Berberis vulgaris extract/β-cyclodextrin complex increases protection of hepatic cells via suppression of apoptosis and lipogenesis pathways

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Abstract. *Berberis vulgaris* (Bv) is well known worldwide for its healing properties. However, limited information is available concerning its mechanism of action and the increased hepatoprotective activity of formulated extracts. This study evaluated the protective effect of Bv bark extract against CCl_4-induced cytotoxicity in Huh7 cells, as well whether β-cyclodextrin complexation of the extract resulted in increased hepatoprotective effects. Huh7 cells were incubated for 48 h with 5, 7.5 and 10 µg/ml of unformulated or formulated Bv extract alone and in co-treatment with CCl_4. The effects on Huh7 cell growth and apoptosis were evaluated by MTT assay, caspase-3/7 activity and caspase-3 expression, whereas fatty acid changes were investigated by Oil red O staining and the detection of peroxisome proliferator-activated receptor-γ (PPARγ) expression using immunofluorescence. Ultrastructural alterations were observed by electron microscopy. The MTT assay showed that co-exposure to CCl_4 and 7.5 µg/ml formulated extract led to a 1.25-fold increase in cell viability compared with the non-formulated extract. Caspase-3/7 activity decreased by 50% and 70% following co-treatment with unformulated or formulated extract, compared with that in cells treated with CCl_4 alone. Furthermore, hepatocyte ultrastructure was protected from CCl_4-induced injury in the two co-treated groups, intracytoplasmic lipid accumulation decreased significantly and PPARγ expression was restored, in comparison with CCl_4-treated cells alone. Formulated and unformulated extracts were efficient against the anti-proliferative and pro-apoptotic actions of CCl_4 through suppression of CCl_4-induced caspase-3 activation and lipid accumulation. The protective effect of the formulated extract was more pronounced than that of the unformulated one, which may be due to its increased solubility.

Introduction

Carbon tetrachloride (CCl_4) is an experimental hepatotoxicant, able to induce acute liver injury characterized by necrosis and steatosis (1,2). Once the metabolism of CCl_4 is initiated, its toxicity is determined by chain chemical reactions that take place in the body. The initial step reaction is a reduction and heterolytic cleavage of CCl_4 to form a trichloromethyl radical (•CCl_3) (3). This radical reacts either directly with cellular macromolecules or with oxygen; reaction with the latter forms the trichloromethylperoxyl radical (•$OOCCl_3$), which acts more rapidly on lipids than does •CCl_3 (4). Production of excessive amounts of reactive oxygen species (ROS) generates oxidative stress which induces major cellular perturbations, such as alterations in the structures of protein and nucleic acids, increases in intracellular free calcium levels, perturbation of membrane permeability, lipid peroxidation and finally cell death (5). In order to minimize the effects of ROS, cells have an antioxidant defense system which includes non-enzymatic antioxidants and enzymes such as superoxide dismutase, catalase and glutathione peroxidase (6). CCl_4 administration destabilizes this defense system and decreases superoxide dismutase, catalase and glutathione peroxidase activity (7).

Berberis vulgaris L. (Berberidaceae) (Bv) has been well known worldwide for its healing properties for >2,500 years (8). The bioactive components are represented by several alkaloid constituents, such as berberine, berbamine and palmatine, which confer healing properties to *Berberis* extracts (9). Berberine is the most important isoquinoline alkaloid, obtained mostly from the roots and bark of *Berberis*

sp. (10). Berberine is known for its multiple pharmacological properties, such as antimicrobial (11), antitumor (12) and anti-inflammatory effects (13,14). Berberine is also known for its dose-dependent hepatoprotective effects on CCl_4-induced liver damage, due to its antioxidant effects (15).

This study was carried out to evaluate for the first time the increased protective effect of a formulation of Bv bark extract in β-cyclodextrin (β-CD) against CCl_4-induced cytotoxicity in Huh7 cells. This natural complex was designed for use in oral formulations, in order to increase the solubility, dissolution, bioavailability, safety and stability of the extract via certain properties of β-CD, including its resistance to hydrolysis by human salivary and pancreatic amylases (16).

Materials and methods

Complex of Bv bark extract and β-CD. Samples of Bv were collected from the Botanical Garden of Vasile Goldis Western University of Arad (Arad, Romania) during October 2008, and certified at the herbarium within the Faculty of Natural Sciences, where a voucher specimen already exists. The synthesis and characterization of the Bv bark extract/β-cyclodextrin complex used in this study have been previously reported by the authors (2).

Cell culture. The study was carried out using as model a Huh7 human hepatoma cell line (American Type Culture Collection, Manassas, VA, USA). The Huh7 cell line was chosen due to its metabolic similarities to normal hepatocytes and in order to avoid the variability and short life spans of primary human hepatocytes (17). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, Steinheim, Germany), and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/ml; PromoCell GmbH, Heidelberg, Germany) in a humidified atmosphere with 5% CO_2, at 37°C.

