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## Hepatoprotective mechanism of lycorine against carbon tetrachloride induced toxicity in swiss albino mice – A proteomic approach

Soundarrajan Ilavenil<sup>1,2</sup>, Dhanaraj Karthik<sup>2</sup>, Mariadhas Valan Arasu<sup>3</sup>, Mayakrishnan Vijayakumar<sup>1</sup>, Srisesharam Srigopalram<sup>4</sup>, Selvaraj Arokiyaraj<sup>5</sup>, Sivanesan Ravikumar<sup>2\*</sup>, Ki Choon Choi<sup>1\*</sup>

<sup>1</sup>Grassland and Forage division, National Institute of Animal Science, South Korea

<sup>2</sup>Department of Biotechnology, PRIST University, Vallam, Thanjavur, Tamilnadu, India

<sup>3</sup>Department of Botany and Microbiology, King Saud University, Saudi Arabia

<sup>4</sup>Department of Horticulture, Sunchon National University, South Korea

<sup>5</sup>Institute of Green Bio-Science and Technology, Seoul National University, South Korea

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### ABSTRACT

**Objective:** To investigate the differential of protein expression in CCl<sub>4</sub> induced mice treated with lycorine. **Methods:** The present study was carried out to identify the differentially expressed protein in carbon tetrachloride (CCl<sub>4</sub>) induced oxidative stress mice treated with lycorine (5 mg/kg. bw) using 2D gel and MALDI-TOF. **Results:** We observed many kinds of differentially expressed protein in experimental liver. Among these, three are much differently expressed protein which is identified as ATP synthase, regucalcin and HSP60; these proteins are involved in the ATP synthesis, calcium regulation and rescue the integrity cellular proteins respectively. **Conclusion:** This investigation provided a molecular mechanism of the lycorine during CCl<sub>4</sub> induced oxidative stress in mice liver.

## 1. Introduction

The liver is the main metabolic organ which metabolizes the toxic substance via xenobiotic metabolism and it's very sensitive to free radical related diseases, hepatitis, cirrhosis, liver cancer and other alcohol related disorders. A major cause of liver disorder is due to exposure of different environmental pollutants and xenobiotics such as Paracetamol, CCl<sub>4</sub> thioacetamide and alcohol, which damage the liver by producing reactive oxygen species (ROS). The ROS is highly toxic which produce the tissue injury through covalent DNA base oxidation, lipid peroxidation and protein oxidation[1].

Antioxidant is an important molecule capable for stabilizing and deactivating the free radical before they attack[2]. At low level of free radical provides beneficial effects on cellular and immune functions, however, excess of free radical generate the oxidative stress which damages the cellular and immune functions[3]. At present CCl<sub>4</sub> is an important substance used to elucidate the mechanism of hepatotoxicity effects[4]. CCl<sub>4</sub> damage the hepatocytes directly by changing the permeability of the plasma, lysosomal and mitochondrial membranes. It generates the highly reactive free radicals through the activation of oxidase reaction by CYP2E1 and leads to the severe centrilobular necrosis[5]. Generally, free radicals can extract hydrogen atoms from the membrane lipids of the liver cells and produce the lipid hydroperoxides. Antioxidants (Super oxide dismutase (SOD), Glutathione peroxidase (GPx), and catalase (CAT) are the first line defense mechanism for scavenging the reactive oxygen species[6]. Imbalance between the reactive oxygen species production and antioxidant defense result in oxidative

\*Corresponding author: Ki Choon Choi, Grassland and Forage Division, National Institute of Animal Science, RDA, Korea.

E-mail: choiwh@korea.kr

Dr. S. Ravikumar Department of Biotechnology, PRIST University, India.

E-mail: drravinikesh@yahoo.co.in

stress which deregulated the cellular function as a result in various pathological conditions like cancer *etc.*. Many studies have been suggested that the natural antioxidants and their mechanism against oxidative stress[7]. The differential protein expression analysis using 2 dimensional electrophoresis (2-DGE) Matrix assisted laser desorption/ ionization time of flight mass spectroscopy (MALDI-TOF-MS) has been used to detect the differential protein expressions for last few decades to present state[8].

