Arctigenin attenuates CCl₄-induced hepatotoxicity through suppressing matrix metalloproteinase-2 and oxidative stress

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Abstract

Background: In spite of the huge advances in recent medicine, there is no effective drug that completely protects the liver from toxic materials. This study was conducted to investigate the hepatoprotective effect of arctigenin from burdock (Arctium lappa) against carbon tetrachloride (CCl₄)-induced liver injury.

Results: Arctigenin pre-administration reduced hepatotoxicity markers significantly as compared to CCl₄ group. In addition, both silymarin and arctigenin declined matrix metalloproteinase-2 (MMP-2) in the serum (1177 ± 176), (978 ± 135) significantly as compared to CCl₄ group (1734 ± 294). The hepatic antioxidant parameters (total glutathione, superoxide dismutase, and glutathione reductase) were significantly decreased after CCl₄ injection, an effect that has been prevented by pre-administration of both silymarin and arctigenin. Histological examinations illustrated that arctigenin reduced CCl₄ damage, where it decreased inflammation, congestion, and ballooning.

Conclusions: Arctigenin exerted a hepatoprotective effect against CCl₄-induced liver damage in terms of suppressing MMP-2 and oxidative stress comparative to that of silymarin.

Keywords: Arctigenin, CCl₄, Hepatoprotective, Matrix metalloproteinase-2, Silymarin

Background

An organ as complex as the liver can be susceptible to a variety of problems. However, in an unhealthy or malfunctioning liver, the outcomes can be dangerous or even fatal. Liver cirrhosis is one of liver serious problems; it is a frequent consequence of the long clinical course of all chronic liver diseases and is characterized by tissue fibrosis and the conversion of normal liver architecture into structurally abnormal nodules [1, 2].

Liver diseases were the 10th leading cause of death for men and the 12th for women in the USA, killing about 27,000 people each year. Also, the cost of liver diseases in terms of human suffering, hospital costs, and lost productivity is very high [3].

The extracellular matrix (ECM), formed by the complex network of proteins and sugars surrounding cells in all solid tissues, is among the most important regulators of cellular and tissue functions in the body [4]. In addition to providing structural support for cells, ECM regulates various cellular functions, such as adhesion, migration, differentiation, proliferation, and survival. Cellular responses are context-dependent, and dysregulation of ECM production and proteolysis is often associated with the development of liver pathology [5]. Matrix metalloproteinases (MMPs) are a family of over 24 zinc-dependent endopeptidases capable of degrading virtually any component of the ECM. MMPs have emerged as essential mediators in defining how cells interact with their surrounding micro-environment in normal liver [6].

Many plants have important roles in human health care. There are some plants that are consumed habitually by humans and that have been proven as
hepatoprotective capacity, for example, artichoke [7–9], milk thistle [10, 11], grapefruit, and chamomile [12, 13].

Arctigenin (AG) is an aglycon of arctiin [14]. It is a bioactive lignan isolated from the seeds of burdock (Arctium lappa) [15], acts as an antioxidant. The phenolic content of burdock is antioxidant too [16, 17]. As previous studies confirmed that Arctium lappa extract has hepatoprotective effect [18], there is no survey related to AG alone (which is one of the constituents of Arctium lappa), has the same effect, knowing that AG has an antioxidant [19], anti-inflammatory [20], and gastroprotective properties [21]. This study was aimed to investigate the hepatoprotective effect of AG on CCl4-induced liver toxicity in experimental rats, in terms of hepatic markers, MMP-2, oxidative stress, and histopathological changes.

Methods
This study was conducted in the Experimental Animal Laboratory of the Faculty of Pharmacy, Al-Ahliyya Amman University, and ethically approved by ethical committee for the care and use of laboratory animals (ethical approval no. AAU-1/14/2017-2018).

Chemicals, reagents, and kits
Arctigenin (Item No. 270652), glutathione (GSH) kit (Item No. 703002), superoxide dismutase (SOD) kit (Item No. 706002), and glutathione reductase kit (Item No. 703202) were purchased from (Cayman chemicals, USA). CCl4 (> 99.9%, Item No. 270652), carboxymethylcellulose (CMC) (Item No. C9481), and metphosphoric acid (MPA) (Item No. 79613) from Sigma Aldrich, USA. Total MMP-2 ELISA kit (Item No. MMP200) and lysis buffer (Item No. 895347) were supplied by RnD Systems, USA. Alanine aminotransferase (ALT) (Item No. 11533), aspartate aminotransferase (AST) (Item No. 11531), alkaline phosphatase (ALP) (Item No. 11592), and bilirubin (Item No. 11515) assay kits were purchased from BioSystems S.A., Barcelona (Spain). Silymarin (Legalon® 70 mg) was kindly provided by Chemical Industries Development CID, Egypt.