Cell treatment. Cells were plated at a density of 10^4 cells/cm² with DMEM medium (high glucose, supplemented with 10% FCS) and allowed to attach overnight at 37°C. The CCl_4 concentration (0.1 mM) used for cell culture co-treatment was previously determined and chosen due to its ability to induce up to 75% cell culture mortality. Three concentrations (5, 7.5 and 10 μg/ml) of unformulated and nanoencapsulated β-CD Bv bark extract were tested for protection against CCl_4-induced cytotoxicity in the Huh7 cell line. Each experiment was performed in triplicate under 48 h of exposure. Stock solutions were prepared fresh to avoid oxidation. The Bv extracts were dissolved in dimethylsulfoxide (DMSO) and diluted with DMEM to the desired concentrations prior to use, while DMSO alone was used as vehicle control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays. The MTT assay was used to detect the cytotoxicity of unformulated and formulated Bv extract. Cells were seeded into 96-well plates and allowed to attach overnight. A series of unformulated and formulated extracts were added (5, 7.5 and 10 μg/ml), alone or together with 0.1 mM CCl_4, followed by 48 h incubation. All experiments were conducted in parallel with a control. The MTT assay was performed using a commercially available MTT assay (MTT base; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. The absorbance (abs) was measured at 565 nm, using a Tecan Infinite F200 microplate reader (Tecan, Männedorf, Switzerland). The cell survival rate was calculated as follows: Survival rate (%) = (Abs treatment - abs blank)/(Abs control - Abs blank) x 100.

Caspase-3 and -7 activities. Total caspase-3 and -7 activities were measured using an Apo-ONE Homogeneous Caspase-3/7 assay kit (Promega Corporation, Madison, WI, USA). Following the various treatments, 100 μl Apo-ONE Caspase-3/7 reagent (substrate and buffer in the ratio of 1:100) were added to each well of a 96-well plate. After 1 h incubation in the dark at room temperature, the fluorescence of each well was measured at 485-520 nm (Fluoroskan Ascent FL; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Immunofluorescence. Expression levels of caspase-3 and peroxisome proliferator-activated receptor-γ (PPARγ) were assessed by immunofluorescence using a rabbit polyclonal caspase-3 antibody (orb10273; Biorbyt Ltd., Cambridge, UK) 1:200 dilution, or PPARγ antibody (sc-7196; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 1:50 dilution and AlexaFluor 488-labeled chicken anti-rabbit IgG secondary antibody (A-21441; Invitrogen; Thermo Fisher Scientific, Inc.). Cells were treated with unformulated and nanoencapsulated β-CD extract for 48 h prior to fixing with cold 100% methanol at -20°C, for 10 min. The cells treated under experimental conditions were fixed with cold 100% methanol at -20°C for 10 min. Blocking of non-specific binding was conducted by incubation with 1% bovine serum albumin (Antibodies Online Inc., Atlanta, GA, USA) and 0.1% Triton X (Sigma-Aldrich; Merck Millipore) for 1 h at room temperature. Following blocking, cells were incubated overnight at 4°C with corresponding dilutions of the primary antibodies. Following overnight incubation, the cells were washed in PBS and incubated with secondary antibody for 1 h at room temperature. Nucleus counterstaining was performed with 10 μg/ml Hoechst 33285 working solution (861405; Sigma-Aldrich, Buchs, Switzerland) for 60 sec, followed by analysis using a Leica TCS SP8 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). The fluorescence intensity of PPARγ- and caspase 3-positive hepatocytes was quantified by automated counting performed using an image analysis system (ImageJ; National Institutes of Health, Bethesda, MD, USA). Three fields were selected randomly from each section, and a total of 100 hepatocytes were evaluated in every field.

