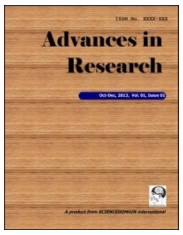

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Antioxidant Mediated Defense Role of the Dietary Phytochemical Ferulic Acid, against Carbon Tetrachloride (CCl4) Induced Toxic Hepatitis

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Authors' contributions

This work was carried out in collaboration between all authors. Author FKK designed the study, wrote the protocol, and wrote the first draft of the manuscript, managed the literature searches, analyses of the study performed the spectroscopy analysis and author WNE managed the experimental process .The two authors read and approved the final manuscript.

Research Article

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ABSTRACT

Ferulic acid (FA) is a dietary phytochemical formed during the metabolism of phenylalanine and tyrosine. Disruption of mitochondria and free radical mediated tissue injury has been reported during liver toxicity induced by carbon tetrachloride, potent hepatotoxic agent. The present study was designed to investigate the antioxidant protective effect of ferulic acid on hepatocytes, liver function and mitochondrial electron respiratory chain enzymes in hepatotoxic rats. Rats were classified into four groups : normal control group, hepatotoxic group subcutaneously injected with CCl4 once weekly for 4 weeks; third group: hepatotoxic group daily administered orally FA with a dose of 20 mg/Kg b.wt., and forth group: hepatotoxic group daily administered orally FA with a dose of 80 mg/Kg b.wt. The model of CCl_4-injected hepatocellular rats elicited declines in liver antioxidant enzyme activities; glutathione peroxidase, superoxide dismutase , Catalase in association of a reduction in reduced glutathione, serum total protein with concomitant

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significant elevations in lipid peroxidation marker , serum nitric oxide,and liver enzymes, lactate dehydrogenase and total bilirubin. CCl_4 resulted in a significant decrease in the activities of mitochondrial respiratory chain enzymes (NADH dehydrogenase and cytochrome *c* oxidase). FA enhanced the attenuation of these functional abnormalities and restored normal mitochondrial function when compared to rats CCl_4 toxicant treated groups , the attenuating effect was more pronounced in FA low dose Hence, these findings demonstrate the ameliorative role of FA on mitochondrial function during CCl_4 induced hepatotoxicity and associated oxidative stress in rats.

Keywords: Ferulic acid; Mitochondria; CCl4; Electron transport chain enzymes.

1. INTRODUCTION

Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) is a dietary phytochemical (Fig. 1) formed during the metabolism of phenylalanine and tyrosine. It occurs primarily in rice, wheat, barley, oat, roasted coffee, tomatoes, vegetables and citrus fruits [1,2]**.** FA functions as an integral component of cell walls, as it becomes covalently bound to polysaccharides. Increasing consumption of phenolics, including hydroxycinnamic acids, by diets that include fresh fruits, vegetables, and whole grains has been found to decrease the incidence of disease [3,4]. Therefore, greater understanding of the biological effects of dietary phenolic acids, and particularly FA, may help in the development of dietary recommendations designed to reduce risk of specific diseases [5,6].

Ferulic acid has been shown to potentially exert several beneficial effects on health. For example, it acted as a peroxyl radical scavenger and increased the resistance of LDL to oxidation and protected against some chronic diseases such as diabetes, Alzheimer's [7]**,** colon and breast cancers [8] and atherosclerosis [9].

The electron transport chain ETC, located in the inner mitochondrial membrane, comprises a series of electron carriers grouped into four enzyme complexes: complex I (NADH dehydrogenase), complex II (succinate ubiquinone reductase), complex III (ubiquinol cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) [10,11,12,13,14]**.**

Carbon tetrachloride (CCl_4) has been used in investigation of acute and chronic hepatic injury showing lesions similar to those seen in most cases of human liver disease. Hepatic injury caused by CCl_4 is characterized by acute responses coordinated by cross-talk between hepatocytes and nonparenchymal cells [15]**.**

With this background, the present study was designed to investigate the possible protective effect of supplementing different low and medium doses of ferulic acid on hepatotoxicity induced by the toxicant CCl_4. And, to determine the effect of ferulic acid (FA) on the mitochondrial dysfunction in rat hepatotoxic livers.