Lycorine is a major alkaloid found in the leaf and root bulb of the amaryllidaceae family plants. This alkaloid has been shown to behave as potent therapeutic agent in numerous experimental models. It possesses many pharmacological activities such as hepatoprotective activity[9, 10], antioxidant[11], anti-inflammatory[12], and antibacterial and analgesics agents[13]. The present study has been designed to identify the proteomic mechanism of lycorine during CCl<sub>4</sub> induced oxidative stress in the swiss albino mice.

## 2. Materials and methods

### 2.1. Chemicals

Required chemicals for 2DE and MS were obtained from Sigma Chemicals [USA] and stored at 2–4 °C and protected from sunlight. All other chemicals were analytical grades and were obtained from standard commercial suppliers.

### 2.2. Experimental design

The Swiss albino mice were randomly divided into four groups each consist of six mice. Group I served as normal and was given olive oil daily for a period of 8 weeks. Group II served as lycorine alone [5 mg/kg]. For inducing hepatotoxicity, animals of groups III and IV were administered orally [1 mL/kg bw] with carbon tetrachloride twice a week for a period of 8 weeks. Group III served as CCl<sub>4</sub> induced group. Group IV administered orally with lycorine[9] [5 mg/kg *i.p.*] daily for a period of 8 weeks. At the end of the experiment, animals were sacrificed by cervical dislocation. Liver samples were dissected out and washed immediately with ice cold saline to remove the blood as possible, and immediately stored at -70 °C until analysis. All the animal experimentations were premeditated and executed in compliance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines [743/03/abc/ CPCSEA dt 3.3.03].

### 2.3. Sample preparation

Approximately 10 mg of tissue samples were cut into small fragments and suspended in phosphate buffer saline (PBS). The suspensions were homogenized using a rotating blade

homogenizer for 50 s followed by sonication for 10 times and then centrifuged at 10 000 rpm, 4 °C for 10min and collect the supernatant, added an equal volume of ice cold acetone with 10% TCA and incubated for overnight at -20 °C. after that centrifuged at 10 000 rpm for 15 min at 4 °C and removed the supernatant and then pellet was rinsed with a fourfold volume of ice cold acetone for five times and then centrifuged. The precipitate was collected and suspended in 200 μL of lysis buffer containing 85 mM Tris [pH 6.8] and 2% SDS stored at -80 °C for further use The extracted protein concentrations was determined as previously reported[14].

### 2.4. High Resolution Two-dimensional gel electrophoresis

350 μg of extracted protein from each tissue was loaded into (160 × 3) mm diameter rod gel with a pH range between 3–10. Isoelectric focusing was conducted using a vertical gel system according to the following procedure: 15 min at 200 V, 30 min at 300 V, 16 h at 400 V. The rod gel was equilibrated with 6 M urea, 6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS and 0.002% bromophenol blue for 25 min with gentle shaking. For second dimensional separation, the rod gel was placed on the top of the SDS-PAGE (12%) gel and sealed with 0.5% agarose in the SDS electrophoresis buffer. Electrophoresis was performed at 10 °C in the vertical PAGE system, according to the following program; 100 V for 30 min, then 200 V gel for 3 h until the bromophenol blue front had migrated to the end of the gel. Gels were stained with colloidal Coomassie blue for overnight. To account for experimental variation, we run and analyzed triplicate gels for each experimental group[15].

### 2.5. MALDI-TOF-Mass Spectroscopy

The selected protein spots were cut and washed twice with 25 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0 and 50% acetonitrile in 25 mM NH<sub>4</sub>HCO<sub>3</sub>, and pH 8.0. For digestion, added 5 μL trypsin solutions (10 ng/μL; sequencing grade, Promega) and incubated at 37 °C for 5 h. The extraction was performed in two steps by the addition of 50% acetonitrile and 1% trifluoroacetic acid and dried. Then it was dissolved in 1:1 ratio of MALDI matrix [5 mg/mL recrystallized 2-cyano-3-[4-hydroxyphenyl] acrylic acid]. In the final step before the MALDI-TOF [time-of-flight] analysis, 1 μL dissolved sample was spotted on the target slides[15].

