

Article

Hepatoprotective Effects of *Berberis vulgaris* L. Extract/ β Cyclodextrin on Carbon Tetrachloride–Induced Acute Toxicity in Mice

Anca Hermenean ^{1,2}, Cristina Popescu ², Aurel Ardelean ², Miruna Stan ³, Nicoleta Hadaruga ⁴, Ciprian-Valentin Mihali ², Marieta Costache ³ and Anca Dinischiotu ^{3,*}

¹ Department of Histology, Faculty of Medicine, Pharmacy and Dentistry, Vasile Goldis Western University of Arad, 1 Feleacului, Arad 310396, Romania; E-Mail: anca.hermenean@gmail.com

² Department of Experimental and Applied Biology, Institute of Life Sciences, Vasile Goldis Western University of Arad, 86 Rebreanu, Arad 310414, Romania; E-Mails: purseaga36@gmail.com (C.P.); aardelean@uvvg.ro (A.A.); mihaliciprian@yahoo.com (C.-V.M.)

³ Department of Biochemistry and Molecular Biology, University of Bucharest, 91-95 Splaiul Independentei, Bucharest 050095, Romania; E-Mails: miruna_stan@yahoo.com (M.S.); marietacostache@yahoo.com (M.C.)

⁴ Food Quality Department, Faculty of Food Processing Technology, Banat's University of Agricultural Sciences and Veterinary Medicine, 119 Calea Aradului, Timisoara 300645, Romania; E-Mail: nico_hadaruga@yahoo.com

* Author to whom correspondence should be addressed; E-Mail: dinischiotu@yahoo.com; Tel.: +40-21-318-1575-108; Fax: +40-21-318-1575-103.

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Abstract: The present study investigated the capacity of formulated *Berberis vulgaris* extract/ β -cyclodextrin to protect liver against CCl₄-induced hepatotoxicity in mice. Formulated and non-formulated extracts were given orally (50 mg/kg/day) to mice for 7 days and were then intra-peritoneally injected with 1.0 mL/kg CCl₄ on the 8th day. After 24 h of CCl₄ administration, an increase in the levels of aspartate-amino-transferase (AST), alanine-amino-transferase (ALT) and malondialdehyde (MDA) was found and a significant decrease in superoxide-dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione-peroxidase (GPx) levels could be detected. This was accompanied by extended centrilobular necrosis, steatosis, fibrosis and an altered ultrastructure of hepatocytes.

Pre-treatment with formulated or non-formulated extract suppressed the increase in ALT, AST and MDA levels and restored the level of antioxidant enzymes at normal values. Histopathological and electron-microscopic examination showed milder liver damage in both pre-treated groups and the protective effect was more pronounced after the formulated extract was administered. Internucleosomal DNA fragmentation induced by CCl₄ was reduced in the group which received non-formulated extract and absent in the group which received formulated extract. Taken together, our results suggest that *Berberis vulgaris*/β-cyclodextrin treatment prevents hepatic injury induced by CCl₄ and can be considered for further nutraceutical studies.

Keywords: *Berberis vulgaris* extract; β-cyclodextrin; hepatoprotection; oxidative stress

1. Introduction

The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism and its portal location within circulation [1].

Several xenobiotics, such as: arsenic, cadmium, chloroform, carbon tetrachloride, pesticides, dioxins, nitrosamines [2] become hepatotoxic through metabolism and bioactivation processes. The first phase reactions (oxidation, reduction or hydrolysis, *etc.*), followed by conjugation ones, play an important role in converting xenobiotics into a form suitable for elimination in order to prevent xenobiotic-induced liver injury [3,4]. But these metabolic processes require multiple biochemical transformations, and in some cases, the intermediates mediate toxic responses. Therefore, the liver is potentially susceptible to injury during the act of performing its function [5].

Carbon tetrachloride (CCl₄) is the best characterized model of xenobiotic-induced hepatotoxicity and commonly used for the screening of hepatoprotective effects of drugs and natural products. Liver damage induced by carbon tetrachloride (CCl₄) is based on metabolism to trichloromethyl radicals (CCl₃· and/or CCl₃OO·) under cytochrome P450 action [6,4].

Many herbs are used by traditional medicine to prevent and treat liver diseases. Several hundred plants have been examined for use in a wide variety of liver disorders, but just a part have been well investigated, such as: *Silybum marianum* (milk thistle), *Picrorhiza kurroa* (kutkin), *Curcuma longa* (turmeric), *Camellia sinensis* (green tea), *Chelidonium majus* (greater celandine), *Glycyrrhiza glabra* (licorice), *Allium sativa* (garlic) [7–9].

Berberine (*Berberis vulgaris* L., family Berberidaceae) is a herb growing in Europe and Asia, which has been less investigated from a therapeutic point of view. The root, bark, leaf and fruit have been used in traditional medicine to treat diarrhea, colitis, gastroenteritis, and hepatic disorders. Several alkaloid constituents with an isoquinolinic nucleus, such as berberine, berbamine and palmatine were isolated [10]. Other compounds like terpenoids lupeol, oleanolic acid, stigmasterol and stigmasterol glucoside [11] as well as polyphenols [12] were also identified. Nevertheless, berberine is the most important alkaloid that is generally claimed to be responsible for their beneficial effects [12]. There are multiple pharmacological effects of berberine, such as antimicrobial [13,14], anti-tumor [15–17], and

anti-inflammatory effects [18–20]. It also has effects on the gastro-intestinal [11,21–23], cardiovascular [24–26] and nervous [27] systems.

Several studies revealed that berberine exerts preventive and curative effects on liver against experimental injuries. Feng *et al* [28] reported a significant decrease of hepatic marker enzymes in CCl₄ treated rats after oral administration of berberine at 80, 120, 160 mg/kg daily doses compared to control animals, whereas a lower dose (4 mg/kg) was not effective [29]. On the other hand, intraperitoneal administration of berberine in rats in a dose of 0.5–5 mg/kg counteracted the damaging effect of tert-butyl hydroperoxide by reducing the generated oxidative stress [30]. A few studies concerning the oral treatment of rats with extract of *Berberis vulgaris* root have been reported [31]. The dose that effectively protected the liver was 900 mg/kg, 30 times higher than average dose (THD) used in some traditional systems of medicine.

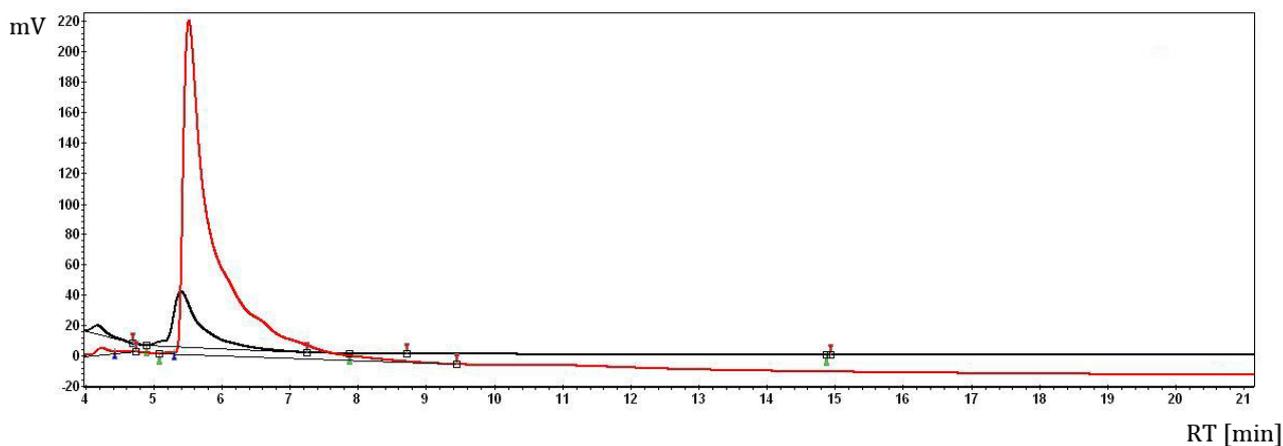
There is no report in the literature on the protective effect of formulated *Berberis vulgaris* L. extract/ β -cyclodextrin against CCl₄-induced liver injury. Therefore, the present study was carried out to evaluate the preventive effect of *Berberis vulgaris* L. extract against CCl₄-induced acute hepatotoxicity in mouse liver as well as the effects of β -cyclodextrin complexation, in hepatoprotective therapeutical formulations.