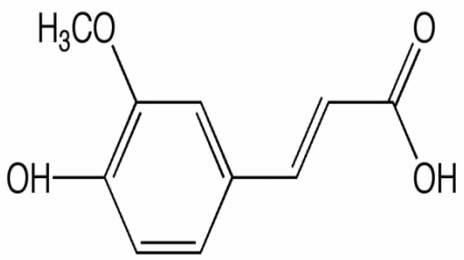

Fig. 1. Structure of Ferulic acid

2. MATERIALS AND METHODS

2.1 Chemicals

Ferulic acid FA was obtained from (Sigma-Aldrich Chemical Co. St. Lous, Mo, USA). Biochemical kits for serum analysis were purchased from Bio-Diagnostic Company for Chemicals, Dokki, Egypt. and Biochain Institute Inc.

2.2 Induction by Hepatotoxicity

Subcutaneous injection with carbon tetrachloride (CCl_4) (200 mg/kg.b.wt) once weekly for 4 weeks [16].

2.3 Rats and Diet

Forty male adult Albino rats supplied from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Helwan, Egypt), weighing 120–130g. They were acclimatized to animal house conditions, fed commercial pellet rat chow and water, *ad libitum*. Rats were randomly divided into 4 groups of 10 animals each. The groups were classified as follows:

G 1: Normal control rats fed on basal diet.
G 2: CCl_4 hepatotoxic group. (Positive control)
G3: hepatotoxic rats, orally administered low dose of FA at 20 mg/kg b.wt./day in (Dimethyl sulphoxide DMSO) for 30 days [17].
G 4: hepatotoxic rats, orally administered high dose of FA at 80 mg/kg b.wt./day for 30 days [18].

All studies were performed in accordance with the guidelines on regulation of scientific experiments on animals in Ain Shams University.

At the end of the study, rats were deprived from food overnight, anesthetized with diethyl ether and blood were collected by cardiac puncture. About 2-4 ml of blood was centrifuged at 5000 rpm for 15 minutes, and the serum was immediately removed from the cells. Livers were isolated, washed with cold saline and blotted dry with filter paper, and then kept for the biochemical analysis and mitochondrial extraction.

2.4 Marker Enzymes

To assess the membrane damage, activities of plasma lactate dehydrogenase [19] and alkaline phosphatase [20] were estimated by using reagent kits.

2.5 Serum Biochemical Determinations

In serum the following parameters were estimated: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [21]. Serum total protein, total and direct bilirubin [22,23]. Serum nitric oxide (NO) levels were measured as total nitrite according to method by [24]. Total nitrite is an index of endogenous nitric oxide production.To evaluate the oxidative stress in livers of the treated groups, one portion of liver was used to prepare 10% homogenate in 1.15% KCl and 5% homogenate in 3% sulfosalicylic acid, centrifuged at 4000 r.p.m at 4ºC for 20 min. and the supernatants were used to obtain the cytosolic fraction which was used for the assay of glutathione peroxidase (GPx) [25], glutathione reductase (GSH) [26], malondialdehyde (MDA) [27] and superoxide dismutase (SOD) [2]. Enzyme activity catalase (CAT) was determined [28].

2.6 Isolation of Mitochondrial Fraction of Liver Tissue

Another portion of liver was used for extraction of mitochondria [29] Fresh liver tissues were homogenized in 1:10 volumes of solution A (10 mM Tris, 0.2 M mannitol, 0.07 M sucrose, 0.1 mM EDTA).