Animals
A total of 24 male Wistar rats (age 6-8 weeks, weight 210-240 g) were provided from the Jordanian University of Science and Technology (JUST), Irbid, Jordan. All animals were kept under observation in Al-Ahliyya Amman University animal house, for 2 weeks prior to the study with free access to commercial rat diet and water ad libitum. Rats were housed at 22 ± 2 °C with a 12 h light-dark cycle. All animals’ handling and treatment were in adherence to the ARRIVE guidelines.

Experimental design
The rats were randomly divided into 4 equal groups (n = 6 rats). Group A (control) and B (toxic), animals were administered the vehicle daily (1% CMC, 4 mL/kg, i.p.). Group C (standard), rats were daily administered silymarin (200 mg/kg, 4 mL/kg, i.p.) [22]. Group D (treatment), rats were daily administered AG (15 mg/kg, 4 mL/kg, i.p.) [19] (Fig. 1). All animals were treated for 6 weeks. The experimental design was approved by the ethical committee in Al-Ahliyya Amman University.

Induction of hepatotoxicity
A single dose of CCl4 (1 mL/kg, i.p.) was chosen according to Kandil et al. [23]. Diluted CCl4 solution was prepared by dissolving CCl4 in olive oil (1:1) to prevent its evaporation. On the last day of the designated period, animals were overnight fasted before the injection with diluted CCl4 (groups of B, C, and D) or olive oil (2 mL/kg, group A). One hour later, they were provided with food. On the next day, animals were fasted for 4 h, lightly anesthetized then sacrificed by cervical dislocation after taking the blood samples.

Blood samples
Twenty-four hours after CCl4 injection, blood samples were withdrawn by heparinized capillary tubes from a
retro-orbital vein under light anesthesia using a piece of cotton immersed in diethyl-ether [24], allowed to clot for 30 min, sera were separated by centrifugation at RCF 1000×g for 10 min. Four aliquots were prepared from each serum and stored at −20 °C until analysis.

Liver tissue specimens
After blood samples collection, animals were sacrificed by cervical dislocation, livers were taken using histological scissors, rinsed with cold saline, dried on a filter paper, and photographed. A portion of each liver was excised, put in 10% formalin solution, and processed as for routine histological evaluation. The remaining part of each liver was stored at −80 °C for later oxidative stress analyses [23].

Liver tissue homogenization
Around 50 mg sample was excised from each liver and homogenized in 1 ml of cell lysis buffer using Teflon homogenizer in ice. The lysate was then cold-centrifuged at RCF 10,000×g for 15 min at 4 °C. Supernatants were distributed into four Eppendorf tubes and stored at −80 °C to be analyzed later.

Histological investigation
Five-micrometer sections were stained with hematoxylin-eosin, examined using a light microscope (Leica), and photographed using MC 170 HD Leica Camera (Switzerland) and LAS EZ software. The histological sections were investigated by 2 of the authors in a blinded fashion.

Serum parameters
Serum ALT, AST, ALP, total bilirubin, and total MMP-2 were analyzed 24 h after induction of hepatotoxicity according to manufacturer instructions.

Oxidative stress
Hepatic total protein was determined in the tissue homogenate according to Lowry method [25], total GSH was assayed according to Eyer et al. [26] method. Briefly, the clear supernatant obtained from the homogenate was first deproteinized using 5% MPA then Ellman’s reagent (5,5′-dithiobis-2-nitrobenzoic acid) was added which is reduced by sulphydryl group of GSH to yield a yellow color with a maximum absorbance at 405-412 nm. The concentration was expressed as nM/mg tissue.

Superoxide dismutase activity was analyzed according to Spits and Oberley [27] utilizing a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Glutathione reductase activity was measured by measuring the rate of NADPH oxidation which is accompanied by a decrease in absorbance at 340 nm [28].

Statistical analyses
All descriptive statistics, analyses, and graphics were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, USA). Data passed the Shapiro-Wilk normality test and were expressed in tables as mean, standard deviation, and standard error of the mean. One-way analysis of variance (ANOVA) followed by Tukey-Kramer post-analysis procedure was used to compare the means of all groups. Differences between means were considered statistically significant at P ≤ 0.05.