2. Results and Discussion

2.1. The Complex of *Berberis vulgaris* Extract and β Cyclodextrin

The presence of the berberine (the main compound from *Berberis vulgaris* L. bark) in the *B. vulgaris* L./ β -cyclodextrin complex was identified by means of HPLC analysis, according to the previous study (Figure 1) [32].

Figure 1. HPLC analysis of the re-extracted (ethanolic solution) *Berberis vulgaris* L. sample from the β -cyclodextrin complex (lower chromatogram—black) in comparison with the berberine standard solution (upper chromatogram—red).



The *B. vulgaris* L. extract/ β -cyclodextrin complex formation is proved by thermal and calorimetric analyses. Thus, the overall profile of the DSC (differential scanning calorimetry) and TG (thermogravimetry) analyses of the complex are very different in comparison with the β -cyclodextrin case; the formation of the complex is especially revealed by the shifting of the water dissociation

calorimetric process to lower temperatures in the DSC analysis. This is explained by the replacing of these water molecules from β -cyclodextrin with the bioactive *B. vulgaris* compounds (such as berberine, which was previously identified by HPLC analysis) (Figure 2). Furthermore, the water concentration in the β -cyclodextrin complex is approximately 3% lower than in the case of initial β -cyclodextrin, which are revealed by both DSC and TG analyses (1200 J/g for *B.v./* β CD complex and 780 J/g for β CD in the DSC analysis; 10.2% mass loss for *B.v./* β CD complex and 13.2% for β CD in the TG analysis; Figures 2 and 3). These means that some water molecules (especially from the β -cyclodextrin cavity) are replaced by *Berberis vulgaris* L. extract compounds (especially by the most concentrated berberine, revealed by HPLC analysis) in the nanoencapsulation process, which leads to the formation of *Berberis vulgaris* extract/ β -cyclodextrin complex.

Figure 2. Differential scanning calorimetry (DSC) analysis of the *Berberis vulgaris* L. extract/ β -cyclodextrin complex (red curve) and the corresponding β -cyclodextrin sample (black curve).

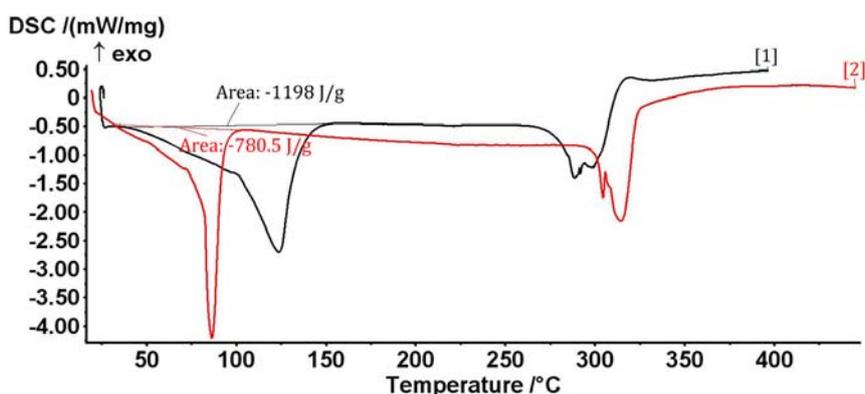
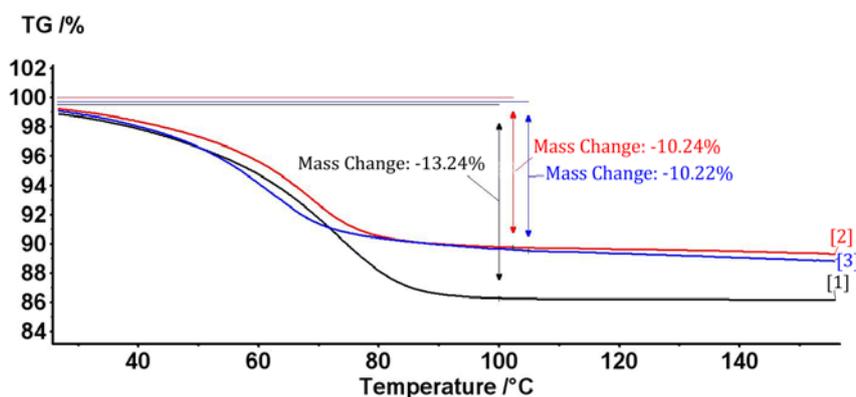


Figure 3. Thermogravimetry (TG) analysis of the *Berberis vulgaris* L. extract/ β -cyclodextrin complexes (upper curves) and the corresponding β -cyclodextrin sample (lower curve).

