deposition of large amounts of collagen fibers resulting in liver fibrosis [47].

Intense caspase-3 immunoreaction in many hepatocytes of group II was confirmed statistically by a highly significant increase in the mean optical density of caspase-3 positive cells in this group as compared to control group.

Martin-Sanchez et al., 2017 studied the effect of Deferasirox on proximal renal tubules and proved that Deferasirox induced mitochondrial stress and caused loss of mitochondrial membrane potential. In addition, it downregulated the antiapoptotic protein BclxL while the levels of proapoptotic Bax were unchanged, leading to a decreased BclxL/Bax ratio which is known to predispose to apoptosis [48]. Stressed mitochondria lead to release of cytochrome c into cytosol that causes activation of caspase-3 and apoptotic cell death [49].

The present data revealed that the group pretreated with curcumin exhibited significant reduction in the values of ALT, AST, ALP, total bilirubin, IL-6 and MDA levels with significant elevation in GSH compared to Deferasirox- treated group. In addition, histological, immunohistochemical and statistical findings exhibited partial improvement as compared to Deferasirox- treated group. These results support the evidence that curcumin possesses an antioxidant and hepatoprotective effects.

Curcumin is rich in curcuminoids which owing to their methoxy group¹, 3 B-diketone moiety and phenolic hydroxyl can inhibit oxidation, and decrease the free radicals generation [50]. In addition, Curcumin was found to inhibit NF-κB, which activates inflammatory cytokines and chemokines, leading to several inflammatory conditions [51]. Also, curcumin is able to inhibit IL1, IL1B, IL6, IL8, tumor necrosis alpha, and cyclooxygenase pathways [52].

Kyung et al. used dimethylnitrosamine (DMN) for induction of liver cirrhosis in rats. The study found curcumin increased conductivity using a magnetic resonance-based electrical conductivity imaging method than cirrhotic tissues in the DMN-group. Curcumin also resulted in significant reduction of fibrosis and decreased inflammation compared with liver tissues treated with DMN [53]. The hepatoprotective and anti-inflammatory effects of curcumin were associated with significant reduction in COX-2 expression in the DMN- group.

Also in agreements with our findings, a study using alcoholic-liver disease found that treatment with curcumin prevented both the pathological and biochemical changes induced by alcohol in an alcohol-induced liver damage (ALD) model as manifested by reduction of activation of NF- κ B and the induction of cytokines, chemokines, COX-2, iNOS, and nitrotyrosine formation [54]. It was suggested that curcumin prevented ALD by inhibiting lipid peroxidation, activation of NF- κ B, and expression of proinflammatory mediators.

In consistent with the present findings, **Ibrahim et al.**, found that the use of curcuminoids crude extract resulted in significant increases in antioxidant enzymes; super oxide dismutase, reduced glutathione, catalase and reduction in liver integrity enzyme biomarkers; aspartate transaminase, alanine transaminase and alkaline phosphatase in comparison to CCL4-induced liver damage [55]. These data support our findings that curcumin preserves liver cell membranes integrity and prevents leakage and release of enzymes such as ALT, AST and ALP and was able to reverse the injurious effects of Deferasirox on the liver. In the present study, curcumin significantly reduced the liver tissue MDA and increased GSH content. This indicates that in addition to protecting the liver against toxicants, curcumin also enhance the antioxidant system of the liver, this will positively enhance the liver capability to fight toxicant.

In another study, it was found that curcuminoids significantly attenuated AFB1-cytotoxicity. The hepatoprotective mechanisms for curcumin against AFB1 toxicity are related to antioxidant response and anti-inflammatory potentials [56]. In addition, curcumin has been reported to inhibit the expression of NF- κ B dependent inflammatory cytokines, such as TNF- α , IL-6, IL-2, TGF- β , and MCP-1, and inflammation-promoting molecules such as COX-2 and iNOS in Kupffer cells [57].

The data of the present study showed that in comparison to Deferasirox group, rats pretreated with silymarin exhibited significant reduction in ALT, AST, ALP, total bilirubin, IL-6 and MDA contents with significant elevation of GSH content. These results were confirmed by the noticeable improvement detected in histological, immunohistochemical and statistical findings.

In accordance with our results, a previous study revealed that CCl4 administration resulted in significant elevation of intracellular enzymes, such as transaminases and serum alkaline phosphatase and an ethanolic plant extract of silymarin produced insignificant decreases in liver functions. The stabilization of these enzymes indicates the improvement of the functional status of the liver [58]. Also, elevation of MDA and reduction of GSH by CCL4 have been reversed upon use of the extract which indicates an antioxidant potential of silymarin.