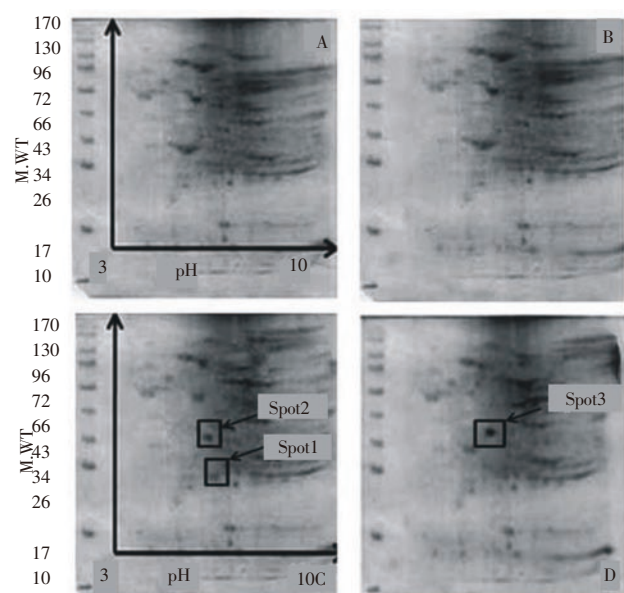
### 2.6. Protein identification

Peptide mass fingerprinting [PMF] was performed by using ultraflex MALDI-TOF mass spectrometer equipped with Linear mode 25KvA, laser shots 150 [337 nm, 50 hz, N<sub>2</sub> laser [Bruker-Daltonics, Germany]. Protein identification was performed by searching for *Mus musculus* proteins in the latest version of the NCBI database using the mascot search engine[15]. The following

parameters were applied: monoisotopic; missed cleavages, 1; allowed variable modifications, oxidation [Met] and fixed modification, Carbamidomethyl [C] and peptide tolerance, 0.5 Da[16].

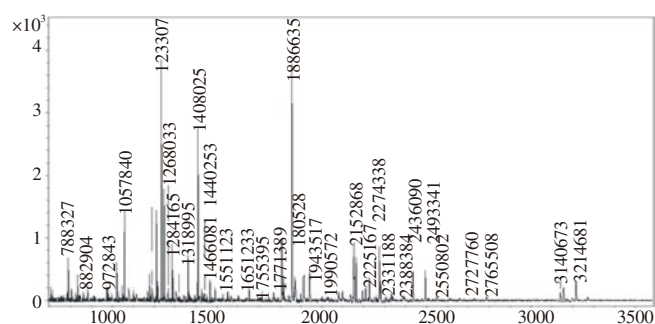
### 3. Results

Figure 1 show images from the IEF and SDS-PAGE which exhibited many protein spots in the experimental liver tissues. The alteration in the liver protein expressions are reproducible and excised the three reliable protein spots from experimental tissues, which are subjected into MALDI-TOF analysis after tryptic digestion. Two protein spots were excised from the lycorine treated mice and another one from the CCl<sub>4</sub> induced group of mouse liver. MALDI spectra were characterized by the presence of several mass signals originated by contaminant keratins and fragment of auto-catalyzed trypsin. The results were shown in Figure 2, 3, 4 and Table 1-4.

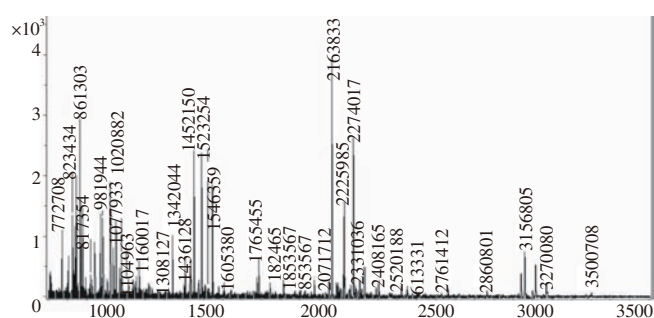


**Figure 1.** Represents the 2D gel analysis of control and experimental animals.

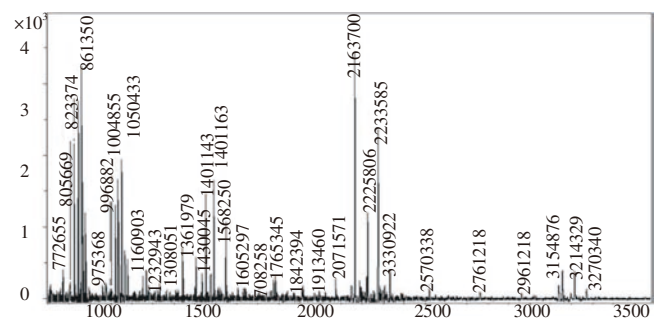
A - Control mice, B – lycorine alone treated mice [5 mg/kg], C – Mice received CCl<sub>4</sub> alone, D – Mice received CCl<sub>4</sub> along with lycorine [5 mg/ kg bw].