Oil Red O staining. Intracellular accumulation of lipid droplets was observed using Oil Red O staining. Cells were plated on Lab-Tek Chamber Slides (Nalge Nunc International, Penfield, NY, USA) for 24 h. After 24 h of seeding, cells were treated with unformulated and β-CD nanoencapsulated Bv extract, respectively, for 48 h. For staining, the cells were fixed with 4% formaldehyde for 10 min at 4°C. The fixed cultures were incubated with Oil Red O working solution for 20 min at room temperature. Oil Red O was provided in a
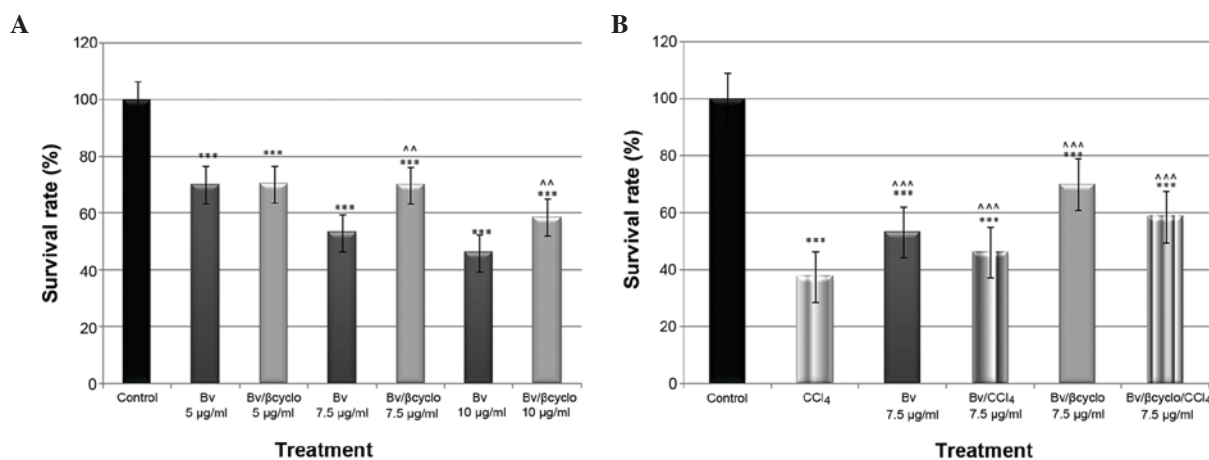
Figure 1. Cell viability analysis by MTT assay. (A) Survival rate of the Huh7 cell line under exposure to unformulated and β-cyclodextrin nanoencapsulated *Berberis vulgaris* extracts. Results are presented as mean ± standard deviation for triplicates. [***]P<0.001 vs. control; [^^]P<0.01 vs. unformulated *Berberis vulgaris* extracts. (B) Survival rate of the Huh7 cell line under co-treatment with CCl_4 and 7.5 μg/ml unformulated and β-cyclodextrin nanoencapsulated *Berberis vulgaris* extracts. The cytoprotective effect of unformulated and β-cyclodextrin nanoencapsulated *Berberis vulgaris* extracts against CCl_4-induced cytotoxicity in Huh7 cells after 48 h treatment was compared with normal and CCl_4-treated controls. Results are presented as mean ± standard deviation for triplicates. [***]P<0.001 vs. normal control; [^^^]P<0.001 vs. CCl_4 control. The IC_{50} value was 10 μg/ml. Survival rate was calculated as follows: Survival rate (%) = (Abs treatment - Abs blank)/(Abs control - Abs blank) x 100. Bv, *Berberis vulgaris;* βcyclo, β-cyclodextrin; CCl_4, carbon tetrachloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

staining kit (04-220923; Bio Optica, Milan, Italy). Nucleus counterstaining was performed with Mayer's hematoxylin provided in the same kit (04-220923; Bio Optica). After washing with tap water, the Permanox slides were mounted. Analysis was performed using an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan).

Electron microscopy. For electron microscopy, 1x10^6 cells were treated with unformulated and β-CD nanoencapsulated Bv extracts for 48 h. Following the drug treatments, cells were collected by trypsinization, followed by centrifugation at 240 x *g*. The obtained pellet was prefixed in 2.7% glutaraldehyde solution in 0.1 M phosphate buffer for 1.5 h, at 4˚C, then 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 1 h at 4˚C. Dehydration was performed in acetone, followed by inclusion in the epoxy embedding resin Epon 812. Blocks 70-nm thick were cut using an LKB ultramicrotome. The sections were doubly contrasted with solutions of uranyl acetate and lead citrate and analyzed under a Tecnai 12 Biotwin transmission electron microscope (FEI, Hillsboro, OR, USA).

Statistical analysis. Statistical analysis of the differences between the means of various treatment groups was performed using one-way analysis of variance. STATA statistical software (version 13.0; StataCorp LP, College Station, TX, USA) was used. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of unformulated and β-CD nanoencapsulated Bv extract on CCl_4-induced cytotoxicity in Huh7 cells. To analyze the protective advantage of β-CD nanoencapsulated Bv extract against CCl_4-induced cytotoxicity, cell viability in
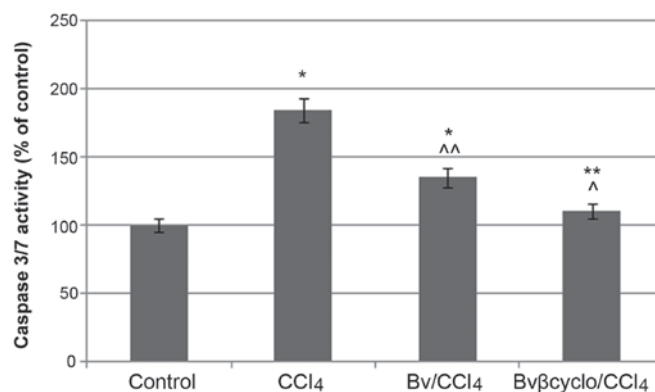

Figure 2. Apoptosis analysis by measurement of caspase-3/caspase-7 activities following 48 h of CCl_4 exposure. Values are expressed as the percentage of control (set as 100%). [*]P<0.05 and [**]P<0.01 vs. normal control; [^]P<0.05 and [^^]P<0.01 vs. CCl_4 control. Bv, *Berberis vulgaris;* Bvβcyclo, Bv complexed with β-cyclodextrin; CCl_4, carbon tetrachloride.

the Huh7 cell line was investigated. The cells were incubated with equivalent doses of formulated and unformulated Bv extract (5, 7.5 and 10 μg/ml Bv extract) for 48 h. As shown in Fig. 1A, the unformulated extract presented a dose-dependent significant cytotoxicity (P<0.001), whereas the formulated one did not. Due to the fact that the dose of 7.5 μg/ml extract presented moderate toxicity, it was chosen for the assessment of antioxidant activity. Following co-exposure of the cells to CCl_4 and 7.5 μg/ml Bv nanoencapsulated extract, the cell viability was 1.25 fold higher compared with that of cells co-exposed to CCl_4 and non-formulated Bv extract (Fig. 1B).

