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# Acot1 and Cyp4a1 Genes are involved in Castor Leaf Extract-Induced Hepatoprotection of Rats

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ABSTRACT

Several plant families were used as medicines for treating various diseases. *Ricinus communis* (Castor) has antioxidant, anti-inflammatory, and hepatoprotective properties that substantiated its use for treating inflammation and liver disorders. In the present study, the hepatoprotective effects of the ethanol extract of Castor leaves against carbon tetrachloride ($CCl_4$)-induced liver injury was investigated in rats. The results indicated that an increase in the levels of liver alanine amino transferase, alkaline phosphatase, and lipid peroxidation, indicated by malondialdehyde activity, in the $CCl_4$-induced liver injury was significantly reduced in the rats pre-treated with Castor leaf extract. Also, the albumin, catalase, and superoxide dismutase levels were markedly increased in the rats pre-treated with Castor leaf extract compared to reduced levels in the $CCl_4$-injured rats. Based on quantitative real-time-PCR data, our results showed that the increase of acyl-CoA thioesterase-1 (Acot1) in the $CCl_4$-injured rats was reduced in the rats pre-treated with Castor leaf extract. However, the decrease of cytochrome P450-4A-1 (Cyp4a1) in the $CCl_4$-injured rats was alleviated by the pre-treatment of injured rats with Castor leaf extract. Compared to silymarin, the current results approve a hepatoprotective action for the ethanol extract of Castor leaves against $CCl_4$-induced liver injury and propose a role for Acot1 and Cyp4a1 gene expressions in the mechanism of hepatoprotection.

***Keywords*:** Carbon tetrachloride, *Ricinus*, Hepatoprotective, Acyl-CoA thioesterase-1, Cytochrome P450-4A-1.

## Introduction

Besides the vital and critical roles of the liver in many physiologic processes such as metabolism, secretion, and storage,[1] it serves in providing the body with nutrients and in detoxifying unwanted substances.[2] Liver injury is a severe health problem as it may develop into further complications including fibrosis, cirrhosis, and liver failure, or even cancers. Some viruses, poisons, alcohols, drugs, and chemical hepatotoxins may induce liver injuries.[3]

Hepatotoxic chemicals (e.g. aflatoxin, $CCl_4$, and chlorinated hydrocarbons) cause lipid peroxidation and generation of reactive oxidative intermediates in the liver.[4] Large levels of reactive oxygen species (ROS) and oxidative stress prompt cell death through necrosis and/or apoptosis, producing cellular and tissue injury.[5]

Antioxidants are used as a curative strategy to help in preventing and curing liver diseases involving oxidative stress,[5] but the drugs used to protect the damaged liver and improve hepatic cells regeneration are very few.[2] Numerous natural products and their bioactive components and medicinal herbs are widely used as alternative and complementary medicines. They have strong antioxidant, anti-inflammatory properties, and free radical scavenging abilities.[6] Various phytoconstituents isolated from several plants were found to have hepatoprotective activities.[7] These plants chemical ingredients such as essential oils, phenols, flavonoids, coumarins, lignans, carotenoids, and glycosides which contribute to the hepatoprotective effects of these plants.[8]

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Silymarin is the active ingredient extracted from the fruits of the Mediterranean herb *Silybum marianum*.[9] Silybin, the most bioactive component of silymarin, plays a fundamental role in protecting the hepatocytes' function and structure through scavenging free radicals, activating the genes associated with antioxidant activity, and regenerating hepatocytes to restore damaged tissue.[10]

*Ricinus communis L.* (Castor) is a shrub or small tree that spreads in tropical and semi-tropical regions[11] and it contains several phytoconstituents like steroids, saponins, alkaloids, flavonoids, and glycosides.[12] The leaves of *Ricinus communis* contain ricinine and N-demethylricinine (alkaloids), six flavones glycosides, and several phenolic compounds (quercetin, gentisic acid, gallic acid, rutin, epicatechin and ellagic acid).[13] *Ricinus communis* was reported to possess several biological and pharmaceutical activities including antimicrobial, antifungal, anticancer, antidiabetic, anti-inflammatory, antimalarial, antioxidant, anticonvulsant, antinociceptive, anti-arthritic, antidandruff and hepatoprotective abilities.[14]

In the cells, there is a class of enzymes called Acyl-CoA thioesterases (ACOTs) that are in charge of the hydrolysis of free acyl-CoA fatty acids into free fatty acids and CoA.[15] ACOTs are expressed in rat liver, brain, kidney, heart, and testis[16] and they are classified into 2 types according to the molecular weight of the enzyme. Cytochrome P 450 (CYP450) is another family of enzymes that are in charge of the metabolism of an enormous number of xenobiotic chemicals including drugs, pesticides, carcinogens, pollutants, and food toxicants plus endogenous compounds like steroids, prostaglandins, and bile acids.[17] CYP450 are predominantly expressed in the liver, lungs, kidneys, intestine, brain, and skin. CYP4A is a subfamily that mainly catalyzes the oxidation of steroids, fatty acids, leukotriene B4, and arachidonic acid.[18]

The current study aimed to (1) investigate the effect of Castor leaf extract against $CCl_4$-induced hepatotoxicity in rats by evaluating the common liver biochemical tests and some antioxidant parameters and (2) investigate whether the enzymes Acot1 and Cyp4a1 are involved in the mechanism of hepatoprotection.

