Therapeutic Effect of Dithiophenolato Chitosan Nanocomposites Against Carbon Tetrachloride-induced Hepatotoxicity in Rats

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Abstract

Our previous study showed that dithiophenolate (DTP) and its chitosan nanoparticles (DTP-CSNPs) have abilities to bind with DNA helixes. So in this study, their lethal doses (LD_{50}) and therapeutic roles against rat liver injuries induced by carbon tetrachloride (CCl_4) were evaluated. The study focused on the determination of the markers of oxidative stress (OS) and apoptosis and compare the results with those of cisplatin treatment. The results revealed that LD50 values of DTP and DTP-CSNPs are 2187.5 and 1462.5 mg/kg, respectively. Treatment with DPT and DPT-CSNPs after CCl4 administration reduced liver injuries, induced by CCl4, and improved liver functions and architecture through the reduction of OS and apoptosis. Where the oxidant marker was decreased with elevations of antioxidant markers. Also, there was an elevation in Bcl 2 value, with decreases in caspase-8, Bax, and Bax/Bcl 2 ratio. DPT-CSNPs treatment gave preferable results than that treated with DPT. Moreover, DTP and DPT-CSNPs treatment gave better results than Cisplatine treatment. The administration of healthy rats with low doses of DTP and DTP-CSNPs for 14 days had no effect. Otherwise, the study on HepG2 cell line showed that DTP and DPT-CSNPs inhibited cell growth by arresting cells in the G2/M phase and inducing cell death. **In conclusion:** DTP and DTP-CSNPs have anti-apoptotic and anti-oxidative stress towards hepatotoxicity induced by CCl4. Moreover, DTP and DTP-CSNPs have anticancer activity against the HepG2 cell line. Generally, DTP-CSNPs are more effective than DTP. So, they can be used in the pharmacological fields, especially DTP-CSNPS.

Introduction

Liver is a crucial organ of metabolism and elimination of foreign substances. Therefore, liver is a preferred target for xenobiotics toxicity (Shaban et al. 2014). Liver injury induced by xenobiotics can impersonate all forms of acute or chronic liver disease. The pathogenesis of xenobiotics-induced liver diseases involve cell stress, mitochondrial feebleness, and definite immune reactions. Mitochondrial feebleness leads to apoptotic and/or necrotic cell death. Apoptosis, as well-referred to as programmed cell death, is an organized form of cell death that comprises distinct biochemical and morphological changes (Youle & Strasser 2008). Apoptosis can be triggered by both internal and external factors (Kvansakul & Hinds 2015, Strasser & Vaux 2018). Internal factors involve misfolded proteins and deregulated signaling, while external factors include the loss of nutrients, heat, radiation, stimulation of the cell surface receptors, and xenobiotics such as carbon tetrachlorid (CCl_4) (Suraweera et al. 2021).

Cellular reactive oxygen species (ROS) are produced endogenously such as in the process of mitochondrial oxidative phosphorylation, or they may be produced from the interactions with exogenous sources as xenobiotic compounds. Oxidative stress (OS) occurs when ROS overcome the cellular antioxidant defense system, whether, via an increase in ROS levels or a decrease in the cellular antioxidant ability. OS performs in direct or indirect ROS-mediated damage of proteins, nucleic acids, and lipids, and has been implicated in diabetes, atherosclerosis, neurodegeneration and carcinogenesis (Ray et al. 2012). The molecular mechanism of hepatotoxicity induced by CCl_4 has been well reported in the rats (Saygili et al. 2017). CCl_4 is bio-transformed by liver cytochrome P450 enzymes to form the trichloromethyl free radical ($^{\bullet}CCl_3$) that reacting rapidly withmolecular oxygen to produce the trichloromethyl peroxyl radical "$CCl_3O_2^{\bullet}$". These toxic

radicals are accountable for the abstraction of hydrogen atoms from unsaturated fatty acids of phospholipids existing in the cell membrane, inducing lipid peroxidation in the hepatocytes and others. It has been authenticated that lipid peroxidation was the master mechanism in the pathogenesis of liver injuries induced by CCl_4 (Shaban et al. 2013).

Chemotherapy is still considered to have a remarkable curative effect with great success in clinical practice. In this regard, there is a significant requirement for novel pharmaceutical agents (Saygili et al. 2017). Nanotechnology is presently involved vastly in human lives with a lot of implementations, especially in sciences, food industry, paints, biology, medicine, diagnosis, drug delivery, etc. Nanomedicines (or nanotherapeutics) are usually intended as miniaturized delivery systems which aim to improve the therapeutic efficiency of currently available chemotherapeutic agents, combining them with a nanoscale delivery component (Baek et al. 2015, Chan et al. 2013). Chitosan nanoparticles **(CSNPs)** (Fig. 1) are perfect drug carriers because of their good biocompatibility and biodegradability and can be easily modified (Saneja et al. 2016). They have attracted rising attention for their broad applications in, such as loading protein drugs, anticancer chemical drugs, and gene drugs, and through different routes of administration involving oral, ocular, nasal, and intravenous (Rosiere et al. 2018, Shah & Rajput 2018, Wang et al. 2011). In our previous studies dithiophenolato ligand N2H2S2\H2 "DTP" (Fig. 1) was prepared (Shaban et al. 2012) as well as DTP chitosan nanocomposite (DTP-CSNPs) was prepared by loading DTP on chitosan-tripolyphosphate nanocomposite (Shaban et al. 2020). Also, their characterization were studied where the study showed that DTP-CSNPs binds strongly to DNA as compared to DTP and both bind via a static quenching mechanism. Therefore, in this study, DTP and DTP-CSNPs were prepared (Shaban et al. 2020) and their lethal doses (LD50: the amount of a material, given all at once, which causes the death of 50% of a group of test animals) were determined. Then the therapeutic roles of both compounds against CCl4 induced-hepatotoxicity in male rats were studied and the results were compared with that resulted from cisplatin treatment (as a standard drug, but has adverse effect). Where the study focused on the determination of the markers of oxidative stress, apoptosis, lipid profile, liver and kidney functions besides liver histopathological examination. Additionally, the anti-tumor efficacies of DTP and DTP- CS against human liver cancer (HepG2) cell line via cell cycle analysis were evaluated.

Materials And Methods

Chemicals

Dulbecco's Phosphate Buffered Saline medium and L-glutamine were obtained from Invitrogen, USA. Trypsin-EDTA, penicillin and streptomycin were purchased from Thermo Fisher Scientific, USA. CCl4, cumene-H2O2 (hydrogen peroxide), 5,5dithiobis (2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH, standard superoxide dismutase (SOD), thiobarbituric acid and tetramethoxypropane (TMP), 99% DMSO, Cisplatin and other analytical grade chemicals were obtained from Sigma Aldrich, USA. Adherent HepG2 were attained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). GIBCO ® Minimum Essential Medium (MEM), was obtained from GIBCO, USA.

Animals

One hundred and twenty Sprague Dawley rats weighing 100- 150 g were obtained from Faculty of Medicine, Alexandria University. The animals were housed in stainless cages under standard laboratory conditions of 12 h light/dark cycle, 55 ±5% air humidity at room temperature of 22±3 °Cand received a standard laboratory diet and tap drinking water for 2 weeks, as an adaptation period.