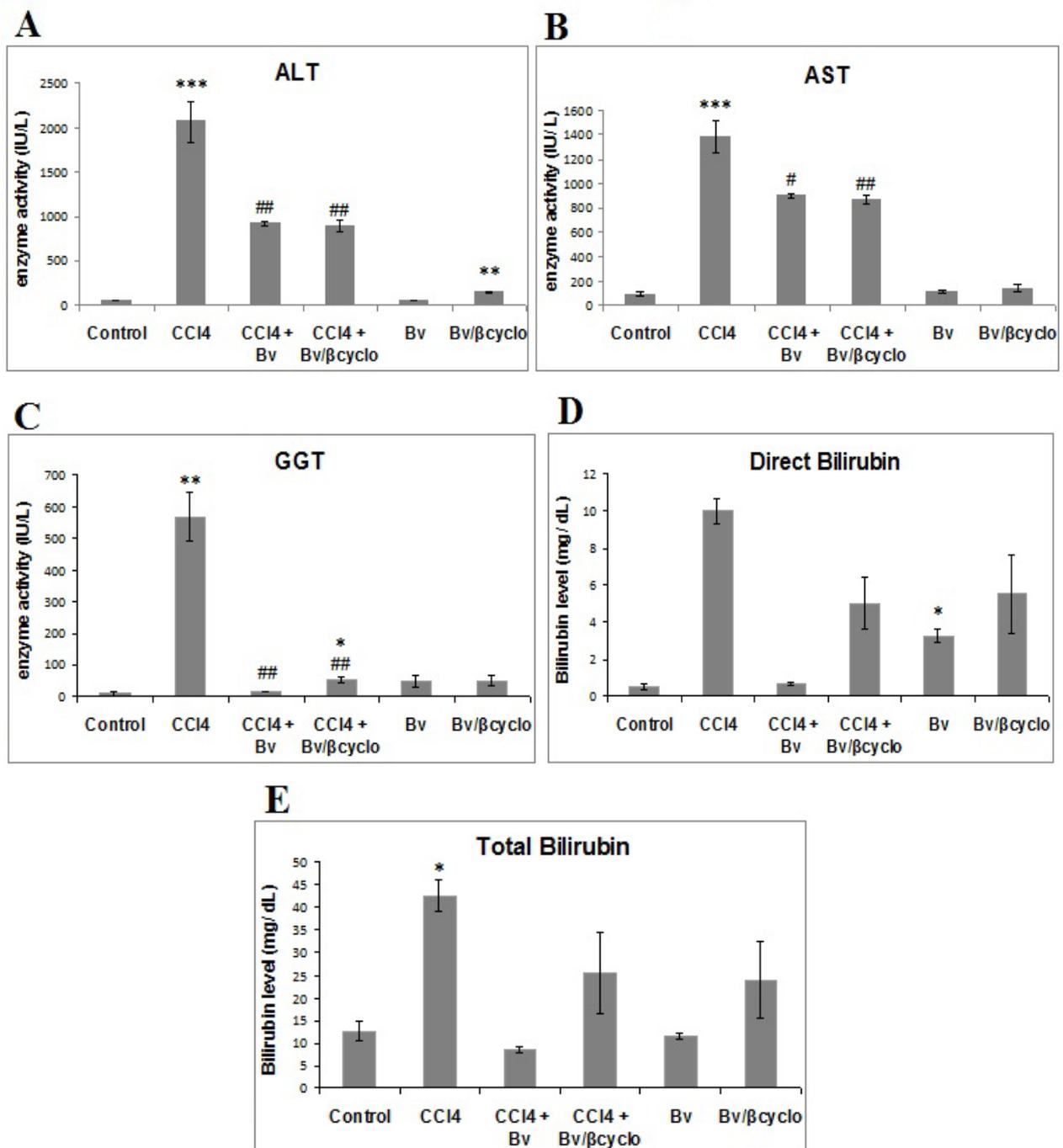


2.2. Effects of *Berberis vulgaris* Extract/ β Cyclodextrin Pre-Treatment on Serum ALT, AST, γ -GT, Total and Direct Bilirubin

Effects of non-formulated and formulated *Berberis vulgaris* extract on serum ALT, AST, γ -GT activities, as well as total and direct bilirubin, in mice from various treatment groups are shown in Figure 4. The dose of 50 mg/kg formulated and non-formulated extract of *Berberis vulgaris* was

chosen according to previous experiments [31], taking into account that the maximum concentration of berberine was found in bark (the plant used for our extract preparation) followed by root, leaves and fruit [32].

Figure 4. The effects of a 50 mg/kg dose of formulated and non-formulated *Berberis vulgaris* extract against liver injuries induced by CCl₄ for 24 h on serum (A) alanine-amino-transferase (ALT); (B) apartate-amino-transferase (AST); (C) γ -glutamyl transferase (γ -GT); (D) direct bilirubin; (E) total bilirubin. Values are expressed as mean \pm SD ($n = 8$). * $p < 0.05$ significantly different from the control group; # $p < 0.05$ significantly different from the CCl₄-treated group.



After 24 h of CCl₄ treatment, the ALT (Figure 4A), AST (Figure 4B) and γ -GT (Figure 4C) serum activities significantly increased ($p < 0.001$) by 40.46, 14.86 respectively 59.82 folds, whereas the total bilirubin and direct bilirubin levels significantly raised by 3.4 and 20 times compared to control group.

Pre-treatment with 50 mg/kg of *Berberis vulgaris* extract/ β -cyclodextrin decreased significantly the CCl₄-induced elevation of serum aminotransferases by 50% for ALT (Figure 4A). The non-formulated *Berberis vulgaris* extract showed no increase of ALT activity whereas the formulated one significantly raised it compared to control group. In the case of AST, both formulated and non-formulated *Berberis vulgaris* extracts reduced the activity elevation induced by CCl₄ treatment by about 35%, whereas in the groups where only one type of extract was administered, AST levels were the same as in the control group (Figure 4B).

In addition, the up-regulation of γ -GT activity determined by CCl₄ exposure was diminished by about 10 times by pre-treatment with formulated and non-formulated extracts (Figure 4C).

In the same time, a significant decrease of serum total and direct bilirubin by about 50% was observed in the pre-treated mice with formulated and non-formulated *Berberis vulgaris* extracts.

It is well known that chemical agents produce liver damage, causing high increases in bilirubin and enzymes activity presented above, which are released into serum [4,33,34]. The elevated activities of ALT, AST and γ -GT as well as total and direct bilirubin are indicative for cellular leakage and loss of the functional integrity of the liver cell membrane [35,36], and mitochondrial disruption respectively [37].

In our study, the pre-treatment with both formulated and non-formulated extracts, suppressed the increment of these parameters induced by CCl₄ administration, results similar with those obtained by 160 mg/kg berberine pre-treatment [28]. It seems that the *Berberis vulgaris* extract entrapped in β cyclodextrin was more efficient at a lower dose than the alkaloid itself.

2.3. Effects of *Berberis vulgaris* Extract/ β Cyclodextrin Pre-Treatment on Antioxidant Status and Lipid Peroxidation

As shown in Figure 5, activities of CAT, SOD and GPx were significantly decreased in CCl₄ treated mice compared to control ones. Formulated and non-formulated *Berberis vulgaris* extract pre-treatment significantly prevented inhibition of these enzymes activities caused by CCl₄, and SOD activity of non-formulated *Berberis vulgaris* extract pre-treatment was slightly decreased compared to formulated extract and CCl₄ treated group. In addition, CCl₄ toxicity was confirmed by depletion of cellular GSH content in liver cells. Pre-treatment with non-formulated and *Berberis vulgaris*/ β cyclodextrin extract determined a significant increase of GSH concentration compared to CCl₄ groups. It should be mentioned that CAT, SOD and GPx activities in mice liver treated only with formulated or non-formulated *Berberis vulgaris* extract were approximately equal to those from control group, and GSH content was higher in these two groups compared to control. The level of this tripeptide, important in redox homeostasis and free radical scavenging detoxification of electrophils, increased probably by stimulation of γ -glutamyl cycle and/or enzymatic regeneration of GSH from oxidized glutathione.

Lipid peroxidation was determined by measuring the concentration of malondialdehyde. As Figure 6 illustrates, after 24 h of CCl₄ treatment, MDA content significantly increased, but its high level was markedly reduced through treatment with formulated and non-formulated *Berberis vulgaris*

extracts to levels almost similar to control group. In the case of treatment with *Berberis vulgaris* extracts alone, MDA levels close to normal group were observed.

Previous studies showed that CCl₄ caused hepatocellular damages through an increase in the formation of DNA strand breaks, 8-oxodeoxyguanosine and MDA-DNA adducts under conditions of increased lipid peroxidation, and high depletion in glutathione content [38].

It is well known that antioxidant enzymes and GSH deficiency enhances the possibility for lipid peroxidation, and may cause irreversible damage to the liver tissue [39]. The protective effects of *Berberis vulgaris* extracts on the liver of CCl₄ treated mice highlighted their elevated antioxidant activity, polyphenolic content, and rich nutritional composition, which sustain redox properties of extracts [40–42]. The antioxidant effects of *Berberis vulgaris* extract shown here were in agreement with a previous report [31].

We have shown that GPx and GSH levels of non-formulated extract pre-treatment group were statistically similar with those measured in the liver of *Berberis vulgaris* extract/ β -cyclodextrin pre-treated mice. The pre-treatment with both *Berberis vulgaris* extracts attenuated CCl₄ toxicity in a very efficient way, improving the antioxidant status of liver tissue.

It seems that they did not stimulate the antioxidant enzymes because CAT, SOD and GPx activities were not increased in mice treated only with formulated or non-formulated *Berberis vulgaris* extract.

Figure 5. Protective effect of a 50 mg/kg dose of non-formulated *Berberis vulgaris* extract and *Berberis vulgaris* extract/ β -cyclodextrin on CCl₄-induced oxidative stress in mice liver. Oxidative stress was assessed by measuring the (A) catalase (CAT); (B) superoxide dismutase (SOD) and (C) glutathione peroxidase (GPx) activity; (D) glutathione (GSH) level. * $p < 0.05$ significantly different from the control group; # $p < 0.05$ significantly different from the CCl₄-treated group.

