Curative Role of Dithiophenolato Titanium (IV)-Chitosan Nanocomposite Complex Against Carbon Tetrachloride-Induced Liver Injuries

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Abstract

Background

Titanium-based compounds have been incorporated as promising antineoplastic metals. In our previous studies, dithiophenolato titanium (IV) Complex "DBT" and its chitosan nanocomposite "DBT-CSNPs" were prepared and we showed that these compounds have antibacterial activities, cytotoxic, and have abilities to bind with DNA helixes. Therefore, in this study, we evaluated the LD50 values of dithiophenolato titanium (IV)-complex (DBT) and its high thermal stable chitosan nanoparticles (DBT-CSNPs). Then their therapeutic effects against liver injuries induced by carbon tetrachloride (CCl₄) were assessed and compared with cisplatin treatment. Additionally, the anti-proliferative activity of DBT and DBT-CSNPs against human liver cancer (HepG2) cell lines through the analysis of the cell cycle was evaluated.

Methods

Nine groups of rats were prepared: normal, DBT, DBT-CSNPs, CSNPs, CCl₄, CCl₄-DBT, CCl₄-DBT-CSNPs, CCl₄-CSNPs and CCl₄-cisplatin. Liver histopathology and the biochemical markers involving oxidative stress, apoptosis, liver and kidney functions, and lipid profile were determined.

Results

The results revealed that the treatment with DBT-CSNPs and DBT after CCl₄ administration abolished liver damage since it reduced the apoptosis induced by CCl₄ via the reduction of DNA fragmentation, Bax and caspase-8 with an elevation of Bcl2 and Bcl2/Bax ratio. Also, these treatments caused nonsignificant changes in the markers of oxidative stress. Therefore, liver histopathology and functions, lipid profile, and kidney functions were improved. Cisplatin treatment reduced liver injury with a degree less than DBT-CSNPs and DBT, but it induced nephrotoxicity. Administration of DBT-CSNPs and DBT to healthy rats for 14 days has no adverse effect. Also, the results showed that DBT-CSNPs and DBT inhibited the proliferation of HepG2 cells by arresting cells in the G2/M phase and inducing cell death.

Conclusion

DBT-CSNPs and DBT have a therapeutic effect against CCl₄-induced liver injuries via the reduction of apoptosis induced by CCl₄. Moreover, both compounds have antineoplastic activities against the HepG2 cell line. In all cases, DBT-CSNPs have a greater effect due to their nanostructure. Therefore, both compounds can be used in the pharmacological fields, particularly DBT-CSNPs.

Highlights

1. LD50 values of DBT and DBT-CS are about 1350 and 1800 mg/Kg, respectively.
2. DBT and DBT-CSNPs showed antiapoptotic effects against CCl₄-induced hepatotoxicity.
3. Treatment with DBT and DBT-CS NPs improved hepato- and nephro-toxicity induced by CCl₄.
4. DBT-CS NPs has a potent therapeutic-effect than DBT and both are better than cisplatin
5. DBT-CS NPs have antiproliferative activities and arrested HepG2 cells at G2/M phase.

1. Introduction

The liver is the largest tough organ in the body and is requisite for survival. The liver has numerous functions including the synthesis of proteins, glucose, bile, and clotting factors, besides breaking down hormones, certain drugs, and xenobiotics [1, 2]. Hepatic metabolism of medicinal agents and toxins as carbon tetrachloride (CCl₄) involves disturbed hepatic cell biochemistry with the augmented generation of free radicals and reactive oxygen species (ROS) and redox imbalance with secondary deterioration to proteins, lipids, carbohydrates, and nucleic acids [3, 4]. The xenobiotic-induced hepatotoxicity ranging from a subclinical anicteric state to severe necroinflammatory hepatitis (acute, recurrent, or chronic) and cirrhosis, relies on the dosage, nature, and period of exposure to the xenobiotics, the antioxidant defense, and attendant exposure to another disease or xenobiotics [1, 2]. ROS and reactive nitrogen species (RNS) are well known for playing a double function as both mischievous and beneficial species. The "two-faced" of ROS is adduced by a growing body of proof that ROS within cells act as secondary messengers in intracellular signaling cascades, which stimulate and preserve the oncogenic phenotype of cancer cells, while, ROS can also prompt cellular senescence and apoptosis and can thus function as antitumorigenic species [5, 6]. The cumulative production of ROS/RNS through either endogenous or exogenous insults is titled oxidative stress (OS) and is popular for numerous types of cancer cells that are connected with altered redox organization of cellular signaling paths. OS prompts a cellular redox imbalance that has been found to be present in different cancer cells as compared with normal cells; the redox imbalance thus may be linked to oncogenic inducement [7].

CCl₄ is an industrial chemical found in refrigerants and solvents for waxes, varnishes and other materials. CCl₄ is one of the most potent hepatotoxins, so it is widely used in scientific research to evaluate hepatoprotective agents [8]. Chronic exposure to CCl₄ can cause liver and kidney damage and could result in cancer [5, 6]. In liver, CCl₄ is metabolized by cytochrome P450 into trichloromethyl radical (•CCl₃) which converts to trichloromethylperoxy radicals (CCl₃OO•) [5, 6].

Liver diseases represent a major cause of serious and public health problems leading to morbidity and mortality in worldwide and Egypt [8, 9]. Therefore, scientists are working on discovering new treatments for liver diseases that are effective and do not have side effects or have minor side effects. During the last 100 years, metals have been used in therapy and other medical applications in a rational manner. A wide variety of metallodrugs have been investigated which showed excellent anticancer results [10–13]. Gallium, titanium, platinum, ruthenium, tin, gold and cobalt have shown effective anticancer activities [10, 13].