Preparation of DTP-CSNPs

DTP-CSNPs were prepared (from **DTP,** sodium tripolyphosphate (TPP) and chitosan) and characterized as mentioned in our previous paper (Shaban et al. 2020). In brief, TPP solution was added to chitosan solution, left at 25^{o}C for 12 hours, **DTP** was added, left for 40 min and the solvent was removed at 40 ^{o}C. The characterization of **DTP-CSNPs** was examined by High Resolution Transmission Electron Microscope (HR-TEM), Scanning Electron Microscope (Chan et al.) with EDX detector, X-Ray Diffraction (XRD), Fourier transforms infrared (FT-IR) and thermographymetric analysis (TGA) (Shaban et al. 2020).

Determination of LD50 values of DTP and DTP-CSNPs

To facilitate the determination of LD50 *in vivo*, the LD_{50} values of **DTP** or **DTP-CSNPs** were estimated first using the values of IC_{50} (µg/ml) according to the regression formula obtained from the Interagency Coordinating Committee on the Validation of Alternative Methods: (ICCVAM) log LD_{50} (mg/kg) = 0.372log IC_{50} (µg/ml) + 2.024 (Wong et al. 2017).

For the determination of LD50 of **DTP** or **DTP-CSNPs**, 48 rats were used after the acclimation period. The rats were divided into 12 groups, six groups for each compound, where six doses of **DTP** and **DTP-CSNPs** (200, 500, 1000, 1500, 2500, 3500 mg/kg bw) were used for the administration once, intraperitoneally (i.p). Any abnormal clinical signs and behavioral changes on the animals were observed for 24 h. In each group, the number of dead rats was recorded (% dead) and LD_{50} was calculated by the arithmetic method of (Kärber 1931).

$$LD50 = LD100 - \sum (a \times b)/n$$

LD100 = Lethal dose causing the 100% mortality.

***n* = g**roup population: total number of animals/ group.

***a* = d**ose difference: the difference between two successive doses of the administered substance.

***b* =** the mean mortality: the average number of dead animals in two successive doses.

The biological effects of DTP, DTP-CSNPs and Cisplatin on hepatotoxicity

DTP and DTP-CSNPs were dissolved in 2% DMSO and their doses were chosen to be safe away from the values of LD50 where these doses were around to that of cisplatin as a standard drug. Seventy-two Sprague Dawley rats were, divided after the adaptation, into nine groups (8 animals/group) (Fig. 2). **Control**

group (C): the rats were treated with 2% DMSO (0.5 ml/kg BW) and Olive oil (0.5 mL /kg B.W) day after day, i.p for 8 weeks (the experimental period); **CSNPs group**: rats were treated (i.p) with 4.41 mg of chitosan (dissolved in 1 ml of 2% DMSO) per kg BW/day for 2 weeks (at 7^{th} and 8^{th} weeks); **DTP group**: the rats were treated (i.p) with 6.71 mg of **DTP** dissolved in 1 ml DMSO per kg BW/day for 2 weaks (at 7^{th} and 8^{th} weeks); **DTP–CSNPs group**: rats were treated (i.p) with 4.41 mg **of DTP-CSNPs** dissolved in 1 ml DMSO per kg BW/day for 2 weaks (at 7^{th} and 8^{th} weeks)**; CCl_4 group**: the rats were injected (i.p) with 0.5 ml of 95% CCl_4 dissolved in 0.5 ml olive oil/kg BW, day after day for the first 6 weeks (Karaca et al. 2017). The groups **(CCl_4-CSNPs), (CCl_4-DTP)** and **(CCl_4-DTP–CSNPs)**: the rats were injected with CCl_4 for the first 6 weeks as in the CCl4 group, then at the 7^{th} and 8^{th} weeks, they were treated with the same doses and periods of CSNPs, DTP, and DTP-CSNPs, respectively. **CCl_4- Cisplatin**: the rats was injected with CCl_4 as in **CCl_4 group**, then at the beginning of the 7^{th} week, they were treated (i.p) with 4 mg of cisplatin dissolved in 0.5 ml olive oil /kg BW/day for 5 consecutivedays (Wang et al. 2010). Fig. 2 shows this experimental design. At the end of the experimental period the rats were fasted overnight and sacrificed after anesthetized by carbon dioxide. Blood was collected from caudal vena cava, kept at room temperature for 15 min, then centrifuged at 3000 rpm for 10 min and serum was stored at -20^{o}C until used. Livers were removed immediately and small portions were fixed in 10% formalin for histopathological examination. The remaining liver was washed with cold saline solution (0.9% NaCl), divided into two parts and kept at -80^{o}C. The first part was used for determination of the expression levels of caspase-8, Bcl-2 and Bax. The second part was homogenized in 9 volumes of cold 0.1 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl, using a glass–Teflon Homogenizer and the homogenate was centrifuged at 4000 rpm for 15 min at 4°C. The supernatant was kept at -80°C till used for determination of malondialdehyde (MDA). SOD activities, GSH levels, glutathione-S-transferase (GST), total glutathione peroxidase (GPx) and glutathione reductase (GR).

Effect of the studied compounds on oxidative stress (OS)

The levels of MDA, GSH and the activities of GPx (EC 1.11.1.19), GR (EC 1.8.1.7), GST (EC 2.5.1.18) and SOD (EC 1.15.1.1) were determined using kits (Bergmeyer et al. 1983, Ellman 1959, Habig et al. 1974, Marklund & Marklund 1974, Ohkawa et al. 1979, Rotruck et al. 1973).

Effect of the studied compounds on apoptotic markers (Bcl2, Bax and caspase- 8)

RNA isolation and quantitative real-time-PCR analyses: Total RNA was isolated from liver tissues via the RNA extraction kit (Thermo Scientific, Fermentas, #K0731). Total RNA was quantified using a NanoDrop™ Q5000 (UV-Vis spectrophotometer Q5000/USA). The complementary DNA was produced using reverse transcription kits (Thermo Scientific, Fermentas, #EP0451). The synthesized cDNA was amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific, USA, # K0221). The primers for Bcl2, Bax, caspase- 8 and β-actin are listed in (Table 1). RT-PCR cycle parameters included 10 min at 95 °C followed by 40 cycles involving the denaturation for 15 s at 95 °C, annealing for 30s at 60 °C and elongation for 30s at 72°C, then final elongation at 72°C for 5 min. qRT-PCR was operated using Step One Plus™ Real-Time PCR System (Applied Biosystems, Life technology, USA). A division curve program was one after each reaction to confirm the purity of the PCR products. The quantities critical threshold (Ct) of target gene was

normalized with quantities (Ct) of housekeeping gene (β-actin) by using the $2^{-\Delta\Delta Ct}$ method to calculate the fold change in target gene.

Liver and Kidney functions and Lipid profile

Liver biomarkers, including serum AST (EC 2.6.1.1), ALT(EC 2.6.1.2) (Reitman &Frankel 1957) and ALP (EC 3.1.3.1) activities (Doumas et al. 1971), STP and albumin besides LTP levels (Gornall et al. 1949) were assayed using commercial kits. Lipid profile (cholesterol, LDL- cholesterol, TGand HDL-cholesterol levels) (Burstein et al. 1970, Fossati & Prencipe 1982) and kidney markers (urea and creatinine levels) were determined using kits (Henry 1974, Patton & Crouch 1977).

Liver Histopathological analysis

Liver tissues were fixed, processed and immersed in paraffin wax. Sections of 5 µm in thickness were obtained and stained with hematoxylin and eosin (H & E) for investigation via light microscope (SUZUKI & SUZUKI 1998).