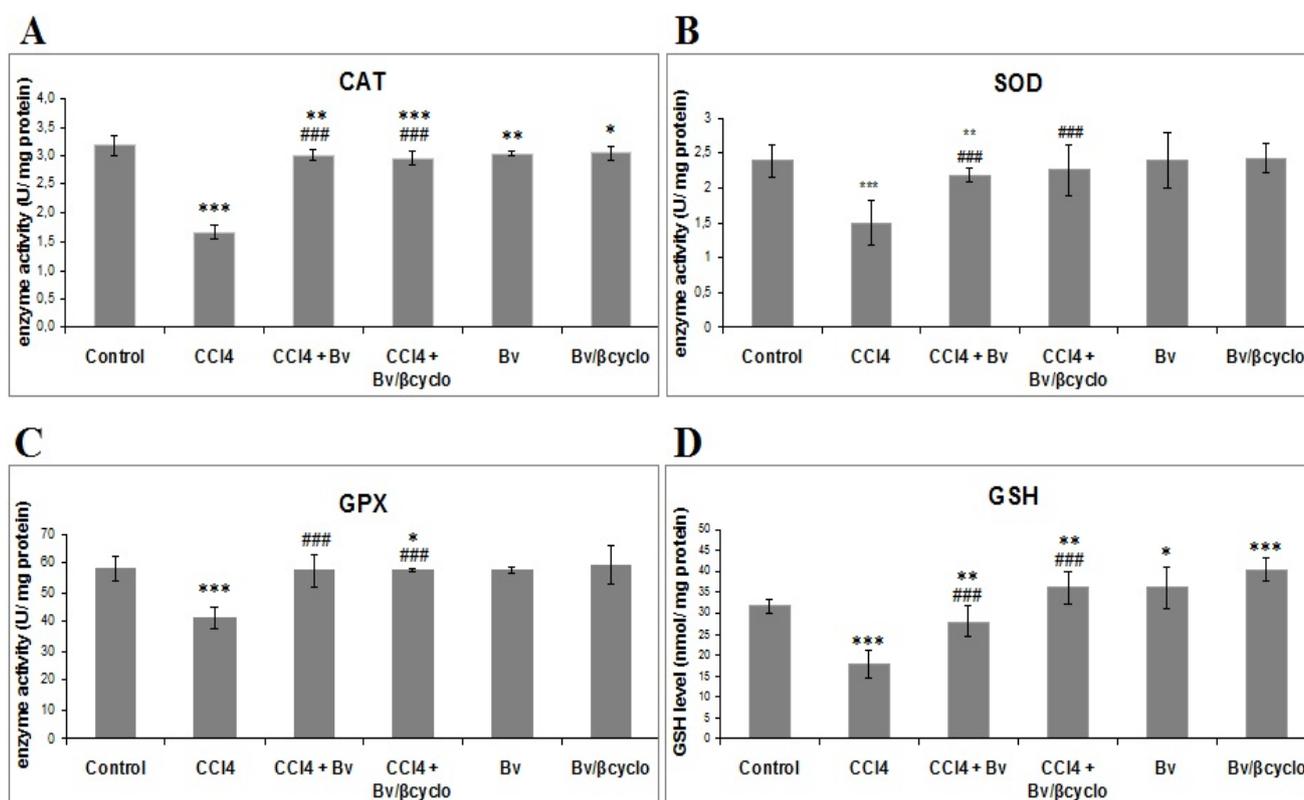
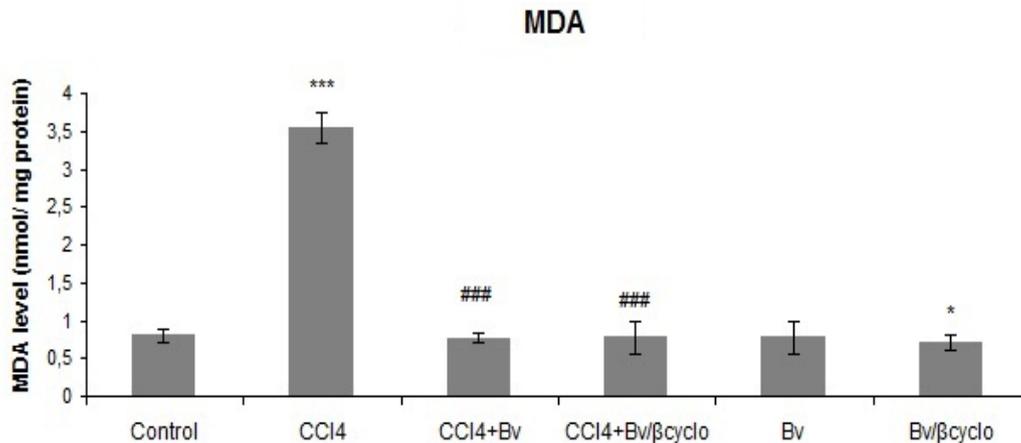


Figure 6. Protective effect of a 50 mg/kg dose of non-formulated *Berberis vulgaris* extract and *Berberis vulgaris* extract/ β -cyclodextrin CCl₄-induced lipid peroxidation in mice liver. * $p < 0.05$ significantly different from the control group; # $p < 0.05$ significantly different from the CCl₄-treated group.



2.4. Histopathology

Light microscopic evaluation of liver tissues showed normal liver architecture (Figure 7A-1) without steatosis (Figure 7A-3) and fibrosis aspects (Figure 7A-2) in control group. Normal glycogen deposits are present into hepatocytes cytoplasm (Figure 7A-4).

Figure 7. Protective effect of a 50 mg/kg dose of *Berberis vulgaris* extract and *Berberis vulgaris* extract/ β -cyclodextrin on structure of hepatocytes against liver injuries induced by CCl₄. (A) Control group; (B) CCl₄ group; (C) *Berberis vulgaris* extract + CCl₄ group; (D) *Berberis vulgaris* extract/ β cyclodextrin + CCl₄ group. **1.** H & E staining (nuclei-blue; cytoplasm-red), **2.** Fouchet Van Gieson staining (nuclei-red; cytoplasm-yellow; collagen- red), **3.** Oil Red staining (nuclei-blue; lipid drops–red), **4.** P.A.S Periodic Acid Schiff staining (glycogen deposits—pink violet). N: centrilobular necrosis; C: collagen proliferation; S: steatosis; G: glycogen deposits depletion. Scale: 50 μ m.

