

## Research Article

## Open Access

Anca Hermenean\*, Miruna Stan, Aurel Ardelean, Luminița Pilat, Ciprian Valentin Mihali, Cristina Popescu, Lajos Nagy, György Deák, Miklós Zsuga, Sándor Kéki, Ildikó Bácskay, Ferenc Fenyvesi, Marieta Costache, Anca Dinischiotu, Miklós Vecsernyés

# Antioxidant and hepatoprotective activity of milk thistle (*Silybum marianum* L. Gaertn.) seed oil

**Abstract:** This study has assessed the protective efficacy of *Silybum marianum* seed oil (SMSO) in the context of  $\text{CCl}_4$ -induced injury and oxidative stress in murine liver. Based on the GC-MS analysis, linoleic and stearic acids, tocopherol, ascorbic acid 2,6 dihexadecanoate and other constituents were identified in SMSO. Swiss mice received oral doses of SMSO daily for 21 days (10 g/kg b.w.) and subsequently injected i.p. with  $\text{CCl}_4$  (50% v/v in olive oil; 1 ml/kg) on the 22<sup>nd</sup> day.  $\text{CCl}_4$  administration induced an elevation of serum amino- and glutamyl transferases activities and an increased peroxidation, as well as a decrease of SOD, CAT, GPx, GR and GST activities in liver. SMSO successfully prevented oxidative stress and restored the biochemical parameters, hepatic architecture and expression of TNF-alpha. These findings suggest that SMSO was effective in counteracting the damaging effects of  $\text{CCl}_4$ -induced injury in hepatocytes, probably due to its inherent antioxidant properties.

**Keywords:** *Silybum marianum*, seed oil,  $\text{CCl}_4$ , oxidative stress, antioxidant, hepatoprotection

\*Corresponding author: **Anca Hermenean:** Department of Histology, Faculty of Medicine, Pharmacy and Dentistry, Vasile Goldis Western University of Arad, Romania, E-mail: anca.hermenean@gmail.com  
**Anca Hermenean, Aurel Ardelean, Ciprian Valentin Mihali, Cristina Popescu:** Institute of Life Sciences, Vasile Goldis Western University of Arad, Romania

**Miruna Stan, Marieta Costache, Anca Dinischiotu:** Department of Biochemistry and Molecular Biology, University of Bucharest, Romania

**Luminița Pilat:** Department of Biochemistry, Faculty of Medicine, Pharmacy and Dentistry, Vasile Goldis Western University of Arad, Romania

**Cristina Popescu:** Department of Pharmaceutical Sciences, Faculty of Medicine, Pharmacy and Dentistry, Vasile Goldis Western University of Arad, Romania

**Lajos Nagy, György Deák, Miklós Zsuga, Sándor Kéki:** Department of Applied Chemistry, University of Debrecen, Hungary

**Ildikó Bácskay, Ferenc Fenyvesi, Miklós Vecsernyés:** Department of Pharmaceutical Technology, University of Debrecen, Hungary

DOI 10.1515/biol-2015-0017

Received March 4, 2014; accepted August 20, 2014

## 1 Introduction

Several xenobiotics are capable of causing liver injury and associated morbidity due to the central role of this organ in xenobiotic metabolism, its portal location within circulation, and its anatomical and physiological structure [1]. Due to the bioactivation of xenobiotics during the detoxification reactions, several water soluble metabolites can become more harmful than the parental compounds [2], causing hepatic degeneration and cellular death [3].

Carbon tetrachloride ( $\text{CCl}_4$ ) has been used as an experimental hepatotoxicant to investigate the induction of fatty liver, fibrosis, cirrhosis and necrosis, as a result of reactive trichloromethyl free radicals formation [4,5]. Oxidative stress, which appears when the pro-oxidant effects of xenobiotics are not counteracted by the cellular antioxidant defense system, is important in the pathogenesis of different liver diseases.

Currently, there is an increasing interest in identifying the potential of various plant products as hepatoprotective/antioxidant and as dietary modulators of toxicity [6,7]. Many seed oil extracts are commonly used as components of traditional healthy food in different countries. To be more specific, it has been shown that seed oil extracts are successful in preventing oxidative stress, as well as restoring the normal level of cholesterol, triglycerides, low density-lipoproteins and hepatic markers related with liver pathologies. Such properties have been associated with tea [8], sesame [9], seabuckthorn [10], or grape [11] seeds. As far as we know, until now, no experiment concerning the antioxidant and hepatoprotective effect of milk thistle (*Silybum marianum*) seed oil has been performed.

*Silybum marianum* has been used medicinally for the treatment of liver diseases in Europe since the first century. Silymarin, the primary active ingredient in the seeds, has been extensively studied for hepatoprotective effects and several putative hepatoprotective mechanisms, including antioxidation and inhibition of lipid peroxidation [12,13], as well as anti-inflammatory [14] and anti-fibrotic effects [15], have been recorded. Although silymarin is orally absorbed, it has very poor bioavailability due to its lower water solubility [16]. Consequently, research efforts have been aimed at developing novel drug delivery systems that can improve the performance of silymarin activity [17-19].

The hepatoprotective effect and antioxidant activity of *Silybum marianum* seed oil (SMSO) have not been previously investigated. The aim of this study was to evaluate the potential of the milk thistle native seed oil to enhance the antioxidant defense system and thus provide protection against  $\text{CCl}_4$ -induced hepatotoxicity in mice.

## 2 Experimental Procedures

### 2.1 *Silybum marianum* seed oil material

The sample of milk thistle seeds (*Silybum marianum* L. Gaertn.) was supplied by a farmer from Arad County (Curtici, Romania) and voucher specimens already exist at the Faculty of Natural Sciences from the Vasile Goldis Western University of Arad.

### 2.2 Preparation and GC-MS study of *Silybum marianum* seed oil extract

The dried *Silybum marianum* seeds were powdered, homogenized and defatted by hexane in a Soxhlet extractor for 6 h. After extraction, two phases were obtained: a solid and a liquid one. The solid phase was the defatted powder (which was dried before further processing) and the liquid phase was the hexane extract. The hexane was evaporated from the extract under vacuum in a rotary evaporator and the residue was a yellow oil (SMSO) that was characterized using a Shimadzu QP-2010 GC-MS instrument. For GC-MS measurements, one mg oil was dissolved in one mL hexane and one  $\mu\text{L}$  of oil solution was injected into the gas chromatograph. Compounds were separated on a Zebron ZB-5MS column ( $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$ ). The initial temperature of the oven ( $60^\circ\text{C}$ ) was maintained for 3 minutes and subsequently heated to  $300^\circ\text{C}$  at a heating rate of  $10^\circ\text{C}/\text{min}$  and maintained at this temperature for 8 minutes. The temperatures of the electron ionization

(EI) ion source and the interface were  $300^\circ\text{C}$ . Split injection was conducted with a split ratio of 10:1, helium was used as the carrier gas and the injector temperature was  $200^\circ\text{C}$ .

### 2.3 Animal treatment

Male Swiss mice ( $25 \pm 3\text{ g}$ ), supplied by the Animal House of the Vasile Goldis Western University of Arad, were used. They were housed under controlled conditions ( $22\text{--}25^\circ\text{C}$ ) on a 12 h light/12 h dark cycle. All experimental procedures were done according to the ethics and regulations of animal experiments of Vasile Goldis Western University of Arad and approved by Institutional Animal Ethical Committee.