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## Materials and Methods

*Animals*

Male albino Wistar rats (140 - 150 g) were housed in plastic boxes under controlled conditions in Faculty of science's animal house in Damietta University, Damietta Governorate. Blood was collected directly by withdrawal from the heart after laparotomy and the sera and plasma were prepared according to the standard protocols. All animals were kept, handled, and manipulated according to the guidelines of the National Institute of Health for the use of laboratory animals and the study was approved by the institute (No. 5/2/2/14).

*Plants*

The leaves of Castor (*Ricinus communis* L.) were collected from New Damietta region with the help of a plant ecologist. *Ricinus communis* specimens are stored in the herbarium of Botany and Microbiology Department, Damietta University, Egypt (Voucher # DAM010218). Plant leaves were washed with water, air dried, and ground in a blender. Extraction was performed by Solid-liquid method. The ground material was incubated in 95% ethanol for 3 days at RT, filtrated, dried for several hours in a rotary evaporator at no more than 40 °C. One gram extract was dissolved in 10 ml of DMSO and each rat received 250 mg extract/Kg b.wt.

*Experimental design*

This study included 7 groups of 7 rats each: Group (1) "untreated" was given basal diet and water. Group (2) "$CCl_4$" was given $CCl_4$ 1.5 mL/kg b.wt in olive oil once (i.p.) at the 5$^{th}$ day. Group (3) "Castor leaves+$CCl_4$" was given Castor leaf extract 250 mg/kg b.wt. for 5 days (p.o.). One hour after the last dose, rats were given $CCl_4$ 1.5 mL/kg b.wt. in olive oil (i.p.). Group (4) "Dimethyl sulfoxide" (DMSO) was given a volume of DMSO equivalent to the volume used in dissolving the extract for the same treatment period. Group (5) "Castor leaves" was given Castor leaves extract 250 mg/kg b.wt. in DMSO for 5 days. Group (6) "Silymarin+$CCl_4$" was given 70 mg/kg b.wt. for 5 days (p.o.) and $CCl_4$ 1.5 mL/kg b.wt. in olive oil (i.p.). Group (7) "Silymarin" was given Silymarin 70 mg/kg b.wt. for 5 days (p.o.).

*Biochemical assessment of $CCl_4$-induced hepatic injury*

After rat scarification, blood was collected from the heart, incubated at 37ºC for 30 min. and then centrifuged at 3000 rpm for 10 min. Important biochemical parameters including alanine amino transferase (ALT), alkaline phosphatase (ALP) and albumin were measured in the plasma using ALT/GPT 4+1 SL kit (ELITech, France), ALP (DEA) SL kit (EllTech, France), and Bromcresol green (Diamond diagnostics, Germany), respectively.

*Estimation of oxidative stress parameters*

Malondialdehyde (MDA) level was estimated in sera using MDA Kit (Bioiagnostic, Egypt). The catalase (CAT) level was estimated in plasma using Catalase kit (Biodiagnostic, Egypt). To estimate the superoxide dismutase (SOD) level, the liver of each rat was dissected out and liver homogenate was prepared in 0.1M potassium phosphate buffer (pH 7.4). The homogenate was then centrifuged at 4ºC at 3000 rpm for 10 min. and the supernatant was used to measure the SOD level using the respective kit (Bioiagnostic, Egypt).

*Quantitative real time PCR*

Easy Red solution (Intron, South Korea) was used to extract total RNA from the liver of 5 rats of each group following the manufacturer's guidelines. Purified RNA was converted into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher, USA) according to the provider's guidelines. Acot1 and Cyp4a1 gene expressions were evaluated by Quantitative Real Time PCR using Maxima SYBR Green PCR Master Mix (Thermo Scientific, USA). The target-specific primers used were Acot1 forward 5'-GGGAACACCATATGCTACAAG-3'; Acot1 reverse 5'-ATATAGTGCCCTGCTTCTGG-3'; Cyp4a1 forward 5'-ATTTCCCAAGTGCCTTTCCTC-3'; and Cyp4a1 reverse 5'-TCCATTCAGCAAGAGCAAACC-3'. The house-keeping gene primers used were GAPDH forward 5'-TGGCCTTCCGTGTTCCTAC-3' and GAPDH reverse 5'-GAGTTGCTGTTGAAGTGGC-3'. Aliquots of 25 µL-scale reactions were loaded on to CFX96 Real Time PCR detection system (Bio-Rad, USA) and cycled as follows: an initial activation step at 95 °C for 10 min. followed by 35 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 60°C for 1 min. The relative mRNA expressions of Acot1 and Cyp4a1 were calculated by the ΔΔCt method and the expression changes were calculated relative to the untreated control.