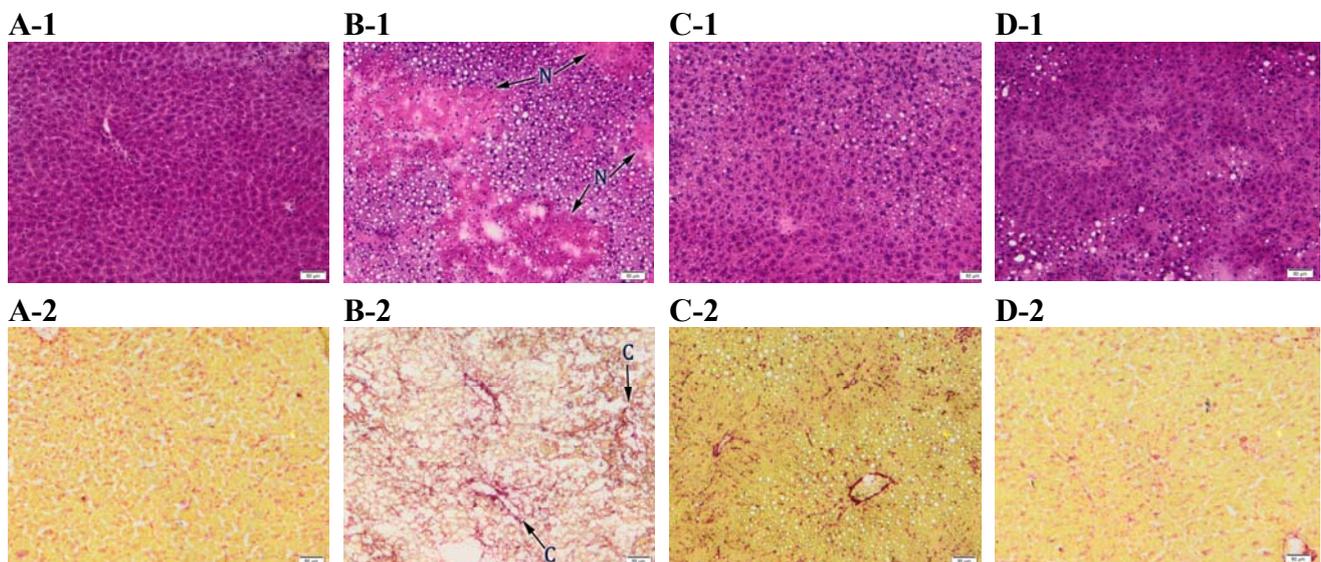
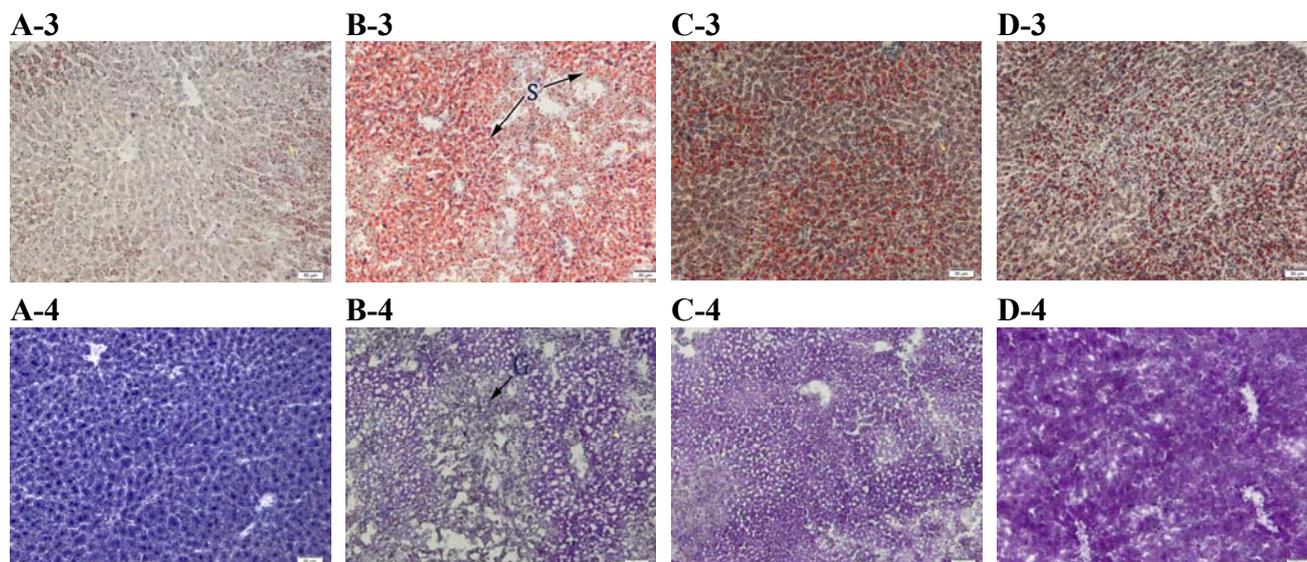


Figure 7. Cont.



After 24 h of CCl_4 exposure, necrobiotic changes of hepatocytes including vacuolar degeneration, nuclear pyknosis and necrosis (Figure 7B-1) were observed, which were in agreement with previous reports [36,43]. The necrosis was more pronounced in the centrilobular area with the formation of bridges from one central vein to another (Figure 7B-1), followed by infiltration of inflammatory cells, sinusoid congestion, macro and microvesicular steatosis of hepatocytes (Figure 7B-3). Inflammatory cell infiltration and slight fibrosis around central vein and sinusoids were detected (Figure 7B-2). Glycogen deposits were severely diminished compared to the control group (Figure 7B-4), probably due to the high-energy demand of the hepatocytes for the possible repair processes in CCl_4 -given animals [44].

The seven-day preventive treatment with 50 mg/kg of formulated and non-formulated *Berberis vulgaris* extract, showed mild to moderate accumulation of lipid drops and reduction of inflammatory infiltrates compared to the CCl_4 group (Figure 7C-3, D-3). In both *Berberis* pre-treated groups, an absence of hepatocellular necrosis areas (Figure 7C-1, D-1) was observed. Vacuolated hepatocytes were seen in some centrilobular areas but to a lesser extent compared to CCl_4 group (Figure 7C-1, D-1). Similarly, Feng *et al.* [28] reported that fatty content change, necrosis and lymphocyte infiltration were improved in the histological sections from 80, 12 and 160 mg/kg berberine pre-treated and post-treated mice in a dose-dependent manner.

Fibrosis around central vein and sinusoids was reduced in both pre-treated groups (Figure 7C-2, D-2). Similar results were observed with berberine that prevented liver fibrosis due to the decreased number of hepatic stellate cells (HSCs) [45] or by regulating the lipid peroxidation and antioxidant system [46].

Also, glycogen deposits were highly restored in the *Berberis vulgaris* extract/ β -cyclodextrin group (Figure 7D-4) than in non-formulated *Berberis vulgaris* extract pre-treatment (Figure 7C-4). Our results are in accordance with previous data which have shown that hepatic glycogen levels increased after 75 mg/kg of berberine were administered to rats [47].

2.5. Electron Microscopy

The ultrastructure of hepatocytes was normal in the control group with regular aspect of nuclear shape and rER's profiles (Figure 8A-2), few lipid globules, and normal glycogen deposits into cytoplasm (Figure 8A-1). In the CCl₄ treated group most of the hepatocytes showed large lipid globules and glycogen loss, according to optic microscopy analysis. Some hepatocytes showed an oedematous cytoplasm matrix with two populations of lipid globules -large and small, degenerated organelles and proliferation of smooth reticulum vesicles (Figure 8B-1). There were irregular lamellar organization and large dilatations with focal breaks in rERs of hepatocytes of CCl₄ treated group in many areas, associated with dilated perinuclear space and enlarged pores (Figure 8B-2). The changes of hepatocytes ultrastructure [48] were probably due to the injuries in membrane structure caused by lipid peroxidation.

Pre-treatment with 50 mg/kg of formulated and non-formulated *Berberis vulgaris* extract significantly reduced the volume and number of lipid globules in the hepatocytes (Figure 8C-1, D-1), more obvious than in non-formulated *Berberis vulgaris* extract (Figure 8C-1). The organelle and cytoplasm structures were widely protected against the effects of CCl₄. Large dilatations with focal breaks in rERs of hepatocytes, associated with dilated perinuclear space and enlarged pores were not seen in either pre-treated group (Figure 8C-2, D-2). Although glycogen deposits are increased both in formulated and non-formulated *Berberis vulgaris* groups compared to CCl₄ (Figure 8C-2, D-2).

Figure 8. Protective effect of a 50 mg/kg dose of *Berberis vulgaris* extract and *Berberis vulgaris* extract/ β cyclodextrin on ultrastructure of hepatocytes against liver injuries induced by CCl₄. **(A)** Control group; normal aspect of nucleus (N), glycogen deposits (G) 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)** *Berberis* extract + CCl₄ group; normal aspect of nuclear shape (arrowhead) and rER (arrow); Mild reduction of number and size of lipid drops (L) and moderate recovery of glycogen deposits (G); **(D)** *Berberis vulgaris* extract/ β cyclodextrin + CCl₄ group; Glycogen deposits recovery (G) and normal aspect of rER (arrow).