Effcts of unformulated and β-CD nanoencapsulated Bv extract on CCl_4.-induced Huh7 cell apoptosis. In order to investigate the anti-apoptotic effects of Bv extracts, the caspase-3/7 activity (Fig. 2) as well as caspase-3 expression in control and experimental variants (Fig. 3) were evaluated.
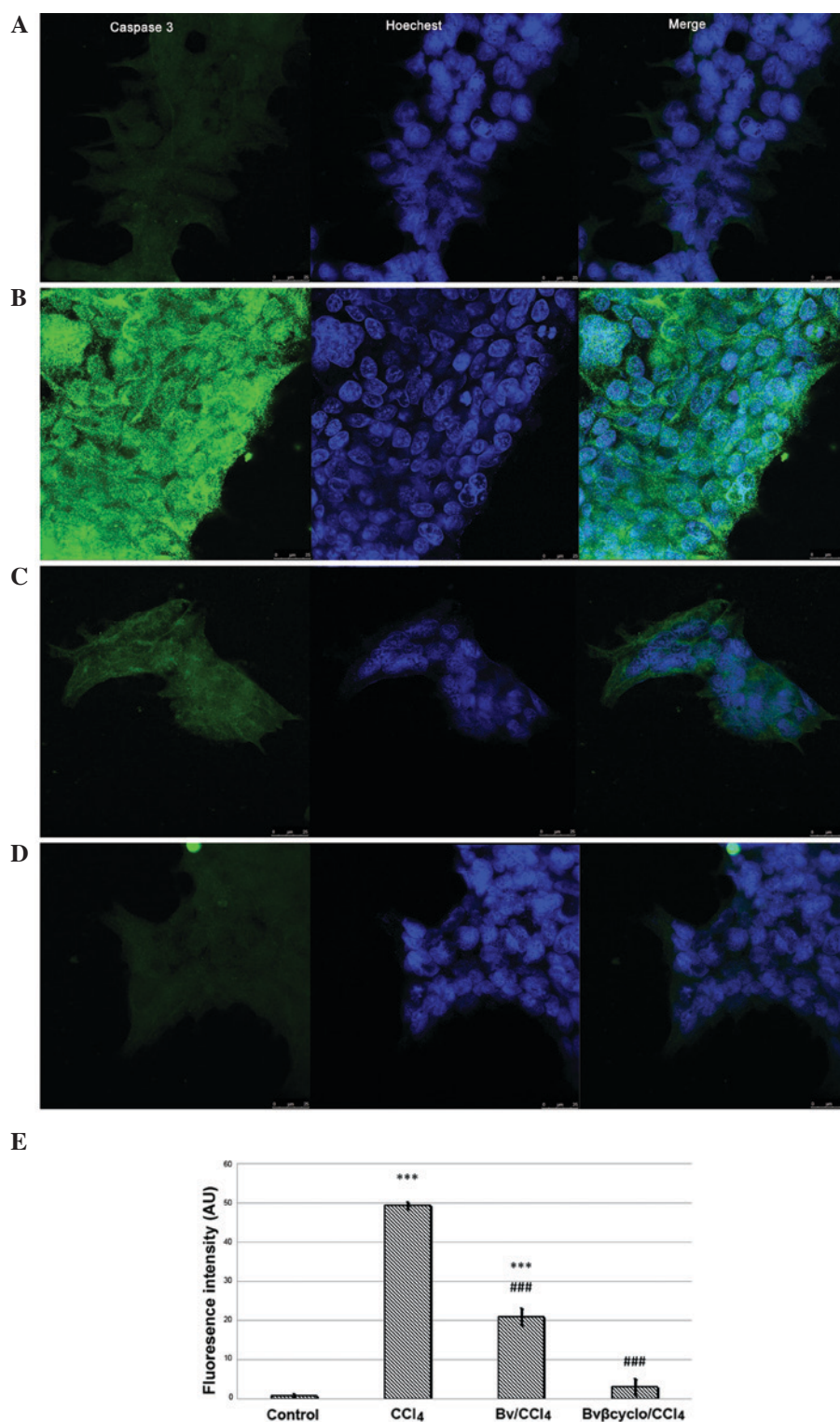
Figure 3. Detection of apoptosis by caspase-3 immunostaining and Hoechst 33285 staining. Representative images (magnification, x620) showing (A) control Huh7 cells (B) Huh7 cells treated with 0.1 mM CCl₄ (C) Huh7 cells treated with 0.1 mM CCl₄ and 7.5 µg/ml unformulated *Berberis vulgaris* extract and (D) Huh7 cells treated with 0.1 mM CCl₄ and 7.5 µg/ml formulated *Berberis vulgaris*/β-cyclodextrin. Nuclear chromatin condensation and apoptotic bodies were visible in Huh7 cells treated with 0.1 mM CCl₄ (arrowhead). Scale bar, 25 μm. (E) Bar chart showing the average fluorescence intensity of the Huh7 cell immunostaining for caspase-3 expression in the experimental variants (A-D). Data are presented as mean ± standard deviation for triplicates. ***P<0.001 vs. the control group; ###P<0.001 vs. the CCl₄-treated group. AU, absorbance units; Bv, *Berberis vulgaris;* Bvβcyclo, Bv complexed with β-cyclodextrin; CCl₄, carbon tetrachloride.