The SMSO dose of  $10\text{ g}/\text{kg}$  b.w. was chosen according to previous experiments with different seed oils [8, 9, 20], as well as the toxicant ( $\text{CCl}_4$ ) dose of  $1.0\text{ ml}/\text{kg}$  b.w. in 50% olive oil [21].

Thirty-two animals were used for the experiment and these were divided into 4 groups, as follows:

Group 1: *Control animals* received isotonic saline solution (ISS) every day for 21 days and on the 22<sup>nd</sup> day were deprived of food for 24 h.

Group 2:  *$\text{CCl}_4$  group* received the ISS every day for 21 days and were subsequently i.p. injected with  $\text{CCl}_4$  at a dose of  $1.0\text{ ml}/\text{kg}$  b.w. in 50% olive oil (1:1) on the 22<sup>nd</sup> day.

Group 3: *SMSO pre-treated group* orally received *Silybum marianum* seed oil (SMSO) in a dose of  $10\text{ g}/\text{kg}$  b.w. for 21 days and were subsequently i.p. injected with  $\text{CCl}_4$  ( $1.0\text{ ml}/\text{kg}$  b.w.) on the 22<sup>nd</sup> day.

Group 4: *SMSO group* orally received SMSO alone ( $10\text{ g}/\text{kg}$  b.w.) daily for 21 days and on the 22<sup>nd</sup> day were deprived of food for 24 h.

After 22 days from the start of the treatment of groups 1 and 4, and at 24 hours after  $\text{CCl}_4$  i.p. injection for group 2 and 3, blood was collected from venae cavae; the mice were subsequently sacrificed by cervical dislocation. Liver samples were used for histopathology, electron microscopy and biochemical analyses.

### 2.4 Assay of serum hepatic markers

The collected blood was placed in heparinized tubes and centrifuged for 15 minutes at  $1,500 \times g$  in order to obtain plasma samples which were used immediately to determine alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) activities; these were measured by spectrophotometric methods using commercially available kits (Roche Reagents, France) according to the manufacturer's instructions.

## 2.5 Tissue homogenate preparation

Mouse livers (0.1 g of tissue) were homogenized in 10 volumes of ice-cold buffer (0.1M TRIS-HCl, 5mM EDTA buffer, pH 7.4) containing a protease inhibitor cocktail (1:100 dilution; Sigma-Aldrich, USA) for 2 min at 16 Hz using a ball mill (type MM 301, Retsch GmbH & Co, Haan, Germany). The homogenates were centrifuged at  $8000 \times g$  for 30 min at 4°C to remove the cell debris. The supernatants were collected and used for biochemical assays.

## 2.6 Antioxidant enzymes assays

Catalase (CAT) activity was measured by the method of Aebi [22]. Changes in absorbance at 240 nm corresponded to  $H_2O_2$  decomposition. One unit of CAT activity was calculated as one  $\mu\text{mole } H_2O_2/\text{min/mL}$ . The determination of liver superoxide dismutase (SOD) activity was performed according to the method described by Paoletti and Mocali [23]. The amount of enzyme that inhibited the oxidation of NADH by 50% at 37°C corresponded to one unit of SOD activity. Glutathione peroxidase (GPx) activity was assessed by a coupled reaction with glutathione reductase that catalyzed NADPH oxidation as described by Beutler [24]. Glutathione reductase (GR) activity was measured by the decrease of NADPH absorbance at 340 nm [25]. One unit of GR activity was equal to one micromole of NADPH oxidized per minute. The total glutathione S-transferase (GST) activity was assayed by measuring the rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with GSH at 340 nm [26]. All enzymatic activities were expressed as specific activities (units/ mg of protein).

## 2.7 Assessment of lipid peroxidation

The hepatic malondialdehyde (MDA) content was determined as a marker of lipid peroxidation using a fluorimetric technique described by Del Rio et al. [27]. This method was based on the reaction of MDA with thiobarbituric acid. Relative fluorescence units (RFU) recorded at Spectrofluorometer FP-6300 JASCO ( $\lambda_{\text{ex}} = 520 \text{ nm}$ ;  $\lambda_{\text{em}} = 549 \text{ nm}$ ) were converted to nmoles malondialdehyde (MDA) using 1,1,3,3-tetramethoxypropane as standard.

## 2.8 Protein concentration measurement

The protein content was determined according to Lowry's method using bovine serum albumin as standard [28].

## 2.9 Histopathology

Freshly prelevated fragments of mice livers were fixed in Bouin solution, dehydrated in ethanol, cleared in toluene and embedded in paraffin. Five mm thick liver sections were deparaffinized and processed routinely for hematoxylin–eosin (H&E) according to Bio Optica staining kit. Frozen sections were cut at 8  $\mu\text{m}$  with the SLEE MNT cryotome, fixed in 10% buffered formaldehyde and stained with Oil Red O kit according to the methods of Bio-Optica staining kits. Mounted slides were examined under a light microscope (Olympus BX43 microscope) and photographed using a digital camera Olympus XC30.

## 2.10 Immunohistochemistry

Immunohistochemical studies were performed on paraffin embedded liver tissues using mouse monoclonal anti-TNF- $\alpha$  antibody diluted 1:100 (Santa Cruz Biotechnology, California, USA), incubated overnight at 4°C, followed by HRP secondary antibodies incubation. The immunoreaction product was visualized by adding DAB solution and counterstained with hematoxylin, dehydrated in a gradient of alcohol and mounted. The specificity of the reaction was confirmed by substituting the primary antibodies with irrelevant immunoglobulins of matched isotype, used in the same conditions and dilutions as the primary antibodies. Stained slides were analyzed by light microscopy (Olympus BX43, Tokyo, Japan).

## 2.11 Electron microscopy

Liver specimens were prefixed in 2.7% glutaraldehyde solution in 0.1 M phosphate buffer for 1.5 hours, at 4°C. Following this, they were washed in 0.15 M phosphate buffer (pH 7.2) and post-fixed in 2% osmic acid solution in 0.15 M phosphate buffer for 1h at 4°C. Dehydration was performed in acetone, and inclusion was done in the epoxy embedding resin Epon 812. The blocks were cut with an ultramicrotome type LKB, at 70 nm thickness. The sections were double contrasted with solutions of uranyl acetate and lead citrate and were analyzed with a TEM Tecnai 12 Biotwin electron microscope.

## 2.12 Statistical analysis

All results have been analyzed for statistical significance using GraphPad Prism software (Version 5; GraphPad Software, Inc., La Jolla, CA) and expressed as mean values  $\pm$  SD ( $n = 8$ ). Comparisons between groups were evaluated

by one-way ANOVA followed by a *post hoc* Bonferroni test. A value of  $p < 0.05$  was considered to be statistically significant.

## 3 Results

### 3.1 GC-MS results of the SMSO

Based on the GC-MS measurements, saturated and unsaturated fatty acids, tocopherol and ascorbic acid 2,6 dihexadecanoate were identified. In addition to these compounds, lignoceric acid methyl ester, squalene and cholesterol could also be detected in the chromatogram. The linoleic acid and the stearic acid appeared with the most prominent peaks in the GC-MS chromatogram. A representative GC-MS chromatogram of SMSO can be seen in Figure 1A. The identified components are indicated on the chromatogram. The spectrum library (NIST05) was used for the identification of the components. The EI mass spectrum of tocopherol (RT 34.5 min) is shown in Figure 1B. The similarity percentages were higher than 90% in all cases concerning tocopherol (75%) and cholesterol (71%).