Titanium Ti(IV) as a biomaterial is generally used for orthopedic implants such as the substitution of teeth and bone [14]. The advantage of titanium compounds lies in their elevated effectiveness and low toxicity. In biological mediums, hydrolysis of Ti(IV) complexes leads to safe and inert titanium oxide (TiO₂) [15]. The next generation of phenolato-based complexes showed markedly improved stability and high activity. Ti(IV) is chiefly accumulated in the cellular nucleic acid-rich areas, mainly in the nucleic chromatin of tumor cells [16] and interacts with the oxygen (hard base) of the phosphoesters and with the nitrogen of the nucleobase in DNA that leads to cell cycle inhibition. Phenolato Ti complexes are considered a novel and efficient anticancer drug with nontoxic effect at extents of the effective doses [17, 18]. In 2012, we synthesized the dithiophenolate titanium (IV)-complex $[\text{Ti}(\text{N}_2\text{H}_2\text{S}_2)(\text{O}^i\text{Pr})_2]$ (DBT: Fig. 1) that contains the thiophenolate and amine donors instead of phenolate donors [19]. DBT complex is stable under physiological conditions, but, it is characterized by low solubility. Therefore, we prepared DBT-CS nanoparticles (**DBT-CSNPs**) by loading DBT on chitosan nanoparticles to improve bioavailability and the therapeutic efficacy of DBT [20]. Chitosan, a natural biomaterial, is a modified biopolymer, derived by partial deacetylation of chitin. It has been investigated for numerous biomedical applications due to its biodegradability, biocompatibility, antimicrobial properties, and functionality [21]. Further, chitosan nanoparticles (CSNPs) have the advantages of chitosan and the properties of nanoparticles such as surface and interface effect, small size and quantum size effects. Therefore, chitosan nanoparticles are greatly studied as nanocarriers for drug, protein, and gene delivery systems [22, 23]. Our previous studies revealed that **DBT** and **DBT-CSNPs** have antibacterial and cytotoxic activities and can bind with the DNA helix via groove binding [20]. Therefore, in this study, and for the first time, the LD50 values of DBT and DBT-CSNPs were determined as well as their therapeutic effects were evaluated against CCl₄-induced liver injuries. The study focused on the determination of the markers of OS, apoptosis, liver functions, lipid profile, and kidney functions besides liver histopathology were determined. Additionally, the effect of **DBT** and **DBT-CSNPs** on cell cycle analysis in normal liver (THLE2) and human liver cancer (HepG2) cell lines were investigated to recognize if these compounds have antitumor activity.

2. Results And Discussion

The results showed that the prepared **DBT-CSNPs** have a spherical morphology with an average particle size of $\sim 85 \pm 2$ nm. The presence of **DBT** in the **DBT-CSNPs** was found to increase the thermal stability of the composite material in comparison to **DBT** [20].

2.1. LD50 of DBT and DBT-CS NPs

The current results showed that LD50 values of **DBT** and **DBT-CSNPs** were about 1350 mg/Kg and 1800 mg/Kg, respectively (Table 2). The previous results revealed that the LD50 values of oral titanium tetrachloride and oral titanium oxide nanoparticles (TiO₂ NPs) are 1780 mg/kg and greater than 12 g/kg, respectively[24].

2.2. Effect of treatments with DBT, DBT-CS NPs and cisplatin on CCl₄-induced hepatotoxicity

2.2.1. Effect of different studied compounds on OS

The present results revealed that CCl₄ injection caused a significant elevation ($P < 0.05$) in the malondialdehyde (MDA) level and GR activity, with a significant decline ($P < 0.05$) in the level of reduced glutathione (GSH) and the activities of glutathione-S-transferase (GST), total glutathione peroxidase (GPx) and superoxide dismutase (SOD) as compared with the control group (Fig. 3). MDA is the main product of peroxidation of polyunsaturated fatty acids and elevated MDA content is a significant indicator for the lipid peroxidation [25]. The formation of lipid peroxides in the liver tissues may be related to the effect of reactive free radicals of CCl₄ metabolites, $\cdot\text{CCl}_3$ and $\text{CCl}_3\text{OO}\cdot$, that accelerated the peroxidation of native membrane lipids. The elevation in the peroxidation of the mitochondrial membrane increased membrane permeability and altered calcium homeostasis, resulted in the loss of cell integrity that contributed to cell death [5, 6]. On the other hand, free radical scavengers are nonenzymatic as GSH and enzymatic such as GPx, glutathione reductase (GR), GST and SOD could protect the biological systems from the deleterious effects of the free radicals [26]. Where, GSH plays an important role against CCl₄-induced lipid peroxidation by covalently binding to $\cdot\text{CCl}_3$ and $\text{CCl}_3\text{OO}\cdot$ radicals[27]. Additionally, GSH acts as a cofactor for GPx and as a nucleophilic scavenger of numerous compounds [26]. The depletion in GSH level after CCl₄ administration could contribute to the stimulation of lipid peroxidation [1, 27]. Otherwise, GR plays a key role in cellular defense against OS by preventing the accumulation of oxidized glutathione (GSSG) and thus maintaining the redox state. The increase in GR activity after CCl₄ administration possibly reflects an adaptation to oxidative condition and this agreed with previous studies [1, 2, 28]. Additionally, SOD is the first line of protection in the body against superoxide radicals [25]. SOD catalyzes the dismutation of the superoxide radical into ordinary molecular oxygen and H₂O₂. GPx, a selenium-containing enzyme, is the second line of protection against hydroperoxides through catalyzing the reduction of H₂O₂ and lipid peroxides, in the presence of GSH, to water and lipid alcohols, respectively, while GSH is transformed into GSSG [1, 27]. Otherwise, GSTs are major phase II detoxification enzymes found mainly in the cytosol. They catalyze the conjugation of GSH to a wide range of electrophilic substrates, usually resulting in detoxification. Subsequently, the reduction in the activities of antioxidant enzymes may be related to their inhibition by CCl₄ and its reactive metabolites through direct interaction with the enzyme molecules. Also the inhibition of GPx and GST activities probably due to GSH depletion. SOD inhibition may be concerned with the oxidation of cysteine residues in the enzyme molecules by superoxide radicals and H₂O₂ [3, 29].