The caspase-3/7 activity increased significantly (P<0.05) by ~80% in the CCl₄-treated cells compared with the control, whereas co-treatment with unformulated and formulated BV extract decreased it by 50 and 70%, respectively.

Analysis of confocal microscopy images showed that caspase-3 immunostaining was significantly increased in CCl₄-treated cells compared with control cells. Under co-exposure to CCl₄ with both formulated and unformulated
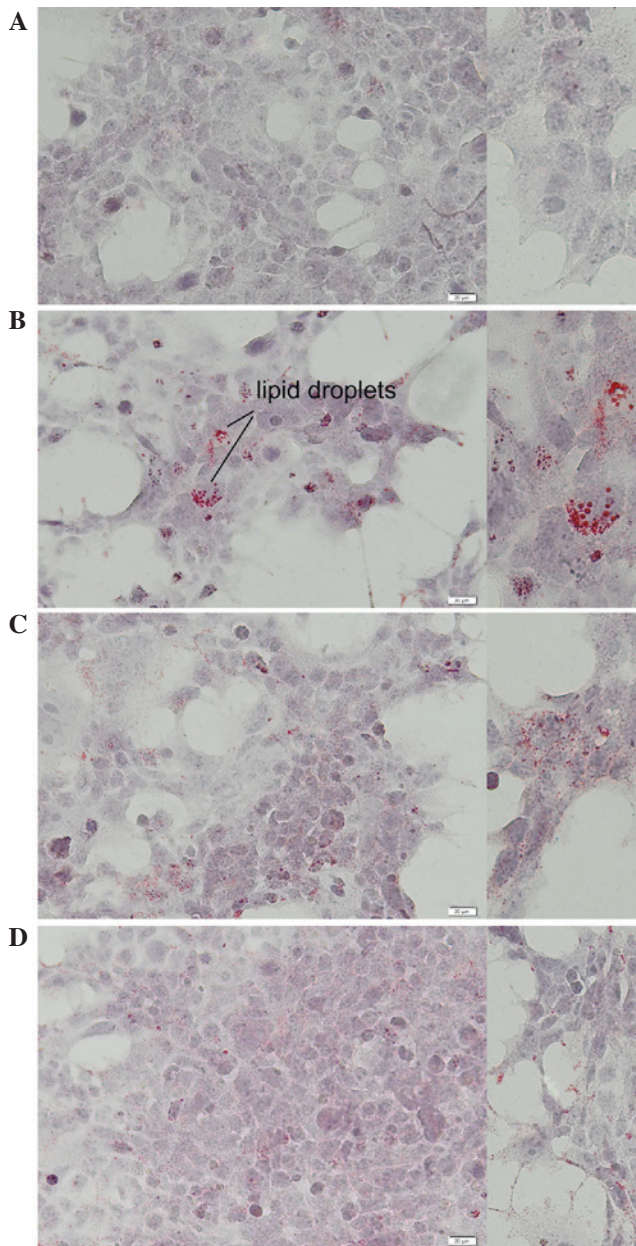
Figure 4. Detection of intracellular lipid droplets after 48 h of CCl_4 exposure. Cells were fixed and stained with Oil Red O and Mayer's hematoxylin. Intracellular lipid droplets are shown as red-stained spheres in the cells, whereas nuclei are stained in blue. (A) Control Huh7 cells, (B) Huh7 cells treated with 0.1 mM CCl_4, (C) Huh7 cells treated with 0.1 mM CCl_4 and 7.5 µg/ml unformulated *Berberis vulgaris* extract and (D) Huh 7 cells treated with 0.1 mM CCl_4 and 7.5 µg/ml formulated *Berberis vulgaris*/β-cyclodextrin complex. Original magnification, x200 and x400, respectively. Scale bar, 20 µm. CCl_4, carbon tetrachloride.