**Figure 2.** MALDI-TOF-MS analysis of tryptic digests of ATP synthase.



**Figure 3.** MALDI-TOF-MS analysis of tryptic digests of Regucalcin.



**Figure 4.** MALDI-TOF-MS analysis of tryptic digests of HSP-60.

**Table 1**

Identified proteins expression in mice liver of control and experimental groups.

Identified proteins	Score	Esti.MW	Esti. PI	II	AI	GRAVY
ATP synthase	30	54621.4	5.10	29.49	102.16	-0.029
Regucalcin	24	33406.8	5.15	39.62	81.47	-0.277
HSP 60	35	56677.8	5.25	21.43	101.97	-0.082

Esti. – Estimated, MW-Molecular Weight, II-Instability Index, pI isoelectric point, AI-Aliphatic Index, GRAVY-Grand Average of Hydrophaticity.

The peptide pattern obtained from each spot were subjected to database analysis using a mascot search engine with allowing one missed cleavage of trypsin per peptide and a mass tolerance of one Dalton. Spot 1 was identified as an ATP synthase, which was 54.62 kDa and has a pI of 5.10. Spot 2 was identified as a Regucalcin, which was 33.40 kDa and has a PI 5.15. Spot 3 was identified as HSP 60 which was 56.67 kDa and has a pI of 5.25.

The cellular locations of these proteins were determined using UniPort and these proteins were associated with many subcellular locations in the cytoplasm and nucleolus of liver cells. The identified protein such as HSP 60 was expressed in CCl<sub>4</sub> induced liver. It indicated that CCl<sub>4</sub> could be induced the oxidative stress in the liver, which stimulates the HSP 60 expression. ATP synthase and regucalcin were expressed in CCl<sub>4</sub> induced rats treated with the lycorine. This result displayed the protective nature of lycorine against CCl<sub>4</sub> induced oxidative stress in swiss albino mice.

#### 4. Discussion

Proteomics database was used to determine the alteration of protein expression patterns in the experimental animals, which might be revealed the metabolic variations in the living system. For example, investigation of reactive oxygen species [ROS] role in the mitochondrial diseases under the normal physiological conditions, approximately 2%-4% of oxygen was consumed by the mitochondria and changed into superoxide anion instead of water due to the unproductive electron transfer within the complex I and III[17]. When the respiratory chain reaction is slow down, more electrons are diverted from their normal pathway which leads to stimulates more ROS production. This kind of situation can be found in the case of ATP synthase deficiency. The limited rate of ATP synthesis results in a high membrane potential which slow down

the electron transfer along with respiratory chain[18]. The previous research revealed that the excess of free radical productions causes the mitochondrial respiratory chain reaction damage which leads to depletion of ATP synthase activity. The present study, ATP synthase expression was observed in the lycorine treatment during the CCl<sub>4</sub> induction in the mice. This condition provides some information about the CCl<sub>4</sub> and lycorine treatment, CCl<sub>4</sub> enhances the ROS production which causes the mitochondrial function and reduced ATP production. But, lycorine treatment to the CCl<sub>4</sub> treated mice may stimulate the ATP synthase which produce more ATP for energy production for neutralizing the generated free radicals or may be reduced free radical activity by the scavenging activity through the antioxidant pathway. Similarly, lycorine have potent antioxidant and hepatocytes protective nature against CCl<sub>4</sub> induced toxicity in Swiss albino mice and it also provides evidence that protective effects are

**Table 2**

Peptide fragment observed in the MALDI mass spectrum of ATP synthase tryptic digest and a comparison with the output of the NCBI database.