2.6 Determination of the Liver Mitochondrial Respiratory Chain Enzymes

2.6.1 Activity of NADH dehydrogenase (complex I)

The mitochondria disrupted in 25 mM potassium phosphate buffer (pH 7.2). Vortex the cell pellet gently. Extract the cells with 100 µL of NADH Extraction Buffer by vortexing 2-3 times for 1 min each with same time intervals or by homogenization using standard techniques. spin the neutralized cell extract at 4000 rpm for 5 min at 4°C. Keep the tube of the cell extract for NADH measurement on ice. Data can be normalized and expressed as nmol NADH /mg of protein [30].

2.6.2 Activity of cytochrome c oxidase (Complex IV)

Determined according to Rustin et al. [31] by monitoring the oxidation of cytochrome c at 550 nm. 1ml reaction mixture contained 25mM KH_2PO_4 buffer (pH 7.2), 2 mg rotenone and 60 µM reduced cytochrome c, 0.05 mg dodecyl maltoside and 50 µL mitochondrial extract was added.

2.7 Statistical Analysis

Data analyses were performed using SPSS software version 14.0 for windows. All data were expressed as mean ± SD. Analysis of variance was used to test for differences between the groups.

3. RESULTS

Table 1 shows the changes in the activities of circulatory marker enzymes, LDH and ALP and NO in control and experimental rats. The activities of both these enzymes were significantly elevated in serum of CCl_4-treated rats when compared with control.

Table 1. Effect of the experimental treatments on the activities of serum LDH, ALP and NO content, ALT, AST, Total protein and Total bilirubin

	G1	G2	G3	G4
ALP (IU/L)	209±27.0	349±32.0	221±8.0	256±20
LDH (U/L)	112.4±0.82	149.41±2.3	134.74±2.7	141.5±2.1
NO (nmol/ml)	32.54±3.5	68.54±4.6	45.43±4.8	53.5±5.3
ALT (IU/L)	89.0 ± 5.3	168 ±19.5	100±9.2	123±12
AST (IU/L)	122±18.6	344 ±22.3	169±14	215±17.5
Total protein (mg/dl)	9.34±1.2	6.8±0.45	9.42±1.54	8.84±1.65
Total Bilirubin (mg/dl)	0.5±0.04	0.24±0.09	0.39±0.9	0.37±0.04

Values represent mean ± S.D.

Results obtained from Table 1 reported that, the levels of serum AST, ALT, and total bilirubin were markedly elevated and that of total protein decreased in CCl_4 treated animals, indicating liver damage.

Table 2. Effect of experimental treatments on hepatic GPx, GSH, SOD, CAT, and MDA

Groups Parameters	G1 control	G2	G3	G4
GPx (nmol/mg tissue)	48.65±1.82	24.28±2.13	42.73±3.1	31.6±2.1
SOD(nmol/mg tissue)	18.32±2.37	9.82±1.41	13.4±2.1	17.9±2.6
CAT(nmol/mg tissue)	62.15±8.5	29.2±4.9	61.87±7.9	53.76±5.6
GSH (nmol/mg tissue)	16.54±7.2	9.42±3.5	15.83±5.5	12.72±5.9
MDA(nmol/mg tissue)	0.391±0.0106	0.518±0.0102	0.411±0.018	0.450±0.065

Values represent mean ± S.D.

It was cleared from Table 2 that the oxidative stress caused by CCl_4 in the liver was assessed by measuring the levels of lipid peroxidation (LPO) product MDA, reduced glutathione (GSH) and the antioxidant defense enzymes GPx, GST, SOD and CAT.

Table 3. Effect of experimental treatments on the activities of complexes I and IV in mitochondrial fraction isolated from livers of rat

Groups Parameters	G1 control	G2	G3	G4
NADH dehydrogenase (nmol/ min/mg protein)	142.6±9.5	78.6±4.6	125.85±6.61	128.18±6.2
Cytochrome C oxidase (nmol/ min/mg protein)	0.369±0.032	0.205±0.021	0.264±0.027	0.261±0.027

Values represent mean ± S.D.