On the other hand, treatment with **DBT** and **DBT-CSNPs** after CCl_4 injection increased MDA level (non-significantly) and GR activity (significantly: $P < 0.05$) but decreased significantly the antioxidants (GSH level, GPx, GST and SOD activities) as compared to the CCl_4 group (Fig. 2). This indicates that **DBT** and **DBT-CSNPs** increased non-significantly lipid peroxidation in liver tissues, in spite of, they improved the malfunctioning liver and kidney functions, and lipid profile as mentioned in (Table 3). The results clarify that **DBT** and **DBT-CSNPs** deteriorated the unwanted hepatocytes via non significant oxidative stress. The induction of OS may be related to the effect of **DBT** and **DBT-CSNPs** and their metabolites, TiO_2 and TiO_2 NPs, respectively. TiO_2 induces the generation of ROS such as $\text{O}_2^{\cdot-}$ and H_2O_2 , resulting in increasing oxidative stress, lipid peroxidation, membrane damage, and increases the apoptosis of the unwanted cells [30]. Also, the thiol groups in the **DBT** and **DBT-CSNPs** are able to oxidize in the presence of H_2O_2 , forming O-S-O [31] resulting in the reduction of H_2O_2 and this leads to the non-significant elevation in the MDA level compared with CCl_4 . Moreover, the reduction in the antioxidant enzymes may be related to the fact that SOD and GPx are Zn- and selenium-dependent enzymes, respectively. Therefore their inhibition may be due to the replacement of zinc and selenium by Ti. Also, the reduction of GSH level may be due to the binding of Ti with the sulfhydryl group and accordingly decreasing GPx and GST activities[32]. These results indicate that **DBT** and **DBT-CSNPs** may promote its antineoplastic effect, though the induction of the oxidative stress, which in consistence with the action of antineoplastic drugs, including doxorubicin and cyclophosphamide [33, 34].

The results showed that the treatment with **CSNPs** after CCl_4 injection increased non-significantly MDA level and GR activity (Fig. 3). While this treatment decreased significantly the antioxidant parameters as compared to the CCl_4 group (Fig. 3). Also, Fig. 3 shows that the administration of **CSNPs**, **DBT-CSNPs** and **DBT** to healthy rats for 14 days caused non-significant changes (increases or decreases) in the levels of markers of lipid peroxidation and the antioxidant markers compared with the control group. This led to nonsignificant changes in the markers of lipid profile, liver and kidney functions. In general, the results revealed that **DBT-CSNPs** had a greater effect than **DBT** and this may be due to the physicochemical properties of the nanoparticles which is distinguished by nano- size, raised surface area/unit mass and surface property effects that facilitated its passage through the cellular membranes, hydrolyzed into TiO_2 NPs and interrupted the biological systems [35–38]. Our results are in agreement with the studies which showed that the nanoparticles as **CSNPs** induce OS and this process is in a dose-dependent manner [39, 40]. Therefore, **DBT** and **DBT-CSNPs** must not be taken without medical indications and must be taken for a short period.

Otherwise, Fig. 3 reveals that treatment with cisplatin (for 4 days) after CCl_4 injection significantly increased OS as shown from the elevation of lipid peroxidation and reduction of antioxidant parameters leading to liver damage resulting in the elevation of serum liver enzymes and changing the lipid profile [41–43].

2.2.2. Effect of different studied compounds on apoptosis

Apoptosis is a basic biochemical process for a selective and controlled removal of cells within multicellular organisms and a key mechanism for the elimination of injured, infected, or mutated cells [44–46]. Apoptosis is tightly regulated by specific genes, including several pro- and anti-apoptotic proteins [44, 45]. Proteins of the Bcl-2 family exert different effects; for example, Bcl-2 and Mcl-1 are anti-apoptotic proteins, while white B-cell lymphoma X protein (bax, Bad and Bak) exhibit pro-apoptotic effects. Dysfunction of apoptosis renders the cancer cell resistant to treatment as well as promotes the tumorigenesis [45, 47]. Bax activation elicits cytochrome-c release, procaspase-3 activation and Poly (ADP-ribose) polymerase cleavage through the stimulation of Apaf-1 (apoptosis protease activating factor-1) which leads to the induction of the apoptotic response and cell death [48]. Caspase-8 is a cysteine protease that initiates apoptotic signaling via the extrinsic pathway. Activation of caspase-8 induces apoptosis through the direct activation of caspase-3 or activation of Bax which in turn activates caspase-3. These activations led to the cleavage of essential substrates for cell viability, inducing cell death [48, 49]. Additionally, cleavage of chromosomal DNA into oligonucleosomal size fragments is an essential part of apoptosis [44–46].

The current results revealed that CCl_4 administration showed a significant ($P < 0.05$) down-regulation of Bcl2 expression with a significant ($P < 0.05$) up-regulation of Bax and caspase-8 expressions as well as reduction in DNAF level compared to the control group (Fig. 4a, b and c "lane 1"). Furthermore, a significant ($P < 0.05$) elevation was observed in the ratio of Bax/Bcl-2. This indicates that CCl_4 administration induced apoptosis where the elevation in Bax/Bcl-2 ratio caused a change in the integrity of the mitochondrial membrane leading to cytochrome C release to the cytoplasm. Where cytochrome C binds to the Apaf-1 resulting in the activation of procaspase 9 which in turn activate procaspases-3 and 7 leading to apoptosis and the cell death[4, 50]. Moreover, as shown from the current results, the elevation in caspase-8 after CCl_4 administration induced apoptosis. Where caspase-8 activates caspase-3 (direct) or activates Bax, which in turn activates caspase-3 (indirect pathway) leading to the cleavage of the fundamental substrates for cell viability, inducing cell death[4, 50, 51]. Otherwise, as shown from our results, CCl_4 increases the ROS, which plays an important role in the activation of apoptosis through intrinsic and extrinsic pathways. The elevation in ROS, leads to the increase of p53 signaling which in turn stimulates the Bax expression, but inhibits Bcl-2 expression[33, 34]. Moreover, DNAF and DNA damage may be related to the reaction of CCl_4 metabolites (CCl_3 and $\text{CCl}_3\text{OO}^{\cdot}$) and other lipid peroxidation products with DNA forming adducts[1, 2] that activated pro-apoptotic factors, promoting apoptosis and cell death [52]. Our results are in agreement with the previous studies which demonstrated that CCl_4 induces rat liver fibrosis through the activation of apoptosis[4, 50, 51].

