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Protective Effect of *Fragaria Ananassa* Methanolic Extract on Cadmium Chloride ($CdCl_2$)-Induced Hepatotoxicity in Rats

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Abstract

This study investigated the protective effect of *Fragaria ananassa* methanolic extract on cadmium chloride ($CdCl_2$)-induced hepatotoxicity in rats. $CdCl_2$ was intraperitoneally injected at a dose of 6.5 mg/kg of body weight for 5 days with or without methanol extract of *Fragaria ananassa* (250 mg/kg). The hepatic cadmium concentration, lipid peroxidation, nitric oxide, glutathione content and antioxidant enzyme activities, including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, were estimated. $CdCl_2$ injection induced a significant elevation in cadmium concentration, lipid peroxidation and nitric oxide and caused a significant depletion in glutathione content compared to controls, along with a remarkable decrease in antioxidant enzymes. Oxidative stress induction and cadmium accumulation in the liver were successfully ameliorated by *Fragaria ananassa* (strawberry) pre-administration. In addition, the pre-administration of strawberry decreased the elevated gene expression of the pro-apoptotic *Bax* gene as well as the protein expression of caspases-3 in the liver of $CdCl_2$-injected rats. In addition, the reduced gene expression of anti-apoptotic *Bcl-2* was increased. Our results show an increase in the expression of tumor necrosis factor α in the liver of rats treated with cadmium. In sum, our results suggested that *Fragaria ananassa* successfully prevented deleterious effects on liver function by reinforcing the antioxidant defense system, inhibiting oxidative stress and reducing apoptosis.

Keywords: *Fragaria ananassa*; Cadmium chloride; Oxidative stress; Apoptosis; Liver

Introduction

Cadmium (Cd) is a widely found environmental and occupational toxic heavy metal that has been released into the environment at a concentration sufficient to induce toxicity. Humans are primarily exposed to Cd from contaminated food and water, smoking, and industrial emissions (El-Habit and Abdel Moneim, 2014). In 2007 according to US Geological Survey, 83% of the global Cd used in batteries, 8% in manufacturing pigments, 8% in plating, 2% used as stabilizer for plastics and other products (USGS, 2008). Cd is classified as a carcinogen for both humans and animals. In addition to the carcinogenicity of Cd, the metal accumulates in soft tissue, especially renal and hepatic tissues where it is bound to the apoprotein metallothionein (Tandon *et al.*, 2001). In hepatic tissue, Cd causes liver failure associated with hepatocyte degeneration, focal necrosis, changes in liver functional biomarkers, and fat deposition (El-Refaiy and Eissa, 2013).

Accumulating evidence has indicated that Cd-induced hepatotoxicity is mediated by reactive oxygen species (ROS) generation (Dkhil *et al.*, 2014). Another possible mechanism for the toxicity of Cd in the liver is the primary injury of hepatocytes resulting from the binding of Cd to sulfhydryl groups in the mitochondria and secondary injury initiated by the activation of Kupffer cells (Arroyo *et al.*, 2012). Oxidative damage is initiated by the inactivation of sulfhydryl groups on critical molecules in the mitochondria, which causes mitochondrial permeability transition and mitochondrial dysfunction-mediated apoptosis (Mari *et al.*, 2009). Kupffer cells may release pro-inflammatory cytokines that stimulate the migration and accumulation of neutrophils and monocytes in the liver. Infiltrating neutrophils can release large amounts of ROS, which can result in oxidative stress and lipid peroxidation (Abdel Moneim, 2016).

There are a number of studies on potential mitigating agents that prevent the hepatotoxicity induced by cadmium exposure. Medicinal plants and herbs have been recognized to have antioxidant activities because they are rich in several antioxidant compounds. Hence, to combat cadmium-induced hepatic injury, medicinal plants might be appropriate due to their relative availability, low cost and minimal side effects. The strawberry (*Fragaria ananassa*) is a widely cultivated hybrid species of the genus *Fragaria* and is an important source of phytochemicals (Giampieri *et al.*, 2014), most of which are natural antioxidants and contribute to the high nutritional value of the fruit (Giampieri *et al.*, 2012b). Flavonoids (mainly anthocyanins, with flavonols and flavanols providing a minor contribution) are the most abundant class of

polyphenolic compounds in strawberry, followed by hydrolyzable tannins (ellagitannins and gallotannins) and phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) together with condensed tannins (proanthocyanidins) as minor constituents. In particular, anthocyanins (cyanidin-3-glucoside, pelargonidin, and pelargonidin-3-rutinoside) have been shown to exhibit a range of biological effects, including antioxidant activity, photoprotection, anticarcinogenesis, induction of apoptosis, and prevention of DNA damage (Giampieri *et al.*, 2012a). Strawberry is also a moderate source of micronutrient carotenoids, particularly β-carotene. The capacity of carotenoids to quench free radicals is well known, and reactions with different radical species have also been documented (Giampieri *et al.*, 2014), including the prevention of lipid peroxidation (Packer, 1993). In folk medicine, strawberry is used as a potential remedy due to its astringent and diuretic properties to heal skin diseases and wounds or for inflammation of the nerves and lungs (Skrovankova *et al.*, 2015). Currently, recent epidemiological studies have indicated that due to their antioxidant properties, strawberries may exert protective effects against a variety of chronic degenerative diseases in which oxidative stress is thought to play a major role.