The protective effects of silymarin may be attributed to different mechanisms including; (1) scavenging of free radicals and chelating free Fe and Cu. (2) inhibition of ROS-producing enzymes and preventing the formation of free radical (3) activating antioxidant enzymes and non-enzymatic antioxidants, via transcription factors, such as Nrf2 and NF- κ B (4) Inhibition of NF- κ B pathways [59]. It was found that silymarin protects the liver from various toxins such as arsenic, CCL-4, mycotoxins, thioacetamide,

cisplatin, manganese, benzopyrene, doxorubicin and ethanol owing to its antioxidant and free radical scavenging properties [60].

Also, our data indicated that silymarin exhibited an anti-inflammatory potential which is evident by significant reduction of inflammatory cytokine IL-6. A large number of studies have demonstrated a regulatory effect of silymarin on the expression of NF- κ B in various in vitro and in vivo models. The drug performs its anti-inflammatory activity by modulating the expression of pro-inflammatory genes such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several cytokines [61].

Silymarin has been shown to have antioxidant, anti-inflammatory and antifibrotic activities in chronic liver disease, liver cirrhosis, non-alcoholic fatty liver and steatohepatitis. Silymarin exerts membrane-stabilizing and antioxidant activities. Furthermore, it reduces inflammatory reactions and inhibits liver fibrogenesis. Silymarin was found to significantly reduce tumor cell proliferation, angiogenesis as well as insulin resistance. In addition, it possesses an anti-atherosclerotic effect and suppresses TNF α -induced protein production and mRNA expression due to adhesion molecules [62].

In addition to its antioxidant and anti-inflammatory potentials, silymarin exhibits an anti-fibrotic property. This effect is associated with its ability to prevent the conversion of hepatic stellate cells into myofibroblasts. Silymarin down-regulates TGF- β 1 mRNA, inhibits NF- κ B, and prevents the stimulation of hepatic stellate cells. These data indicate that Silymarin is able to slow down the progression of early fibrosis [63].

Declarations

Ethics approval

The authors declared that all experimental procedures described in this study comply with the ethical principles and guidelines for care and the use of laboratory animals adopted by the “Research Ethics Committee” Faculty of Medicine, Tanta University (NIH publication NO.8023, revised 1978) to minimize animal suffering. Research Ethics Committee (Federal Wide Assurance (FWA) 00022834) of Faculty of Medicine, Tanta University, Egypt has approved the study on 21/4/2020 and the study was given an approval number 34876/4/20 . The current study is in accordance with ARRIVE guidelines.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study will be available from the corresponding author upon request

Competing interests

The authors declared that they have no competing interests.

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Authors' contributions

Enass Y. Osman was responsible for the statistical analysis, writing the abstract, results and discussion and revised the article and submission of the manuscript.

Souzan E. Abo-Elnasr was responsible for histopathology and immunohistochemistry examination (images comments and statistics) and writing the material and method of histopathological examination.

Shimaa Mohammed Zaher was responsible for histopathology and immunohistochemistry examination (images comments and statistics) and writing the material and method of histopathological examination.

Norhan Ahmed AbuoHashish was responsible for the study design, animal handling, experimental design and measuring biochemical parameters. She also wrote introduction, material and methods parts.

All authors approved the submitted version of the manuscript.

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Figures

Figure 1

Comparison of serum alanine amino transferase (ALT) level (u/ml) in different studied groups .Values are expressed as mean±SD. n=8* Significant difference from control group at p< 0.05 & Significant difference from Deferasirox group at p< 0.05. # Significant in-between Curcumin and Silymarin groups at p< 0.05.

Figure 2

Comparison of serum Aspartate amino transferase (AST) level (u/ml) in different studied groups .Values are expressed as mean $\pm$ SD. n=8^{*} Significant difference from control group at p< 0.05 & Significant difference from Deferasirox group at p< 0.05. # Significant in-between Curcumin and Silymarin groups at p< 0.05.

Figure 3

Comparison of serum alkaline phosphatase (ALP) level (IU/L) in different studied groups. Values are expressed as mean $\pm$ SD. n=8* Significant difference from control group at $p < 0.05$ & Significant difference from Deferasirox group at $p < 0.05$. # Significant in-between Curcumin and Silymarin groups at $p < 0.05$.

Figure 4

Comparison of serum total bilirubin content (mg/dl) in different studied groups. Values are expressed as mean $\pm$ SD. n=8* Significant difference from control group at $p < 0.05$ & Significant difference from Deferasirox group at $p < 0.05$. # Significant in-between Curcumin and Silymarin groups at $p < 0.05$.

Figure 5

Comparison of tissue interleukin-6 (IL-6) content (pg/ml) in different studied groups. Values are expressed as mean $\pm$ SD.n=8* Significant difference from control group at $p < 0.05$ & Significant difference from Deferasirox group at $p < 0.05$. # Significant in-between Curcumin and Silymarin groups at $p < 0.05$.