In contrast, treatment with **DBT** and **DBT-CSNPs**, and cisplatin after CCl_4 injection caused a significant ($P < 0.05$) elevation in the Bcl-2 expression level associated with a significant ($P < 0.05$) decline in the expression levels of Bax and caspase-8 as well as reduction in the Bax/Bcl-2 ratio and DNAF level compared to the CCl_4 group (Fig. 4a-c). Also, treatment with **CSNPs** after CCl_4 injection caused a nonsignificant elevation in all markers of apoptosis when compared with the CCl_4 group. This indicates that the treatment with **DBT-CSNPs** after CCl_4 was related to the effect of DBT not to that of **CSNPs**. **DBT**, **DBT-CSNPs**, and their metabolites as well as cisplatin reduced the apoptosis induced by CCl_4 . A possible mechanism for such effect is the reduction of Bax/Bcl2 ratio led to the stabilization of the mitochondrial membrane and prevented the releasing of cytochrome C. Interestingly, treatment with **DBT-CSNPs** showed a greater effect than **DBT** and this may be due to the differences in the physicochemical properties of these compounds and their metabolites as mentioned before. These results agree with the previous studies which showed that TiO_2 NPs have anti-apoptotic action greater than TiO_2 [53]. Also, the studies showed that nanoparticles act as anti-apoptotic effect depending on their doses and period as well as cellular types [50].

Otherwise, treatment with **DBT-CSNPs** and **DBT** gave better results than cisplatin treatment. Where treatment with cisplatin (for 4 days) after CCl₄ injection significantly increased OS and lipid peroxidation leading to liver damage resulting in the elevation of serum liver enzymes and changing the lipid profile (Table 3) which in accordance with Silici [54] and Ateyya [55].

On the other hand, the administration of each **DBT**, **DBT-CSNPs**, or CSNPs for the healthy rats caused non-significant changes in all markers of apoptosis as compared with the control group (Figs. 3 and 4). This indicates that **DBT-CSNPs** and **DBT** have no effect on the apoptotic markers in normal animals.

2.2.3. Liver and kidney functions and lipid profile

The results showed that CCl₄ administration caused significant ($P < 0.05$) elevation in the activities of serum ALT, AST and ALP with significant reductions in serum albumin and TP and liver TP levels compared to the control group (Table 3). Also, there were significant increases ($P < 0.05$) in serum cholesterol, TG and LDL-cholesterol with a significant decrease in serum HDL-cholesterol level (Table 3). This may be due to liver damage induced by CCl₄ which led to the leakage of liver enzymes into the blood circulation and decreased protein biosynthesis [56]. Additionally, blood urea and serum creatinine levels in rats injected with CCl₄ were significantly increased ($P < 0.05$, Table 3) indicating that CCl₄ induced nephrotoxicity and these results are in accordance with the results of Safhi [57].

Otherwise, treatment with **DBT** and **DBT-CSNPs** after CCl₄ administration improved liver functions, lipid profile and kidney functions with different degrees as shown from the levels of all parameters (Table 3). This indicates that treatment with **DBT** and **DBT-CSNPs** reduced the hepato- and nephrotoxicity induced by CCl₄. Also, treatment with **DBT-CSNPs** showed a better effect than **DBT**. Treatment with CSNPs after CCl₄ administration caused nonsignificant changes in all markers of liver and kidney functions and lipid profile.

Treatment with cisplatin after CCl₄ administration improved liver function and lipid profile, but to a lesser degree than those treated with **DBT** and **DBT-CSNPs** (Table 3). Furthermore, cisplatin treatment caused non-significant improvement in kidney functions compared to the CCl₄ group. These results agree with the previous studies which showed that treatment of different cancers with cisplatin causes nephro-, neuro-, cardio- and hepatotoxicity, due to its accumulation in the liver, kidney and other organs leading to production of ROS and induces OS resulting in kidney failure and liver damage [71].

On the other hand, Table 3 shows that **DBT** and **DBT-CSNPs** administration for the healthy rats for 14 days caused significant changes ($P < 0.05$) in some markers of liver and kidney functions and lipid profile as compared to the control group. These effects on healthy rats may be related to the long period (14 days) of the administration of **DBT** and **DBT-CSNPs** and the accumulation of their metabolites. The results showed that CSNPs administration to healthy rats for 14 days caused nonsignificant changes in liver and kidney functions and lipid profile as compared to the control group.

2.2.4. Histopathological analysis

The histopathological characteristics of liver tissues from the different studied groups are shown in Fig. 5 (A-K). Administration of the healthy rats with each CSNPs, **DBT** and **DBT-CSNPs**, separately had no effect on normal liver architecture. While, treatment with **DBT** and **DBT-CSNPs** after CCl₄ administration improved liver histopathology induced by CCl₄ indicating the therapeutic roles of **DBT** and **DBT-CSNPs** that confirm the biochemical analysis.

2.3. BT and DBT-CSNPs toxicity and cell cycle arrest

The viability of the HepG2 and THLE2 cells was measured under the effect of different concentrations of each **DBT** and **DBT-CSNP** for 24 h (Fig. 6a). The results showed that the cell viability of HepG2 and THLE2 cells was decreased with increasing the concentration of these compounds. Also, both **DBT** and **DBT-CSNP** showed selective cytotoxic, where **DBT-CSNP** exhibits greater cytotoxicity against HepG2 cells than **DBT** cytotoxicity (their IC₅₀ values were 44.04 and 31.76 µg/ml, respectively). While, **DBT-CSNP** exhibits lower cytotoxicity towards the normal THLE2 cells than **DBT** cytotoxicity (their IC₅₀ values were 1915 and 2094 µg/ml, respectively).