This study was designed to investigate the protective impact of strawberry on cadmium chloride-induced hepatic injury in rats as an experimental animal model of cadmium toxicity.

Materials and methods

Chemicals

All chemicals and solvents were of analytical grade. Anhydrous cadmium chloride ($CdCl_2$; CAS Number 10108-64-2) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin assay kits were supplied by Randox Laboratory (Crumlin, UK). Tris-HCl and sucrose were purchased from Fluka Chemie (Buchs, Switzerland). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). RevertAid H minus Reverse Transcriptase was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA), PCR primers were synthesized by Jena Bioscience GmbH (Jena, Germany).

Fruit material

Fresh strawberry (*Fragaria ananassa*) fruit was collected from a market in Cairo, Egypt from April-May, 2016. The fruit material was authenticated by the Botany Department, Faculty of Science, Helwan University, on the basis of taxonomic characteristics and by direct comparison with the herbarium specimens available at the herbarium of the Botany Department. Five hundred grams of fruits were homogenized in 2000 ml (1 : 10 w/v) of a 70% methanol aqueous solution for 48 h with mixing every 2 h. After 48 h, the homogenate was filtered and methanol was evaporated to dryness in a vacuum evaporator (IKA, Germany). The semi-liquid residues (~28.4 g) were dissolved in distilled water. The clear solution of the extract was stored in an air tight bottle at -20 °C and designated as strawberry methanolic extract (SME).

HPLC analysis, total phenolics, total flavonoids, and total anthocyanins contents

The analysis of bioactive phenolics and flavonoids compounds present in the SME was performed using a Perkin Elmer Series 200 liquid chromatography (PerkinElmer, USA) according to the method described in the published work of Hamed et al. (2016). Whereas, the total anthocyanins content in SME was determinate by the pH differential method as described by Van De Velde et al. (2013).

Animal treatment

Thirty two adult (8-9 weeks old, 160–180 g) male Wistar rats were purchased from the Animal facility of VACSERA **(**Cairo, Egypt). The rats were housed at the Zoology department, Helwan University (Cairo, Egypt) under standard laboratory conditions with a 12 h light/dark cycle and a fixed temperature (22±25 ºC) with access to pelleted rodent feed and water *ad libitum*. To

investigate the hepatoprotective activity of strawberry on cadmium chloride ($CdCl_2$)-induced hepatotoxicity, randomly selected rats (n=8) were divided into four groups. Control (Con) rats were intraperitoneally (i.p.) injected with 0.9% physiological saline daily for 5 days. The $CdCl_2$ group was injected i.p. with 6.5 mg/kg $CdCl_2$ for 5 days. The SME group was orally administered a 250 mg/kg strawberry methanolic extract, and the SME+$CdCl_2$ group was pre-administered with SME one hour before $CdCl_2$ i.p. injection for 5 days. $CdCl_2$ was dissolved in 0.9% physiological saline (NaCl). SME was orally administered at a dose of 250 mg/kg according to a preliminary study in which this dose yielded no signs of toxicity, while $CdCl_2$ was i.p. injected at 6.5 mg/kg according to Dkhil et al. (2014). Rats were sacrificed by decapitation 24 h after the last administration, and their livers were dissected, weighed and immediately homogenized in an ice-cold medium of 10 mM phosphate buffer (pH 7.4) to yield a 10 % (w/v) homogenate for biochemical analysis. Blood was collected using cardiac puncture method for serum analysis. All of these protocols and animal handling of this study were approved by the Research Ethics Committee of Zoology department, Faculty of Science, Helwan University for the Laboratory Animal Care and were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals 8th edition (NIH Publication No. 85-23 revised 1985).

Cadmium level

The cadmium level in the liver tissue was determined using a standard method. Briefly, liver tissue samples were weighed and ashed with hot 1 M nitric acid at 150 °C for 2 h. The ashed samples were diluted with deionized water to 50 ml. The levels of cadmium were analyzed by atomic absorption spectrophotometry (Perkin Elmer 3100) in a graphite furnace at 228.8 nm. The cadmium values are expressed on a wet liver tissue basis in µg/g.

Biochemical assay

Serum alanine aminotransferases (ALT) and aspartate aminotransferases (AST) were estimated according to the method of Reitman and Frankel (1957). The level of alkaline phosphatase (ALP) was assayed based on the method of Belfield and Goldberg(1971), while the method of Schmidt and Eisenburg was used to determine total serum bilirubin (1975). Lipid peroxidation was measured by determining the amount of malondialdehyde (MDA) formed by the method of Ohkawa et al. (1979). Nitric oxide was determined by using the Griess reagent (Green *et al.*, 1982). The content of glutathione (GSH) in the liver homogenate was estimated using the

method described by Ellman (1959). Catalase (CAT) activity was determined by measuring the rate of decomposition of hydrogen peroxide at 570 nm as described by Aebi (1984). SOD activity was determined using the method of Nishikimi et al. (1972), while the activities of glutathione peroxidase and reductase were measured using the methods of Paglia and Valentine (1967) and De Vega et al. (2002), respectively.