Start - End	Observed	Calculated(MW)	Peptide sequence
001 - 017	1842.3940	1841.3867	MGIQAAEISAILKEQIK.N
014 - 021	9630.8100	962.8027	K.EQIKNFGK.D
022 - 040	1913.4400	1912.4327	K.DAEVAEVGRVLSVGDGIAR.V
061 - 080	2146.6740	2145.6667	R.GMALNLEVDNVGIVIFGDDR.D
081 - 089	1004.8550	1003.8477	R.DIKEGDTVK.R
140 - 151	1267.9780	1266.9707	R.KGVHEPMATGLK.A
141 - 161	2163.7000	2162.6927	K.GVHEPMATGLKAVDAMIPIGR.G
152 - 164	1399.0650	1398.0577	K.AVDAMIPIGRGQR.E
176 - 199	2551.1110	2550.1037	K.TAVALDITLNLQKVYNDAAGDDESK.K
213 - 220	845.3710	844.3637	R.STVAQLVK.K
265 - 275	1320.9870	1319.9797	R.HALMIYDDLKQ
265 - 281	1994.5450	1993.5377	R.HALMIYDDLKQAVAYR.Q
276 - 281	0707.5160	706.5087	K.QAVAYR.Q
276 - 288	01548.259	1547.2517	K.QAVAYRQMSLLLR.R
282 - 288	0861.3500	860.3427	R.QMSLLLR.R
282 - 288	0877.3300	876.3227	R.QMSLLLR.R
294 - 306	1553.0980	1552.0907	R.EAYPGDVFFYLHSR.L
376 - 386	1066.4130	1065.4057	R.VGSSAQTSAMK.S
423 - 431	1034.4520	1033.4447	R.GARLTELKQ
480 - 490	1308.0610	1307.0537	K.ALLDDMTKNDR.K

**Table 3**

Peptide fragment observed in the MALDI mass spectrum of Regucalcin tryptic digest and a comparison with the output of the NCBI database.

Start - End	Observed(MW)	Calculated(MW)	Peptide sequence
002 - 011	1191.0380	1189.6489	M.SSIKVECVLR.E
038 - 051	1775.3890	1773.8944	K.IICRWDTVSNQVQR
042 - 051	1233.0070	1231.5946	R.WDTVSNQVQR.V
052 - 064	1284.1550	1282.7245	R.VAVDAPVSSVALR.Q
102 - 112	1175.9950	1174.5731	R.FNDGKVDPAGR.Y
113 - 129	1856.5350	1854.8822	YFAGTMAEETAPAVLE
145 - 163	2281.1270	2279.0859	YFDQVDISNGLDWSLD
146 - 163	2152.8680	2150.9909	YFDQVDISNGLDWSLD
227 - 234	915.82900	914.48210	R.LDPETGKRL
254 - 264	1394.0770	1393.5642	K.DYSEMYVTCAR.D
265 - 274	1057.9400	1056.5564	R.DGLNAEGLLR.Q
265 - 283	2028.8870	2027.0436	DGLNAEGLLRQPDAG
284 - 299	1567.1190	1565.8453	K.ITGLGVKGIAPYSYA

**Table 4**

Peptide fragment observed in the MALDI mass spectrum of HSP 60 tryptic digest and a comparison with the output of the NCBI database.

Start - End	Observed	Calculated(MW)	Peptide sequence(MW)
064- 075	13 99.1200	1398.1127	K.DPFENMGAQMVK.E
064- 080	1913.5670	1912.5597	K.DPFENMGAQMVKEVASK.T
106- 117	1342.0440	1341.0367	K.YVASGMNPMDLK.R
106- 118	1513.2270	1512.2197	K.YVASGMNPMDLKR.G
123- 142	2163.8330	2162.8257	K.AVHAVIKELQTLSPVTNSK.E
143- 160	1775.4510	1774.4437	K.ETAQVAALSANSDEAIGK.I
161- 168	877.3760	876.3687	K.IIADAMDK.V
161- 168	893.3350	892.3277	K.IIADAMDK.V
161- 171	1175.9620	1174.9547	K.IIADAMDKVKGK.E
198- 210	1529.2370	1528.2297	R.GYLSPYFITDPEK.Q
287– 321	3500.7090	3499.7017	LEDIAILTGGTVIAEETGLTLEKAGLAELGA.
312- 321	917.3540	916.3467	K.AGLAELGSAK.R
328- 339	1232.9850	1231.9777	K.ENTTIIDGAGDK.A
369- 380	1169.0170	1168.0097	R.VAKLSGGVAVIR.I
381- 390	1047.8860	1046.8787	R.IGAATEVEMK.E
394- 404	1268.0620	1267.0547	K.DRVDDALHATR.A
424- 430	839.4130	838.40570	R.AHIKELK.G
502- 529	2761.4120	2760.4047	R.TALQNAASIASILTLTDTATVAEAGQDSK.A
502- 531	2960.6010	2959.5937	TALQNAASIASILTLTDTATVAEAGQDSKAK.A
530- 538	977.8610	976.8537	K.AKAPAELDY.-