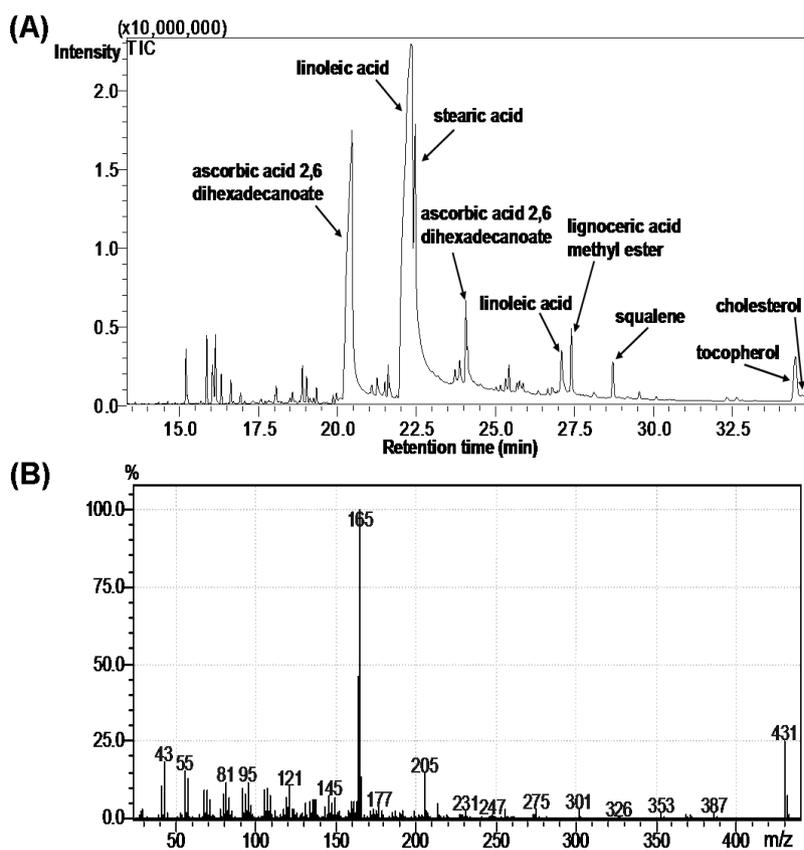
### 3.2 Effects of SMSO on the serum hepatic markers

The treatment with  $\text{CCl}_4$  increased the serum activity of AST by 67.8 fold, ALT by 67.5 folds and GGT by 2.7 fold, respectively, compared to untreated individuals. The pretreatment for 21 days with SMSO oil before  $\text{CCl}_4$  administration significantly decreased the elevated levels of the AST, ALT and GGT activities by 42.45%, 50% and 32%, respectively, by comparison to the  $\text{CCl}_4$ -treated group. In addition, there were no significant changes of these enzymatic activities in the plasma of individuals treated with SMSO only (Figure 2).

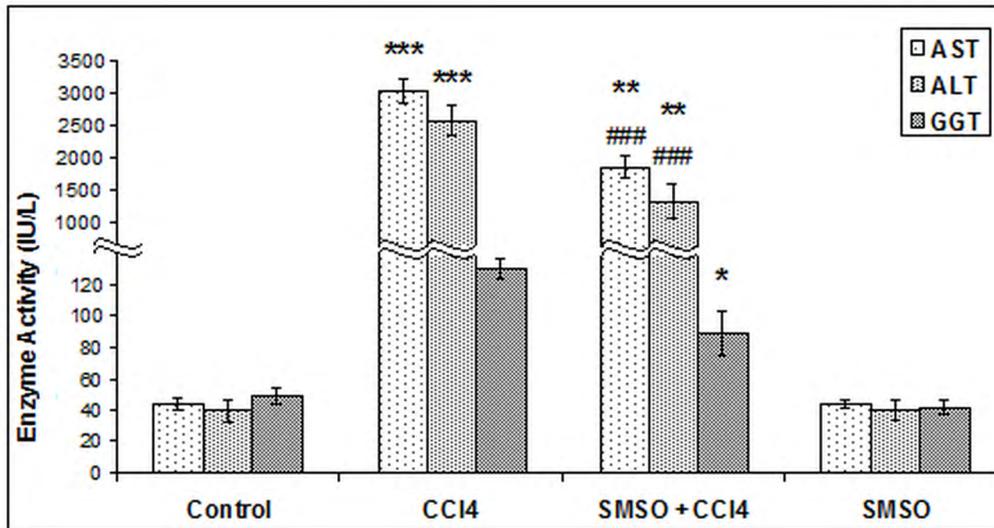
### 3.3 Effects of SMSO on hepatic lipid peroxidation and antioxidant enzymes

The level of MDA was significantly increased by 2.2 fold ( $p < 0.001$ ) in the livers of individuals treated with  $\text{CCl}_4$  compared to control. By contrast, the pretreatment with SMSO significantly diminished the MDA concentration, lowering it to control levels (Figure 3).

The exposure to  $\text{CCl}_4$  induced a reduction in CAT and SOD specific activities in mice liver ( $p < 0.001$ )



**Figure 1.** (A) GC-MS chromatogram of SMSO where the identified components are indicated; (B) Electron-ionization mass spectrum of tocopherol (RT 34.5 min).



**Figure 2.** Protective effect of native SMSO (dose of 10 g/kg b.w.) on serum AST, ALT and GGT activities. Values are expressed as means ( $\pm$  SD) ( $n = 8$ ). \*, \*\*, \*\*\* Statistical significance at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, compared to control. ### Statistical significance at  $p < 0.001$  compared to CCl<sub>4</sub>-treated group.

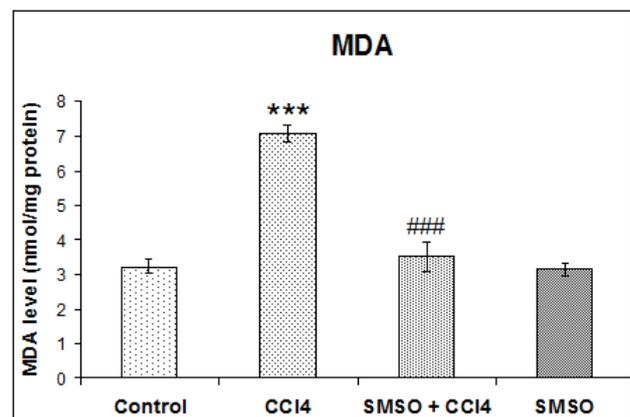
by 44% and 47.5%, respectively, compared to control (Figure 4). Nevertheless, in the SMSO pretreated group, these enzymatic activities were restored to control levels (Figure 4).

As far as hepatic enzymes involved in reduced glutathione metabolism are concerned, the decrease of GPX, GST and GR activities by approximately 44%, observed in the CCl<sub>4</sub> exposed group, was abolished in the group pretreated with SMSO (Figure 4).

### 3.4 Histopathologic and immunohistochemical examination

Light microscopic evaluation of liver tissues from the control group revealed normal cellular architecture (Figure 5 A1). Liver samples from 24 hours CCl<sub>4</sub> exposed group showed necrotic changes of hepatocytes including vacuolar degeneration, especially in the centrilobular area with infiltration of inflammatory cells (Figure 5 B1). Sinusoid congestion, macro and microvesicular steatosis of hepatocytes were observed (Figure 5 B2). Preventive SMSO treatment generated a marked reduction of hepatocellular necrosis, vacuolization, steatosis and inflammatory infiltrate, in comparison with the intoxicated group (Figure 5 C1-2).

