Antioxidant Activity and Hepatoprotective Potential of Black Seed, Honey and Silymarin on Experimental Liver Injuries Induced by CCl₄ in Rats

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Abstract: The possible antioxidant activity and hepatoprotective potential of black seed honey and silymarin on CCl₄ induced liver injuries in rats was investigated. Fifty male rats were used in this study and divided into five groups, 10 rats each. Group 1 served as a control; group 2 injected 1 mL kg⁻¹ day⁻¹ CCl₄ intraperitoneally 3 times a week for 4 weeks, groups 3, 4 and 5 subjected to the same injection of CCl₄ and co-treatment with black seed, honey and silymarin (50 mg kg⁻¹ b.wt.), respectively, daily by stomach tube for 4 weeks. Blood and tissue samples were taken for biochemical and histopathological studies. The results revealed that CCl₄ administration caused significant elevations in the levels of MDA, NO, MMP-2, AST and ALT. Histopathological observations showed severe damage in the liver. Its fibrotic areas were measured using Image Analyzer. Combined treatment with CCl₄ and black seed, honey and silymarin showed marked improvement in antioxidant status and in histopathological findings as well as reductions in the fibrotic areas. These results concluded that black seed, honey and silymarin have protective characteristics against CCl₄-induced rat liver injury through potentiation of antioxidant capacity of liver cells and prevention of oxidative stress that accompanied with CCl₄ hepatotoxicity. The protective effect was higher in silymarin followed by black seed then honey.

Key words: Liver, antioxidants, black seed, honey, silymarin, CCl₄, rats

INTRODUCTION

Liver is the main organ involved in the metabolism of biological toxins and medicinal agents. Such metabolism always associated with the disturbance of hepatocyte biochemistry and generation of Reactive Oxygen Spices (ROS) (Fernandez-Checa and Kaplowitz, 2005). Lots of liver damages ranging from subclinical icteric hepatitis to necroinflammatory hepatitis, cirrhosis and carcinoma have been proved to associate with the redox imbalance and oxidative stress (Vrba and Modriansky, 2002). Therefore, a potential novel approach, namely developing antioxidant drugs to treat and protect liver injury and liver disease, has been proposed by Bansal et al. (2005). One of these drugs is silymarin, which was chosen in the present study in addition to black seed and honey.

Black seed (Nigella sativa) is an herbaceous plant known to have many properties in traditional medicine and used as a natural remedy for a variety of complications including liver diseases (El-Dakhakhny et al., 2002). Black seed with its active principle Nigellone found to have an antioxidant activity and may reduce the hepatotoxicity resulted from many insults (Mabrouk et al., 2002). It was reported as hepatoprotective agent against CCl₄-induced liver fibrosis (Turkdogan et al., 2001, 2003), possess antiviral effect in viral infected model (Salem and Hossain, 2000), have anticestode and antinematode action (Mahmoud et al., 2002), prevent lipid peroxidation through the decrease in MDA, increase in antioxidants, prevent liver damage (Ramadan et al., 2003) and have anti-inflammatory activity (Arifah et al., 2004).

Honey is one of honeybees' products which are used in medicine in many cultures since ancient times. Honey is the main source of concentrated sweetness in the diet of many people and contained about 80% carbohydrate, 20% water and traces of protein and ash (Mahdy and Morsy, 2001). Honey is known to exhibit a broad spectrum of activities including antiviral, antibacterial and immunostimulant (Molan, 2002; Mato et al., 2003). It was found to have antioxidant activity due to its high content of flavonoids (Mabrouk et al., 2004; Aljadi and Kamaruddin, 2004).

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Silymarin is among the drugs that are used in the treatment of hepatic dysfunction. Silymarin is a potential mixture of antioxidant flavonolignans, extracted from the seed of *Silybum marianum* (Shalan *et al.*, 2005). It is used as hepatoprotective agent against hepatic injury caused by many toxic substances such as CCl₄ (Muriel *et al.*, 2005), aflatoxin B₁ (Mekala *et al.*, 2006) and galactosamine (Dhanabal *et al.*, 2006).

