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Research article

# Hesperidin an antioxidant flavonoid prevents carbon tetrachloride-induced hepatic toxicity in male albino rats

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**Key words:** Hesperidin (HDN), Antioxidants, Hepatic Toxicity, CCl4.

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**Abstract**

Through this research work, an experimental study was conducted to evaluate the protective effects of an antioxidant (Hesperidin) on carbon tetrachloride-induced hepatic toxicity. This effect was evaluated through assessment of liver functions as well as histopathological changes in livers of rats exposed to Hesperidin prior to carbon tetrachloride. Thirty two male albino rats (160-200 gm) were chosen as an animal model for this study and distributed to four equal groups each of 8 rats (Treated for 10 days). After the ten days of treatment, the following were assessed: liver enzymes, tumour necrosis factor –alpha, oxidant parameters as malondialdehyde and antioxidant parameters as glutathione, superoxide dismutase, and total antioxidant capacity. Histopathological examination of the liver tissues was conducted. Hesperidin in the dose of 100 and 200 mg/kg produced a significant decrease in the levels of liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), tumour necrosis factor–alpha(TNF-α), and oxidant parameters as malondialdehyde (MDA). Antioxidant parameters as glutathione (GSH), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC), also have shown significant increase. These findings were confirmatory to histopathology. Hesperidin in a dose of 100 and 200 mg/kg offers significant protection against hepatotoxicity produced by $CCl_4$ in albino rats, but this protection is dose-dependent.

## Introduction

The liver is the main organ involved in metabolism of biological toxins and medicinal agents. Such metabolism is associated with disturbance of hepatocyte biochemistry and generation of reactive oxygen species (ROS) [1]. Among free radicals (FRs), reactive oxygen species (ROS) are derived from oxygen. ROS contains FR and reactive form of oxygen. Other FR families exist such as reactive nitrogen species (RNS) and reactive sulphur species (RSS) [2]. Molecular ground-state oxygen can be activated to ROS by means of energy transfer (e.g., under the influence of ultraviolet radiation), forming singlet oxygen ($1O_2$). Or by electron transfer, forming reduction products i.e. the superoxide anion radical (•O-2). Oxidative stress, resulting from an imbalance in generation of free radicals and antioxidant defense molecules, affects biological macromolecules causing their structural alterations that lead to cell damage and death [3]. This phenomenon is considered a major factor in the pathogenesis of a variety of liver diseases. In this regard, reduction of oxidative stress may be a good target for prevention and treatment of hepatic and renal toxicity [4]. Chemical structure of Hesperidin is shown in Figure 1.

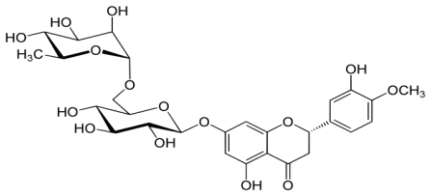


**Figure 1. Chemical structure of Hesperidin (Hesperitin-7-rhamnoglucoside) [5].**

The antioxidant activity of hesperidin is well known. The objective of work is to know the antioxidant and anti-inflammatory effects of hesperidin using two different concentrations (100, 200 mg/kg) which are affected by doses on hepatic cells by measuring different parameters, histopathological examinations of hepatocytes were also considered.

## Materials and methods

### Materials

### Experimental Animals

This study was conducted on 32 male albino rats. Their weight ranged between 160-200 gm. Rats were housed as 4 groups with 8 rats each in clean capacious macrolane cages under standard laboratory conditions. The animals

were handled according to the guidelines of local ethical committee which comply with the international laws for use and care of laboratory animals. Animal Ethics Committee Approval No. Ethics/research/OMU/2018-87.