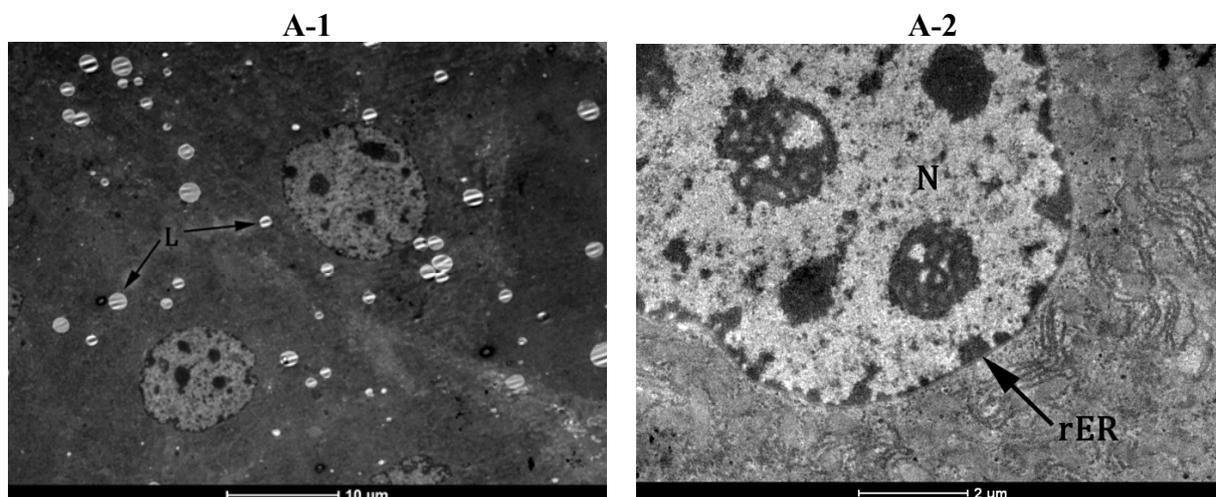
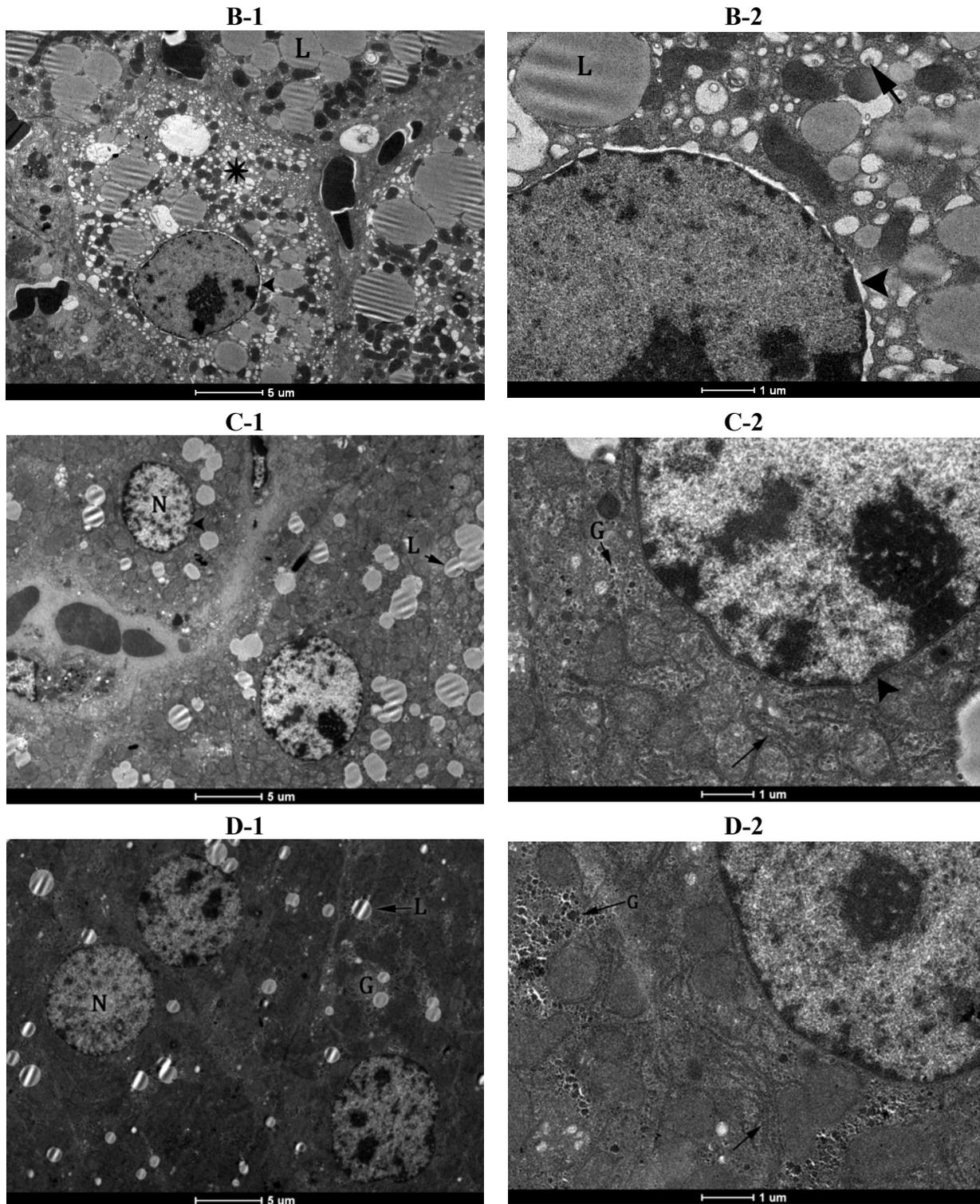


Figure 8. Cont.



2.6. Effects of *Berberis vulgaris* Extract/ β -Cyclodextrin Pre-Treatment on DNA Damage

DNA internucleosomal fragmentation was seen in liver samples of CCl_4 treated group and a reduced level of it appeared in the mice group pre-treated with non-formulated *Berberis vulgaris* extract. At the same time, the pre-treatment with *Berberis vulgaris* extract/ β -cyclodextrin completely

suppressed this process (Figure 9A). Also, no DNA internucleosomal fragmentation was induced by either formulated and non-formulated *Berberis vulgaris* extracts in the absence of CCl₄ treatment.

The methyl-green pyronin (MGP) histochemical investigation of liver tissue was in agreement with DNA fragmentation assay. Large pale green areas of damaged DNA were seen in the CCl₄ group especially in the centrilobular area with the formation of bridges from one central vein to another (Figure 9B-2) which overlap with areas of the liver affected by necrobiosis observed in H & E micrographs. These areas are more reduced in the group pre-treated with non-formulated *Berberis vulgaris* extract (Figure 9B-3) and are absent in the group pre-treated with *Berberis vulgaris* extract/ β -cyclodextrin (Figure 9B-4), as in the case of normal mice, where intense pink areas of RNA are also present (Figure 9B-1).