The protective effects of SMSO on the TNF- $\alpha$  expression, elevated by CCl<sub>4</sub> pro-inflammatory activity, are shown in Figure 5. The liver slides of the control mice did not show substantial TNF $\alpha$  immunopositivity (Figure 5 A3). The TNF- $\alpha$  expression in the SMSO only group was similar to the controls (Figure 5 D3). By contrast, strong

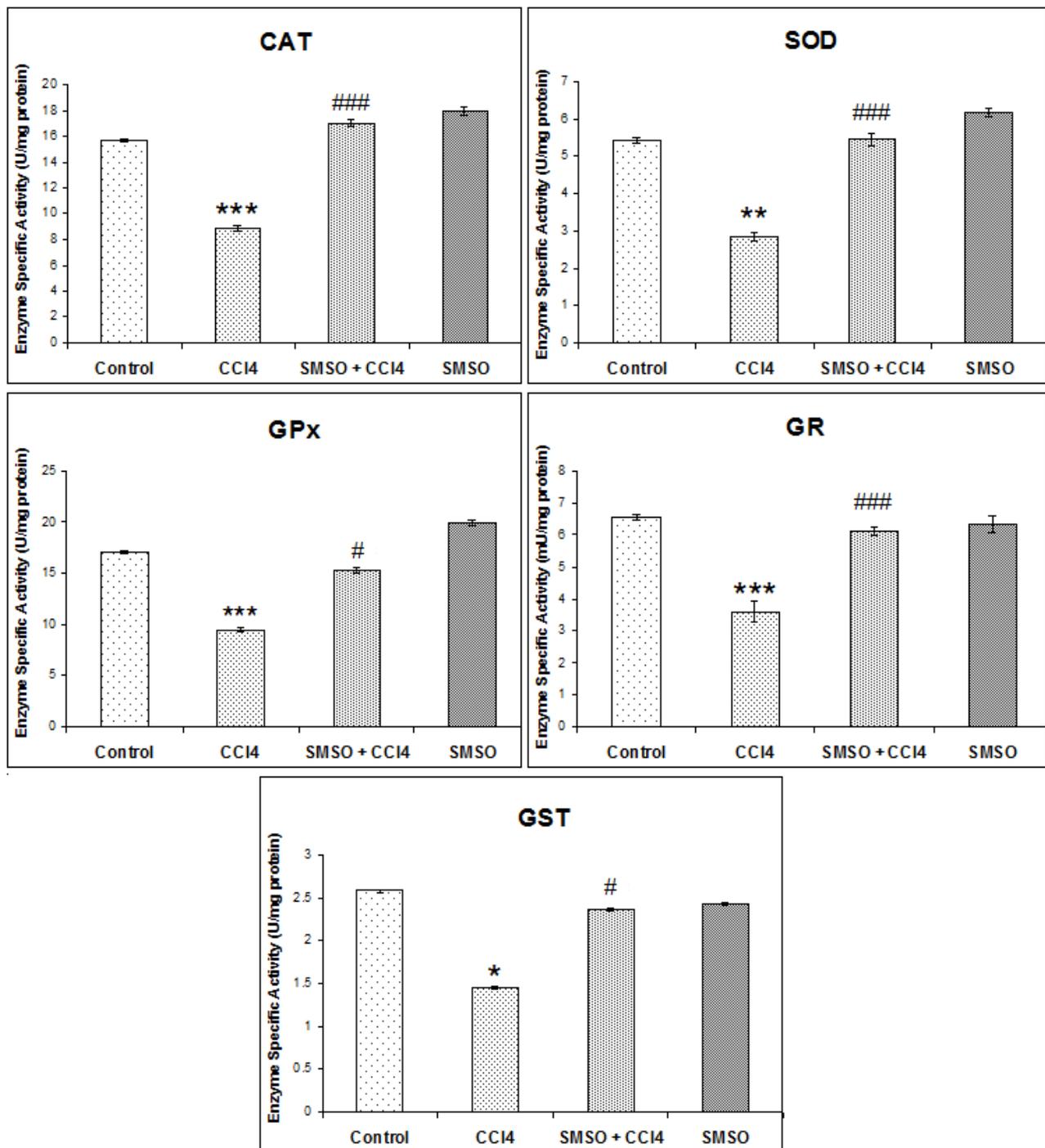


**Figure 3.** Effect of native SMSO (dose of 10 g/kg) on MDA levels induced by CCl<sub>4</sub> exposure in murine liver. Values are expressed as means ( $\pm$  SD) ( $n = 8$ ). \*\*\* Statistical significance at  $p < 0.001$  as compared to control. ### Statistical significance at  $p < 0.001$  as compared to CCl<sub>4</sub>-treated group.

TNF- $\alpha$  expression was observed for the CCl<sub>4</sub> group. TNF- $\alpha$  immunoreactivity was mainly detected in the vicinity of centrilobular veins, with the bridges formation between neighboring veins appearing as a predominantly brown staining (Figure 5 B3). This effect was in the liver of CCl<sub>4</sub>-intoxicated mice pretreated with SMSO (Figure 5 C3).

### 3.5 Electron microscopic examination

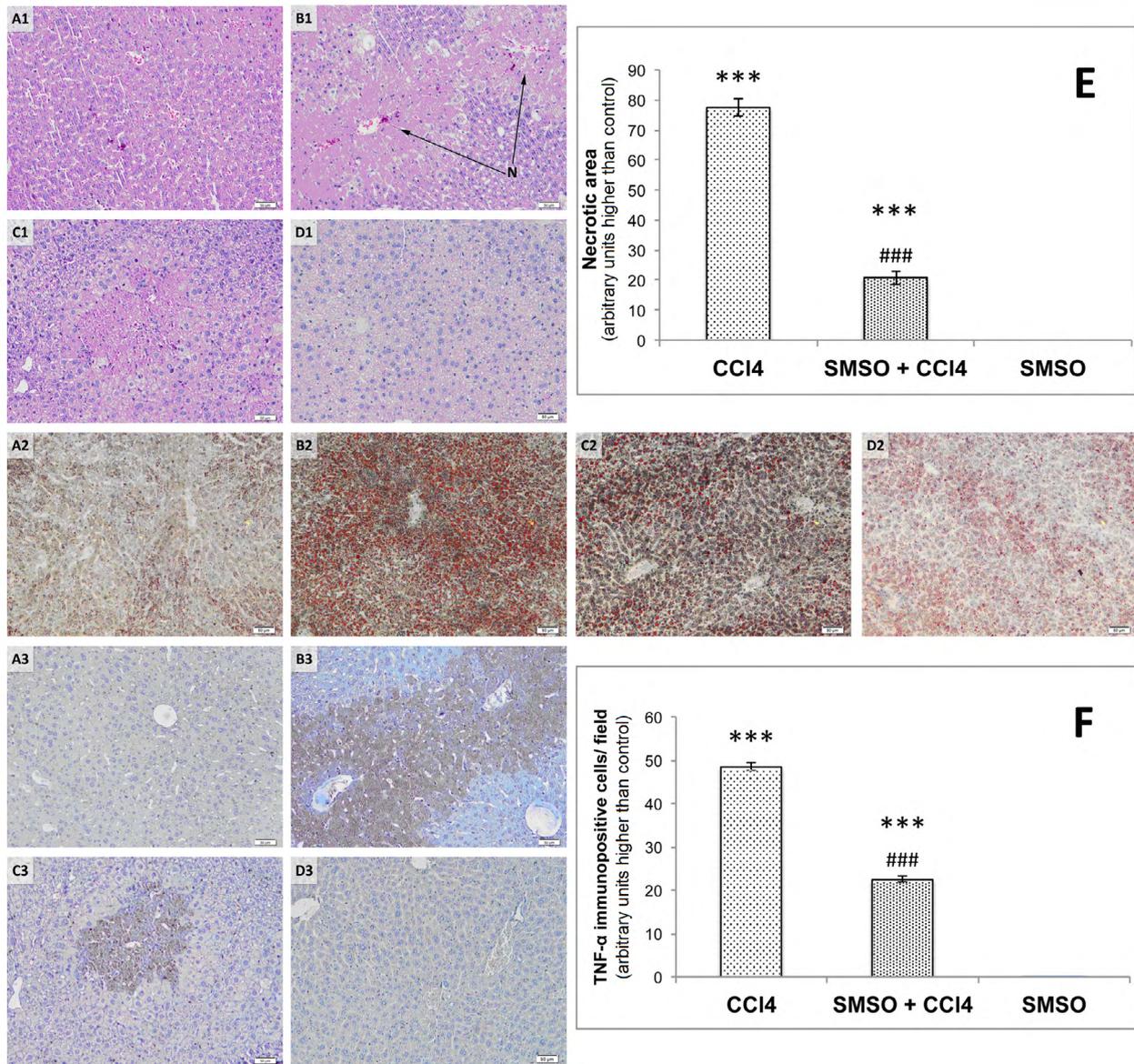
The hepatocyte ultrastructure was normal in the control group. In the CCl<sub>4</sub> treated group, most of the hepatocytes showed two populations of lipid droplets (large and small), degenerated organelles and smooth reticulum