Cell growth and proliferation are controlled by the cell cycle regulation, and an imbalance between them causes apoptosis that is implicated in the growth and progression of most tumors [58]. Therefore, anticancer agents that interfere with the cell division and halt the uncontrolled proliferation of cancer cells are considered an important treatment strategy [58, 59]. The current results showed that treatment with **DBT** and **DBT-CSNPs** caused a significant decrease in the population of HepG2 cells in G₀/G₁ and S phases compared to the normal cells (Fig. 6b). High populations of HepG2 cells were arrested at G₂/M checkpoint as compared to untreated cells. Also, the results showed that the cells treated with **DBT-CSNPs** showed the lowest levels of G₀/G₁ and S phases and the highest level of G₂/M phase (Fig. 6b). Cell cycle arrest in the G₂/M phase leads to disruption of the tubulin-microtubule equilibrium and allows the time for the repair of DNA damage [60]. This means that these complexes have anticancer activities and this may be related to the effect of the **DBT** and **DBT-CSNPs** and their hydrolyzates; (TiO₂ and TiO₂NPs), respectively. It has been reported that TiO₂ NPs may induce OS in HepG2 cells causing DNA damage and p53 activation which lead to apoptosis [30, 37]. The results also indicate that each **DBT** and **DBT-CSNPs** inhibited cell growth in S- and G₂/M-phases with different degrees and **DBT-CSNPs** has a potent effect than **DBT**, this may be due to the simplicity of these NPs which can pass throughout the cellular membranes and disturb the biological systems.

In general, our results revealed that **DBT-CSNPs** and **DBT** treatments decreased rat liver apoptosis induced by CCl₄ resulting in the improvement of liver architecture and functions.

In contrast, the results showed that **DBT-CSNPs** and **DBT** prevented the proliferation of the HepG2 cell line, where they arrested the cell cycle in the G₂/M phase. Also, these compounds showed pro-apoptotic effects in HepG2 cells. These results indicate that both complexes are characterized by selectivity, i.e. they have an ability to differentiate between liver injuries and cancer cell lines. These results are in agreement with the previous studies which reported that the effect of some drugs and xenobiotics are not matched in vivo and in cell lines [67, 68]. These results are in agreement with the previous studies which reported that the effect of some drugs and xenobiotics in vivo and in cell lines are unlike each other [61, 62]. Where, these studies revealed that tumor cell lines have

important limits, and there are some variations between cell lines and tumor tissues *in vivo* including gene expression. Also, Cell lines lack interactions with the other cell types, their growth is not affected by cytokines and other cell signaling molecules, and the native tissue architecture is lost [61, 62].

3. Conclusion

LD50 values of **DBT** and **DBT-CSNPs** are 1350 mg and 1800 mg, respectively. **DBT** and **DBT-CSNPs** have a therapeutic effect against CCl₄- induced liver injuries by reducing apoptosis through the reduction of DNAF, caspase 8, and the Bax/Bcl2 ratio as well as remediation of the antioxidant parameters. **DBT-CSNPs** "due to their structure" have a greater effect than **DBT** and both compounds have greater effects than cisplatin. The administration of healthy rats with low doses of **DBT** and **DBT-CSNPs** for 14 days has no effect on apoptotic markers and non-significant effect on the oxidative stress. Otherwise, **DBT** and **DBT-CSNPs** have antineoplastic activities against the HepG2 cell line, but **DBT-CSNPs** has a greater effect due to their nanostructure. Therefore, the pharmacokinetics of **DBT-CSNPs** and **DBT** should be evaluated to know their pharmacological roles.

4. Materials And Methods

4.1 Chemicals

Adherent HepG2 were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). GIBCO® Minimum Essential Medium (MEM), was purchased from GIBCO, USA. 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. Cisplatin, CCl₄, dimethylsulfoxide (DMSO) and other analytical grade chemicals were purchased from Sigma Aldrich, USA. MTT assay kit and RNA extraction kit were obtained from Eugene, Oregon, USA and Thermo Scientific, Fermentas, respectively. All other analytical grade chemicals were obtained from Sigma (Germany) and Merck (Germany). All dilutions were made with high purity deionized water, obtained from a Milli-Q® system (Merck Chemicals GmbH, Darmstadt, Germany).

4.2 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 °C and received a standard laboratory diet and tap drinking water for 2 weeks, as an adaptation period. All animal methodology was accomplished following the Institutional Animal Care and Use Committee (IACUC) and approved via the Committee of the Animal Care and Use in Alexandria University (Ethical approval reference number: AU 04 20 06 20 2 02).

4.3 Preparation of DBT-CSNPs

DBT-CSNPs were prepared from **DBT**, sodium tripolyphosphate and chitosan[20]. In brief, sodium tripolyphosphate solution was added to chitosan solution, left at 25°C for 12 hours, **DBT** was added, left for 40 min and the solvent was removed at 40°C. The characterization of **DBT-CSNPs** was examined by High Resolution Transmission Electron Microscope (HR-TEM), Scanning Electron Microscope (SEM) with EDX detector, X-Ray Diffraction (XRD), Fourier transforms infrared (FT-IR) and thermogravimetric analysis (TGA) [20].

4.4 Determination of LD50 values of DBT and DBT-CSNPs

For determination of LD50 of **DBT** or **DBT-CSNPs**, 48 rats were used. The animals were divided into 12 groups and six doses of each **DBT** or **DBT-CSNPs** dissolved in 2% DMSO (200, 400, 800, 1200, 2000 and 3000 mg/kg) were used for the administration once, intraperitoneally (i.p). The animals were examined for any abnormal clinical signs and behavioral changes for 24 h. The number of dead rats in each group was recorded (% dead) and LD₅₀ was calculated by the arithmetic method of Kärber [63].