*Statistical analysis*

The statistical significance of the differences among groups for each tested parameter was evaluated by One-Way Analysis of Variance (ANOVA) followed by Tukey's HSD test. P values were considered significant if ≤ 0.05.

## Results and Discussion

*Liver biochemical and oxidative stress parameters*

The levels of ALT, ALP, and albumin were estimated in the plasma of different rat groups ("Untreated", "$CCl_4$-injured", "Castor leaf extract protected and $CCl_4$-Injured", "Castor leaf extract treated", "DMSO-treated", "Silymarin-protected and $CCl_4$-injured", and "Silymarin-treated") as biomarkers for liver functionality. There was no significant difference in the levels of ALT ($P$=1.00), ALP ($P$=0.061), and albumin ($P$=0.822) between the "Untreated" and "DMSO-treated" groups. In the "$CCl_4$-injured" group, the levels of ALT and ALP were significantly increased ($P$ = 0.0001) and the level of albumin was significantly decreased ($P \leq 0.0001$) when compared with the "Untreated" group (Figs. 1, 2, and 3). The disturbance in these physiological parameters indicated $CCl_4$-induced liver injury. While in the "Castor leaf extract protected and the $CCl_4$-injured" group, the levels of ALT and ALP were significantly reduced ($P \leq 0.0001$ for both) and albumin level was markedly increased compared to the "$CCl_4$-injured" group (Figures 1, 2, and 3). In the "Silymarin-protected and $CCl_4$-injured" group, the levels of ALT and ALP were significantly decreased ($P \leq 0.0001$ for both) and the albumin level was significantly increased ($P \leq 0.0001$) compared to the "$CCl_4$-injured" group (Figures 1, 2, and 3).

To evaluate the lipid peroxidation state, the hepatic MDA level was determined in the above-mentioned rat groups. There was no significant difference (P = 1.000) in the MDA level between the "Untreated" and "DMSO-treated" groups. The MDA level was significantly increased (P ≤ 0.0001) in the "CCl4-injured" group compared to the "untreated" group (Figure 4). In the "Castor leaf extract protected and CCl4-injured" and "Silymarin-protected and CCl4-injured" groups, the MDA level was significantly reduced (P ≤ 0.0001) compared to "CCl4-injured" group (Figure 4). Castor leaf extract alone slightly decreased the MDA level in "Castor leaf extract treated" group compared to the "DMSO-treated" one. The levels of the antioxidant activity markers, CAT and SOD, did not differ significantly (P = 0.850 and $P$ = 0.054), respectively, between the "untreated" and "DMSO-treated" groups (Figures 5 and 6). The activity of CAT and SOD was significantly reduced ($P \leq 0.05$ and $P \leq 0.0001$), respectively, in the "$CCl_4$-injured" group compared to the "Untreated" group. While in the "Castor leaf extract protected and $CCl_4$-injured" groups, the CAT and SOD levels were significantly increased ($P \leq 0.0001$ and $P \leq 0.05$, respectively), compared to the "$CCl_4$-injured" group (Figs. 5 and 6). A similar significant increase in both CAT and SOD levels was detected in the "Silymarin-protected and $CCl_4$-injured" group compared to the "$CCl_4$-injured" group (Figs. 5 and 6).

In the current study, liver injury was induced by administrating $CCl_4$ and the injury was evidenced by a significant increase in the ALT and ALP levels and a significant decrease in the albumin level. Also, $CCl_4$ administration caused excess ROS and lipid peroxidation as indicated by the significant increase in the MDA level in $CCl_4$-injured rats when compared with the untreated rats. On the other hand, the antioxidant

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activity decreased in $CCl_4$-injured rats as indicated by the significant decrease in CAT and SOD levels. The current results agree well with previous studies.[19-21] $CCl_4$ causes liver damage through the production of free radicals, ROS, and lipid peroxidation.[22] Liver damage affects the structural rigidity of the hepatic cell membrane causing leakage of the cytosolic enzymes into the blood circulation.[23] Therefore, measuring the level of the biochemical parameters of the liver (leakage enzymes, liver function deficiency, and cholestatic-induction parameters) is a clinical way to investigate the injury of the liver.[24] The elevated level of MDA is a result of the lipid peroxidation induced by $CCl_4$,[25] and the reduced levels of SOD and CAT are because of the excess production of free radicals and ROS that overcome the body's natural antioxidant defense mechanisms.[26]

In the current study, we confirm the hepatoprotective role of the ethanol extract of Castor leaves against $CCl_4$-induced liver injury. Pretreatment of rats with the ethanolic extract of Castor leaves significantly reduced the ALT and ALP levels and markedly increased the albumin level. Because of the antioxidant activity and ability to scavenge free radicals, the pre-treatment with ethanolic extract of Castor leaves significantly decreased MDA level and significantly elevated CAT and SOD levels. The methanolic and ethanol extract of castor leaves show significant anti-inflammatory properties, membrane stabilizing and antiperoxidative effects because of the presence of flavonoids.[12,27] Also these extracts exerts a hepatoprotective effect by inhibiting the increase in serum transaminases, alkaline phosphatase, and lipid peroxidation and improving the antioxidant mechanisms by elevating SOD level and catalase activity.[28, 29]