Detection of the anticancer activities of DTP and DTP-CSNPs against HepG2 cells

Cell cycle analysis

The distribution of HepG2 cells in the dissimilar cell cycle phases (G0/G1, S and G2/M)were measured (Shanmugasundaram et al. 2017). In brief, HepG2 cells were untreated (George & Abraham) and treated with 40 µg/mlof **DTP** and **DTP-CSNPs** separately for 24 h,then cells were harvested and fixed in ice cold 70% ethanol for 12 hours at 4 $^{\circ}$C and centrifuged for 5 min at 1000 rpm. The pellets were resuspended in propidium iodide(0.05 mg/ ml) and RNase (100 U/ml) in phosphate buffer (pH 7.4), incubated at 37 ºC for 30 min and DNA was analyzed by flow cytometer (Attune® acoustic focusing flow cytometer, Thermo Scientific, USA). Finally, the cell cycle data was analyzed using cellquest software.

Statistical analysis

The data were expressed as means ± SD (standard deviation). One-way analysis of variance (ANOVA) via SPSS, 18.0 Software, 2011 and the individual comparisons were acquired by Duncan's multiple range test (DMRT). Values were considered statistically significant as $P \leq 0.05$.

Results

The results investigated that the prepared **DTP-CSNPs** have a spherical morphology with an average particle size of ~150 ± 5 nm. The conversion of DTP into nanoparticles form **"DT-CSNPs"** was found to increase the thermal stability of the composite material in comparison to **DTP** (Shaban et al. 2020).

LD 50 of DTP and DTP-CSNPs

Karber's method for determination of LD50 showed that LD50 values of DTP and DTP-CSNPs were about 2187.5 and 1462.5 mg /kg, respectively (Table 2).

Effect of different treatments on the oxidative stress

Administration of CCl_4 caused a significant ($P \leq 0.05$) increase in MDA level and GR activity with significant ($P \leq 0.05$) decreases in the level of GSH and the activities of GPx, GST and SOD when compared with the control group. However, treatment with DTP and DTP-CSNPs after CCl_4 administration decreased significantly ($P \leq 0.05$) MDA levels and GR activity, while, GSH level and GPx, GST and SOD activities were increased significantly ($P \leq 0.05$)when compared with the CCl_4 group (Fig. 3). Whereas, the animals treated with CSNPs only after CCl_4 administration showed non-significant effect on the oxidative stress parameters when compared with the CCl_4. In contrast, treatment with cisplatin after CCl_4 administration increased significantly ($P \leq 0.05$) MDA level and GR activity as compared to the CCl_4 group but decreased significantly ($P \leq 0.05$) GPx, GST and SOD activities and GSH level when compared to the CCl_4 group (Fig. 3).

On the hand, the administration of CSNPs, DTP and DTP-CSNPs to healthy rats caused non significant changes (increased or decreased) in levels of MDA, GSH and the activities of GR, GPx, GST and SOD compared to the control group (Fig. 3).

Effect of different treatments on the apoptotic markers

Administration of CCl_4 investigated a significant ($P \leq 0.05$) down-regulation of the Bcl-2 gene expression with a significant up-regulation of Bax and caspase-8 gene expressions as compared with the control group (Fig. 4a). Treatment with DTP, DTP-CSNPs and cisplatin after CCl_4 injection showed a significant down regulation in the gene expression levels of Bax and caspase-8 with a significant ($P \leq 0.05$) up regulation in Bcl-2 gene expressions when compared with the CCl_4 group (Fig. 4 a). While, the animals treated with CSNPs only after CCl_4 administration showed non-significant impact on the apoptotic markers as compared with the CCl_4 group. Also, there were non-significant changes in all parameters in the healthy rats after administration with CSNPs, DTP, or DTP-CSNPs when compared with the control group (Fig. 4 a). Figure 4 b shows that CCl_4 injection increased Bax/Bcl 2 ratio, while treatment with DTP, DTP-CSNPs and cisplatin after CCl_4 injection reduced this ratio with different degrees.

Effect of different compounds on lipid profile and liver and kidney functions

Administration of CCl_4 increased significantly ($P \leq 0.05$) serum cholesterol, TG and LDL-cholesterol levels but decreased significantly ($P \leq 0.05$) HDL-cholesterol level as compared with the control group (Fig. 5a). However, their levels were significantly ($P \leq 0.05$) improved in rats treated with DTP-CSNPs over than DTP and cisplatin as compared to the CCl_4 group. While, the animals treated with CSNPs after CCl_4 administration showed non-significant changes in lipid profile as compared with the CCl_4 group (Fig. 5a).

CCl_4 administration resulted in significant ($P \leq 0.05$) increases in the activities of ALT, AST and ALP with a significant decrease in the levels of STP and LTP compared to control group (Fig. 5 b-d). In contrast, their levels were significantly ($P \leq 0.05$) improved in rats treated with DTP-CSNPs, DTP and cisplatin after CCl_4 injection as compared to the CCl_4 group, where DTP-CSNPs gave better results than DTP and cisplatin. We

noticed that cisplatin treatment decreased STP and LTP as compared with the CCl_4 group. Urea and creatinine levels were increased after CCl_4 injection as compared to control group, while their levels were improved after treatment with DTP-CSNPs over than DTP and cisplatin as compared to CCl_4 group. However, the animals injected with CSNPs after CCl_4 administration revealed non-significant changes in the markers of liver functions, lipid profile, kidney functions as compared with the CCl_4 group (Fig. 5).

Administration of CSNPs, DTP, and DTP-CSNPs for the healthy rats caused nonsignificant changes in the levels of liver functions, lipid profile and kidney functions when compared to the control group (Fig. 5).

Histopathological analysis

The histopathological characteristics of liver tissues of the different examined groups are shown in Fig. 6. The histopathological results revealed that treatment with DTP and DTP-CSNPs after CCl_4 administration improved liver histopathology induced by CCl_4 indicating their therapeutic roles which confirm with the biochemical analysis. The results revealed no changes in liver histology of rats administered with CSNPs, DTP, and DTP-CSNPs when compared with the control group.

Induction of cell cycle arrest by DTP and DTP-CSNPs

Treatment with DTP and DTP-CSNPs resulted in a significant decrease in the population of HepG2 cells in G0/G1 and S phases as compared to the control cells (Fig. 7). Moreover, high populations of HepG2 cells were arrested at the G2/M checkpoint as compared with untreated cells. Cells treated with DTP-CSNPs showed the lowest levels of both phases, G0/G1 and S with the highest level in the G2/M phase when compared with that treated with DPT (Fig. 7).

Discussion

DTP and DTP-CSNPs treatment diminished liver injuries induced by CCl_4

The present results showed that LD_{50} values of **DTP** and **DTP-CSNPs** were about **2187.5** and **1462.5mg /kg**, respectively, (Table 1). So in this study, the rat hepatotoxicity induced by CCl_4 administration was treated with safe doses of DTP and DTP-CSNPs (6.71 and 4.41 mg/kg BW, respectively).