Carbon tetrachloride (CCl₄) is widely used as hepatotoxic compound for screening the anti-hepatotoxic/hepatoprotective activity of drugs in experimental model systems, because CCl₄-induced hepatotoxicity is regarded as an analogue of liver injury caused by a variety of hepatotoxicity in man. It has been generally reported and accepted that CCl₄-induced hepatotoxicity due to its hepatotoxic metabolites and trichloromethyl free radicals (●CCl₃) induced lipid peroxidation (Basu, 2003; Lee *et al.*, 2005). Therefore, one of the therapeutic strategies against liver injury is to find antioxidant compounds that are able to block liver injury through scavenging of trichloromethyl free radical generated by CCl₄ (Lee *et al.*, 2005). Accordingly the present work was designed to elucidate the antioxidant activity and hepatoprotective potential of black seed, honey and silymarin (a standard drug for liver fibrosis) on experimental liver injuries induced by CCl₄ in rats to known the possible mechanisms(s) targeted by these natural antioxidants in hepatoprotection against CCl₄ toxicity.

**MATERIALS AND METHODS**

**Black seed, honey and silymarin used:** Black seed and honey were purchased from the local market at Cairo, Egypt. The black seed was washed, dried in sun and ground and then suspended in water before use. The honey used is a cotton flower honey, was diluted with water (1/4 V/V) before use. Silymarin was obtained from the pharmacy as 10 sachets (instant) produced by SEDICO pharmaceutical Co., 6 October City, Egypt. Each sachet contains 140 mg silymarin (calculated as silybin). It was prepared by dissolving the content of each sachet in water (50 mL) and administered immediately. All the treatments were given to rats by oral using stomach tube.

**Animals and diets:** Fifty male albino rats of the Sprague-Dawley strains, weighing 90-130 g each, were left under normal healthy conditions at the Animal House of the National Research Centre. Animal were fed on basal diets (Reeves *et al.*, 1993; NRC, 1995) and water was supplied ad libitum.

**Experimental design:** The animals were segregated into five groups each of 10 rats as follows:

- **Normal controls.**
- **CCl₄ intoxicated group:** Rats received 1 mL kg⁻¹ CCl₄ (10% v/v olive oil) intraperitoneally three times a week for 4 week.
- **CCl₄ and black seed supplemented group:** Rats treated with 1 mL kg⁻¹ CCl₄ and received orally 50 mg kg⁻¹ black seed daily for 4 week.
- **CCl₄ and honey supplemented group:** Rats treated with 1 mL kg⁻¹ CCl₄ and received orally 50 mg kg⁻¹ honey daily for 4 week.
- **CCl₄ and silymarin supplemented group:** Rats treated with 1 mL kg⁻¹ CCl₄ and received orally 50 mg kg⁻¹ silymarin daily for 4 week.

**Collection of blood samples:** At the end of the experiment, blood samples were collected after 16 h fasting using the orbital sinus technique of Sanford (1954). Blood samples were left to clot in clean dry test tubes and then centafugated at 3000 rpm for 10 min. The clear supernatant serum was then frozen at -20°C for the biochemical analysis.

**Biochemical methods:** Serum alpha Glutathione-S-Transferase (α-GST), was estimated by the enzymatic immunoassay method using kit produced by Biotrin International – Ireland, according to the method described by Meister (1985). Serum Malondialdehyde (MDA) was estimated by the enzymatic immunoassay method using kit of Oxis Research, Inc. USA, according to the method described by Liu *et al.* (1991). Nitric Oxide (NO) and Matrix Metalloprotease-2 (MMP-2) were estimated in serum by the immunoassay technique, using kit of R and D systems, Inc. USA, according to the method of Conner and Grisham (1995) and Parks and Mecham (1999), respectively. Serum liver function tests, AST and ALT were estimated using the kit of Sentinel-Italy, according to the method described by Reitman and Frankel (1957).