With respect to silymarin effect, our results are consistent with previous studies indicating the hepatoprotective action of silymarin against $CCl_4$-induced liver injury. The levels of liver enzymes (ALT and ALP), albumin, and oxidative stress markers (MDA, CAT and SOD) were significantly modulated into non-harmful levels in the rats pretreated with silymarin before $CCl_4$ injury. Our results are consistent with the previous studies in the ability of silymarin to return the ALT, AST, ALP and MDA levels into the normal range.[30,31] At the mRNA level, the expression levels of oxidative related genes; CAT, SOD, and glutathione peroxidase (GPx); were upregulated after administration of silymarin.[32]

*Acot1 and Cyp4a1 gene expressions*

To investigate whether $CCl_4$-induced liver injury and the alleviation of this injury by Castor leaf extract involve a change in Acot1 and Cyp4a1 gene expression, Acot1 and Cyp4a1 mRNA levels were evaluated by quantitative real-time PCR in the hepatocytes of 5 rats of each of the studied groups. Fold changes of Acot1 and Cyp4a1 mRNA levels normalized to GAPDH were calculated by ΔΔCt method (Figures 7 and 8).

With respect to Acot1 gene expression (Fig. 7), Acot1 mRNA level increased 3.4 folds in the "$CCl_4$-injured" group compared to the "Untreated" group. The increase in Acot1 expression was ameliorated in the "Castor leaf extract protected and $CCl_4$-injured" group to 1.7 folds. Compared to the "Untreated" group, DMSO slightly increased Acot1 expression (0.8 folds). Compared to DMSO, Castor leaf extract alone ameliorated the increase in Acot1 mRNA level to 0.6 folds. In "Silymarin-protected and $CCl_4$-injured" group, the increase in Acot1 gene expression was reduced to 1.3 folds only. Compared to the "Untreated" group, Silymarin only increased Acot1 expression slightly (0.8 folds). In the current study, Acot1 gene expression was significantly increased in the CCl4-injured rats and the Castor leaf extract ameliorated the increase in mRNA level ~50% in the $CCl_4$-injured rats. In the non-injured rats, Castor leaf extract itself slightly decreased Acot1 mRNA level. Protection with Silymarin had a similar effect to Castor leaf extract as it reduced Acot1 gene expression ~70% in $CCl_4$-injured rats.

Acot1 was reported to be involved in fatty acids (FA) trafficking during periods of increased hepatic FA influx and oxidation,[33] and its upregulation was supposed to maintain the rate of FA oxidation.[34] According to Franklin et al., Acot1 knockdown increases oxidative stress and inflammation and probably leads to fibrosis in response to high-fat feeding.[35] In mice with diabetic cardiomyopathy, ACOT1 was overexpressed in cardiomyocytes to reduce FA oxidation and ROS production.[36] In the current study, Acot1 gene expression increased 3.4 times in the $CCl_4$-injured group compared with the untreated group. This increase in Acot1 mRNA level may be a way to overcome and to protect the liver from ROS produced as a result of $CCl_4$ administration and its metabolism. Because of the antioxidant activity of *Ricinus communis* and its ability to scavenge different radicals (2, 2-dipehyl-1-picrylhydrazyl (DPPH), nitric oxide (NO) and superoxide radicals),[37,38] the production of ROS was attenuated. This may explain the 50% decline in Acot1 mRNA level in the rats protected with Castor leaf extract before the administration of $CCl_4$.

Silymarin showed a protective and antioxidant activities and a role in decreasing the ROS production.[39] Similar to the effect of Castor leaf extract, Silymarin also caused a 70% decrease in Acot1 mRNA level. The decrease in the Acot1 expression in rats protected with Castor leaf extract or with Silymarin approves their antioxidant activity against $CCl_4$-induced liver injury.

Concerning Cyp4a1 gene expression (Fig. 8), Cyp4a1 mRNA level decreased 2.5 times in the "$CCl_4$-injured" group compared to the "Untreated" group. The decrease in Cyp4a1 expression was ameliorated in the "Castor leaf extract protected and $CCl_4$-injured" groups to 0.8 folds. Compared to the "Untreated" group, DMSO decreased Cyp4a1 expression 1.7 folds. Compared to DMSO, Castor leaf extract alone ameliorated the decrease in Cyp4a1 mRNA level to 0.8 folds. In "Silymarin-protected and $CCl_4$-injured" group, the decrease in the Cyp4a1 mRNA level was alleviated to 0.7 folds. Compared to the "Untreated" group, Silymarin increased Cyp4a1 expression to 1.6 folds. In the current results, Cyp4a1 mRNA level decreased significantly in the $CCl_4$-injured rats. This decrease was ameliorated ~70% in the Castor leaves-protected-$CCl_4$-injured rats. In the non-injured rats, Castor leaf extract alone caused a slight increase in Cyp4a1 gene expression. Similar to the effect of Castor leaf extract, protection with Silymarin increased Cyp4a1 mRNA level ~70% in $CCl_4$-injured rats.