Results
Hepatotoxicity markers
As shown in Table 1, a single injection of CCl₄ significantly increased all hepatotoxicity markers as compared to control group, an effect that was inhibited by pre-administration of both silymarin and AG.

Table 1 Hepatotoxicity markers in all studied groups

| Parameter       | Control | CCl₄ | Silymarin | Arctigenin |
|-----------------|---------|------|-----------|------------|
| ALT (U/L)       | 27.2 ± 8.67 | 920 ± 137**** | 644 ± 128****/*** | 658 ± 144***/**** |
| AST (U/L)       | 42.1 ± 9.41 | 1390 ± 262**** | 805 ± 194**** | 753 ± 185****/**** |
| ALP (U/L)       | 71.5 ± 26.4 | 169 ± 20.7**** | 106 ± 18.9**** | 119 ± 26.5**** |
| Total bilirubin (mg/dl) | 0.61 ± 0.18 | 1.82 ± 0.27**** | 1.03 ± 0.25**** | 1.11 ± 0.21**** |

a Significantly different from the control group
b Significantly different from CCl₄ group
P values: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001
Higher GSH levels in the hepatic tissues. However, AG and silymarin GSH levels were close to each other and lied between those of control and CCl₄.

Hepatic SOD activity (U/mg protein) of CCl₄ group was diminished significantly (1.65 ± 0.48) when compared to the control group (3.60 ± 0.25). AG injected-group showed SOD activity (2.34 ± 0.17) higher than that of CCl₄ but lower than the control group. Also, silymarin pretreatment induced SOD activity (2.71 ± 0.38).

Single CCl₄ injection lowered the glutathione reductase activity in the hepatic tissues (2.90 ± 1.35) significantly as compared to the control group (6.78 ± 1.55); this effect of CCl₄ was prevented by the administration of both silymarin (5.49 ± 1.46) and AG (4.59 ± 0.78).

**Histopathological results**

Figure 4a shows a section in control liver tissue which demonstrates normal histology; central vein and each
lobule are bordered by the “portal triad” consisting of a (branch of the hepatic artery, portal vein, and bile duct), in addition to hepatocytes surrounding it which are in sinusoids.

Ballooning (degeneration of hepatocytes) is obvious in CCl₄-injected group section (Fig. 4b). Cells are pale (lightly stained); parenchymal cells show necrotic and apoptotic alterations, in addition to an increase in the number of inflammatory cells (WBCs), and congestion (RBCs).

Figure 4c shows that silymarin repaired some of the damage which is caused by CCl₄ when they were injected respectively. Silymarin group section shows less inflammation and less ballooning. AG also protected the liver tissue from CCl₄ damage (Fig. 4d); it is obvious that ballooning, congestion, and inflammation were reduced.

Discussion
Due to its large size, exclusive structure, and essential roles in maintaining homeostasis, the liver is subjected to many types of diseases and toxic agents [1]. CCl₄ is commonly used for free radical-induced liver injury as many experimental and clinical studies consider it as a classical hepatotoxic agent that induces liver cirrhosis, fibrosis, and necrosis [29]. People may easily be exposed to it by inhalation or skin absorption due to its various usage such as in fire extinguisher and in refrigerant gas [30].

It is now generally accepted that CCl₄ toxicity results from bioactivation of CCl₄ into trichloromethyl free radical by cytochrome P₄₅₀ system in liver microsomes and consequently causes lipid peroxidation of membranes that leads to liver damage. The free radicals generated by CCl₄ metabolism attack polyunsaturated fatty acids in cell membranes forming (fatty acid) free radicals and induce lipid peroxidation with the production of reactive aldehydes, which lead to oxidative stress [31]. Lipid peroxidation causes cell membrane disruption leading to increased cell membrane permeability and enzyme leakage. This, in turn, activates cellular proteases, phospholipid, and protein degradation leading to cytotoxicity and inflammatory response [32]. Antioxidants and anti-inflammatory agents play a critical role against CCl₄ intoxication by scavenging active oxygen and free radicals and neutralizing lipid peroxides [33].