### Drugs
$CCL_4$ (El-Nasser Pharmaceuticals chemical company, Egypt) and Hesperidin (HDN: Sigma, Aldrich).

### Chemicals
Saline (El-Nasser Pharmaceuticals chemical company, Egypt), Phosphate buffered saline (Hi-media- Lab. Pvt. Inc., USA), SOD, MDA and GSH reduced kits (Biochemical Enterprise, Italy), ALT/AST kits (Centronic_Gmbh, Germany) TNF-α, ELISA kit (Ray Biotech, Inc., USA), T-AOC assay kit, (Cayman Chemical Company, USA).

### Experimental design
Animals were divided into 4 groups, each consisted of 8 rats. Animals were fed on commercial pellet food, water was supplied freely. Group-I (Control negative): rats received 5% carboxymethyl cellulose orally (as a vehicle) for 10 days and were injected by olive oil subcutaneously in the 8th day [6]. Group-II (Control positive): These animals received 5% carboxymethyl cellulose orally for 10 days and were challenged with $CCl_4$, 2 ml/kg/SC (40 % v/v in olive oil) on 8th day [7]. Group-III (HDN 100): These rats received HDN 100 mg/kg/PO daily for 10 days. On the 8th day they received $CCl_4$ 2ml/kg/SC in olive oil once. HDN was further continued for 2 more days [6]. Group-IV (HDN 200): These rats received HDN 200 mg/kg/PO daily for 10 days. On the 8th day they received $CCl_4$ 2ml/kg/SC in olive oil once. HDN was further continued for 2 more days [6].

### Procedures
Blood sampling: At the end of the experiment, rats were sacrificed and blood samples were collected from the retro-orbital vein of each animal, under light anaesthesia by diethyl ether, according to the method of Cocchetto, and Bjornsson, (1983) [8]. Blood samples were then centrifuged and the serum from each animal was kept in epindorff tubes in the deep freezer at (-20°C) until analyzed for liver functions, TNF-α, and T-AOC.

### Preparation of liver homogenate
Animals were sacrificed; livers were immediately excised, rinsed from blood in ice cold saline and blotted dry by filter papers. Small piece of each liver was fixed in 10% phosphate-buffered formalin for histological examination. About 0.5 gm of each liver was homogenized by ultrasonic homogenizer in 5 ml ice-cold phosphate buffered saline (PBS) to obtain ultimately10% (w/v) whole liver homogenate [9]. The homogenate was centrifuged at 3000 rpm for 15 min and the resultant supernatant was stored at (-20°C) until used for determination of reduced Glutathione (GSH), Malondialdehyde (MDA), Superoxide dismutase (SOD).

### Determination of liver function
Commercial kit Purchased from (Centronic_Gmbh, Germany) based on the method described by Thomas, (1998) [10] was used for determination of ALT & AST activity.

**Determination of serum tumor necrosis factor alpha (Pg /ml)** by using rat specific ELISA kit (Cat#: ELR-TNFalpha-001).

### Determination of serum total antioxidant capacity (µmol/l) by Koracevic, *et al.* (2001) [11]
The determination of T-AOC is performed by the action of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide ($H_2O_2$). The antioxidants in the sample eliminate a certain amount of the provided $H_2O_2$.The residual $H_2O_2$ is determined calorimetrically by an enzymatic reaction with involves the conversion of 3,5,dichloro-2-hydroxy benzene sulphonate to a colored product. The absorbance were recorded spectro-photometrically immediately at 505 nm.

### Determination of hepatic reduced glutathione (mg/g tissue)
The method based on the reduction of 5, 5' dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm [12].

### Determination of hepatic superoxide dismutase activity (U/g tissue)
This assay relies on the ability of the enzyme to inhibit the phenazinemethosulphate-mediated reduction of nitrobluetetrazolium dye [13].

### Determination of hepatic lipid peroxide (Malondialdehyde) (nmol/g tissue)
It was determined colorimetrically according to Ohkawa,*et al.*, (1979) [14].