Figure 5. PPARγ expression after 48 h of CCl_4 exposure. Representative images showing (A) control Huh7 cells, (B) Huh7 cells treated with 0.1 mM CCl_4, (C) Huh7 cells treated with 0.1 mM CCl_4 and 7.5 µg/ml unformulated *Berberis vulgaris* extract and (D) Huh7 cells treated with 0.1 mM CCl_4 and 7.5 µg/ml formulated *Berberis vulgaris*/β-cyclodextrin complex. PPARγ expression decreased visibly in CCl_4-treated cells, compared with control cells, and the reduction was attenuated in cells treated with CCl_4 and unformulated/β cyclodextrine *Berberis vulgaris* extracts. Original magnification, x200. (E) Bar chart showing the average fluorescence intensity of the Huh7 cell immunostaining for PPARγ expression in experimental variants (A-D). Data are presented as mean ± standard deviation for triplicates. [*]$P<0.05$ and [**]$P<0.01$ vs. the control group; [##]$P<0.01$ vs. the CCl_4-treated group. PPARγ, peroxisome proliferator-activated receptor γ; Bv, *Berberis vulgaris;* Bvβcyclo, Bv complexed with β-cyclodextrin; CCl_4, carbon tetrachloride; AU, absorbance units.

Bv extract, signals were significantly reduced ($P<0.001$) compared with those in the CCl_4-treated cells, and were reduced most strongly with the formulated Bv extract ($P<0.001$; Fig. 3).

Effects of unformulated and β-CD nanoencapsulated Bv extracts on CCl_4.-induced fatty accumulation in Huh7 cells. Light microscopic evaluation of Huh7 cells stained with Oil Red O showed normal morphology of Huh7 cells and few accumulated intracellular lipid droplets in the control (Fig. 4A). 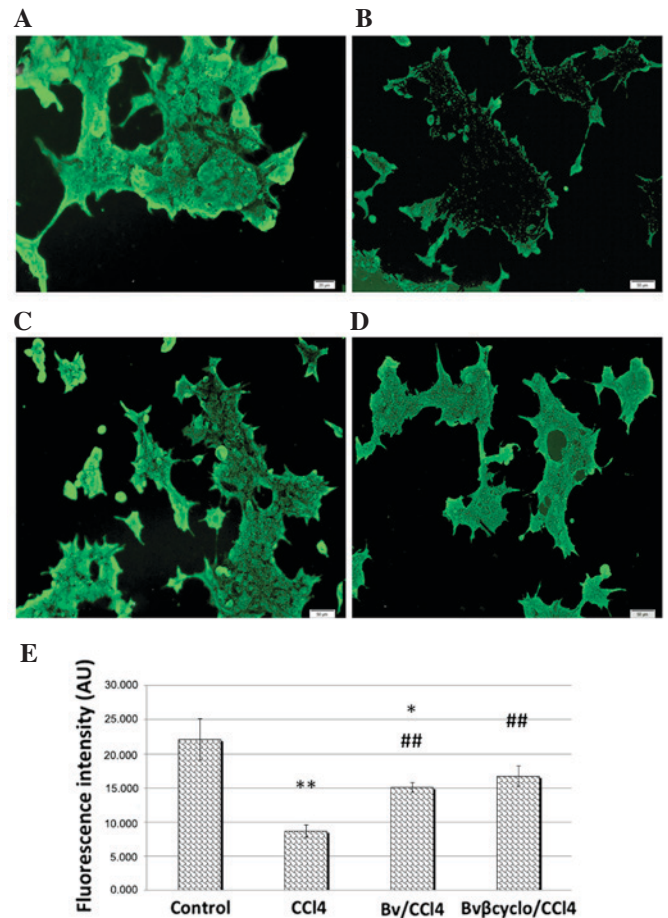Intracytoplasmic lipids increased significantly in the CCl_4-treated cells without Bv extract, compared with the control (Fig. 4B). After 48 h of co-treatment with CCl_4 and either Bv extract, HuH7 cells accumulated fewer lipid droplets compared with cells treated with CCl_4 alone (Fig. 4C and D). In addition, the level of PPARγ expression decreased significantly ($P<0.01$) in CCl_4-treated cells (Fig. 5), compared with control cells, and this reduction was attenuated in the cells co-treated with unformulated or formulated Bv extracts (Fig. 5).