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

LD100 = Lethal dose causing the 100% mortality.

n = Group population: total number of animals/ group.

a = Dose difference: the difference between two successive doses of the administered substance.

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

4.5 Biological effects of DBT, DBT-CSNPs and cisplatin on CCl₄-induced hepatotoxicity

The doses of **DBT** and **DBT-CSNPs** were chosen to be safe, away from their LD50 values where these doses approach that of cisplatin. **DBT** and **DBT-CSNPs** and CSNPs were dissolved in 2% DMSO [64, 65]. Seventy two Sprague Dawley rats were divided into nine groups (8 animals/group).

Fig. 2 shows the experimental design, since the period of the experiment was 12 weeks comprehensive 2 weeks the adaptation period. **Control group (C)**: the rats were treated with 0.5 ml DMSO (2%) for 14 days (at 9th and 10th weeks), **CSNPs group**: the rats were treated (i.p) with 3.0 mg CSNPs / kg BW/day for 14 days (at 9th and 10th weeks), **DBT group**: the rats were treated (i.p) with **DBT** (4.5 mg/kg BW/day for 14 days (at 9th and 10th weeks), **DBT-CSNPs group**: the rats were treated (i.p) with **DBT-CSNPs** (3.0 mg/ kg BW/day for 14 days (at 9th and 10th weeks), **CCl₄ group**: the rats were injected (i.p) with 0.5 ml of 99.9%

CCl₄/kg BW, with equal amount of olive oil, day after day for 6 weeks (at 3rd and 8th weeks) [66]. **CCl₄-CSNPs, CCl₄-DBT, CCl₄-DBT-CSNPs** groups: the rats were injected with CCl₄ for 6 weeks, then they treated with the same doses and periods of **CSNPs, DBT** and **DBT-CSNPs**, respectively. **CCl₄- Cisplatin**: the rats were injected with CCl₄ for 6 weeks then they treated (i.p.) with 4 mg of cisplatin/kg BW/day for 5 consecutive days [67]. 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°C until used. The livers were removed immediately where small portions were taken and fixed in 10% formalin for the histopathological examination. The remaining livers were washed with cold saline solution (0.9% NaCl), divided into two parts and kept at -80°C. The first part was used for determination of the expression levels of caspase-8, Bcl-2, Bax and DNA fragmentation (DNAF). 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 the markers of OS [malondialdehyde (MDA) and reduced glutathione (GSH) levels, and the activities of glutathione-S-transferase (GST), total glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD)].

4.5.1 Effect of the studied compounds on OS markers

The level of malondialdehyde (MDA, as oxidant) and the antioxidants {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 according to the methods of Ohkawa [68] Ellman [69], Rotruck [70], Bergmeyer [71], Habig [72] and Marklund and Marklund [73], respectively.

4.5.2 Effect of the studied compounds on apoptotic markers

4.5.2.1 Determination of gene expressions of Bcl2, Bax and caspase- 8

Total RNA was isolated from liver tissues using 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 Bax, Bcl2, 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 at 95 °C for 15 s, annealing at 60 °C for 30s and elongation at 72°C for 30s, then final elongation at 72°C for 5 min. qRT-PCR was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life technology, USA). A division curve program was employed after each reaction in order to verify 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.

4.5.2.2 Determination of DNAF

It was determined using agarose gel electrophoresis technique [74]. Liver tissues were homogenized in five volumes of 50 mM Tris-HCl buffer containing 50 mM EDTA and 20% sucrose, pH 7.6. The genomic DNA was separated using the DNA purification kit (G-spin™ Total, Cat. No.17045, Korea). Then 15 µg of DNA/lane was loaded and separated by electrophoresis for 2h on 1% agarose gel containing ethidium bromide (10 µg/ml). Finally, the DNA bands were visualized using trans-illumination with ultraviolet light (Consort, Turnhout, Belgium).

4.5.3 Effect of the studied compounds on liver and kidney functions and lipid profile

Liver biomarkers involving; serum AST (EC 2.6.1.1), ALT (EC 2.6.1.2) and ALP (EC 3.1.3.1) activities, serum total protein (TP) and albumin and liver total protein levels were assayed using commercial kits (Biosystem, S.A., Spain). Lipid profile (serum triglycerides, cholesterol, HDL and LDL levels) and kidney markers (urea and creatinine levels) were determined using kits (Biosystem, S.A., Spain).

4.5.4 Liver histopathological analysis

Liver tissues were fixed, processed and embedded in paraffin wax. Sections of 5 µm in thickness were cut and stained with hematoxylin and eosin (H & E) for examination using light microscope [75].

4.6 Disclosure of the antitumor activities of DBT and DBT-CSNPS

4.6.1 Cell viability

The anti-proliferative activity of the DBT and DBT-CS nanocomposite was studied against HepG2 and THLE2 cell lines using methyl thiazolyl tetrazolium (MTT) assay. MTT assay is used to quantify the number of viable cells, depends on the reduction of the yellow tetrazolium salt MTT to a purple MTT-formazan crystal by viable cells with active metabolism [76]. HepG2 and THLE2 Cells were plated in 96-well plates at 10⁴ cells/well and treated after 24 h with serial dilutions (0, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/ml DMSO (0.1%) of DBT and DBT-CS nanocomposite separately. Each concentration was repeated in 4 wells. After incubation, 10 µL of MTT solution (5 mg/ml) in phosphate- buffered saline (PBS) was added to each well and incubated for another 4 h at 37 °C. After the medium was discarded, 100 µL of DMSO was added to each well to dissolve the purple formazan crystal formed at the bottom of the wells. Absorbance at 490 nm was measured in a BioTek Instruments Elx800 Universal Microplate Reader (USA) and the percentage of cell inhibition was determined.

