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 (CCl₄) 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 and restored the biochemical parameters, hepatic architecture and expression of TNF-alpha. These findings suggest that SMSO was effective in countering the damaging effects of CCl₄-induced injury in hepatocytes, probably due to its inherent antioxidant properties.
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 CCl₄-induced hepatotoxicity in mice.

2 Experimental Procedures

2.1 Sylilbum 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 μL of oil solution was injected into the gas chromatograph. Compounds were separated on a Zebron ZB-5MS column (30 m × 0.25 mm × 0.25 μm). The initial temperature of the oven (60°C) was maintained for 3 minutes and subsequently heated to 300°C at a heating rate of 10°C/min and maintained at this temperature for 8 minutes. The temperatures of the electron ionization (EI) ion source and the interface were 300°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°C.

2.3 Animal treatment

Male Swiss mice (25±3 g), supplied by the Animal House of the Vasile Goldis Western University of Arad, were used. They were housed under controlled conditions (22–25°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 g/kg b.w. was chosen according to previous experiments with different seed oils [8, 9, 20], as well as the toxicant (CCl₄) dose of 1.0 ml/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 22nd day they were deprived of food for 24 h.
- **Group 2:** CCl₄ group received the ISS every day for 21 days and were subsequently i.p. injected with CCl₄ at a dose of 1.0 ml/kg b.w. in 50% olive oil (1:1) on the 22nd day.
- **Group 3:** SMSO pre-treated group orally received Silybum marianum seed oil (SMSO) in a dose of 10 g/kg b.w. for 21 days and were subsequently i.p. injected with CCl₄ (1.0 ml/kg b.w.) on the 22nd day.
- **Group 4:** SMSO group orally received SMSO alone (10 g/kg b.w.) daily for 21 days and on the 22nd day they 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 CCl₄ 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 × 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 × 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 H₂O₂ decomposition. One unit of CAT activity was calculated as one µmole H₂O₂/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 thiorbituric acid. Relative fluorescence units (RFU) recorded at Spectrofluorometer Jasco FP-6300 (λ_ex = 520 nm; λ_em = 549 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 mm 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-α 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 ± 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 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 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 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 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 CCl$_4$ induced a reduction in CAT and SOD specific activities in mice liver ($p < 0.001$)
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 CCl₄ 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 CCl₄ 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-a expression, elevated by CCl₄ pro-inflammatory activity, are shown in Figure 5. The liver slides of the control mice did not show substantial TNF-a immunopositivity (Figure 5 A3). The TNF-a expression in the SMSO only group was similar to the controls (Figure 5 D3). By contrast, strong TNF-a expression was observed for the CCl₄ group. TNF-a 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 CCl₄-intoxicated mice pretreated with SMSO (Figure 5 C3).

### 3.5 Electron microscopic examination

The hepatocyte ultrastructure was normal in the control group. In the CCl₄ treated group, most of the hepatocytes showed two populations of lipid droplets (large and small), degenerated organelles and smooth reticulum.
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 CCl₄ (Figure 6 C1-2).
Antioxidant and hepatoprotective activity of milk thistle (Silybum marianum L. Gaertn.) seed oil

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.

4 Discussion

The hepatotoxicity following CCl₄ administration in rodent cells, as well as the generation of oxidative stress, is well documented by previous studies [29,30]. In CCl₄ metabolism, the trichloromethyl free radical formation (CCl₃) under cytochrome P₄₅₀ 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 (CCl₃OO). 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 CCl₄. (A) Control group; normal aspect of nucleus (N) and nuclear envelope (arrowhead), and few lipid drops (L); (B) CCl₄ 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 + CCl₄ 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 corrobate 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], squalen, 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 CCl₄ 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, CCl₄ 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 CCl₄-induced hepatic injury, as evidenced by decreased plasma AST (by 42.45%), ALT (by 50%) and GGT (by 32%) compared to CCl₄ 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 CCl₄-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 CCl₄ 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 CCl₄ treated group.

The first targets of reactive oxygen species are polyunsaturated fatty acids from phospholipids and lipid peroxidation, quantified as MDA concentration, as 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 CCl₄, 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 centrlobular steatosis and necrosis after exposure to CCl₄ [21,57].

Inflammatory cells were also observed, being highlighted by a significant release of TNF-α from Kupffer cells especially, localized around the central vein and...
extending to neighboring veins. Similarly, other studies have shown that TNF-α played an important role in the pathogenesis of CCl₄-induced hepatotoxicity [58].

Previous studies have recognized the antioxidant and anti-inflammatory properties of ascorbic acid, α-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 CCl₄-induced oxidative stress and liver injury. These findings suggest that the SMSO was effective in counteracting the damaging effect of CCl₄ in murine hepatocytes and indicate that the preventive effects of SMSO against CCl₄-induced liver damage are related to its antioxidant properties, which are likely due to the presence of tocopherol and ascorbic acid 6-hexadecanoate.

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Conflict of interest: Authors declare nothing to disclose.

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