Effects of unformulated and β-CD nanoencapsulated Bv extract on CCl_4.-induced ultrastructural injuries of Huh7 cells. The ultrastructure of Huh7 cells was normal in the control group. Control cells showed integrated nuclear membranes, relatively homogeneous chromatin, and organelles with a normal appearance (Fig. 6A). Following treatment with 0.1 mM CCl_4 for 48 h, Huh7 cells were characterized by
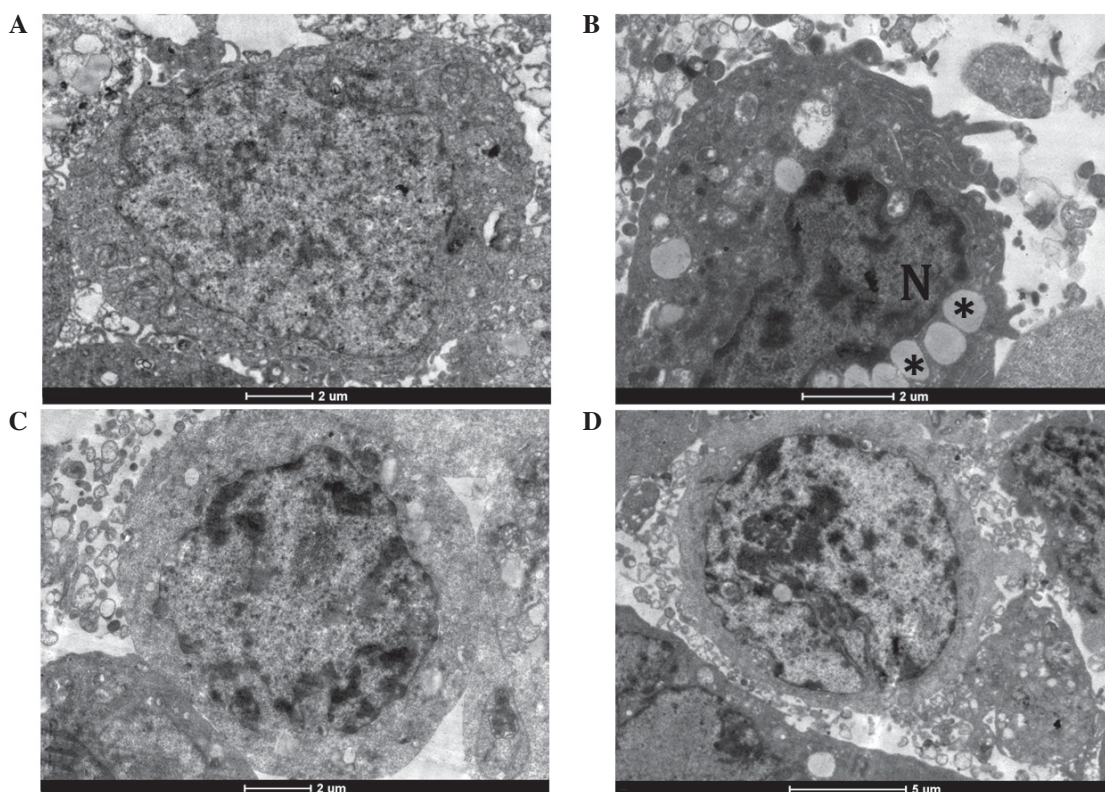
Figure 6. Apoptosis of Huh7 cells observed with a transmission electron microscope after 48 h of CCl_4 exposure. (A) Huh7 control cells, (B) Huh7 cells treated with 0.1 mM CCl_4 showing a condensed nucleus (N), lipid droplets (*) and altered mitochondria (arrow), (C) Huh7 cells treated with 0.1 mM CCl_4 and 7.5 µg/ml unformulated *Berberis vulgaris* extract and (D) Huh7 cells treated with 0.1 mM CCl_4 and 7.5 µg/ml formulated *Berberis vulgaris*/β-cyclodextrin complex. CCl_4, carbon tetrachloride.

irregular nuclear shapes, chromatin condensation into dense granules or blocks, vacuolar cytoplasm, and lipid droplet accumulation, together with altered mitochondria and rarefied cristae (Fig. 6B). Co-treatment with unformulated and formulated Bv extracts protected the hepatocyte ultrastructure from CCl_4-induced injury; in the two BV groups the appearance of the Huh7 cells was similar to that of the control cells (Fig. 6C and D).

Discussion

Numerous herbs are used in traditional medicine to prevent and treat liver diseases as a result of hepatotoxicant action. Barberry root, bark, leaves and fruit have been used in traditional medicine to treat hepatic disorders, and berberine is generally thought to be responsible for their beneficial effects (18). Berberine itself is considered a potent antioxidant with a wide spectrum of applications (19); the biological activities and levels of safety should be established for the whole Bv extract and its formulations. Another important compound in barberry is berbamine, a bis-benzylisoquinoline alkaloid, which inhibits chemically-induced carcinogenesis (20).

To demonstrate the increased protective potential of β-CD nanoencapsulated Bv extract compared with unformulated Bv extract against CCl_4-induced cytotoxicity, an MTT assay was performed in the present study. The notable cell mortality observed with formulated and unformulated extracts could be attributable to their content of berbamine, which inhibits Ca^{2+}/calmodulin-dependent protein kinase II in

liver cancer cells (21). CCl_4 caused a reduction in cell viability of 62.43% compared with control. It is likely that a 7.5 µg/ml dose of unformulated or formulated extract presented a sufficient quantity of berberine to act as an antioxidant. The β-CD nanoencapsulated Bv extract displayed 1.25-fold higher protective effects compared with the unformulated form, probably due to its increased solubility. Previously, it has been demonstrated that the solubility of berberine hydrochloride was increased 5.27-fold by cyclodextrin complexation (22). In addition, Ziolkowski *et al* (23) have shown depletion of mitochondrial cholesterol in the presence methyl-β-cyclodextrin, which was associated with impaired bioenergetics and increased resistance to calcium chloride-induced swelling and subsequently prevented changes associated with early stage apoptosis.

In the present study 7.5 µg/ml Bv extract/β-CD complex exhibited no cytotoxicity whereas Kiss *et al* (24) showed that significant cell toxicity was induced by 50 mM β-CD; this difference in cytotoxicity may be explained by the CD concentration used being 75-fold lower than that in the previous study.