**Histological studies:** The liver of rats of different groups were removed and fixed in 10% formal saline, 5 μm thick paraffin sections were stained with haematoxylin and eosin (Drury and Wallington, 1980) and examined by light microscope. Quantitative measurement of fibrotic areas was achieved by using computerized image analysis (Leica Qwin 500 Image) in Image Analyzer Unit, Pathology.
Department, National Research Center. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Ten fields were chosen in each specimen and the mean values were obtained.

Statistical analysis: The data obtained in the present work are represented as average (mean) ± standard error. Statistical analysis was evaluated using the student t-test. p<0.05 were treated as statistically significant (Armitage, 1971).

RESULTS

Biochemical results: The results obtained indicated that, CCl4 treated rats exhibited significant increases in MDA, NO, MMP-2, AST and ALT levels in comparing with control group. The group of rats co-administered with CCl4 and black seed revealed significant increase in serum α-GST as compared with control group and significant decrease in the levels of MDA, NO, MMP-2, AST and ALT when compared with the group of rats treated with CCl4 only. The group of rats co-administered with CCl4 and honey exhibited significant increase in serum α-GST as compared with control group and significant decreases in the levels of MDA, AST and ALT when compared with the group of rats treated with CCl4 only. The co-administered rats with CCl4 and silymarin exhibited significant increase in serum α-GST as compared with control or CCl4 groups and significant decreases in the levels of serum MDA, NO, MMP-2, AST and ALT when compared with CCl4 treated group (Table 1).

Histological results: The liver of control rats revealed normal characteristic hepatic architecture as presented in (Fig. 1A). The treatment rats with CCl4 showed moderate fibrosis, massive vacuolar degeneration; minute fatty changes and many pyknotic nuclei was also found. The dilated blood sinusoids are filled with red blood cells (Fig. 1B, C). The liver of CCl4 administered rats that protected by black seed for 4 week showed some protective effects as compared to CCl4-administered group. Examination of liver sections showed moderate fibrosis and minute fatty change. Focal necrosis and some pyknotic nuclei could be noticed (Fig. 1D). The liver of CCl4 administered rats that protected by honey for 4 week showed more improvement in the pathological changes in the form of diminution of vacuolar degeneration and fibrosis as compared to CCl4-treated group. However, small haemorrhagic areas, many pyknotic nuclei and cellular infiltration were still present (Fig. 1E). The liver of CCl4 administered rats that protected by silymarin for 4 week showed some obvious pathological changes, but these changes were some what less than those of CCl4-administered group. Examination of liver sections showed that dilated blood sinusoids are filled with red blood cells, focal necrosis and hydropic degeneration. Large haemorrhagic areas were also showed (Fig. 1F).

Image analysis of liver fibrosis: Areas of liver fibrosis were assessed by hepatic morphometric analysis which has been considered as the gold standard for quantitative of fibrosis. Significant increase in the area of fibrosis was observed in the group of rats treated with CCl4 only as compared with control group. The liver of CCl4 administered rats that protected by black seed, honey and silymarin showed significant decrease (p<0.05) in the area of fibrosis as compared with CCl4 treated group (Table 2).

| Table 1: Serum levels of fibrosis markers and liver function tests in control, CCl4-exposed rats with or without black seed, honey and silymarin treatment for 4 week |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Serum levels   | Control group (n=10) | CCL treatment group (n=10) | CCL+ black seed treatment group (n=10) | CCL+ honey treatment group (n=10) | CCL+ silymarin treatment group (n=10) |
| α-GST (µg L⁻¹) | 9.11±0.46        | 10.16±0.60       | 11.34±0.82³     | 11.23±0.73¹     | 12.32±0.76⁶     |
| MDA (µg L⁻¹)   | 3.10±0.02        | 4.19±0.08²       | 3.17±0.01³      | 5.20±0.01¹      | 3.12±0.05⁵      |
| NO (µM L⁻¹)    | 61.80±2.37       | 101.50±3.52      | 84.40±3.20⁵     | 90.10±2.97⁶     | 88.90±3.75⁶     |
| MMP-2 (µg mL⁻¹)| 108.10±2.81      | 126.40±2.59      | 118.90±2.79⁴    | 125.40±2.59⁵    | 119.20±2.91⁶    |
| AST (µL⁻¹)     | 98.90±1.35       | 119.30±1.08      | 110.60±1.99⁴    | 113.60±1.16⁴    | 99.40±2.35⁵     |
| ALT (µL⁻¹)     | 65.60±1.47       | 94.80±0.92²      | 79.30±1.03⁴     | 85.10±1.52⁵     | 65.40±1.38⁵     |