Our results showed that CCl4 administration caused hepatotoxicity leading to severe liver injuries as shown from the histopathological results, which confirmed by the biochemical results involving the markers of liver functions, lipid profile, oxidative stress and apoptosis in liver tissues. The mechanism of hepatotoxicity by CCl_4 may be related to the deleterious effect of CCl_4 and its highly reactive metabolites (CCl_3* and CCl_3O_2*). Since these free radicals induced oxidative stress as shown from the elevation of MDA level, the end product of lipid peroxidation, and GR activity with the reduction in the levels of antioxidants, GSH, GPx, GST and SOD. The lipid peroxidation of poyunsaturated fatty acids of the hepatocytes membrane led to the disturbance in Ca^{2+} homeostasis resulting in the destruction of the cells and their intracellular organelles and also protein content leading to hepatocellular injuries. The elevation in GR activity may be related to the

adaptation to the elevation in lipid peroxidation. Free radical scavengersmay protect biological systems from the deleterious effects of the free radicals induced by xenobiotics including CCl_4 (Kurutas 2016). GSH plays an important role against CCl_4-induced lipid peroxidation by covalently binding to $CCl_3^{\cdot}$ radicals and enhancing the activities of GR (Shah et al. 2017). Also, GSH acts as a cofactor to GPx besides its action as a nucleophilic scavenger of numerous compounds via enzymatic and chemical mechanisms (Kurutas 2016). The reduction in GSH level after CCl_4 administration may be regard to the reactions through oxidation and/or conjugation, leading to the elimination of the products of lipid peroxidation, peroxides and aldehydes. GSH depletion may lead to the inhibition of GPx activity and the elevation of lipid peroxidation (Shaban et al. 2014, Shah et al. 2017). Furthermore, SOD is considered the most effective antioxidant in the body against superoxide radicals (Alkreathy et al. 2014) and its inhibition may be owed to the action of superoxide radicals theirself or after their conversion to H_2O_2, by oxidation of the cysteine in the enzyme (Ighodaro & Akinloye 2017).

Apoptosis is a form of cell death in which a programmed sequence of proceedings leads to the removal of unnecessary cells without releasing harmful substances into the surrounding area.Apoptosis is regulated by specific genes, including many pro-and anti-apoptotic proteins. Additionally, caspase-8 is a member of the cysteine proteases, which are involved in apoptosis and cytokine processing. Caspase-8 is synthesized as an inactive enzyme and is activated by proteolytic cleavage. Our results revealed that CCl_4 administration caused up regulation of pro-apoptotic proteins Bax and caspase 8, with down regulation of anti-apoptotic proteins Bcl2. The changes in Bax and Bcl 2 levels led to the disturbance in the ratio of Bax/Bcl2 which became very greater than that of the control group. The elevation in Bax/Bcl2 ratio causing permeabilization of the outer membrane of the mitochondria, and releasing cytochrome C into the cytoplasm. Where it binds with the Apaf-1 (apoptosis protease activating factor-1) leading to the activation of procaspase 9 which in turn activates procaspases-3 leading to induction of apoptosis and cell death (Campbell &Tait 2018, Pistritto et al. 2016). Moreover, CCl_4 caused apoptosis through the activation of caspase-8. Where caspase 8 activates caspase 3 directly or indirectly via activation of Bax, which in turn activates caspase-3 resulting in the cleavage of the essential substrates for cell viability inducing apoptosis and cell death (Kalkavan & Green 2018, Pistritto et al. 2016). In addition, the raise in ROS after CCl_4 administration leads to an increase of p53 signaling which in turn activates Bax expression, but inhibit Bcl2 expression (i.e. the ratio of Bax/Bcl2 was elevated) leading to apoptosis (Eltahir &Nazmy 2018). Also, the previouds studies revealed that CCl_4 induced apoptosis via induction of DNA fragmentation (Eidangbe et al. 2020). Generally, our biochemical results showed that CCl4 administration induced oxidative stress and apoptosis, and this led to severe liver injuries where these results are in harmony with the histopathological results. Also, liver injuries after CCl_4 administration are confirmed by the elevation of the activities of liver enzymes (ALT, AST and ALP), lipid profile (TC, TG and LDL-cholesterol) in serum with a decline of albumin, STP, LTP and HDL-cholesterol. Since the liver damage leads to the leakage of liver enzymes into the blood circulation and decreases the protein biosynthesis. In addition, CCl4 administration induced nephrotoxicity as shown from the elevation of creatinine and urea levels in serum. These results agree with the previous (Krithika & Verma 2019, Safhi 2018).

On the other hand, the current results showed that LD_{50} values of **DTP** and **DTP-CSNPs** were about **2187.5** and **1462.5mg /kg**, respectively, (Table 1). The results showed an improvement of the hepatic histopathology in rats treated with DTP and DTP-CSNPs after CCl_4 administration as shown from the liver architecture (Fig. 6). These results were confirmed by the improvement of liver functions and lipid profiles. Where AST, ALT, and ALP levels, as well as total cholesterol, TG, LDL-cholesterol, became lower than those of CCl_4 group, while STP, LTP, and HDL-cholesterol became greater. Also, the renal functions were improved as revealed from the levels of urea and creatinine, which became lower than their corresponding in the CCl_4. All these positive results may be related to the therapeutic effects of DTP and DTP-CSNPs where they reduced liver injuries induced by CCl_4 through the reduction of both oxidative stress and apoptosis induced by CCl_4. Firstly, these treatments reduced the oxidative stress and lipid peroxidation induced by CCl_4 as MDA levels and GR activities became lower than those in the CCl_4 group. However, the levels of antioxidants (GSH, GPx, GST and SOD) became greater than those of the CCl_4 group. This indicates that DTP and DTP-CSNPs have antioxidant activities against the oxidative damage induced by CCl_4. This antioxidant activity may be related to the effect of dithiol groups (2 SH) in both compounds which may be oxidized forming an S-S bond (Fig.1). This means that the presence of the dithiol groups makes these compounds similar to cysteine and glutathione. Additionally, previous studies showed that the metabolism of sulfur-containing xenobiotics can be produced bisulfite (HSO^{3-}) and sulfite (SO_3^{2-}) (Shaban et al. 2010). The bisulfite can be oxidized by both one- and two-electrons forming sulfur trioxide radical anion ($SO_3^{\bullet-}$) and sulfate (SO_4^{2-}). Then $SO_3^{\bullet-}$ reacts with a molecule of oxygen forming an $O_2^{\bullet-}$ and peroxysulfate radical anion ($SO_5^{\bullet-}$) (Shaban et al. 2010). The results in Fig. 3 showed that the toxic effects of the reactive metabolites were lower than the useful effects of the antioxidants present in the dithiol groups of DPT and DPT. Secondly, the reduction of apoptosis is obviously from the reduction of pro-apoptotic marker levels, Bax and caspase 8, and elevation of anti-apoptotic marker (Bcl-2) which in turn changed the ratio of Bax/Bcl2 and became lower than that of the CCl_4 group. In addition, treatment with DTP, DTP-CSNPs reduced the oxidative stress induced by CCl_4 and this led to the reduction of apoptosis. This indicates that DTP, DTP-CSNPs act as anti-apoptotic action against the apoptosis induced by CCl_4. A possible mechanism for such effect is by elevation of the anti-apoptotic proteins and the reduction of pro-apoptotic proteins causing the stabilization of the mitochondrial membrane which in turn preventing the leakage of cytochrome C into the cytoplasm resulting in preventing the activation of caspase 3 leading to the prohibition of apoptosis and cell death. Additionally, the reduction in oxidative stress via treatment with DTP and DTP-CSNPs leads to a reduction of apoptosis induced by CCl_4. Nevertheless, treatment with DTP-CSNPs gave preferable results than DTP treatment and this may be owing to the simplicity of the DTP-CSNPs which can pass via the cellular membranes. Additionally, the large number of DTP-CSNPs leads to an increase in their active surface area which may raise the absorption rate resulting in troubles in the biological systems (Mohammed et al. 2017).