Figure 9. Effects of *Berberis vulgaris* extract/ β -cyclodextrin on CCl₄-induced DNA damage. (A) Lane 1-DNA isolated from normal liver; Lane 2: DNA isolated from CCl₄ intoxicated liver; Lane 3: DNA isolated from liver treated with *Berberis vulgaris*; Lane 4: DNA isolated from liver pre-treated with *Berberis vulgaris* extract followed by i.p. CCl₄ injection; Lane 5: DNA isolated from liver treated with *Berberis vulgaris* extract/ β cyclodextrin; Lane 6: DNA isolated from liver pre-treated with *Berberis vulgaris* extract/ β cyclodextrin followed by i.p. CCl₄ injection; Lane 7: Marker (1-kb DNA ladder); (B) Methyl-green pyronin staining of liver. B-1: Control group; B-2: CCl₄ group; extended ssDNA areas (asterisk); B-3: *Berberis vulgaris* extract + CCl₄; semnificative reduction of ssDNA areas (arrow); B-4: *Berberis vulgaris* extract/ β cyclodextrin + CCl₄; ssDNA areas loss and normal RNA distribution (arrowhead) (DNA—pale green; RNA—pink).

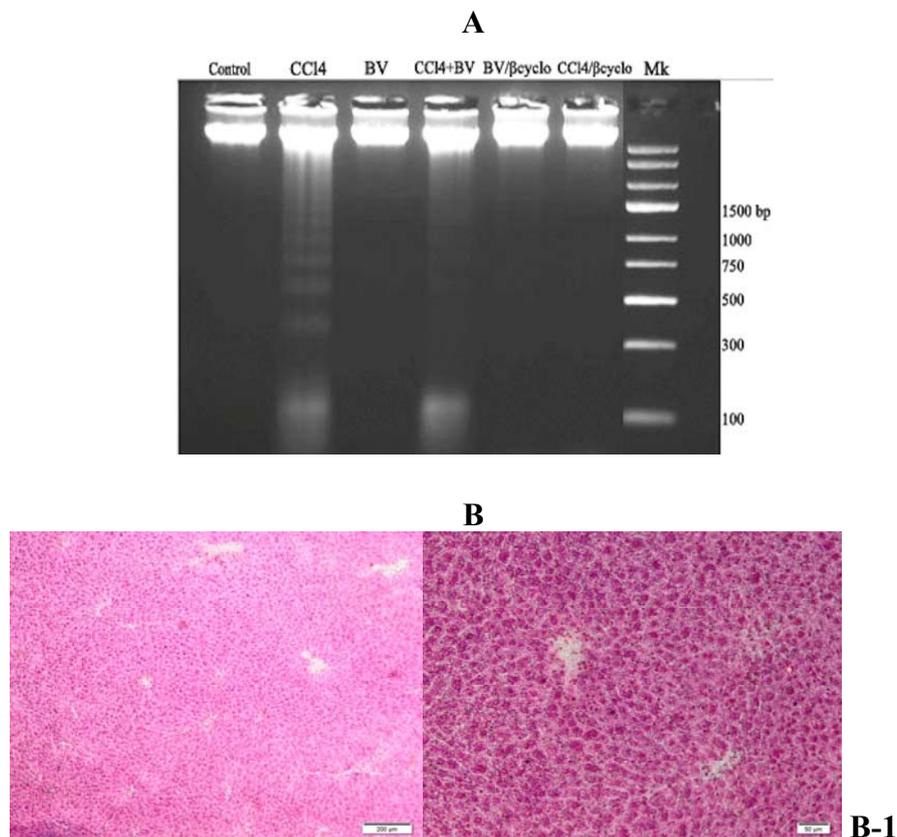
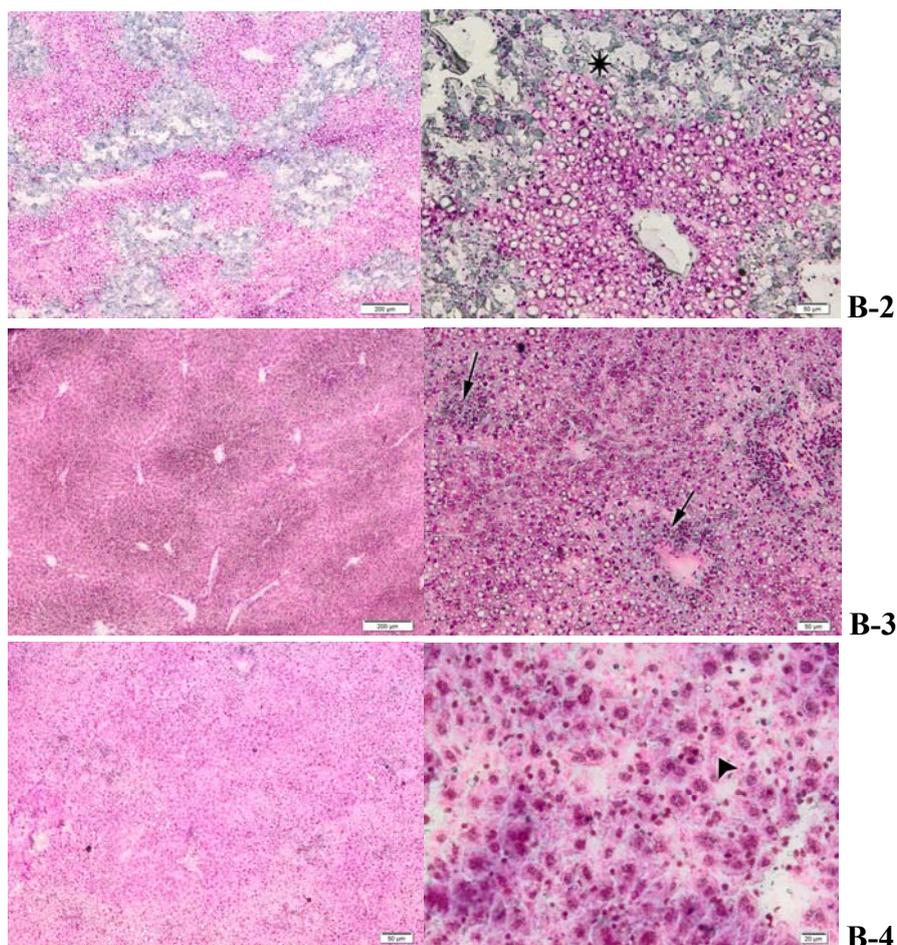


Figure 9. Cont.



DNA strand breaks are a cause of direct modification of DNA by chemical agents or their metabolites, which occurs when reactive oxygen species (ROS) interact with DNA [49,50]. In our study, the type of cell death induced by CCl_4 was investigated by DNA fragmentation assay and methyl-green pyronin (MGP) histochemical staining, which highlights the presence of RNA and ssDNA into liver tissue [51–53]. Internucleosomal DNA fragmentation was observed in liver samples of 1.0 mL/kg CCl_4 intoxicated group, which is the indication that cells were undergoing apoptotic or necrotic death as in previous studies [54–56]. The absence of DNA ladder and of MGP staining in the liver of mice pre-treated with *Berberis vulgaris* extract/ β -cyclodextrin and intoxicated with CCl_4 indicated the hepatoprotective role of the formulated extract. The non-formulated extract counteracted the damaging effect of CCl_4 but to a lesser extent, possibly due to the increased bioavailability of β cyclodextrin formulation as already it was proved in the case of liver injuries [57–59].

3. Experimental Section

3.1. *Berberis vulgaris* Extract/ β -Cyclodextrin Formulation

Samples of *Berberis vulgaris* L. were collected from the Botanical Garden of Vasile Goldis Western University of Arad during October 2008 and certified at the herbarium in the Department of Botany, Faculty of Natural Sciences, where a voucher specimen already exist. The bark of

Berberis vulgaris L. was separated and frozen until extraction. Bioactive compounds from *Berberis vulgaris* samples were separated by solid-liquid extraction in 96% ethanol, according to the method described by Hadaruga *et al* [32]. The final extract was subjected to HPLC analysis [32] followed by β -cyclodextrin nanoencapsulation [32,60]. The evaluation of *Berberis vulgaris*/ β -cyclodextrin complex formation was proved by differential scanning calorimetry (DSC) and thermogravimetry (TG) according to the previous study [32]. The dried form of the formulated and non-formulated extract, dissolved in distilled water was administered orally (50 mg/kg b.w.) to mice so as to check hepatoprotective activities.