4.6.2 Cell cycle analysis

The distribution of HepG2 cells in the different cell cycle phases (G0/G1, S and G2/M) were estimated [77]. Briefly, HepG2 cells were untreated (control, received only DMSO, 0.1%) and treated with 40 µg/ml of **DBT** and **DBT-CSNPs** separately for 24 h, then cells were harvested and fixed in ice cold 70% ethanol

at 4 °C for 12 hours and centrifuged at 1000 rpm for 5 min. The pellets were resuspended in 0.05 mg/ml propidium iodide (0.05 mg/ ml) and RNase (100 U/ml) in phosphate buffer (pH 7.4), incubated at 37 °C for 30 min and DNA was analyzed by flow cytometer (Attune® acoustic focusing flow cytometer, Thermo Scientific, USA). Finally, cellquest software was used to analyze the cell cycle data.

4.7 Statistical analysis

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

List Of Abbreviations

ALP: alkaline phosphatase; ALT: alanine aminotransferase; Apaf-1: apoptosis protease activating factor-1; AST: aspartate aminotransferase; CCl₄: carbon tetrachloride; CSNPs: chitosan nanoparticles; DBT: dithiophenolato titanium (IV); DBT-CSNPs: : dithiophenolato titanium (IV)- chitosan nanoparticles; DMSO: dimethylsulfoxide; DMRT: Duncan's multiple range test; DNAF: DNA-fragmentation; EDTA: ethylenediamine-tetraacetic acid; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; GST: glutathione-S-transferase; H₂O₂: hydrogen peroxide; HDL: high density lipoprotein; HepG2: human hepatoma G2; LD50; lethal dose causing the 50% mortality; LD100: lethal dose causing the 100% mortality; LDL: low density lipoprotein; MDA: malondialdehyde; MTT: methyl thiazolyl tetrazolium; OS: oxidative stress; RNS: reactive nitrogen species; ROS: reactive oxygen species; SOD: superoxide dismutase; TG: triacylglycerol; Ti(IV):titanium; TiO₂: titanium oxide; TiO₂ NPs: titanium oxide nanoparticles; TP: total protein.

Declarations

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. **Salah A. Yehia** 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. **Shaban Y. Shaban** participated in the supervision of the preparation of the studied complexes, read and approved the final manuscript. **Samar R. Saleh** participated in the suggestion of this study and its design, supervised on the experimental part and the statistical analysis, and contributed to writing and reviewing the manuscript.

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Competing interests

All authors have no conflicts of interest.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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Ethical conduct of research

All animal procedures were carried out in accordance with the Ethics Committee of the Faculty of Science, Alexandria University, where we have obtained institutional review board approval from Alexandria University for the research described (Approval No. AU 04 20 06 20 2 02).

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Tables

Table 1. Primers used in the RT-PCR

Gene	Forward primer (5' — 3')	Reverse primer (3' — 5')
Bcl-2	ATCGCTCTGTGGATGACTGAGTAC	AGAGACAGCCAGGAGAAATCAAAC
Bax	ACACCTGAGCTGACCTTG	AGCCCATGATGGTTCTGATC
Caspase-8	CTGGGAAGGATCGACGATTA	CATGTCCTGCATTTTGATGG
β -actin	AAGTCCCTCACCCCTCCCAAAG	AAGCAATGCTGTACCTTCCC

Table 2: 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	-	-
2	400	200	4	1	0.5	100	0	-	-
3	800	400	4	1	1	400	1	0.5	200
4	1200	400	4	2	1.5	600	1	1	400
5	2000	800	4	3	2.5	2000	2	1.5	1200
6	3000	1000	4	4	3.5	3500	4	3	3000
Sum of product						6600			4800
LD50				LD50 = 3000 – (6600/4) = 1350 mg /kg			LD50 = 3000 – (4800 /4) = 1800 mg /kg		

Table 3. Effect of different compounds on liver and kidney functions and lipid profile

Parameters	C	CSNPs	DBT	DBT-CSNPs	CCl ₄	CCl ₄ -CSNPs	CCl ₄ -DBT	CCl ₄ -DBT-CSNPs	CCl ₄ -Cisplatin
ALT (U/L)	117.72±2.54	119.32±2.56	131.58±3.74*	123.58±2.81*	265.52±4.33*	258.31±3.89* ^a	188.33±2.74* ^{#a}	157.92±6.09* [#]	210.81±6.81*
AST (U/L)	146.19±3.86	148.3±3.69	155.78±5.4*	151.7±4.7	302.1±4.27*	293.61±3.89* ^a	224.1±3.25* ^{#a}	187.49±4.14* [#]	259.58±6.07* ^{#a}
ALP (U/L)	252.85±2.08	256.25±2.09	267.8±4.1*	257.85±4.29	456.31±5.58*	446.49±5.41* ^a	352.16±4.05* ^{#a}	314.9±3.44* [#]	375.24±5.74*
TP (g/dl)	5.89±0.042	5.81±0.037	5.77±0.035	5.86±0.037	4.99±0.033*	5.06±0.039* ^a	5.57±0.045* [#]	5.67±0.035* [#]	5.08±0.091*
Liver TP (mg/g tissue)	146.1±7.38	142.6±3.92	136.8±4.34	141.2±3.77	91.03±5.88*	87.21±5.22* ^a	105.2±7.15* [#]	121.1±5.23* [#]	83.5±4.75* ^c
Albumin (g/dl)	4.99±0.33	4.78±0.28	4.29±0.063*	4.47±0.057*	3.48±0.15*	3.57±0.14* ^a	3.91±0.025* [#]	3.77±0.057* [#]	3.35±0.054*
Cholesterol (mg/dl)	88.75±2.39	90.3±2.31	98.17±3.98*	96.08±2.16*	165.44±2.32*	160.7±4.21* ^a	131.11±2.61* [#]	128.94±2.57* [#]	153.78±2.39* ^{#a}
TG (mg/dl)	87.49±1.58	89.2±1.62	96.08±2.16*	93.07±2.89*	148.03±2.27*	145.9±3.26* ^a	128.75±1.67* ^{#a}	123.84±2.78* [#]	133.3±1.9*
LDL-cholesterol (mg/dl)	55.61±1.5	56.69±1.54	64.19±3*	58.69±1.54*	93.1±1.87*	90.2±2.83* ^a	78.28±2.2* ^{#a}	70.79±1.45* [#]	86.59±2.0*
HDL-cholesterol (mg/dl)	47.61±1.45	45.2±1.42	42.37±1.51*	43.88±1.86*	27.95±2.09*	28.9±1.93* ^a	40.22±1.17* [#]	41.42±0.87* [#]	33.74±1.74*
Urea (mg/dl)	43.79±2.16	45.2±2.19	48.74±2.35*	45.07±3.96	73.21±2.98*	71.6±1.09* ^a	65.76±3.19* ^{#a}	58.74±2.35* [#]	69.74±2.26*
Creatinine (mg/dl)	0.95±0.02	0.950.13	1.13±0.108*	1.07±0.04*	1.5±0.068*	1.42±0.071* ^a	1.38±0.049* ^{#a}	1.21±0.027* [#]	1.44±0.04*
Values represent the values mean ± SD of 8 rats. One-way ANOVA followed with Tukey's test was used (* <i>P</i> < 0.05 vs. saline control group, # <i>P</i> < 0.05 vs. CCl ₄ group & <i>aP</i> < 0.05 vs. DBT-CSNPs treated group).									