### Statistical analysis
Statistical analysis was done using the computer program (prism 5). The quantitative data were presented in the form of mean ± standard error (S.E) of mean. Statistical analysis of the difference between groups was performed by using One –way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

## Results

Serum levels of liver enzymes (AST and ALT) and TNF-α were significantly increased in group-II, but when treated with HDN (100 mg/kg/day) there was a significant decreased in activity of the two enzymes, The decrease in activities of the two enzymes was significant in rats treated with an increase HDN dose (200 mg/kg/day). Also hesperidin (100, 200 mg / kg/day) exerted a significant decrease TNF-α, Malondialdehyde level was increased significantly in rats treated with $CCl_4$ (group-II) and decreased significantly in rats treated with HDN (200mg/kg/day). Glutathione, Superoxide dismutase, and total antioxidant capacity were decreased significantly in group-II and increased significantly in rats treated with HDN (100, 200 mg/ kg/ day) (Table 1 and figure 2).

**Table 1. Showing comparison of results of biochemical tests among groups (I, II, III and IV).**

| Groups / Parameters (Mean±SE) | Group (I) Control negative No $CCL_4$ No HDN n = 8 | Group (II) Control positive $CCL_4$ NO HDN n = 8 | Group (III) HDN (100 mg/kg) n = 8 | Group (IV) HDN (200 mg/kg) n = 8 |
|---|---|---|---|---|
| **Malondialdehyde (nmol/gm tissue)** | 49.013 ± 1.03 | 82.763 ± 0.91 | 81.625 ± 0.68* | ##50.2 ± 0.38 |
| **Glutathione (mg/gm tissue)** | 5.088 ± 0.06 | 2.88 ± 0.048 | 3.09 ± 0.067* | ##5.025 ± 0.072 |
| **Superoxide dismutase (U/gm tissue)** | 107.888 ± 0.56 | 89.688 ± 0.45 | 90.863 ± 0.26* | ##107.013 ± 1.77 |
| **AST(Aspartate aminotransferase) (IU/L)** | 48.725 ± 0.47 | 163.875 ± 2.99 | #111.375 ± 1.78** | ##71.375 ± 1.71* |
| **ALT(Alanine aminotransferase) (IU/L)** | 38.5 ± 0.76 | 87.875 ± 1.46 | #57.375 ± 1.28** | ##46.5 ± 0.94* |
| **TNF-α (Tumor necrosis factor-α) Pg /ml** | 35.75 ± 1.816 | 131.417 ± 3.150 | #81.766 ±2.994** | #73.666 ±2.689** |
| **T-AOC (Total antioxidant capacity) µmol/l** | 116.050 ± 2.024 | 35.400 ± 1.864 | #56.566 ± 5.587** | ##70.166 ± 2.734** |

*means statistical significance at **P < 0.05** as compared to group (**I**); **n =** number of rats
#means statistical significance at **P < 0.05** as compared to group (**II**)
** means **P < 0.001** which indicates high significance as compared to group (**I**)
## means **P < 0.001** which indicates high significance as compared to group (**II**)
Both doses of HDN have prominent prevention of hepatic damage which was assessed microscopically, but this prevention is dose dependent (figures 5 and 6).