*: Significant differences vs control group (p<0.05); ¹: Significant differences vs CCL4 group (p<0.05)

| Table 2: Mean values of fibrotic areas in liver of CCl4-exposed rats with or without black seed, honey and silymarin treatment for 4 weeks |
|----------------|----------------|----------------|----------------|----------------|
| Parameters     | CCL treatment group | CCL + black seed treatment group | CCL + honey treatment group | CCL + silymarin treatment group |
| Area (µm²)     | 518.37±44.46      | 203.10±40.68²   | 345.88±61.94²   | 171.78±17.70⁴  |
| Area fraction  | 0.28±0.025       | 0.10±0.02²      | 0.16±0.03³      | 0.10±0.01⁴     |
| Area (%)       | 28.90±2.49        | 9.11±1.83       | 15.69±2.71      | 10.11±1.15     |

*: Significant difference vs CCL4 group (p<0.05)
Fig. 1: Sections in the liver of (A) Control rat showing normal histological structure of hepatic lobules and central vein (CV), (B) Rat administered with CCl₄ for 4 weeks showed moderate fibrosis (arrow), (C) Rat administered with CCl₄ for 4 weeks showing massive vacuolar degeneration (arrow) and the dilated blood sinusoids are filled with red blood cells (double arrows), (D) Rat administered with CCl₄ and protected by Black seed showing pyknosis in some hepatocytes (arrow) and moderate fibrosis (double arrows) and minute vacuolar degeneration (arrowhead), (E) Rat administered with CCl₄ and protected by honey showing small haemorrhagic area (arrow) and cellular infiltration (double arrows). Many pyknotic nuclei could be noticed (arrow head) and (F) Rat administered with CCl₄ and protected by Silymarin showing dilated blood sinusoids are filled with red blood cells (arrow). Large haemorrhagic areas are noticed (double arrows) (Hx and E x 200)
DISCUSSION

In this study we investigated the possible protective effects of black seed, honey and silymarin on liver injury induced by CCl₄ to know the possible mechanism(s) targeted by these natural antioxidants in hepatoprotection and prevention of the oxidative stress that accompanied with hepatotoxicity. We focused on the biochemical changes elicited in the liver including antioxidants capacity and oxidative damage, in addition to the histopathological changes in liver cells.

The present results showed significant increase in the levels of serum MDA in CCl₄-administered group after 4 week. These results are in accordance with many reports that found increase level of MDA in hepatic cirrhosis induced by CCI₄ (Cabre et al., 2000; Cremonese et al., 2001) and in heptotomized cirrhotic rats (Andiran et al., 2003). The increase in MDA induced by CCI₄ has been explained by Socha et al. (1992), they postulated that the rise of MDA in liver disease may be attributed to the chronic pathology of the liver lead to disturbance in circulation and oxygenation which in turn cause lipid peroxidation and subsequently increase MDA concentration. Therefore, lipid peroxidation may cause severe damage and play a key role in pathogenesis of several human diseases. It was found that also the elevated MDA was found in serum with staging of fibrosis and in tissue mainly around periportal area (Mahmood et al., 2004).

In the present study, histopathological examinations of CCl₄-exposed rats for 4 week showed a massive vacuolar degeneration and micro-fatty changes. These results are in agreement with Turkdogan et al. (2003) who reported that the treatment of rats with CCl₄ caused hepatocellular necrosis, vacuolar degeneration and advanced fibrosis. Coinciding with Germano et al. (2001) and Al-Ghamdi (2003) whom reported that the administration of CCl₄ induced hepatic lesions including fatty change, ballooning infiltrate in the form of neutrophils and mononuclear cells. However, the pathological changes observed in liver of rats due to administration of CCl₄, may be attributed to lipid peroxidation and glutathione depletion (Meki and Hussein, 2001).