**Figure 4.** Protective effect of native SMSO (10 g/kg b.w.) on specific activities of CAT, SOD, GPx, GR and GST in murine livers. Values are expressed as means ( $\pm$  SD) ( $n = 8$ ). \*, \*\*, \*\*\* Statistical significance at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, compared to control. #, ### Statistical significance at  $p < 0.05$  and  $p < 0.001$ , compared to CCl<sub>4</sub>-treated group.

vesicles proliferation (Figure 6 B1). We also observed large dilatations with focal breaks in the rERs of hepatocytes in many areas, associated with dilated perinuclear space and enlarged pores (Figure 6 B2). In the pre-treated group,

SMSO administration reduced the volume and number of the lipid droplets in the hepatocytes and the organelles and cytoplasm structure were widely protected from the effects of CCl<sub>4</sub> (Figure 6 C1-2).



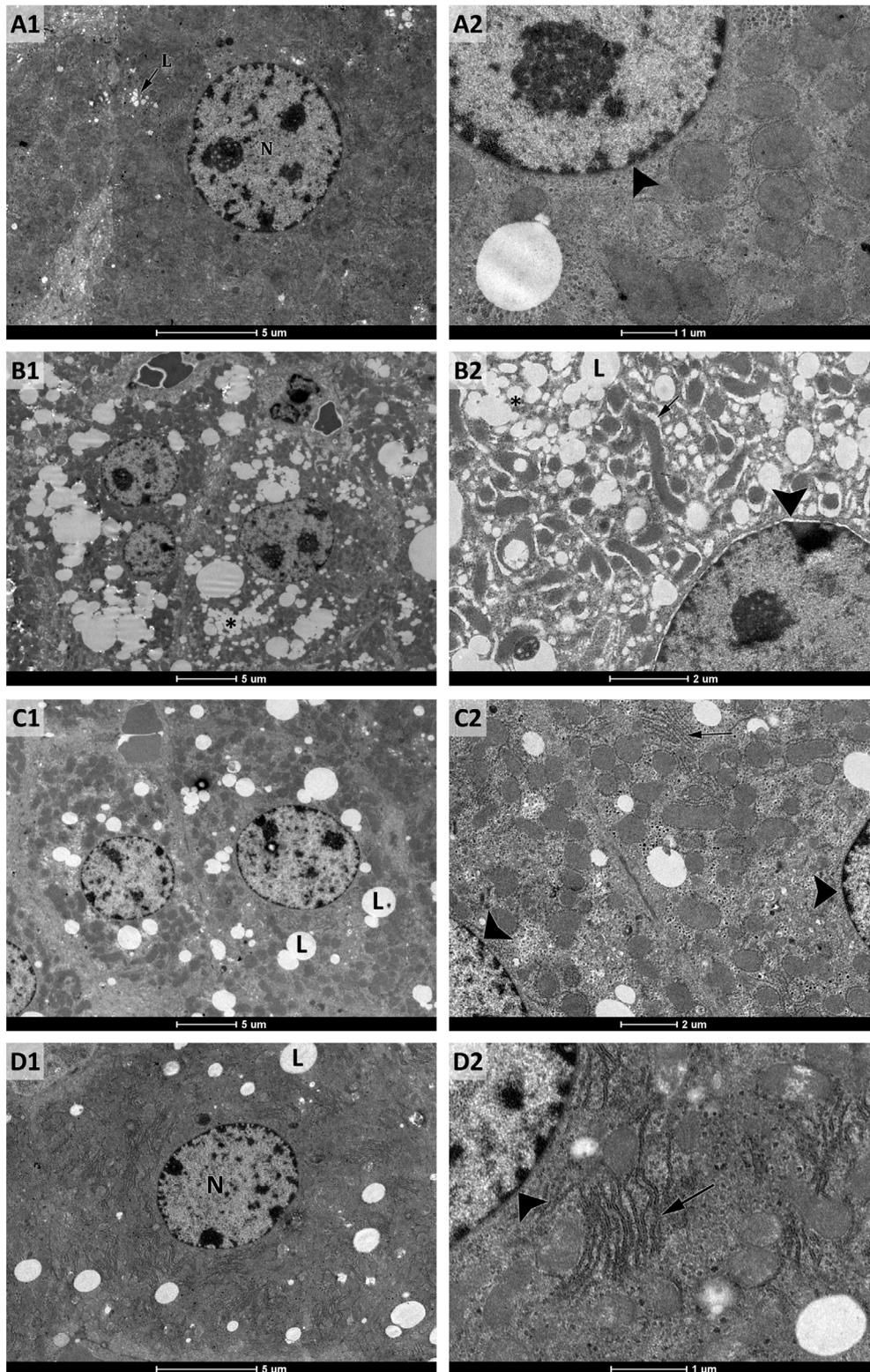
**Figure 5.** The effect of SMSO on histological and immunohistochemical changes in the liver of CCl<sub>4</sub>-treated mice; (A) Control group; (B) CCl<sub>4</sub> group (C) SMSO+CCl<sub>4</sub> group (D) SMSO group; 1.H&E stain (N-centrilobular necrosis); 2 Oil Red O stain (lipid drops – red); 3.TNF-α immunohistochemical stain (E) The percentage of necrotic area in the livers in the livers; (F) TNF-α immunopositive hepatocytes/field (units above control). Values are expressed as means (± SD) (n = 8). \*\*\*  $p < 0.001$  significantly different from the control group; ###  $p < 0.001$  significantly different from the CCl<sub>4</sub>-treated group.