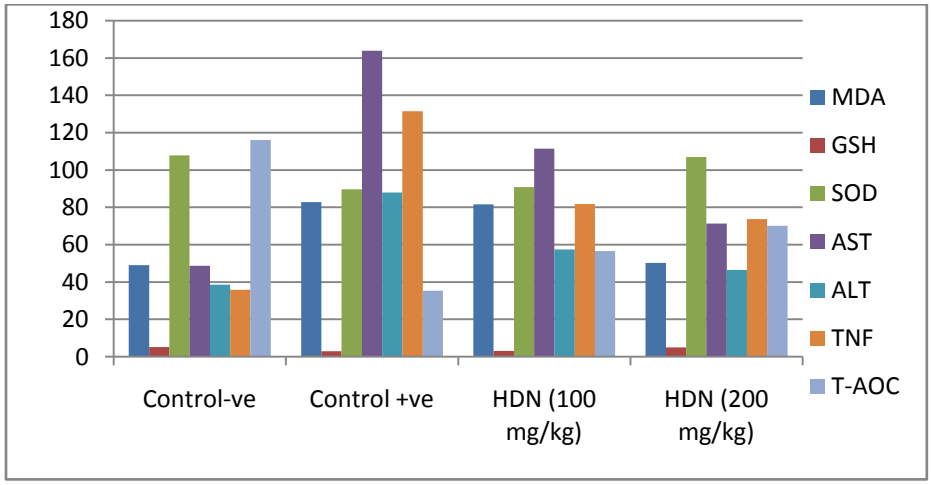


**Figure 2. Showing comparison of results of biochemical tests among groups (I, II, III and IV).**

## Histopathological results

Group (I) Control negative: were fed on 5% carboxymethyl cellulose (as a vehicle) only for 10 days & were injected by olive oil S.C. in the 8th day.

Normal (liver tissue, architecture, rows, cellular appearance and apparent nuclei) No "Inflammatory cell infiltrate" (Figure 3).

Group (II) Control positive: fed on 5% Carboxymethyl cellulose (as a vehicle) only for 10 days & were injected by $CCl_4$ in olive oil (2 ml/kg) SC in the 8th day. Extensive damage, very severe vasculitis, inflammatory cell, infiltration, disruption of the latticenature of hepatocytes, damaged hepatocyte cell membrane, irregular architecture (damaged sinusoids, rows and disintegrated central vein) and degenerated nuclei (Figure 4).

Group (III): Treated with HDN as 100 mg/kg in the vehicle for 10 days and were injected by $CCl_4$ in olive oil (2 ml/kg) SC in the 8th day. Vaculation occurs but less than Control positive group, more eosinophilic infiltration than Control positive group. (Figure 5).

Better viability and less damage than control positive group, nuclei are healthier than positive control group, less disruption of the lattice nature of hepatocytes and less damaged hepatocyte cell membrane, more regular architecture and rows than control positive.

Group (IV): Treated with HDN as 200 mg/kg in the vehicle for 10 days and were injected by $CCl_4$ in olive oil (2 ml/kg) SC in the 8thday. Faded vaculation (very mild), architecture and rows are so close to normal, normal viability, less infiltration by the inflammatory cells than treated groups by (HDN100), normal nuclei and cell membranes, normal central vein and sinusoids (Figure 6).