In the present study the treatment of rats with CCl₄, only showed significant increase in AST and ALT. These results are in parallel with the results of Lee et al. (2007) who reported that the treatment of rats with CCl₄ at a dose level of 0.15 mL kg⁻¹ b.wt. three time a week for 8 week showed high significant increase in AST and ALT levels and with the results of Ichi et al. (2007) who found that the treatment of rats with CCl₄, at dose level of 4 mL kg⁻¹ b.wt. showed severe elevation in plasma AST and ALT after 6 h of administration.

The present results showed significant increase in serum NO level in case of rats treated with CCl₄ alone. These results are in agreement with the previous reports that found elevation in NO level via Inducible Nitrile Oxide Synthetase (iNOS) in animal with advanced cirrhosis associated with endothelial dysfunction, portal hypertension and ascites after CCl₄ administration (Nelson and Eichinger, 2001). NO could protect the liver from lipid peroxidation by interacting with superoxide anion and other free radicals to produce less toxic species (Muriel, 1998). Also, NO was found to modulate pulmonary vasoreactivity observed in cirrhotic rats induced by CCl₄ (Nelson and Eichinger, 2001), inhibit Hepatic Satellite Cells (HSCs) proliferation after DimethylNitrosamine (DMN)-induced liver fibrosis (Svegliati-Baroni et al., 2001) and mediate the abnormalities associated with cirrhosis in rats induced by bile duct ligation (Ortiz et al., 2001). The elevated levels of MDA and NO and the insignificant changes of α-GST proved that one of the mechanisms involved in the process of liver fibrogenesis induced by CCl₄, is the imbalance between antioxidants and reactive oxygen species as well as the development of oxidative stress.

In the present histological examination, the treatment of rats with CCl₄ only showed moderate fibrosis in liver. Coinciding with the results of Luo et al. (2004) and Morsy et al. (2004) that reported that CCl₄, administration to rats for 8 week induced liver fibrosis. The liver exhibited a marked increase in the extracellular matrix content and displayed bundle of collagen surrounding the lobules, which resulted in a large fibrosis septa and distorted tissue architecture. The liver damage varied from one area to another and ranged from moderate fibrosis to cirrhosis.

In the present study, the treatment rats with CCl₄, caused significant increase in MMP-2 level. These results are in accordance with the previous reports that showed an increase in MMP-2 level during CCl₄-induced liver fibrosis in rats (McCudden and Iredale, 2000) and in Schistosoma Mansoni-induced liver fibrosis in mice (Vailiant et al., 2001). MMP-2 is a very important member of MMPs family. This enzyme was synthesized by activated HSCs and involved in degrading the native form of type IV collagen, the major component of the basement membrane (Yang et al., 2003).

The co-treatment of CCl₄-exposed rats with black seed showed significant improvement and decreased in the levels of MDA, NO, MMP-2, AST and ALT in
comparing with group of rats treated with CCl\textsubscript{4} only, while 
significant increase in serum α-GST level was obtained, as 
compared with control group. According to Murali \textit{et al.} 
(2001) black seed was found to prevent lipid peroxidation 
induced liver damage in experimentally diabetic rabbits 
assessed by decreased MDA and increase antioxidant 
defense system. Also it was found that the treatment of 
CCl\textsubscript{4} exposed rats with black seed was able to protect liver 
from damage by decrease MDA and increase GSH. 
Furthermore the histological examination, showed 
improvement in hepatocytes in the form of diminution of 
liver fibrosis and reduction in the fibrotic areas, as 
compared to CCl\textsubscript{4}-administered group. These results are in 
agreement with Kanter \textit{et al.} (2005) who reported that 
black seed prevent liver fibrosis induced by CCl\textsubscript{4} in 
experimental animals by decreasing lipid peroxidation, 
increasing antioxidants defense and enhancing liver 
enzymes. It was also reported that the treatment of rats 
with black seed might at least partly by successful in 
prevention of liver fibrosis in rabbit (Turkdogan \textit{et al.}, 
2001). Coinciding with Al-Ghamdi (2003) who reported that 
the treatment of rats with black seed along with CCl\textsubscript{4}, 
the comparative histopathological study of liver exhibited 
amost normal architecture. It was reported also that the 
rats treated with black seed along with CCl\textsubscript{4} showed 
non of the serious histopathological findings except for 
sparse coagulation necrosis in periaccinar regions 
(Turkdogan \textit{et al.}, 2003).