## 4 Discussion

The hepatotoxicity following CCl<sub>4</sub> administration in rodent cells, as well as the generation of oxidative stress, is well documented by previous studies [29,30]. In CCl<sub>4</sub> metabolism, the trichloromethyl free radical formation (CCl<sub>3</sub>) under cytochrome P<sub>450</sub> action is one of the first steps. This free radical reacts very rapidly with an oxygen molecule to yield a highly reactive trichloromethyl peroxy radical (CCl<sub>3</sub>OO). Both radicals are capable of binding to proteins or lipids, thus initiating lipid peroxidation and

protein oxidation in the liver, resulting in membrane damage and liver injury. Several phytochemicals are antioxidants and have the capacity to protect cells and biomolecules, neutralizing free radicals and preventing oxidative degradation and certain human diseases [31].

*Silybum marianum* seed oil is rich in phytochemicals such as tocopherol and shows antioxidant effects [32]. The chemical composition of the oil obtained by us was studied by several methods. In more detail, the oleic and linoleic acids were identified as the predominant components of the oil based on gas chromatography



**Figure 6.** Protective effect of SMSO (dose of 10 g/kg b.w.) on the ultrastructure of hepatocytes against liver injuries induced by  $\text{CCl}_4$ . **(A)** *Control group*; normal aspect of nucleus (N) and nuclear envelope (arrowhead), and few lipid drops (L); **(B)** *CCl<sub>4</sub> group*; oedematous cytoplasm matrix with sER proliferation (asterix); dilated rER profiles (arrow) and enlarged nuclear space (arrowhead); increased number and size of lipid drops (L); **(C)** *SMSO + CCl<sub>4</sub> group*; normal aspect of nuclear shape (arrowhead) and rER (arrow); Reduction of number and size of lipid drops (L); **(D)** *SMSO group*; normal aspect of nucleus (N) and nuclear envelope (arrowhead) and rER (arrow), few lipid drops (L).

measurements [33]. Beside the saturated and unsaturated fatty acids, other compounds such as tocopherol, sterols and sterylglucosides were also identified by HPLC and capillary GLC [32]. These results highly corroborate with our GC-MS results. In addition, the presence of ascorbic acid 2,6 dihexadecanoate in SMSO was also identified, for the first time. Besides tocopherol and ascorbic acid 2,6 dihexadecanoate, the sterols of SMSO may be potent antioxidants due to their possibility to form resonance-stabilized structures [34]. On the other hand, sterylglucosides dissolve better in polar solvents because the glucose moiety is polar and hydrophilic; while these might play a role as antioxidants in the cellular hydrophilic environment [35], squalene, which is an isoprenoid compound could function as a highly effective oxygen scavenging agent [36].

It is well known that chemical agents inducing hepatic injury can lead to significant increases in ALT and AST activity [5]. One of the most sensitive indicators of liver injury after  $\text{CCl}_4$  administration is the release of intracellular enzymes in the circulation, such as aminotransferases. Becker et al. [37] stated that elevated activities of these enzymes are indicative of cellular leakage and loss of the functional integrity of liver cell membrane. The elevation of plasma enzymes concentration is generally regarded as one of the sensitive markers of hepatic damage [38]. In our study,  $\text{CCl}_4$  was found to cause significant increases in plasma AST, ALT and GGT levels compared to control ( $p < 0.001$ ). Pre-treatment with 10 g/kg b.w. of SMSO effectively protected the animals against  $\text{CCl}_4$ -induced hepatic injury, as evidenced by decreased plasma AST (by 42.45%), ALT (by 50%) and GGT (by 32%) compared to  $\text{CCl}_4$  group. The antioxidant effect of all these natural products (tocopherol and ascorbic acid 6 hexadecanoate, sterols, sterylglucosides and squalene), which are present in SMSO, could diminish the membrane peroxidation as well as the AST, ALT and GGT leakage in plasma [39].

In the case of  $\text{CCl}_4$ -induced hepatopathy, a 2.5-fold higher production of superoxide at the level of complex I of the mitochondrial respiratory chain compared to the control was registered [40]. This radical is transformed in hydrogen peroxide in the reaction catalyzed by SOD. Beside dismutation, the Cu/Zn SOD isoenzyme catalyzes surrogate reactions, such as the production of hydroxyl radicals, using anionic scavengers and hydrogen peroxide [41]. The very reactive hydroxyl radicals react rapidly with a wide range of biological macromolecules, causing oxidative damage [42] and a decrease in activity/level of basic cellular enzymatic and non-enzymatic antioxidants [43]. Recent studies proved that in  $\text{CCl}_4$  exposed mice the level of SOD, CAT and GPX mRNAs decreased significantly

[44]. In addition, glutathione reductase may be vulnerable to oxidative damage itself [45]. Also, some isoenzymes of GST, such as pi class, can be deactivated by oxidation of a cysteine residue near the active centre [46]. These could be the reasons for which total SOD and CAT activities, as well as those of the enzymes involved in glutathione metabolism, i.e. GPX, GST and GR, decreased in the  $\text{CCl}_4$  treated group.

The first targets of reactive oxygen species are polyunsaturated fatty acids from phospholipids and lipid peroxidation, quantified as MDA concentration, an end product of this process. It seems that the significant decrease of this biochemical parameter in the case of pretreatment with SMSO was also due to tocopherol, ascorbic acid 6-hexadecanoate, sterols, sterylglucosides, squalene and linoleic acid presence. The linoleic acid could be transformed in the mice gastrointestinal tract in certain isomers of conjugated linoleic acids (CLA) that could induce reduced glutathione synthesis through a mechanism independent of ROS production. CLA suppress lipoperoxidation [47] and as a result, probably, MDA decreased. Tocopherol [39,48], sterols, sterylglucosides, and squalene could protect cell and organelle membrane polyunsaturated fatty acids and lipoproteins from oxidation by reactive free radicals and might inhibit lipid peroxidation. Being a chain breaking antioxidant, tocopherol can directly interact with free radicals and it is transformed in tocopheryl radical which can be reduced to tocopherol by ascorbic acid 6-hexadecanoate. Subsequently, the dehydroascorbic acid formed in this reaction [49] can be transformed in ascorbic acid by reduced glutathione [50].

The treatment of mice with SMSO only induced an insignificant increase of liver CAT and SOD activities. In the individuals pretreated for 21 days with SMSO and then treated with  $\text{CCl}_4$ , a recovery of these enzymatic activities, as well as of GPX, GST and GR levels, was noticed; this could be due to the antioxidant effects of tocopherol from SMSO [51], but also of CLA [52], ascorbic acid 6-hexadecanoate [53], sterols [54] and sterylglucosides [55].

Membrane damage probably resulted in changes in lipoprotein releasing and subsequently induced lipid droplets accumulation into hepatocytes [56]. Consequently, hepatocyte vacuolization could be due to lipid accumulation that was detected ultrastructurally as large lipid droplets buildup in these cells. Several studies have reported hepatic centrilobular steatosis and necrosis after exposure to  $\text{CCl}_4$  [21,57].

Inflammatory cells were also observed, being highlighted by a significant release of TNF- $\alpha$  from Kupffer cells especially, localized around the central vein and

extending to neighboring veins. Similarly, other studies have shown that TNF- $\alpha$  played an important role in the pathogenesis of CCl<sub>4</sub>-induced hepatotoxicity [58].

Previous studies have recognized the antioxidant and anti-inflammatory properties of ascorbic acid,  $\alpha$ -tocopherol [59] and sterols [60]. In our case, the histological and electron microscopy examination revealed that pre-treatment with SMSO suppressed the acute hepatic damage and was consistent with the improvement of plasmatic and tissue biological parameters for hepatotoxicity.