possible due to increases of free radicals scavenging activities and restored the antioxidant status in the hepatic tissues[9].

Calcium ion [Ca<sup>2+</sup>] plays a very important role in the regulation of numerous cellular functions through its Ca<sup>2+</sup> binding proteins[19]. The Ca<sup>2+</sup> effect has been modulated by the Ca<sup>2+</sup> binding proteins, regucalcin is a calcium binding protein which plays an important role in the regulation of intracellular Ca<sup>2+</sup> homeostasis and the activity of several proteins which are involved in the signaling pathways such as kinases, phosphates etc[20]. Oral administration of carbon tetrachloride has been shown to be down regulated the regucalcin mRNA expression in hepatic cells, which is known as chemical inducer of liver injury[21]. The protein and their mRNA levels of regucalcin in the liver were significantly down regulated at 6, 8 and 30 days after CCl<sub>4</sub> treatment[22]. The present study identified the regucalcin expression in the CCl<sub>4</sub> induced mice treated with lycorine. This investigation, demonstrated that the lycorine may stimulate the regucalcin expression by the scavenging CCl<sub>4</sub> produced free radicals. Many reports claimed that the regucalcin mRNA expression was down regulated by the carbon tetrachloride, Phenobarbital, galactosamine, ethanol[20] and streptozotocin-diabetic state, which cause a disorder of liver metabolism. The decrease in regucalcin leads to an acceleration of signal transduction from the cytoplasm to nucleus, and such a decrease weakens the suppressive effect of regucalcin on the over expression of liver cell proliferation and protein synthesis.

Heat shock proteins are most evolutionarily conserved proteins[23]. The amino acid sequence of the HSP60 was almost similar to its homolog in plants, bacteria and humans[24]. These proteins are primarily responsible for maintaining the integrity of cellular proteins especially, in response to environmental changes, temperature, imbalance of concentration, pH changes and other toxins can induce the heat shock proteins for maintaining the

conformation of the cellular proteins. In this study, we identified the HSP-60 protein expression in CCl<sub>4</sub> treated mouse liver. This result evidenced that CCl<sub>4</sub> administration stimulates the HSP-60 expression liver of mice, because CCl<sub>4</sub> administration can be making more stress to the liver cells, therefore, HSP-60 synthesized for preventing CCl<sub>4</sub> mediated oxidative stress. The HSP-60 expression did not show the visible expression in the lycorine treated and control mouse. The up regulation of HSP60 expression allows for the maintenance of other cellular processes occurring in the cell, especially during stressful times. HSP60 constitutes approximately 15%-30% of all cellular proteins[25]. The HSP60 expression protects the cells from hydrogen peroxide or indomethacin induced cellular injury under the both necrotic and apoptotic conditions[26]. Mice treated with DOPA stimulate the HSP-60 up regulation in the mitochondria and HSP-70 in the cytoplasm. It concluded that the heat shock signal pathway serving as the basic defense mechanism for neurotoxicity induced by the oxygen and nitrogen species free radical [27].

The present study, we identified the differentially expressed proteins in the liver of experimental animals. Mass spectra analysis and database searching shows three differentially expressed proteins like ATP synthase, regucalcin and HSP60 which are associated with liver functions such as energy productions, calcium regulation and maintaining integrity of cellular proteins. These results showed that lycorine could restore the ATP synthase, regucalcin and HSP60 protein expression during the CCl<sub>4</sub> administration by its antioxidant mechanism.

### Conflict of interest statement

We declare that we have no conflict of interest.

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