Cyp4a1 is a member of the CYP4A subfamily (Family: CYP450) that catalyzes the ω-hydroxylation of fatty acids[40] which is considered a minor path way (4-15%) in the metabolism of fatty acids but very important in susceptibility of human to genetic, environmental, and metabolic diseases associated with lipid metabolism.[41] In treated rats with $CCl_4$, the hepatic protein level of CYP1A2, CYP2B1, CYP2C6, CYP2E1 and CYP3A2 was reduced. This was attributed to the destruction of these enzymes during their role in $CCl_4$ metabolism.[42] The results of the current study agree with these finding as the mRNA expression level of CYP4A1 was reduced 2.5 folds in the rats injured with $CCl_4$ compared to the untreated ones.

Different cytokines (IL-6, INF-α, INF-γ, and TNF-α) are thought to mediate the inflammatory effects on the regulation of CYP450 family.[43] IL-6 and IL-1β have been reported to decrease the CYP4A1 apoprotein content and CYP4A1 mRNA level.[44] $CCl_4$ induces the activation of the immune system and induces several immunological reactions and the interleukins (IL-1, IL-6 and IL-10), TNF-α, prostaglandins and various cytokines are released from Kupffer cells.[45] These results may support the decrease in mRNA expression level of Cyp4A1 in the $CCl_4$-injured rats in the current study. The anti-inflammatory ability of *Ricinus communis* could have contributed to the rise of mRNA expression level of Cyp4A1 to protect the liver from hepatotoxicity in the current study. Silymarin significantly inhibited the production of the inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10) associated with oxidative stress, decreases established fibrosis, and inhibits downstream effects of IL-13 on fibrogenesis.[46, 47] The mechanism envisaged above for the role of Castor leaves in rising Cyp4a1 mRNA level explains how could silymarin, in the current study, increase the mRNA expression level of Cyp4A1 and ameliorate its decrease in the $CCl_4$-injured rats pretreated with silymarin.

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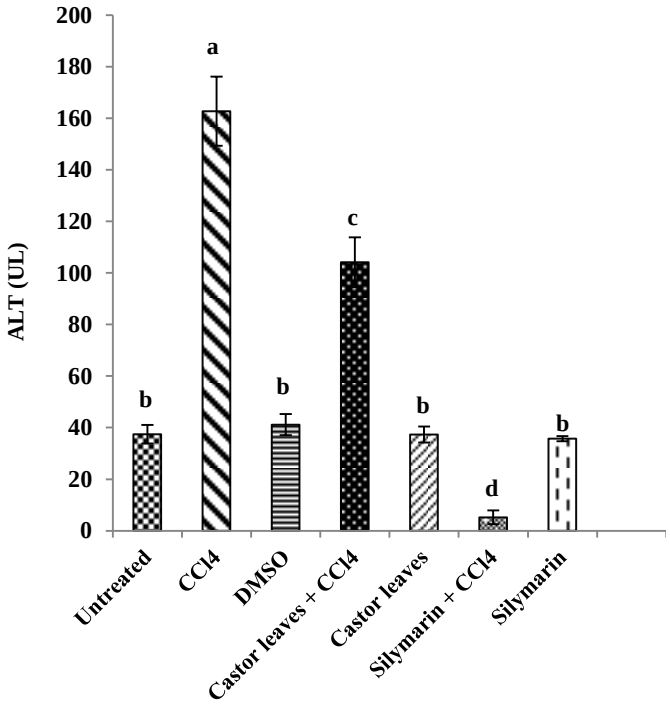


**Figure 1:** ALT levels in the untreated and differently treated rat groups.
Standard errors are indicated on the bars. There is no significant difference between the values with same letter. Values with different letters are significantly different.

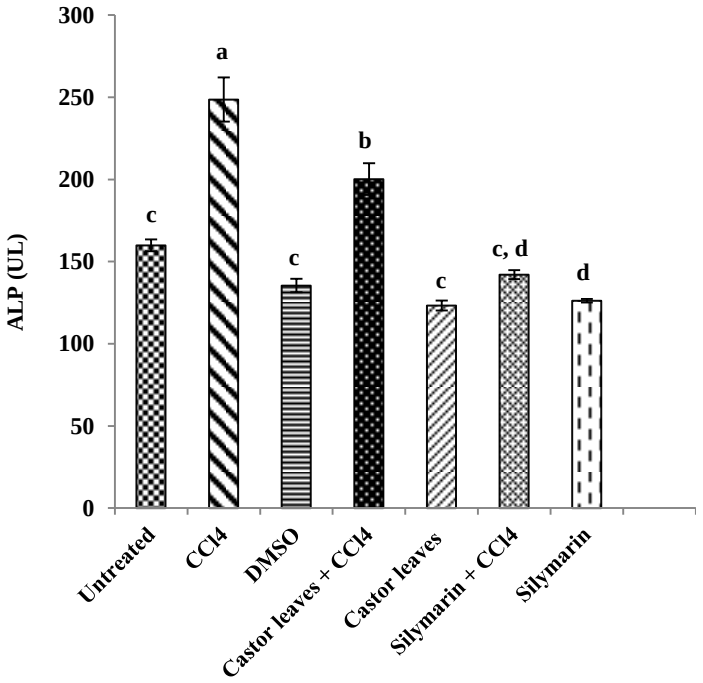


**Figure 2:** ALP levels in the untreated and differently treated rat groups.
Standard errors are indicated on the bars. There is no significant difference between the values with same letter. Values with different letters are significantly different.