Figure 6

Comparison of tissue malondialdehyde (MDA) content (nmol/g.tissue) in different studied groups. Values are expressed as mean $\pm$ SD. n=8* Significant difference from control group at $p < 0.05$ & Significant difference from Deferasirox group at $p < 0.05$. # Significant in-between Curcumin and Silymarin groups at $p < 0.05$.

Figure 7

Comparison of tissue reduced glutathione (GSH) content ($\mu\text{mol/g. tissue}$) in different studied groups. Values are expressed as mean $\pm$ SD. $n=8$ Significant difference from control group at $p < 0.05$ & Significant difference from Deferasirox group at $p < 0.05$. # Significant in-between Curcumin and Silymarin groups at $p < 0.05$.

Figure 8

a: A Photomicrograph of a liver section from the control group showing normal classic hepatic lobular architecture with cords of hepatocytes radiating around a central vein (CV) and a portal area (P) at its corner. b: A Photomicrograph of a liver section from the control group showing the central vein (CV) at the center of the classic hepatic lobule. Hepatocytes appear with acidophilic cytoplasm and vesicular nuclei (arrows). Some cells appear binucleated (bifid arrows). Blood sinusoids (S) separate hepatocyte cords and are lined by flat endothelial cells (arrow heads). (H&E x400) c: A photomicrograph of a liver section from group II (deferasirox-treated group) showed disturbed hepatic lobular architecture with intralobular inflammatory cellular infiltration (curved arrow). d: A photomicrograph of a liver section from group II (deferasirox-treated group) showing extensive inflammatory cellular infiltration (curved arrows) in between hepatocytes and around central vein (CV) and portal area (P). e: A photomicrograph of a liver section from group II (deferasirox- treated group) showing proliferation of bile duct (B). f: A photomicrograph of a liver section from group II (deferasirox- treated group) showing hepatocytes appear swollen with highly vacuolated cytoplasm (wavy arrows) and shrunken deeply stained pyknotic nuclei (arrows). g: A photomicrograph of a liver section from group III (deferasirox + curcumin treated group) showing some inflammatory cellular infiltration (curved arrow) around the portal area (P). h: A photomicrograph of a liver section from group III (deferasirox+curcumin treated group) showing hepatocytes appear with acidophilic less vacuolated cytoplasm and vesicular nuclei (arrows). Cords of hepatocytes are radiating around slightly dilated central vein (CV). (H&E x400) i: A photomicrograph of a liver section from group III (deferasirox+ curcumin treated group) showing cytoplasm of some

hepatocytes appear highly vacuolated (wavy arrows). Notice intralobular inflammatory cellular infiltration (curved arrows). j: A photomicrograph of a liver section from group IV (deferasirox+ silymarin treated group) showing classic hepatic lobular architecture appear normal with hepatocytes radiating around central vein (CV) and few inflammatory cellular infiltration (curved arrow) around the portal area (P). k: A photomicrograph of a liver section from group IV (deferasirox+silymarin treated group) showing hepatocytes with acidophilic less vacuolated cytoplasm and vesicular nuclei (arrows). Some hepatocytes appear binucleated (bifid arrows). Hepatic cords are separated by blood sinusoids (s). Notice endothelial cells (arrow heads) lining the central vein (CV).

Figure 9

a: A photomicrograph of a liver section from the control group showing minimal deposition of collagen fibers around the central vein (green arrows) and in the portal area (yellow arrows). b: A photomicrograph of a liver section from group II (deferasirox- treated group) showing excessive deposition of collagen fibers (green arrows) around central veins. Notice vacuolated cytoplasm of hepatocytes (wavy arrows) and dilated congested blood sinusoids (S). c: A photomicrograph of a liver section from group II (deferasirox-treated group) showing excessive deposition of collagen fibers (yellow arrows) in the portal area. d: A photomicrograph of a liver section from group III (deferasirox+curcumin treated group) showing deposition of relatively moderate amount of collagen fibers in the portal area. e: A photomicrograph of a liver section from group IV (deferasirox+silymarin treated group) showing deposition of relatively small amount of collagen fibers in the portal area.

Figure 10

a: A photomicrograph of a liver section from the control group showing few hepatocytes with faint cytoplasmic & nuclear immunoreactivity (thick arrows). b: A photomicrograph of a liver section from group II (deferasirox-treated group) showing many hepatocytes with intense nuclear & cytoplasmic immunoreactivity (thick arrows). c: A photomicrograph of a liver section from group III (deferasirox+curcumin treated group) showing some hepatocytes with moderate immunoreactivity

mainly nuclear (thick arrows). d: A photomicrograph of a liver section from group IV (deferasirox & silymarin treated group) showing some hepatocytes with faint nuclear immunoreactivity (thick arrows).

Figure 11

Comparison between different groups as regards the optical density of caspase3 positive cells.

Figure 12

Comparsion between different groups for the mean of area percentage of collagen fibers.