## 5 Conclusions

Taken together, our data, including biochemical markers, pathological histology and electron microscopy, showed that SMSO pre-treatment could prevent CCl<sub>4</sub>-induced oxidative stress and liver injury. These findings suggest that the SMSO was effective in counteracting the damaging effect of CCl<sub>4</sub> in murine hepatocytes and indicate that the preventive effects of SMSO against CCl<sub>4</sub>-induced liver damage are related to its antioxidant properties, which are likely due to the presence of tocopherol and ascorbic acid 6-hexadecanoate.

**Acknowledgments:** This work was financially supported by the Hungary-Romania Cross-Border Co-operation Programme 2007-2013 (HURO/0901/058/2.2.2.). MS Stan acknowledges the strategic grant POSDRU/159/1.5/S/133391 within the project “*Doctoral and Post-doctoral programs of excellence for highly qualified human resources training for research in the field of Life sciences, Environment and Earth Science*” co-financed by the European Social Fund within the Sectorial Operational Program Human Resources Development 2007 – 2013”.

**Conflict of interest:** Authors declare nothing to disclose.

## References

- [1] Jones A.L., Anatomy of the normal liver., Hepatology: a text book of liver disease, 3rd ed., Zakin D and Boyer TD, Philadelphia: W.B. Saunders, 1996
- [2] Sturgill M., Lambert G.H., Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function, Clin. Chem., 1997, 43 (8), 1512-1526
- [3] Oesterreicher C.H., Trauner M., Xenobiotic-induced liver injury and fibrosis, Expert Opin. Drug Metab. Toxicol., 2012, 8(5), 571-80
- [4] Clawson G.A., Mechanism of carbon tetrachloride hepatotoxicity, Pathol. Immunopathol. Res., 1989, 8(2), 104-112
- [5] Weber L.W., Boll M., Stampfl A., Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model, Critical Rev. Toxicol., 2003, 33 (2), 105-136
- [6] Muriel P., Rivera-Espinoza Y., Beneficial drugs for liver diseases, J. Appl. Toxicol., 2008, 28(2), 93–103
- [7] Vitaglione P., Morisco F., Caporaso N., Fogliano V., Dietary Antioxidant Compounds and Liver Health, Critical Rev. Food Sci. Nutrition, 2004, 44(7-8), 575–586
- [8] Lee C.P., Shih P.H., Hsu C.L., Yen G.C., Hepatoprotection of tea seed oil (*Camellia oleifera* Abel.) against CCl<sub>4</sub>-induced oxidative damage in rats, Food Chem Toxicol., 2008, 45(6), 888–895
- [9] Prasanthi K., Muralidhara M., Rajini P.S., Fenvalerat-induced oxidative damage in rat tissues and its attenuation by dietary sesame oil, Food Chem Toxicol., 2005, 43(2), 299-306
- [10] Hsu Y.W., Tsai C.F., Che W.K., Lu F.J., Protective effects of seabuckthorn (*Hippophae rhamnoides* L.) seed oil against carbon tetrachloride-induced hepatotoxicity in mice, Food Chem. Toxicol., 2009, 47(9), 2281–2288
- [11] Maheswary M.U., Rao P.G.M., Antihepatotoxic effect of grape seed oil in rat, Indian J. Pharmacol., 2005, 37(3), 179-182
- [12] Haddad Y., Vallerand D., Brault A., Haddad P.S., Antioxidant and hepatoprotective effects of silibinin in a rat model of nonalcoholic steatohepatitis, Evidence-Based Complementary and Alternative Medicine, 2011, ID 647903, 10 pages
- [13] Shaker M.E., Zalata K.R., Mehal W.Z., Shiha G.E., Ibrahim T.M., Comparison of imatinib, nilotinib and silymarin in the treatment of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis, Toxicol. Appl. Pharmacol., 2011, 252(2), 165–175
- [14] Au A.Y., Hasenwinkel J.M., Frondoza C.G., Silybin inhibits interleukin-1b-induced production of pro-inflammatory mediators in canine hepatocyte cultures, J. Vet. Pharmacol. Therap., 2010, 34(2), 120–129
- [15] Tsai J.H., Liu J.Y., Wu T.T., Ho P.C., Huang C.Y., Shyu J.C., et al., Effects of silymarin on the resolution of liver fibrosis induced by carbon tetrachloride in rats, J. Viral Hepatitis, 2008, 15(7), 508–514
- [16] Kshirsagar A., Ingawale D., Ashok P., Vyawahare N., Silymarin: A Comprehensive Review, Pharmacognosy Rev., 2009, 3(5), 126-134
- [17] Abrol S., Trehan A., Katare O.P., Comparative study of different silymarin formulations: formulation, characterisation and In Vitro/In Vivo evaluation, Curr. Drug Delivery, 2005, 2(1), 45-51
- [18] Passerini N., Perissutti B., Albertini B., Franceschini E., Lenaz D., Hasa D., et al., A new approach to enhance oral bioavailability of *Silybum marianum* dry extract: Association of mechanochemical activation and spray congealing, Phytomedicine, 2012, 19(2), 160–168
- [19] Wang Y., Zhang Z., Liu Z., Liu G., Duan C., Jia L., et al., *In vitro* and *in vivo* evaluation of silybin nanosuspensions for oral and intravenous delivery, Nanotechnology, 2010, 21, 155104
- [20] Kasdallah-Grissa A., Nakbia A., Koubaa N., El-Fazaâb S., Gharbib N., Kamounb Aet al., Dietary virgin olive oil protects against lipid peroxidation and improves antioxidant status in the liver of rats chronically exposed to ethanol, Nutrition Res., 2008, 28(7), 472–479
- [21] Hermenean A., Popescu C., Ardelean A., Stan M., Hadaruga N., Mihali C.V., et al., Hepatoprotective Effect of *Berberis vulgaris*