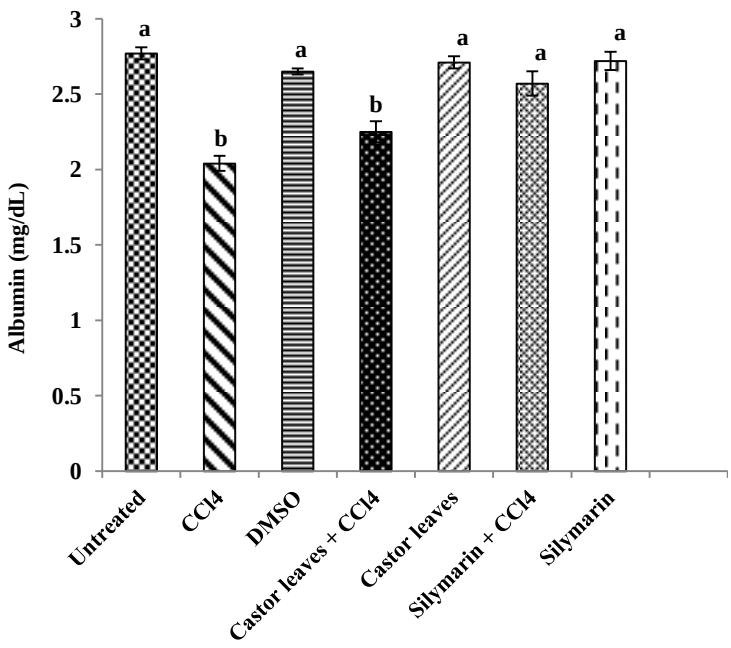


**Figure 3:** Albumin levels in the untreated and differently treated rat groups.
Standard errors are indicated on the bars. There is no significant difference between the values with same letter. Values with different letters are significantly different.

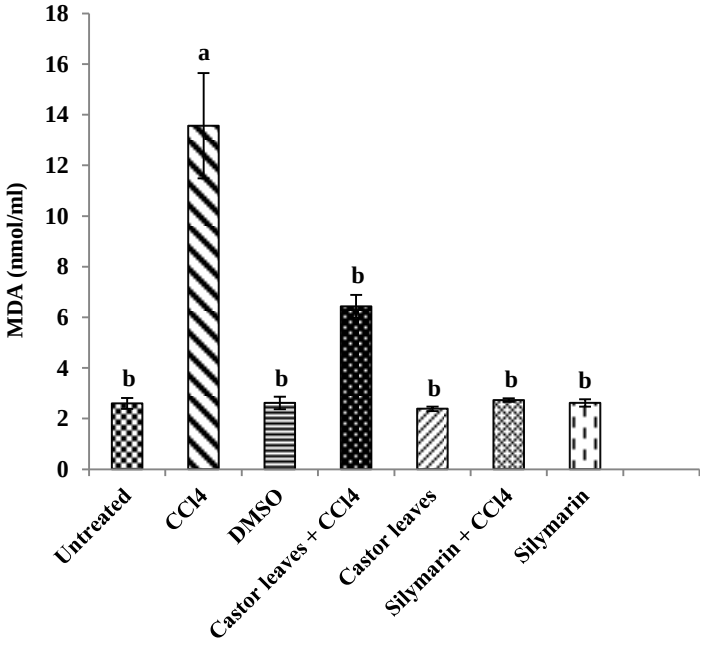


**Figure 4:** MDA levels in the untreated and differently treated rat groups.
Standard errors are indicated on the bars. There is no significant difference between the values with same letter. Values with different letters are significantly different.