Results in Table 3 showed that, CCl_4 treatment resulted in a significant decrease in the activities of respiratory chain enzyme (NADH dehydrogenase and Cytochrome *c* oxidase). Oral administration of either low or high dose of FA significantly enhanced the attenuation of these functional mitochondrial abnormalities restoring their levels near normal when compared to CCl_4 group.

4. DISCUSSION

Acute liver diseases constitute a global concern, and medical treatments for these diseases are often difficult to manage and have limited efficacy. Therefore, there has been considerable interest in the role of complementary and alternative medicines for treatment of liver diseases**.** Development of therapeutically effective agents from natural products may reduce the risk of toxicity [32]**.**

In the present study, drastic alterations in the level of serum marker enzymes AST, ALT, ALP and LDH were noted, indicating CCl_4 mediated hepatic damages. CCl_4 is responsible for oxidative stress and lipid peroxidation through cytochrome P450-mediated generation of highly reactive radicals, leading to eventual damage characterized by hepatocellular necrosis [33,34,35] .These changes were significantly attenuated by FA, indicating that FA may have potential clinical application for treatment of liver diseases. These results are in accordance with the previous report by Srinivasan et al. [36,37].

The study showed that, CCl_4 toxicant reduced the serum total protein concentration, while FA supplementing increase the level. The phenolic groups enable FA to act as the natural antioxidant, and FA can bind to some proteins, modifying their structural properties and altering their biological activities [29]. In liver tissue, dietary treatments with 1% FA or FAEE did not induce GST specific activities. In contrast, oral administration of FA increased antioxidant enzyme activities in azoxymethane-exposed rat liver tissue [38].

Lipid peroxidation is a chemical mechanism capable of disrupting the structure and function of the biological membranes that occurs as a result of free radical attacking on lipids. When reactive oxygen species (ROS) begin to accumulate, hepatic cells exhibit a defensive mechanism by various antioxidant enzymes [39,40]. Results showed that, CCl4 treatment significantly elevated the lipid peroxidation as evident from the increased MDA level in the liver tissue. Antioxidant enzymes GPx, SOD and CAT were significantly reduced by CCl_4 intoxication, besides reduction of GSH. GSH is a co-factor for several detoxifying enzymes of oxidative stresses such as glutathione peroxidase and glutathione transferase and scavenges hydroxyl radicals and singlet oxygen species directly and detoxifying hydroperoxides and lipid peroxides [41]. GPx and CAT, which act as preventative antioxidants and SOD, a chain breaking antioxidant, play a vital role in protection against the deleterious effects of lipid peroxidation [42,43].

In the present study, FA exhibited protective effects by reducing CCl_4-mediated oxidative stress through decreased production of free radical derivatives, as evidenced by the decreased MDA level.

If the CAT and GSH activities are not sufficiently enhanced to metabolize hydrogen peroxide, this can lead to increased hydrogen peroxide and TBARS levels. It is noteworthy that, in mice supplemented with ferulic acid and clofibrate, the changes of anti-oxidant enzyme activities resulted in significant reduction of hepatic TBARS levels compared with the control group. As a result, enhanced anti-oxidant enzyme activities in the liver by ferulic

acid may play a protective role against ROS, thereby preventing the formation of lipid peroxidation. Thus, it seems reasonable that ferulic acid is effective in preventing hepatic damage [44].

Hiroe et al. [45] showed that the main function of FA is to protect cells against ischemic reperfusion injury by inhibiting the oxidative action *in vivo*. Dietary FA was previously found to be protective against CCl_4-induced toxicity in rat kidney and this effect was associated with increases in GPX and SOD levels [46,47]**.** Dietary FA also increased GSH in renal tissue of 20-month old male Sprague–Dawley rats [48]. A study by Ronchetti et al. [49] noticed prominent decrease of GSH levels in cancer cells treated with radiation plus FA. It is implicated that FA might act with inhibitors of de novo synthesis of GSH to sensitize tumor cells to radiation.