Otherwise, treatment with cisplatine for 4 days, after CCl_4 administration increased oxidative stress as shown from the markers of MDA and antioxidants, so the MDA level and GR activity became greater than those of CCl_4, while, the antioxidants became lower. However, cisplatine treatment reduced the apoptosis with a degree lower than those induced by treatment with DTP and DTP-CSNPs. Therefore, cisplatine

treatment improved liver and kidney functions and lipid profile with a degree lower than those resulted from DTP-CSNPs and DTP.

On the other hand, the results showed that administration of DTP, DTP-CSNPs for healthy rats for 14 days had no effects on the studied apoptotic markers as compared with the control group. However, they caused non significant changes in the some markers of oxidative stress as shown from the markers in Fig. 3 and this may be related to the effects of their reactive metabolites ($O_2^{\bullet-}$ and $SO_5^{\bullet-}$) (Shaban et al. 2010) which may be accumulated in the liver. Therefore, we noticed some changes (increase or decrease) in the markers of lipid profile and liver and functions, but there were no changes in liver histopathology when compared with the control.

DTP and DTP-CSNPs have antitumor activities against HepG2 cells

Cell growth and proliferation are controlled by the cell cycle management, so its interruption causes the development and progression of most tumors due to an imbalance between proliferation and apoptosis (Hong et al. 2019). Our previous study showed that the DTP and DTP-CSNPs have the ability to bind to DNA and also they have cytotoxic effects, so, in this study, we examined their antiproliferative effect against HepG2 cell line via cell cycle analysis to elucidate if these compounds have antitumor activities. Accordinglly, the current results showed that the treatment of HepG2 cells with DTP and DTP-CSNPs resulted in a significant reduction in the population of HepG2 cells in G0/G1 and S phases as compared with the control cells. This indicates that these compounds interfered with DNA synthesis and disrupted the progression of the cell cycle, leading to apoptosis (Yuan et al. 2015). Furthermore, both DTP and DTP-CSNPs arrested high populations of HepG2 cells at the G2/M checkpoint as compared with untreated cells, indicating that the cell cycle arrest led to disruption of the tubulin-microtubule equilibrium and allows the time for the repair of DNA damage, or allowed cells to survive with persistent DNA damage (Sosnowska et al. 2019, Yuan et al. 2015). In general, DTP and DTP-CSNPs have anticancer activities through interfering with the cell division and arresting the uncontrolled proliferation of cancer cells, initiating apoptosis and this is considered to be an important strategy. These results agree with our previous studies which demonstrated that DTP and DTP-CSNPs have an strong ability to interact to DNA helix (Fouad et al. 2016). The antiproliferative and apoptotic activities of DTP and DTP-CSNPs brobably may be due to the cytotoxicity of these compounds and their metabolites "bisulfite (HSO^{3-}) and sulfite (SO_3^{2-}) "(Fouad et al. 2016, Shaban et al. 2010). These results agree with the behavior of numerous anticancer agents such as vinblastine and Taxol (Hong et al. 2019, Li et al. 2007). Additionally, treatment with DTP-CSNPs gave better results than that of DTP and this may be due to the naturalness of the DTP-CSNPs as we mentioned before. In addition, previous studies revealed that the nanoparticles induce apoptosis (Mohammed et al. 2017).

In general*, in vivo,* treatment with DTP-CSNPs and DTP reduced rat liver injuries induced by CCl4 through diminishing apoptosis and oxidative stress induced by CCl4 resulting in the improvement of liver architecture and function as well as decreasing the nephrotoxicity. Moreover, DTP-CSNPs and DTP showed anticancer activities against HepG2 cell lines where they prevented the proliferation of HepG2 cell line by increasing apoptosis via arresting cell cycle in the G2/M phase. These results designate that these complexes are characterized by selectivity. (i.e.) they have an ability to differentiate between liver injuries

and cancer cell lines. These results agree with the previous studies which showed that the effect of drug (or xenobiotics) are not matched in vivo and *in vitro* (Mirabelli et al. 2019, Weinstein 2012).

Conclusion

DTP-CSNPSsand DTP exposed their therapeutic effect against liver injuries induced by CCL4 by reducing both oxidative stress and apoptosis. Where, LD50 values of DTP and DTP-CSNPs are 2187.5 and 1462.5 mg/kg, respectively. DTP-CSNPs has greater effect than DTP and both compounds are greater effect than Cisplatin. The administration of healthy rats with low doses of DTP and DTP-CSNPs for 14 days has no effect on apoptotic markers and causes nonsignificant changes in oxidative stress markers. Furthermore, DTP-CSNPs and DTP showed anticancer activities against HepG2 cell lines. So, the pharmacokinetics of DBT-CSNPs and DBT must be studied to evaluate their clinical applications.

Declarations

Ethics approval and consent to participate

HepG2 cell lines and all animal methodology were done following the Institutional Animal Care and Use Committee (IACUC) and approved via the Committee for Animal Care and Use in Alexandria University (ethical approval reference number: **(AU 042008 15 3 01).**

Consent for publication: Not applicable

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Competing interests: "The authors declare that they have no competing interests"

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Authors' contributions

Nadia Z. Shaban suggested this study, designed and organization and participated in the sequence arrangement, wrote and reviewed and approved manuscript, and agreement to be accountable for all aspects of the work to be accountable for all aspects of the work for ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Ahmed M. Aboelsaad participated in the suggestion of this study, participated in its design, carried out the experimental part in vitro and *in vivo*, performed the statistical analysis and drew the figures, and was a major contributor in writing and reviewing the manuscript. Doaa Awad participated in the statistical analysis. Shaymaa A. Abdulmalek participated in supervision on the experiments *in vitro*. Shaban Y. Shaban participated in the supervision of the preparation of the studied complexes, read and approved the final manuscript."

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Tables

Table 1. Primers used in the synthesis of cDNA

Gene	Forward primer (5- - - - - - 3)	Reverse primer (5- - - - - - 3)
Bcl-2	ATCGCTCTGTGGATGACTGAGTAC	AGAGACAGCCAGGAGAAATCAAAC
Bax	ACACCTGAGCTGACCTTG	AGCCCATGATGGTTCTGATC
Caspase- 8	CTGGGAAGGATCGACGATTA	CATGTCCTGCATTTTGATGG
β-actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

Table2: Karber's method for determination of LD50 of DBT and DBT-CSNPs on rats

Group	Dose (mg/kg)	Dose difference (a)	No of rats	LD50 of DBT			LD50 of DBT-CSNPs		
				No of dead	Mean Mortality (b)	Product (a x b)	No of dead	Mean Mortality (b)	Product (a x b)
1	200	-	4	0	0	0	0	0	0
2	500	300	4	0	0	0	1	0.5	150
3	1000	500	4	0	0	0	2	2	1000
4	1500	500	4	1	0.5	250	2	2	1000
5	2500	1000	4	2	1.5	1500	3	2.5	2500
6	3500	1000	4	4	3.5	3500	4	3.5	3500
Sum of product						5250			8150
LD50				LD50 = 3500 – (5250/4) = 2187.5 mg /kg			LD50 = 3500 – (8150 /4) = 1462.5mg /kg		

Figures

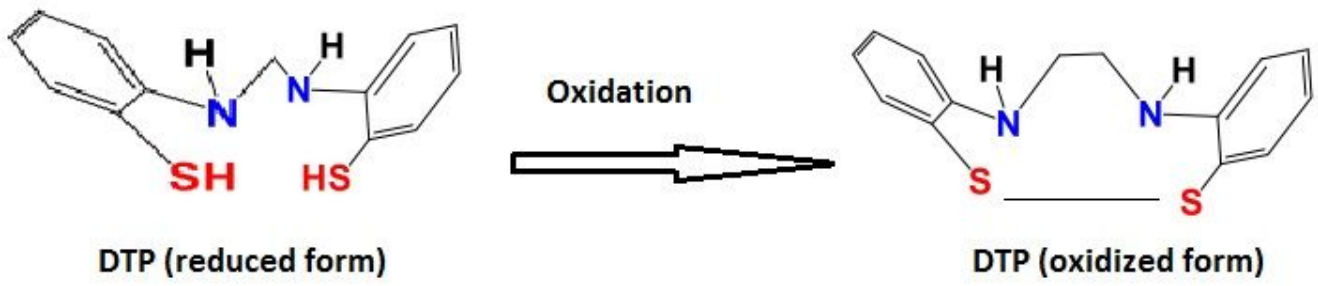

Figure 1

Figure 1

Structure of the reduced and oxidized DTP.