Apoptosis has been shown to be important in CCl_4-induced hepatotoxicity (25). In the present study, apoptosis was confirmed by the detection of caspase-3 activation and ultrastructural analysis. CCl_4 induced activation of the apoptotic signaling pathway via caspase-3, as shown in Figs. 2 and 3, consistent with previous *in vivo* studies that have demonstrated a similar apoptotic mechanism for this toxic agent (26). In addition, cell death could be initiated by the ability of CCl_4 to arrest Huh7 cells in the G0/G1 phase. Also, it could decrease

the proportion of cells in the S phase, which is associated with inhibition of DNA synthesis (27). Co-treatment with Bv/β-cyclodextrin complex significantly increased (P<0.001) the percentage of viable cells, which suggests that it reversed the cell cycle by increasing the proportion of cells in the S phase and inhibited the apoptotic process by suppression of CCl$_4$-induced caspase-3 activation.

ROS generated by CCl$_4$ exposure can cause changes in the inner mitochondrial membrane and loss of the mitochondrial transmembrane potential (28). As a result, cytochrome c and other mitochondrial proteins are released into the cytosol (29), activating caspase-9 and subsequently caspase-3, which cleaves a set of vital proteins and promotes the apoptotic degradation phase including DNA damage and morphological changes (30).

Apoptosis was also investigated by electron microscopy in the present study. The ultrastructural changes that were observed indicated the apoptosis of Huh7 cells due to the presence of CCl$_4$, results that are in agreement with other results on HepG2 hepatocarcinoma cells exposed to fatty acid ethyl esters (31) or sarsasapogenin (32). In the present study, both formulated and unformulated Bv extracts were able to prevent ultrastructural injuries induced by CCl$_4$ exposure.

Lipid droplets represent intracellular compartments able to store neutral lipids as triglycerides and cholesteryl esters. Each lipid droplet is surrounded by a phospholipid monolayer (33). The accumulation of neutral lipids (mainly triglycerides and cholesterol esters) into hepatocyte cytoplasm is associated with hepatic steatosis. This abnormal accumulation of lipid droplets may result from the release of free fatty acids (FFAs) by adipocytes, dietary intake, diminished hepatic export of FFAs, increased de $novo$ lipogenesis or impaired β-oxidation of FFAs (34). In the present study, a massive accumulation of lipid droplets in the cytoplasm was observed after 48 h of CCl$_4$ toxic exposure. The high increase in lipid concentration within the CCl$_4$-treated Huh7 cells can be attributed to a reduction in fatty acid activation and cholesterol catabolism (35). Toxic injuries due to poisoning with compounds like CCl$_4$ are expressed by fatty infiltration of the liver due to changes in low-density lipid synthesis (3,5,7). The use of unformulated and formulated Bv extracts exhibited a very good protective effect against the formation of lipid droplets due to CCl$_4$ exposure, with slightly increased protective effects in the case of formulated Bv extracts.

PPARγ plays an important role as a regulator of lipid metabolism and is a key mediator of lipid storage and inflammation (36). The results of the current study present a number of intriguing observations. Firstly, in the CCl$_4$-treated group intracellular lipid accumulation was observed, whereas a reduction in PPARγ expression was evidenced. It has previously been demonstrated that the PPARγ2 isoform regulates intracellular lipid stores (37), whereas PPARγ1 regulates the expression of inflammatory cytokines through inhibition of nuclear factor (NF)-κB (37). Studies have shown a decreased number of PPARγ-positive hepatocytes (26) and an increased number of hepatocytes expressing NF-κB and tumor necrosis factor (TNF)-α following CCl$_4$ treatment (38). The severity of liver lesions has been found to be correlated with a reduction in the number of hepatocytes expressing PPARγ (38). In addition, results of another study indicated that the PPARγ1 isoform

was suppressed while, in contrast, PPARγ2 levels remained unchanged and finally peaked at 8 weeks after CCl$_4$ exposure (37). Considering the results of the present and previous studies, it may be speculated that the decreased expression of PPARγ due to CCl$_4$ toxic activity could be followed by an increased inflammatory response mediated via NF-κB and TNF-α, which leads subsequently to indirect intracellular lipid accumulation and apoptosis. A recovery of PPARγ expression was observed following co-exposure with Bv extract, particularly with nano-encapsulated Bv extract, which may also contribute to the protective effect against CCl$_4$-induced apoptosis, whereas protection of the mitochondrial membrane potential by PPARγ overexpression through the upregulation of anti-apoptotic Bcl-2 family proteins has previously been demonstrated (39).

Formulated and unformulated Bv extract were both efficient against the anti-proliferative and pro-apoptotic actions of CCl$_4$ through suppression of CCl$_4$-induced caspase-3 activation and lipid accumulation. The protective effect was more pronounced following exposure to the formulated β-CD extract compared with the unformulated one, which is probably due to the increased solubility of the complex form, which assured that mitochondrial bioenergetics were balanced.

In conclusion, Bv extract complexed with β-CD protects Huh7 cells against apoptosis and cytotoxicity induced by CCl$_4$ and represents a more potent liver protector against toxic chemical substances and drugs than does the pure extract.

Acknowledgements

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