It possesses antioxidant property by virtue of its phenolic hydroxyl group in its structure. The hydroxyl and phenoxy groups of FA donate electrons to quench the free radicals. Thus, in our study by quenching the free radicals that are produced by nicotine, FA mightm have reduced the extent of lipid peroxidation, activities of marker enzymes, DNA damage, and restored the endogenous antioxidant status [50].

CCl_4 produces ROS that not only directly cause damage to tissues, but also initiate inflammation. Redox change activates Kupffer cell by the NADPH oxidase pathway or intracellular ROS-dependent kinase activation under pathological conditions [51,52]. NO is a highly reactive oxidant that is produced through iNOS, and it can augment oxidative stress by reacting with ROS and forming peroxynitrite [53]. Results of this study suggest that FA suppresses CCl_4-induced production of inflammatory mediators at the transcriptional level. Mitochondrial ROS is produced by electron leakage from ETC complexes during normal respiration particularly in complex I and III [54].

Based on the hypothesis that altered mitochondrial function might be involved in the hepatic encephalopathy, in the present work we evaluated the activities of mitochondrial respiratory chain complexes (NADH dehydrogenase and cytochrome c-oxidase) in the liver of rats submitted to acute administration of carbon tetrachloride. We also evaluated the effects of the administration of different doses of ferulic acid on these enzymes.

In normal conditions, mitochondrial ATP production is coupled with oxygen consumption [55]. Cytochrome *c* consists of a single polypeptide chain of 104 amino acid residues. This protein has three major functions, namely, electron transfer, apoptosis, and antioxidation. First, cytochrome *c* acts as an electron carrier transferring electrons from complexes III to IV in the respiratory chain of mitochondria. Secondly, cytochrome *c* plays a vital role in apoptosis, the programmed cell death, and during apoptosis, it is released from intermembrane space of mitochondria into the cytosol where it triggers the caspase-dependent machinery. Thirdly, cytochrome *c* plays an antioxidative role by acting on the generation and elimination of O_2 and H_2O_2 in mitochondria [56]. Decline in cytochrome *c* oxidase activity can cause an increase in H_2O_2 production. It may be speculated that decline in cytochrome *c* oxidase activity results in partial blockage of electron flow, which alters reducing potentials of some electron carriers favoring their autoxidation and consequent generation of O_2. NADH-dehydrogenase, a flavin-linked dehydrogenase, constitutes complex I of the electron transport chain, which passes electrons from NADH to coenzyme Q [57].

An increased lipid peroxidation has been reported to alter the lipid environment of the membrane thus affecting the activity of some respiratory chain enzymes like NADH dehydrogenase and cytochrome c-oxidase [58]. On the other hand, the enhanced lipid peroxidation can cause opening of mitochondrial membrane permeability transition (MPT) pores that attributes to cell death [59,60].

FA possesses distinct structural motifs that can also possibly contribute to the antioxidant property of this compound [61].

The next functionality is the carboxylic acid group in FA with adjacent unsaturated C=C double bond that can provide additional attack sites for free radicals and thus prevent them from attacking the membrane. In addition, the carboxylic acid group also acts as an anchor of FA by which it binds to the lipid bilayer providing some protection against lipid peroxidation. Thus the presence of electron donating substituents enhances the antioxidant properties of FA [16]**.** Despite the direct scavenging of ROS, FA can chelate the ferrous ion and decrease the formation of hydroxyl radical via inhibition of iron-dependent Fenton's reaction [22]**.** Hence, these findings demonstrate the synergistic ameliorative potential of FA on mitochondrial function under oxidative stress.

5. CONCLUSION

FA and synergistically extenuates the mitochondrial dysfunction via restoring the elevated electron transport chain enzymes during CCl_4 induced hepatotoxicity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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