We have used CCl₄ rat model to investigate the heptoprotective effect of AG in terms of hepatotoxic markers, antioxidant activities, MMP-2, and histopathological outcomes. Male Wistar rats were the animals of choice due to their higher ability to withstand CCl₄-induced hepatotoxicity and to avoid any hormonal changes that may interfere with study outcomes when use females [23]. Silymarin (200 mg/kg) was used as a standard drug due to its reported hepatoprotective benefits [34].

Inflamed or injured hepatocytes leak high amounts of its contents including enzymes into the bloodstream;

![Fig. 4](https://example.com/fig4.jpg)

**Fig. 4** Histology of the liver from rats receiving only vehicle a, CCl₄ b, silymarin c, AG d (H & E stain). CV, central vein; ballooning degeneration (black arrow); infiltration of inflammatory cells (white arrow)
these enzymes are perfect indicators for diagnosis of hepaticellular damage [35].

After injection of CCl₄, hepatotoxicity parameters ALT, AST, ALP, and bilirubin, were significantly increased as compared to the control group. Results that are supported by many previous studies [36, 37]. In our study, previous silymarin and AG administration demonstrated decreased levels in ALT, AST, ALP, and bilirubin. The same results were obtained by Lee et al. and Talwar et al. [37, 38] when they tested silymarin efficacy and found that it suppressed CCl₄ damage and normalized hepatotoxicity markers due to its free radical scavenging effect.

According to oxidative stress parameters, there was a significant drop in GSH, SOD, and glutathione reductase in the liver of CCl₄-injected animals compared to the control group. This effect agrees with Abdel-Moneim et al.'s study, when they investigated CCl₄ efficacy on rat models too [36]. On the other hand, pretreatment with AG or silymarin expressed higher levels in these main liver antioxidant parameters. GSH, SOD, and glutathione reductase were normalized by silymarin and which could diminish oxidative stress produced by ethanol gavage in mice. It was able to enhance mitochondrial metabolic processes and electron transport chain, to increase intracellular SOD activity, which led to the drop of intracellular ROS levels to improve mitochondrial function [34].

Matrix metalloproteinase-2 is also an important indicator of liver impairment. Our results elucidated the upregulation of this enzyme in rats injected with CCl₄. Liang et al. proved this effect after the application of CCl₄ in rats too, owing that to the activation of hepatic stellate cells (HSCs) by CCl₄ which in turn increases MMP-2 expression. This enzyme increase resulted in hepatocytes matrix degradation, which ends in basement membrane destruction and initiating inflammatory cells to recruit to the injured site [39]. Feher and Lengyel found that silymarin has a beneficial effect on liver carcinogenesis explained by attenuating MMP-2 which is involved in invasion and angiogenesis [40].

When AG and silymarin were injected into rats in the current study, MMP-2 level was declined; findings that are supported by Kara et al. where silymarin lowered total MMP-2 activity knowing that total MMP-2 activity increases in hepatic decay [41]. In addition, Cichici et al. reported that administration of silymarin reduced inflammatory mediators including MMP-9 and liver fibrosis [42].

**Conclusion**

In conclusion, this study emphasized that AG played an obvious protective role from the harmful effect of CCl₄ on rats’ liver. In addition to that, AG protective efficacy with a dose of 15 mg/kg/day, is very close to that of silymarin 200 mg/kg in the term of hepatotoxicity markers, oxidative stress parameters, MMP-2, and histopathological observations.

**Abbreviations**

AG: Arctigenin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; ANOVA: Analysis of variance; AST: Aspartate aminotransferase; CCl₄: Carbon tetrachloride; CMC: Carboxymethylcellulose; ECM: Extracellular matrix; GSH: Glutathione; HSCs: Hepatic stellate cells; JUST: Jordan University of Science and Technology; MMP: Matrix metalloproteinase; MPA: Metphosphoric acid; NADP⁺: Nicotinamide adenine dinucleotide phosphate; RBCs: Red blood corpuscles; RCF: Relative centrifugal force; ROS: Reactive oxygen species; SD: Standard deviation; SE: Standard error; SOD: Superoxide dismutase; WBCs: White blood cells

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**Authors’ contributions**

GK performed the analytical methods. IA contributed in the proposal and manuscript writing. YK was a major contributor in performing the analytical methods, collecting and analyzing data, and writing the manuscript. All authors have read and approved the manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

This study was ethically approved by ethical committee for the care and use of laboratory animals at Al-Ahliyya Amman University (ethical approval no. AAU-1/14/2017-2018).

**Consent for publication**

Not applicable

**Competing interests**

The authors declare that they have no competing interests.

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