Figure 2

An illustration of the experimental design. Where all animals received the normal diet throughout the whole experimental period.

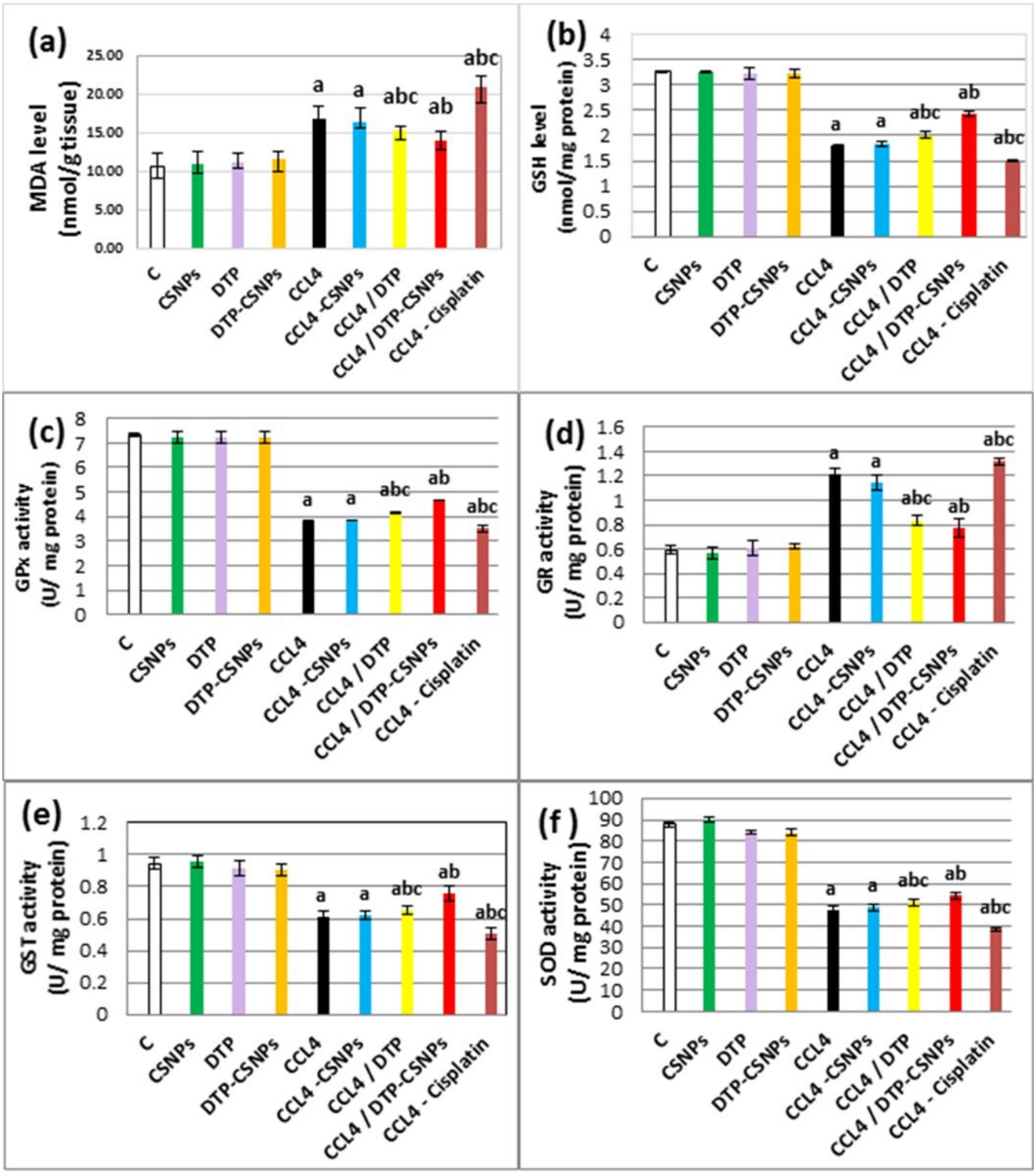

Figure 3

Effect of different studied compounds on oxidative stress parameters. (a) MDA, (b) GSH, (c) GPx, (d) GR, (e) GST, and (f) SOD. Values represent the values mean ± SD of 8 rats. One-way ANOVA followed by Tukey's test was used (a P≤ 0.05 versus control group, b P≤ 0.05 versus CCl4 group &c P≤ 0.05 versus DTP-CSNPs treated group).