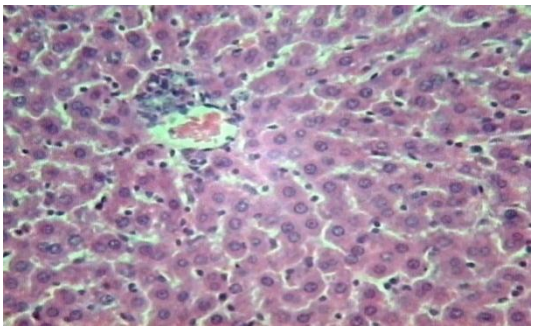

**Figure 3. Liver tissue of group (I: Control negative).**

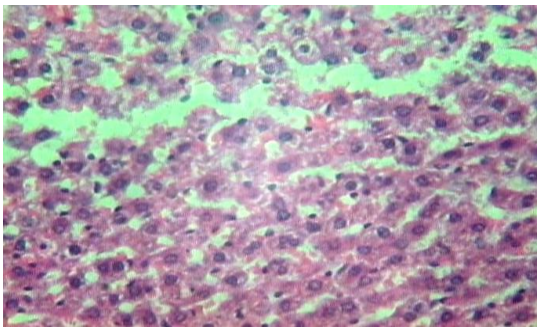

**Figure 4. Liver tissue of group (II: Control positive).**

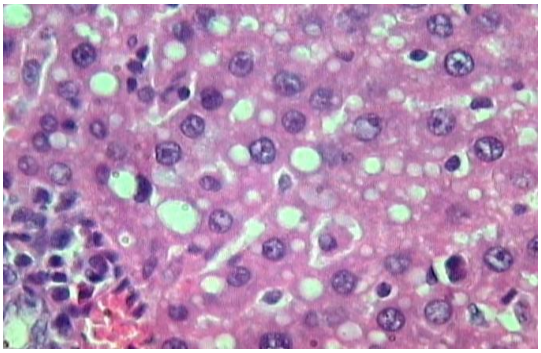

**Figure 5. Liver tissue of Group (III) treated with HDN (100 mg/kg).**

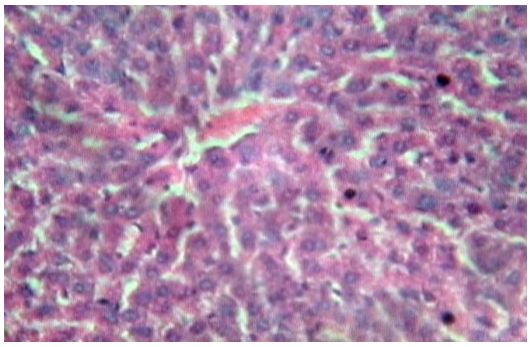

**Figure 6. Liver tissue of Group (IV) treated with HDN(200 mg/kg).**

## Discussion

Hepatotoxicity implies chemical-driven liver damage induced by certain medicinal agents and other chemical agents [15]. There are increasing evidences that free radicals and reactive oxygen species play a crucial role in the various steps that initiate and regulate the progression of liver diseases independently of the agent in its origin [16].

Oxidative stress in hepatotoxicity, resulting from increasing generation of reactive oxygen species (ROS) and other reactive intermediates as well as by decreased efficiency of antioxidant defenses, actively contributes to excessive tissue remodeling [17].

In the present study, induction of acute hepatic toxicity in Wister male albino rats was done by SC injection of $CCl_4$ 2 ml/kg (40% v/v in olive oil) characterized model for acute hepatic toxicity has been extensively performed and revealed microscopically in the liver as extensive damage, very severe vaculation, inflammatory cells infiltration, irregular architecture (damaged sinusoids, rows and disintegrated central vein) and degenerated nuclei [7].

The mechanism of hepatotoxicity undergoes two phases. The first resulted from its metabolic conversion to trichloromethyl free radical ($CCl_3^+$) by cytochrome P450 mainly (CYP2E1 and CYP2B1) which react very rapidly with oxygen to produce more reactive trichloro-

methylperoxy ($CCL_3OO$) free radical [18]. These free radicals attack microsomal lipids, DNA and proteins in the endoplasmic reticulum leading to initiating a chain of lipid peroxidation, cell necrosis and liver fibrosis [19]. $CCl_4$ not only initiates lipid peroxidation but also depletes tissue GSH and SOD [20].

In the present study, $CCl_4$ induces a severe hepatic damage as represented by markedly elevated levels of ALT, AST and TNF-α.

Usually, the extent of hepatic damage is assessed by increased level of cytoplasmic enzymes (ALT and AST). This was associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver [21].

$CCl_4$ induced a severe hepatic damage as represented by markedly elevated levels of ALT and AST coupled with a marked hepatic oxidative stress [6]. Injection of the rats with $CCl_4$ decreased the antioxidant capacity of liver as evidenced by the decreased GSH level and activities of SOD, and T-AOC [22]. The results of the present study have been showed that; subcutaneous injection of $CCl_4$ lead to increase Malondialdehyde (MDA) level. Free radicals causes generate lipid peroxidation of the living cell, MDA is one of final product of lipid peroxidation, so increase the level of MDA indicate occurring of oxidative stress [23].

Indeed, oxidative stress, presumably by favouring mitochondrial permeability transition, is able to promote hepatocyte death (necrotic and/or apoptotic). In some of clinically relevant conditions, generation of ROS within hepatocytes may represent a consequence of an altered metabolic state with ROS being generated mainly by mitochondrial electron transport chain or through the involvement of selected cytochrome P450 isoforms like (CYP2E1) [24].