- L.extract/beta-cyclodextrin on carbon tetrachloride – induced acute toxicity in mice, *Int. J. Mol. Sci.*, 2012, 13(7), 9014-934
- [22] Aebi H., Catalase, *Methods of Enzymatic Analysis*, Bergmeyer, H.U., Ed. Academic Press: New York, NY, USA., 1974, 673–677
- [23] Paoletti F., Mocali A., Determination of superoxide dismutase activity by purely chemical system based on NADP(H) oxidation, *Meth. Enzymol.*, 1990, 186, 209–221
- [24] Beutler E., *Red Cell Metabolism, A Manual of Biochemical Methods*, Beutler E. Ed., Grune and Stratton: Orlando, FL, USA. 1984, 68–73
- [25] Goldberg D.M., Spooner R.J., Glutathione Reductase, *Methods of Enzymatic Analysis*, Bergmeyer H.U. Ed., Verlag Chemie: Weinheim, Germany, 1983, 258–265
- [26] Habig W.H., Pabst M.J., Jakoby W.B., Glutathione S-transferases. The first enzymatic step in mercapturic acid formation, *J. Biol. Chem.*, 1974, 249(22), 7130–7139
- [27] Del Rio D., Pellegrini N., Colombi B., Bianchi M., Serafini M., Torta F., et al., Rapid fluorimetric method to detect total plasma malondialdehyde with mild derivatization conditions, *Clin. Chem.*, 2003, 49(4), 690-692
- [28] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, 193, 265–275
- [29] Johnson D.E., Kroenung C., Mechanism of early carbon tetrachloride toxicity in cultured rat hepatocytes, *Pharmacol. Toxicol.*, 1998, 83(6), 231-239
- [30] Slater T.F., Lipid peroxidation, *Biochem. Soc. Transactions*, 1983, 10, 70-71
- [31] Rubalya V.S., Neelameagam P., Gayathri K., Estimating antioxidant stability in sunflower and rice bran oil on heating using in vitro analysis, *Biomedicine*, 2009, 29, 31-36
- [32] El-Mallah M.H., El-Shami S.M., Hassanein M.M., Detailed studies on some lipids of *Silybum marianum* (L.) seed oil, *Grasas y Aceites.*, 2003, 54(4), 397-402
- [33] Khan I., Khattak H.U., Ullah I., Bangash .FK., Study of the physicochemical properties of *Silybum marianum* seed oil, *J. Chem. Soc. Pak.*, 2007, 29(6), 545-548
- [34] Wang T., Hicks K.B., Moreau R., Antioxidant activity of phytosterols, oryzanol, and other phytosterol conjugates, *J. Am. Oil Chem. Soc.*, 2002, 79, 1201-1206
- [35] Weber N., Murkherjee K.D., Plant sterols and steryl esters in functional foods and nutraceuticals, Shahidi F. (Ed.), *Nutraceutical and specialty lipids and their co-products*, CRC Press, Taylor & Francis Group, 2006, 483-508
- [36] Kabuto H., Yamamishi T.T., Janjua N., Takayama F., Mankura M., Effects of squalene/squalane on dopamine levels, antioxidant enzyme activity and fatty acid composition in the striatum of Parkinson's disease mouse model, *J. Oleo. Sci.*, 2013, 62(1), 21-28
- [37] Becker E., Messner B., Berndt J., Two mechanisms of CCl<sub>4</sub>-induced fatty liver: lipid peroxidation or covalent binding studied in cultured rat hepatocytes, *Free Radic. Res. Commun.*, 1987, 3 (-5), 299-308
- [38] Manna P., Bhattacharyya S., Das J., Ghosh J., Sil P., Phytomedicinal role of *Pithecellobium dulce* against CCl<sub>4</sub>-mediated hepatic oxidative impairments and necrotic cell death, *Evidence-Based Complementary and Alternative Medicine*, 2011, 832805, 17 pages
- [39] Yavuz T., Delibas N., Yildirim B., Altuntas I., Candir O., Cora A., et al., Vascular wall damage in rats induced by methidathion and ameliorating effect of vitamins E and C, *Arch. Toxicol.*, 2004, 78(11), 655–9
- [40] Shiryaeva A., Arkandjeva A., Emelyanova L., Sakuta G., Morozov V., Superoxide anion production by the respiratory chain of hepatocytes of rats with experimental toxic hepatitis, *J. Bioener. Biomembranes*, 2009, 41(4), 379-385
- [41] Yim M.B., Chock P.B., Stadtman E.R., Enzyme function of copper, zinc superoxide dismutase as a free radical generator, *J. Biol. Chem.*, 1993, 268, 4099-4105
- [42] Ames B.M., Shigenaga M.K., Hagen T., Oxidants, antioxidants and the degenerative diseases of aging, *Proc. Nat. Acad. Sci.*, 1993, 90(17), 7915-7922
- [43] Siemieniuk E., Kolodziejczyk L., Skrzydlewska E., Oxidative modifications of rat liver cell components during *Fasciola hepatica* infection, *Toxicol. Mechanism Meth.*, 2008, 18(6), 519-524
- [44] Chen S., Zou L., Li L., Wu T., The protective effect of glycyrrhetic acid on carbon tetrachloride-induced chronic liver fibrosis in mice via up-regulation of Nrf-2, *PLOS One*, 2013, 8(1), e53662
- [45] Starke D.W., Chen Y., Bapna C.P., Lesnefsky E.J., Mieyal J.J., Sensitivity of protein sulfhydryl repair enzymes to oxidative stress, *Free Rad. Biol. Med.*, 1997, 23(3), 373-384
- [46] Tamai K., Satih K., Tsuchida S., Hatayama I., Maki T., Sato K., Specific inactivation of glutathione S transferase in class Pi by SH-modifiers, *Biochem. Biophys. Res. Commun.*, 1990, 167, 331-338
- [47] Arab K., Rossary A., Souler L., Steghens J.P., Conjugated linoleic acid unlike other unsaturated fatty acids, strongly induces glutathione synthesis without any lipoperoxidation, *Brit. J. Nut.*, 2008, 96(5), 811-819
- [48] Prabu S.M., Shagirtha K., Renugadevi J., Amelioration of cadmium-induced oxidative stress, impairment in lipids and plasma lipoproteins by the combined treatment with quercetin and  $\alpha$ -tocopherol in rats, *J. Food Sci.*, 2010, 75(7), 132-140
- [49] Chen L.H., Interaction of vitamin E and ascorbic acid, *In vivo*, 1989, 3(3), 199-209
- [50] Bandopadhyay D., Das D., Banerjee R.K., Reactive oxygen species: Oxidative damage and pathogenesis, *Curr. Sci.*, 1999, 77(5), 658-666
- [51] Sudheesh N.P., Ajith T.A., Janardhanan K.K., Hepatoprotective effects of DL- $\alpha$ -lipoic acid and  $\alpha$ -Tocopherol through amelioration of the mitochondrial oxidative stress in acetaminophen challenged rats, *Toxicol. Mechanism Meth.*, 2013, 23(5), 368–376
- [52] Belury M.A., Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action, *Ann. Rev. Nutr.*, 2002, 22, 505-531
- [53] Klein E., Weber N., *In vitro* test for the effectiveness of antioxidants as inhibitors of thiyl radical-induced reaction fatty acids, *J. Agric. Food Chem.*, 2001, 49(3), 1224-1227
- [54] Conforti F., Sosa S., Marrelli M., Menichini F., Statti G.A., Uzunov D., et al., In vivo anti-inflammatory and in vivo antioxidant activities of Mediterranean dietary plants, *J. Ethnopharmacol.*, 2008, 116, 144-151
- [55] Mirmiran P., Bahadoran Z., Azizi F., Functional foods-based diet as a novel dietary approach for management of type 2 diabetes and its complication: A review, *World J. Diabetes*, 2014, 5(3), 267-281

- [56] Ozturk F., Gul M., Ates B., Ozturk I.C., Cetin A., Vardi N., et al., Protective effect of apricot (*Prunus armeniaca* L.) on hepatic steatosis and damage induced by carbon tetrachloride in Wistar rats, *British J. Nutr.*, 2009, 102(12), 1767–1775
- [57] Domitrovic R., Jakovac H., Blagojevic G., Hepatoprotective activity of berberine is mediated by inhibition of TNF-alpha, COX-2, and iNOS expression in CCl<sub>4</sub>-intoxicated mice, *Toxicology*, 2011, 280, 33–43
- [58] Chamulitrat W., Blazka M.E., Jordan S.J., Luster M.I., Mason R.P., Tumor necrosis factor- $\alpha$  and nitric oxide production in endotoxin-primed rats administered carbon tetrachloride, *Life Sci.*, 1995, 57(24), 2273–2280
- [59] Garcia-Bailo B., Roke K., Mutch D.M., El-Soheby A., Badawi A., Association between circulating ascorbic acid,  $\alpha$ -tocopherol, 25-hydroxyvitamin D and plasma cytokine concentrations in young adults: a cross-sectional study, *Nutr. Met.*, 2012, 9, 102-110
- [60] Micallef M.A., Garg M.L., Anti-inflammatory and cardioprotective effects of n-3 polyunsaturated fatty acids and plant sterols in hyperlipidemic individuals, *Atherosclerosis*, 2009, 204(2), 476-482