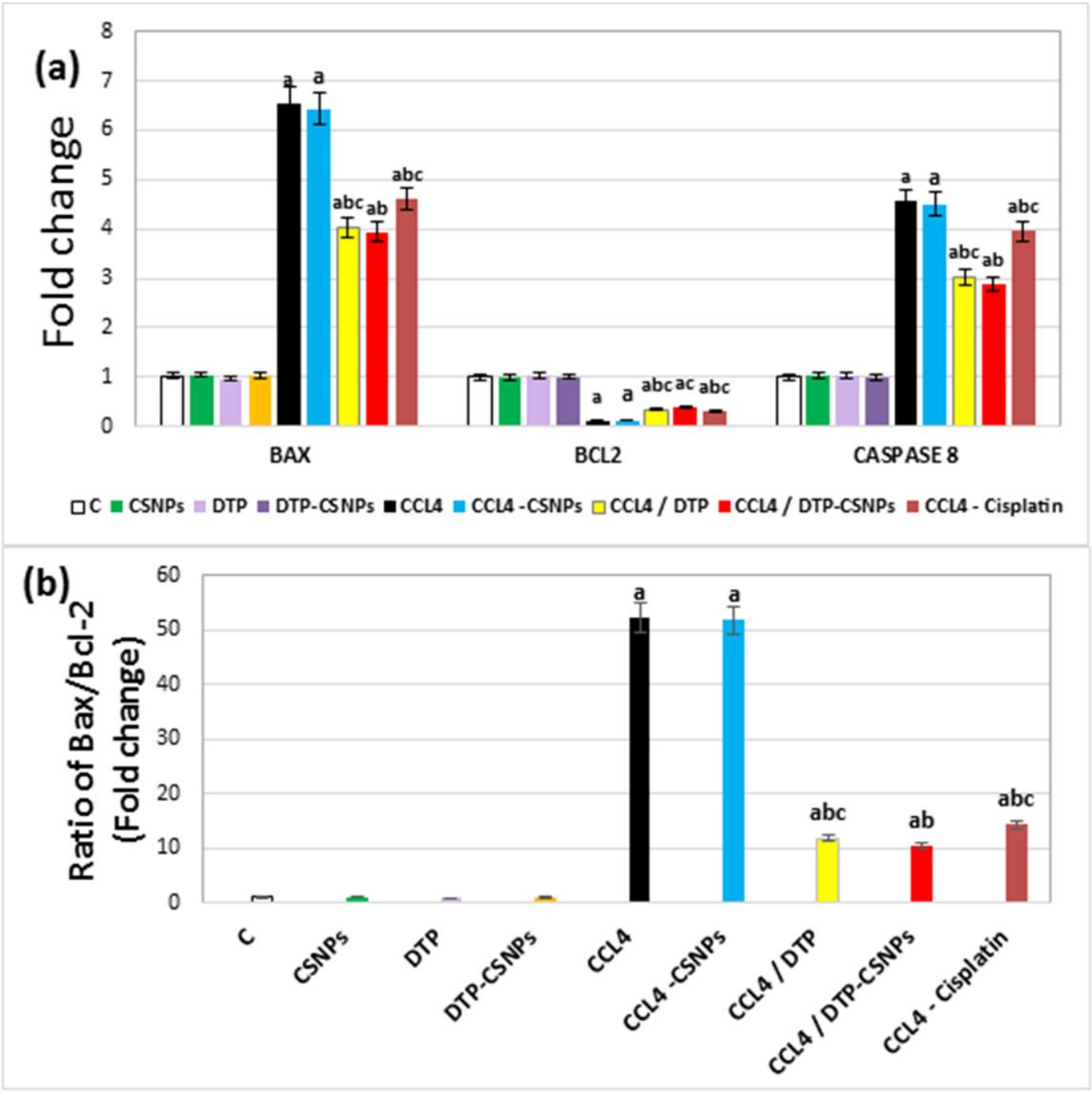

Figure 4

Effect of different studied compounds on apoptotic markers. (a) The mRNA levels of Bax, Bcl-2 and caspase-8. (b) Relative ratio of Bax/Bcl-2 mRNA. Gene expression was normalized to β-actin. Data are expressed as mean ± SD of three rats. One-way analysis of variance (ANOVA) followed by Tukey's test was used (a P≤ 0.05 versus the control group, b P≤ 0.05 versus CCl4 group & c P≤ 0.05 versus DTP-CSNPs treated group).

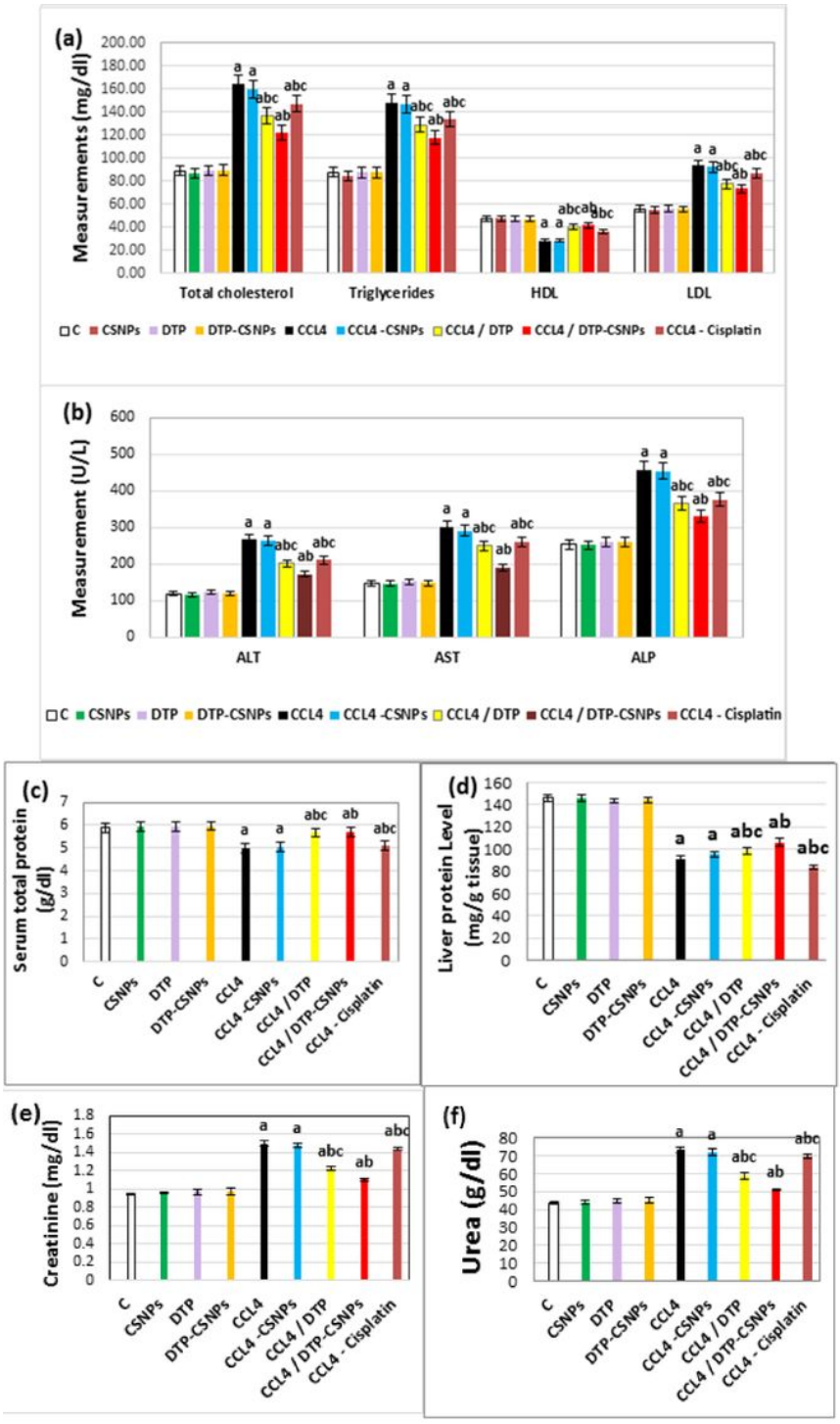

Figure 5

Effect of different studied compounds on liver functions, lipid profile and kidney functions. Where, lipid profile is represented by (a): cholesterol level, TG, HDL-cholesterol and LDL-cholesterol, liver functions are represented by (b): serum ALT, AST and ALP activities, (c) serum total protein and (d) liver total protein level; and kidney functions are represented by (e): Creatinine serum level (f) Blood urea level. The values represent the values mean ± SD of 8 rats. One-way ANOVA followed by Tukey's test was used (a P≤ 0.05 versus control group, b P≤ 0.05 versus CCl4 group & c P≤ 0.05 versus DTP-CSNPs treated group).