The results of the present study showed that oral administration of HDN (100 mg/kg) and (200 mg/kg) significantly decrease the ALT and AST in $CCl_4$-treated rat and in the group of the dose (200 mg/kg) produces more decrease in ALT and AST. Also decrease in serum (TNF-α) level. But increase T-AOC [25]. The different flavonoid-rich extracts have the ability to decrease oxidative stress by promoting T-AOC [26-27].

Hesperidin shows a marked protective effect against inflammatory disorders, both in *vivo* and in *vitro,* possibly through a mechanism involving an inhibition of eicosanoid synthesis and/or antioxidant free radical scavenger activity [28].

The results of the present study showed that oral administration of HDN (100 mg/kg) causes insignificant decrease in MDA and insignificant increase in hepatic GSH and SOD levels.

Also showed that oral administration of HDN (200 mg/kg) causes significant decrease in MDA and significant increased hepatic GSH and SOD levels.

Hepatic MDA levels were also highly significantly increased in $CCl_4$ treated group, showing an increased oxidative stress compared to control group. The increased MDA level suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals [29]. Glutathione is an important intracellular antioxidant that also plays a role in the detoxification and elimination of potential carcinogens and toxins. Studies in animals have found that glutathione synthesis and tissue glutathione levels are significantly lower in aged animals than in younger animals, leading to decreased ability of aged animals to respond to oxidative stress or toxin exposure [30].

SOD catalyzes the destruction of the $O_2^-$ free radical ($2O^{2-} + 2H^+ \rightarrow O_2 + H_2O_2$). It protects oxygen-metabolizing cells against harmful effects of superoxide free-radicals [31]. $CCl_4$ challenge significantly decreased the levels of SOD and catalase in liver, by alteration in gene expression and depletion of SOD and catalase levels [32].

Antioxidants are agents that inhibit or neutralize potentially harmful elements known as free radicals [33, 34].

Flavonoids are naturally occurring polyphenolic compounds in plants that are thought to have positive effects on human health [35].

Hesperidin administration ameliorates the increased level of lipid peroxidation after $CCl_4$ treatment, able to show improvement in the levels of endogenous antioxidant enzymes SOD and improvement of hepatic GSH levels in HDN-treated rats in comparison to $CCl_4$ intoxicated rats, thereby, this demonstrates the antioxidant effect of HDN [6].

Flavonoids are known to operate via direct scavenging of ROS, chelation of redox active transition metal ions, inhibition of enzymes involved in ROS production, regeneration of endogenous antioxidants [33, 36].

The radical scavenging power of flavonoids is thought to be related to their structure. Flavonoids in general, scavenge oxidizing radicals preferentially via their B-ring catechol; in particular the ortho-dihydroxy structure in the B-ring gives a higher stability during the formation of aroxyl radicals and participation in electron dislocation. The presence of the 3' and 5' OH functions together give a maximum radical -scavenging potential; this property is found in Hesperidin [37-39].

The results of the present study showed that; oral administration of HDN (100 and 200 mg/kg) significantly improves hepatic architecture microscopically in a dose-dependent manner as the group of HDN administration (100 mg/kg) shows slight improvement while the group of HDN administration (200 mg/kg) show no difference with control normal group. Also can improve hepatic architecture in nicotine –treated rats [40].

## Conclusion

The present study suggested that the imbalance between production of oxygen free radicals and the endogenous antioxidant defense system, as a result of the effect of CCl4, so antioxidant properties of HDN might be the main factor responsible for its strong protective action on $CCl_4$-induced hepatotoxicity, through its ability to inhibit the lipid peroxidation and increase the activity of cellular antioxidant enzymes. Based on this study HND at these doses are safe and effective antioxidant also has cytoprotective property in dose depended manner.

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