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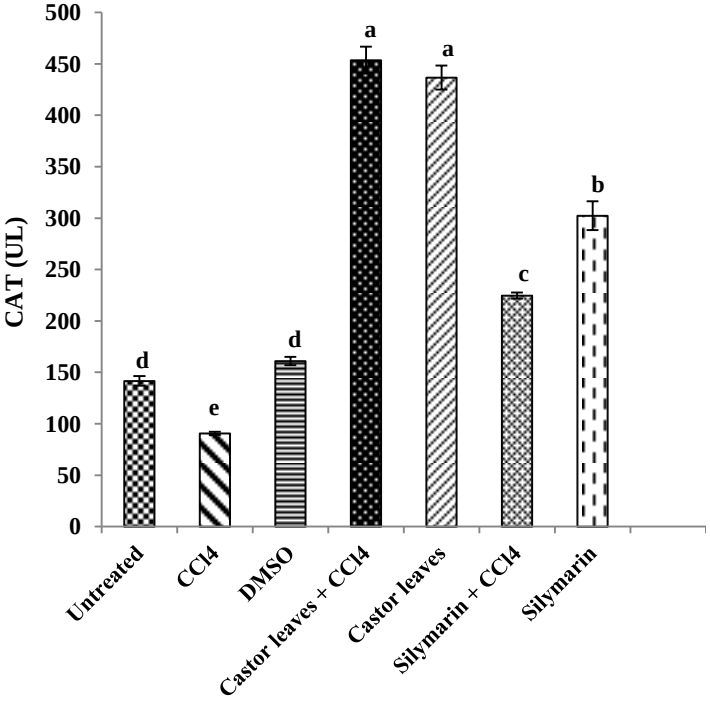


**Figure 5:** CAT levels in the untreated and differently treated rat groups.
Standard errors are indicated on the bars. There is no significant difference between the values with same letter. Values with different letters are significantly different.

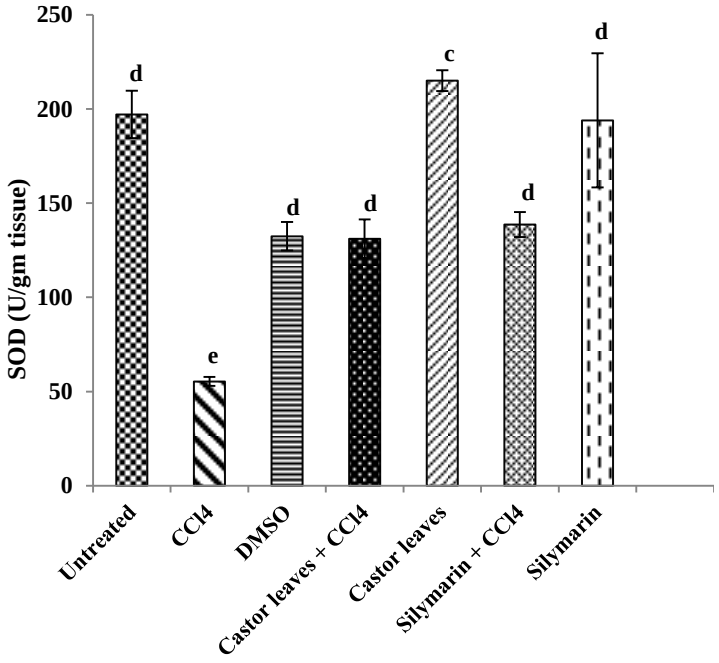


**Figure 6:** SOD levels in the untreated and differently treated rat groups.
Standard errors are indicated on the bars. There is no significant difference between the values with same letter. Values with different letters are significantly different.