Concerning the hepatoprotective activity of honey 
against CCl\textsubscript{4}-induced liver fibrosis, it was observed that 
significant increase in serum α-GST level than control 
group and significant decreases in MDA, AST and ALT 
levels as compared to CCl\textsubscript{4}-administered group. It was 
found that honey reduce lipid peroxidation and nitric 
oxide and greatly improved liver enzymes and lipid profile 
in mice implicated with carcinoma cells referred to its 
anthioxidant activity (Antony \textit{et al.}, 2000). Honey have a 
hepatoprotective activity against methyl nitrosoourea 
(MNU)-induced oxidative stress and inflammatory 
response in rats by 100% via keeping normal defense 
system and decrease NO and MDA (Mabrouk \textit{et al.}, 2002, 
2004) and to have a hepatoprotective activity against 
CCl\textsubscript{4}-induced liver damage in sheep (Al-Waili, 2003) and 
in mice (Resende \textit{et al.}, 2003) by improving liver 
functions. Moreover, the histopathological examination 
showed that honey leads to some improvement in 
pathological changes in the form of diminution of fibrosis 
and vascular degenerations and reduction in the fibrotic 
areas, as compared to CCl\textsubscript{4}-treated group. These results 
are in agreement with Al-Waili (2003) who reported that 
the intravenous injection of honey had a hepatoprotective 
effect against CCl\textsubscript{4}-induced liver injury.

Regarding, the hepatoprotective effect of silymarin 
against CCl\textsubscript{4}-induced liver injuries in rats, It was found 
that silymarin leads to significant increase in serum α-GST 
level and prevent the elevation of MDA than control 
group and significant improvement and decreases were 
obtained in the levels of NO, MMP-2, AST and ALT. 
These results are in agreement with previous studies that 
obtained silymarin, significantly reduced lipid 
peroxidation, liver enzymes and increase glutathione 
content in rats (Murali \textit{et al.}, 2005) or in mice 
(Chungoo \textit{et al.}, 1996) exposed to CCl\textsubscript{4}. The protective 
effect of silymarin against CCl\textsubscript{4} induced lipid peroxidation 
in experimental animals have been explained by its free 
radical scavenger property that prevent lipid peroxidation 
and making cells more resistance to osmotic lyses 
(Halim \textit{et al.}, 1997). In addition silymarin has a potential 
antifibrotic property through inhibition of HSC's 
proliferation that is the central event of liver fibrosis 
(Shency \textit{et al.}, 2001). Furthermore, the present study 
showed that the treatment of rats with silymarin along 
with CCl\textsubscript{4} exhibited more improvement in pathological 
changes in the form of diminution of fibrosis and 
reduction in the fibrotic areas, as compared to CCl\textsubscript{4}- 
administered group. According to Jeong and co-author 
(Jeong \textit{et al.}, 2005) silymarin is well known as a protective 
agent against hepatotoxin. Silymarin has the ability to 
reduce the collagen content (Murali \textit{et al.}, 2005).

In conclusion, the present results demonstrate that 
black seed, honey and silymarin acted as potent 
protective agents against liver toxicity induced by CCl\textsubscript{4} in 
rats through potentiation of antioxidant capacity of liver 
cells by increasing α-GST level, prevention of NO, MDA 
and MMP-2 release, as well as improvement in liver 
functions and reduction in the fibrotic areas. The 
protective effect was higher in silymarin followed by black 
seed then honey.

REFERENCES