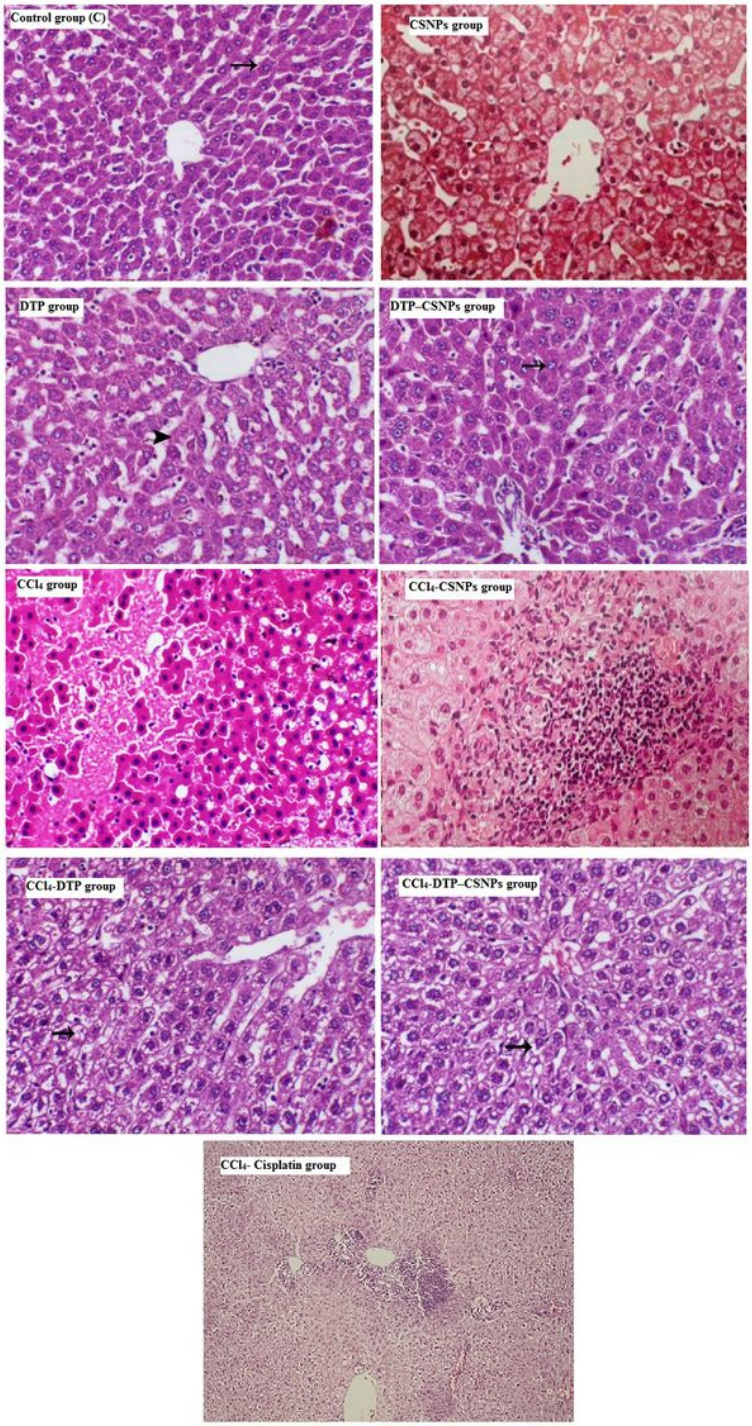

Figure 6

Histopathological analysis of liver tissue samples from the different examined groupsH&E, X200. Liver tissues in rats after CCl4 administration showed cellular infiltration congestion of hepatic sinusoids and portal vein, multiple hemorrhage and center lobular hepatic necrosis. While, after treatment with DTP confirmed a marked decrease of hepatic degeneration with granular hepatic vacuolation (arrow), and also after DTP-CSNPs treatment, liver tissues revealed a marked decrease of hepatic degeneration with mild to moderate hepatic vacuolation (arrow). CSNPs a portal tract infiltrated by inflammatory cells, mainly

lymphocytes. The infiltrate extends into the adjacent parenchyma. Some hepatocytes exhibit ballooning. Otherwise, after treatment with cisplatin, a preprortal inflammatory reaction with degenerated hepatic cord and disrupts cell plates were observed in liver tissues. Liver tissues in healthy rats after administration of each CSNPs, DTP and DTP-CSNPs exhibited normal hepatocytes (arrow) around the central vein.

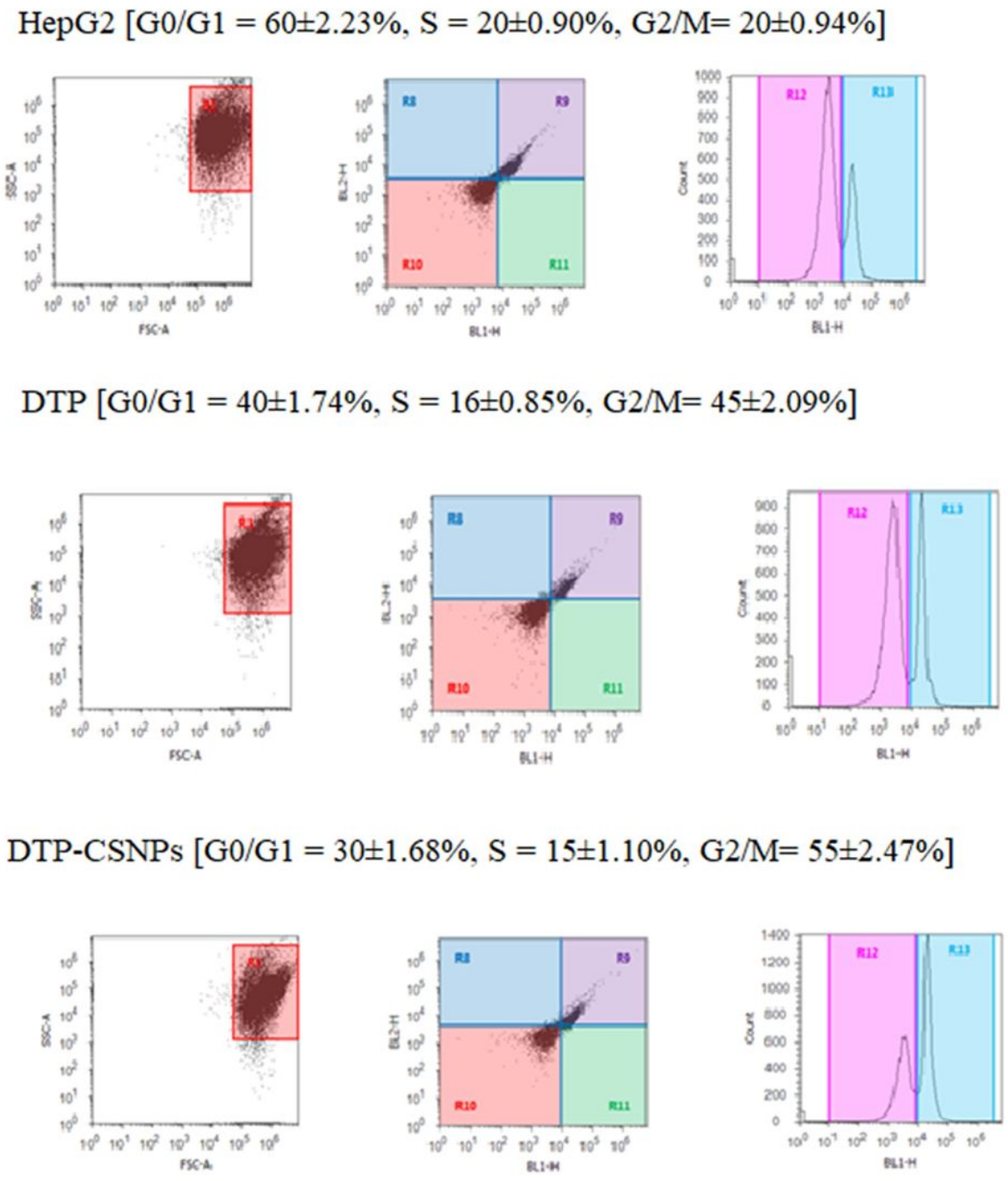

Figure 7

Flow cytometric analysis of the control and treated HepG2 cells. (a) Control (b) DTP-treated HepG2 cells and (c) DTP-CSNPs-treated HepG2 cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AbstractFigure.jpg