3.2. Animals and Experimental Procedure

Adult male Swiss mice weighing 25 ± 3 g were obtained from Animal House of the Vasile Goldis Western University of Arad. The animals were left for 2 days for acclimatization to animal room conditions maintained on standard pellet diet and water *ad libitum* at a temperature of 20–25 °C under a 12 h light/dark cycle throughout the experiment. The food was withdrawn on the day before the sacrifice. All animals received human care and study protocols complied with the guidelines of the Animal House. Throughout the experiments, animals were processed according to the international ethical guidelines for the care of laboratory animals.

Forty-eight animals were divided into 6 groups, as follows: group 1 (control group), group 2 (CCl₄ treated group), group 3 (pre-treated with 50 mg/kg b.w. of *Berberis vulgaris* extract and subsequently intraperitoneally injected with CCl₄), group 4 (pre-treated with 50 mg/kg b.w. of *Berberis vulgaris* extract/ β -cyclodextrin and subsequently i.p. injected with CCl₄), group 5 (treated with 50 mg/kg b.w. of *Berberis vulgaris* extract), group 6 (treated with 50 mg/kg b.w. of *Berberis vulgaris* extract/ β -cyclodextrin). Non-formulated *Berberis vulgaris* extract and *Berberis vulgaris* extract/ β -cyclodextrin complex were orally administrated to mice from groups 3 and 5 and to groups 4 and 6 for 7 days. Mice from groups 2, 3, and 4 were intraperitoneally (i.p.) injected with CCl₄ at a dose of 1.0 mL/kg body weight in 50% olive oil (1:1) on the 8th day. Control and CCl₄ treated groups received the equivalent volume of distilled water orally for 7 days.

24 h after CCl₄ i.p. injection the mice were sacrificed by cervical dislocation. Serum and tissue samples were used for histopathology, electron microscopy and biochemical analyses.

3.3. Animals and Experimental Procedure

Homogenates (prepared as 1 g of tissue per 10 volumes of buffer) of mice livers were prepared in ice-cold buffer (0.1 M TRIS-HCl, 5 mM EDTA buffer, pH 7.4) and homogenized for 2 min at 16 Hz using a ball mill (type MM 301, Retsch GmbH & Co, Haan, Germany). The homogenate was centrifuged at 10,000 rpm for 30 min in a Hettich centrifuge at 4 °C to remove the cell debris. A few crystals of the protease inhibitor phenylmethylsulfonyl fluoride were added in the homogenates. The supernatant was decanted and used for biochemical assays.

Blood was collected from retro-orbital venous plexus. The tubes with whole blood were incubated for 60 min at room temperature to allow clotting. They were then centrifuged for 15 min at 2000 rpm. The supernatant (serum) was aliquoted and used immediately for biochemical analyses.

3.4. Histopathology

Frozen liver sections were cut at 8 μm at SLEE MNT cryotome, fixed in 10% buffered formaldehyde and stained with hematoxylin-eosin, oil red, P.A.S. periodic acid Schiff, Fouchet van Gieson and methyl-green pyronin, 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.

3.5. Electron Microscopy

For ultrastructural investigations, liver samples were prefixed in 2.7% glutaraldehyde solution in 0.1 M phosphate buffer for 1.5 h, at 4 °C. Then they were washed in 0.15 M phosphate buffer (pH 7.2). Postfixation was performed in 2% osmic acid solution in 0.15 M phosphate buffer for 1 h at 4 °C. Dehydration was performed in acetone, and inclusion in the epoxy embedding resin Epon 812. The blocks have been cut at an ultramicrotome type LKB, at 70 nm thickness. The sections were doubly contrasted with solutions of uranyl acetate and lead citrate and were analyzed with TEM Tecnai 12 Biotwin electron microscope.

3.6. Biochemical Assays

3.6.1. Activities of Serum Hepatic Markers

The serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transferase (γ -GT), direct and total bilirubin were evaluated by the spectrophotometric method using commercially available kits (Roche reagents, Meylan, France) according to the manufacturer's instructions.

3.6.2. Assesment of Antioxidant Status

Hepatic catalase (CAT) activity was determined by the Aebi method [61] which records the decomposition of H_2O_2 by decrease in the absorbance at 240 nm. One unit of CAT activity is equal to the decomposition of one μmole $\text{H}_2\text{O}_2/\text{min}/\text{mL}$. Liver superoxide dismutase (SOD) activity was measured by the method described by Beauchamp and Fridovich [62]. One unit of SOD activity is defined as the amount of enzyme that inhibits the oxidation of NADH by 50% at 37 °C. The measurement of GPx activity was performed according to the method of Beutler *et al.* [63] which is based on monitoring of the oxidation of NADPH at 340 nm (V-530 JASCO spectrophotometer). All enzymatic activities, calculated as specific activities (units/mg of protein) were expressed as % from controls.

GSH content was measured with a commercial kit (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's instructions. The absorbance was measured at 405 nm using a microtiter plate reader (GENIOS Tecan) and the concentration was expressed in nmoles GSH/mg protein.

3.6.3. Assay of Lipid Peroxidation

The hepatic malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed using a fluorimetric method described by Del Rio *et al.* [64]. Briefly, the sample (200 μL) was incubated with 0.1 M HCl (700 μL) for 20 min at room temperature. A volume of 900 μL of 0.025 M thiobarbituric acid was added, and the mixture was maintained at 37 °C for 65 min. Then the samples were subjected to fluorescence analysis ($\lambda_{\text{ex}} = 520 \text{ nm}$; $\lambda_{\text{em}} = 549 \text{ nm}$) (Spectrofluorometer FP-6300 JASCO). Relative fluorescence units (RFU) were converted to nmoles malondialdehyde (MDA) using 1,1,3,3-tetramethoxypropane as standard.

3.6.4. Protein Concentration Measurement

The protein content was determined using Lowry's method with bovine serum albumine as standard [65].

3.7. DNA Fragmentation Assays

Following the method of Zhou *et al.* [66], 0.15 g of frozen liver was homogenized in 1.5 mL lysis buffer [10 mM Tris-HCl pH 7.5; 100 mM EDTA; 0.5% SDS; 0.1 mg/L proteinase K]. RNA-free DNA from each sample was extracted twice with phenol, once with phenol-chloroform-isoamyl alcohol (PCI 25:24:1) and once with chloroform. After centrifugation, DNA was precipitated with cold ethanol and sodium acetate. The pellet was dissolved in TE buffer (10 mM Tris-HCl; 1.0 mM EDTA) and incubated with RNase (0.1 mg/L) at 37 °C for 45 min. Subsequently the suspension was re-extracted with PCI and chloroform and precipitated as described above. A quantity of 3 μg of DNA was loaded on 1.5% agarose gel and run at 100 V. The gel was stained with ethidium bromide and photographed under UV light using UVP BioDoc-IT Imaging System.

3.8. Statistical Analysis

All results were expressed as mean \pm SEM. The data were analyzed for statistical significance using Student's *t* test. A value of $p < 0.05$ was considered significant: *; distinct significant at $p < 0.01$: **; very significant at $p < 0.001$: ***.

4. Conclusions

In conclusion, this study showed that *Berberis vulgaris* extract/ β -cyclodextrin presented better hepatoprotective effects than free extract on oral administration probably due to its increased bioavailability. It seems that formulated extract could be used as a low cost phytotherapeutical supplement suitable for acute or chronic conditions of liver diseases or as a supportive treatment in addition to conventional therapies of serious hepatic diseases. Taken together, our results suggest that *Berberis vulgaris* extract/ β -cyclodextrin should be further investigated regarding clinical applications.

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