Figures

Figure 1

Dithiophenolato titanium (IV)-complex $[\{Ti(N_2H_2S_2) (OiPr)\}_2] (DBT)$.

Two weeks adaptation	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8	WEEK 9	WEEK 10
C							•DMSO (2%, 0.5 ml/day)	
CSNPs							•CSNPs (3.0 mg/kg, i.p)	
DBT							•DBT (4.5 mg/kg, i.p)	
DBT-CSNPs							DBT-CSNPs (3.0 mg/kg, ip)	
CCl ₄	•CCl ₄ (0.5 ml/kg BW, with equal amount of olive oil, i.p, day after day)							
CCl ₄ -CSNPs	•CCl ₄ (0.5 ml/kg BW, with equal amount of olive oil, i.p, day after day)						CSNPs (3.0 mg/kg, ip)	
CCl ₄ -DBT	•CCl ₄ (0.5 ml/kg BW, with equal amount of olive oil, i.p, day after day)						DBT (4.5 mg/kg, i.p)	
CCl ₄ -DBT-CSNPs	•CCl ₄ (0.5 ml/kg BW, with equal amount of olive oil, i.p, day after day)						DBT-CSNPs (3.0 mg/kg, ip)	
CCl ₄ -Cisplatin	•CCl ₄ (0.5 ml/kg BW, with equal amount of olive oil, i.p, day after day)						Cisplatin (4 mg/kg, i.p)	

Figure 2

The experimental design

Figure 3

Effect of different compounds on oxidative stress parameters. (a) malondialdehyde (MDA), (b) glutathione (GSH), (c) glutathione reductase (GR), (d) glutathione peroxidase (GPx), (e) glutathione-S-transferase (GST), and (f) superoxide dismutase (SOD) activities. Values represent the values mean $\pm$ SD of 8 rats. One-way ANOVA was used (* $P < 0.05$ vs. saline control group, # $P < 0.05$ vs. CCl₄ group & α $P < 0.05$ vs. DBT-CSNPs treated group).

Figure 4

Effect of different compounds on apoptotic markers. (a) The mRNA levels of Bax, Bcl-2 and caspase-8. (b) Relative ratio of Bax/Bcl-2 mRNA. (c) DNAF. Gene expression was normalized to β -actin. Data are expressed as mean $\pm$ SD of three rats. One-way analysis of variance (ANOVA) was used (* $P < 0.05$ vs. saline control group, # $P < 0.05$ vs. CCl₄ group & $\alpha P < 0.05$ vs. DBT-CSNPs treated group). In DNAF, ethidium bromide-stained agarose gel showing fragmentation patterns of the extracted DNA in the studied groups. G1: control, G2: DBT, G3: DBT-CSNPs, G4: CCl₄, G5: CCl₄-DBT, G6: CCl₄-DBT-CSNPs and G7: CCl₄-cisplatin.

Figure 5

Effect of different compounds on the liver histopathology. H & E stained sections of liver tissue from the different examined groups. A: control, B: DBT, C: DBT-CSNPs, D-G: CCl₄, H: CCl₄-DBT, I: CCl₄-DBT-CSNPs and J: CCl₄-cisplatin (X200). Liver tissues of the control group showed normal lobular architecture, individual hepatocytes disclosed no pathology and the portal triad was unremarkable. Liver tissues of DBT and DBT-CSNPs groups exhibited normal hepatocytes around the central vein (Figure 5 B and C). Tissue sample of CCl₄ intoxicated group indicated cellular infiltration, congestion of central vein, mild portal inflammation, hemorrhage as well as centrilobular hepatic necrosis and focus of lytic necrosis & dispersed apoptotic bodies both intra & extracellular in location (Figure 5 D-G). The specimen of DBT treated rats confirmed a mild degree of hepatic degeneration represented by cell swelling (arrow), Figure 5 H. On the other hand, liver tissue of DBT-CSNPs treated rats revealed a marked decrease of hepatic degeneration unless single cells degeneration (arrow), Figure 5 I. Moreover, a periportal inflammatory reaction with degenerated hepatic cord and disrupted cell plates were observed in tissue of the cisplatin treated group, Figure 5 J. These histopathological results revealed the hepatoprotective effects of DBT and DBT-CSNPs that confirm the biochemical analysis.

Figure 6

Flow cytometric analysis of control and treated HepG2 cells. (a) Control (b) DBT- treated HepG2 cells and (c) DBT-CSNPs- treated HepG2 cells. Values represent the values mean $\pm$ SD (n=3).

Supplementary Files

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