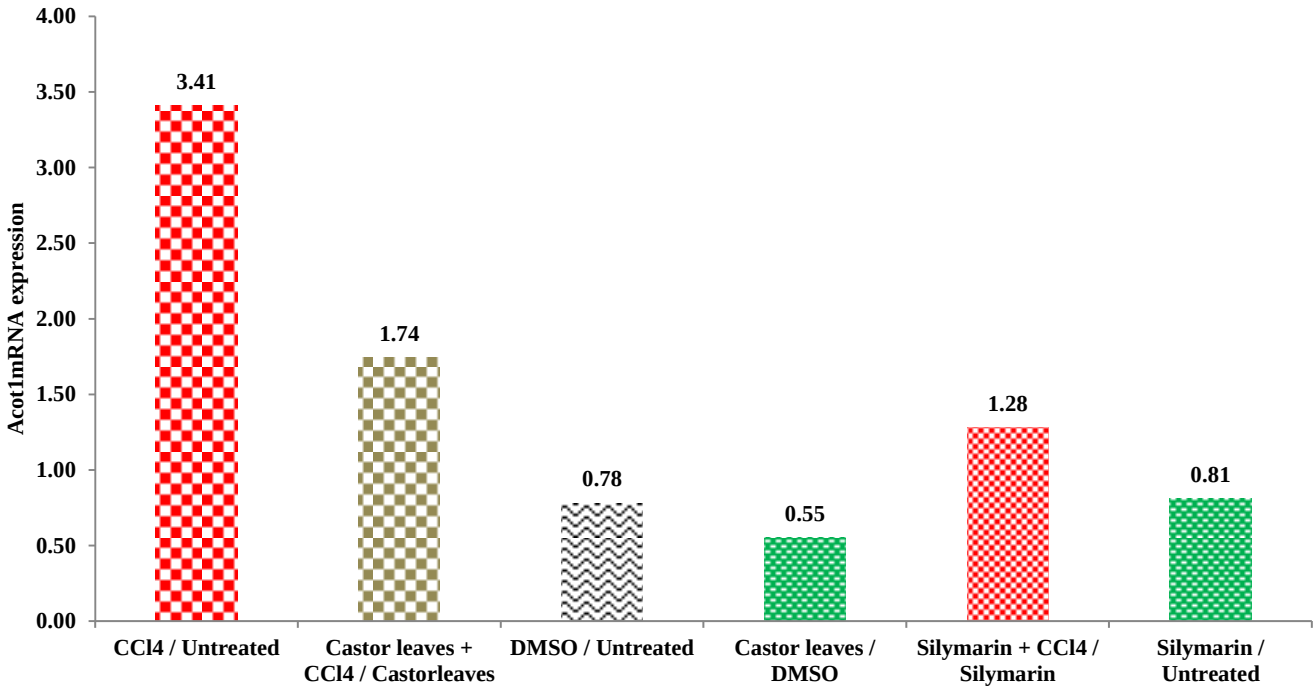


**Figure 7:** Expression fold change of Acot1 mRNA in different rat groups: "$CCl_4$-injured" rats compared to "Untreated" rats, "Castor leaves-protected and $CCl_4$-injured" rats compared to "Castor leaves-treated" rats, "DMSO-treated" compared to "Untreated" rats, "Castor leaves-treated" rats compared to "DMSO-treated" rats, "Silymarin-protected and CCl4-injured" rats compared to "Silymarin-treated" rats, and "Silymarin-treated" compared to "Untreated" rats.

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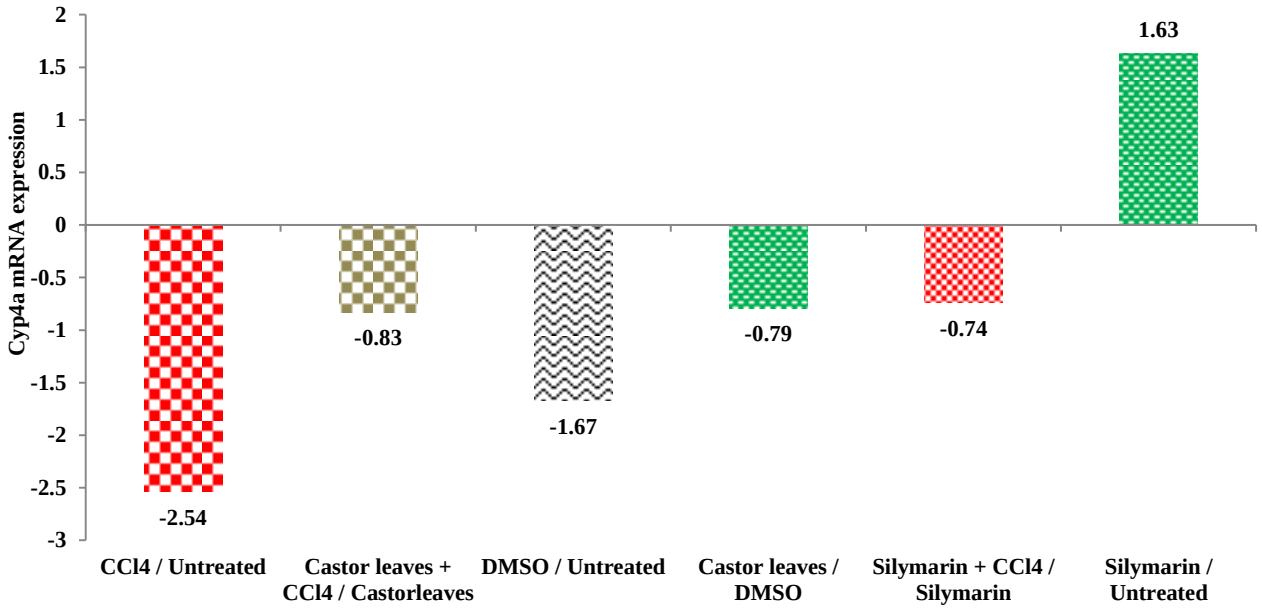


**Figure 8:** Expression fold change of Cyp4a1 mRNA in different rat groups: "$CCl_4$-injured" rats compared to "Untreated" rats, "Castor leaves-protected and $CCl_4$-injured" rats compared to "Castor leaves -treated" rats, "Castor leaves-protected and $CCl_4$-injured" rats compared to "Castor leaves-treated" rats, "DMSO-treated" compared to "Untreated" rats, "Castor leaves-treated" rats compared to "DMSO-treated" rats, "Silymarin-protected and CCl4-injured" rats compared to "Silymarin-treated" rats, and "Silymarin-treated" compared to "Untreated" rats.

## Conclusion

Castor leaf ethanol extract exerted a hepatoprotective activity against $CCl_4$-induced liver injury in rats. Hepatoprotection was indicated by the significant change in the levels of the important liver enzymes and other function parameters, including ALT, ALP, albumin, MDA, CAT, and SOD levels towards the normal values. Pre-treatment of rats with Castor leaf ethanol extract decreased ACOT1 expression level and increased the CYP4A1 expression levels indicating potential roles for these genes in the mechanism of hepatoprotection.

## Conflict of interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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