Quantitative reverse transcriptase-polymerase chain reaction analysis

Total hepatic RNA was extracted from frozen samples with TRIzol reagent. Approximately 5 μg of total RNA was converted to cDNA. The sense and antisense primers for selected genes are listed in Table 1. The Power SYBR® Green Master Mix kit was used for real-time PCR analysis. The relative differences in gene expression between groups were expressed using cycle time (Ct) values, and the relative differences between groups were expressed as relative increases by setting the control as 1-fold. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a control and was shown to be unchanged by any treatment.

Preparation of histological sections

Specimens of the liver were taken and fixed in 10% formaldehyde/PBS for 24 h at room temperature and processed for light microscopy. The specimens were embedded in paraffin and sectioned at a thickness of 4-5 μm. The sections were stained with hematoxylin and eosin.

Immunohistochemistry analysis

Formaldehyde/PBS-fixed, paraffin-embedded sections (4 μm) were mounted on glass slides. Sections were deparaffinized and blocked with methanol containing 0.1% H_2O_2 for 10 min to quench endogenous peroxidase activity. After blocking, the sections were stained with a polyclonal rabbit anti-TNF-α antibody at 4 °C overnight. The sections were then washed with phosphate buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody at 37°C for 30 min. The antibody biding sites were visualized by incubation with DAB-H_2O_2 at room temperature for 10 min. Images were taken at an original magnification of 400× (Nikon Eclipse E200-LED, Tokyo, Japan).

Statistical analysis

Values were expressed as the mean ± standard error of the mean (SEM) of seven rats. Data were analyzed with one-way analysis of variance (ANOVA) with post hoc Duncan multiple tests. P values <0.05 were considered statistically significant.

Results

Phenolics and flavonoids fingerprint detected at 280 nm for the SME is presented in Figure 1. The chromatogram shows the presence of many peaks with retention times between 0 and 8 min. Based on the UV-Visible spectral data and their retention time, the SME has UV band at 280 nm characteristic for phenolics and flavonoids compounds, may be gallic acid, p-coumaric acid, cyanidin, pelargonidin, ellagic acid, and quercetin. Total phenolics content (ranged from 98.3 to 112.7 mg/100 g SME), total flavonoids content (ranged from 44.2 to 57.1 mg/100 g SME) and total anthocyanin content (ranged from 12.6 to 25.3 mg/100 g SME).

Rats receiving $CdCl_2$ alone had significant ($p<0.05$) decrease in the final body weight, associated with a significant ($p<0.05$) increase in liver weight were observed compared with the control rats. Interestingly, rats pre-administered with the SME produced a significant improvement in the final body weight and relative liver weight and these effects were significantly changed compared with the control rats (Figure 2).

$CdCl_2$ treatment in rats markedly ($p < 0.05$) increased the concentration of Cd in hepatic tissue compared to control rats. The increased Cd concentration in the liver was significantly ($p < 0.05$) attenuated by administration of SME in $CdCl_2$ injected rats (Figure 3).

Table 2 shows the significant ($p < 0.05$) elevation in liver enzyme biomarkers, ALT, AST and ALP, in addition to total bilirubin in $CdCl_2$ injected rats compared to controls. Elevated outflow of hepato-specific enzymes upon injection with $CdCl_2$ was significantly ($p < 0.05$) prevented by SME compared to the $CdCl_2$ group.

$CdCl_2$ injection significantly ($p < 0.05$) increased the oxidative stress markers MDA and NO and decreased the GSH content in liver tissue compared to control rats. SME administration at 250 mg/kg counteracted the oxidative stress induced following $CdCl_2$ injection by significantly ($p < 0.05$) increasing the GSH content and decreasing the MDA and NO levels in hepatic tissue (Figure 4).

The antioxidant enzyme activities of SOD, CAT, GPx and GR were significantly ($p < 0.05$) reduced in the liver of rats injected with $CdCl_2$ (Figure 5). SME pre-treatment enhanced the activities of antioxidant enzymes, and those enzymes showed higher activities that those in $CdCl_2$ injected rats. In parallel to the biochemical findings, the mRNA expression levels of *SOD2*, *CAT*, *GPx1* and *GR* were significantly down-regulated ($p < 0.05$) in $CdCl_2$ injected rats. However, the data illustrated in Figure 5 showed that SME administered 1 h before $CdCl_2$ reversed the decrease in hepatic antioxidant enzyme expression in $CdCl_2$ intoxicated rats.

Histological examination of the liver tissue showed no abnormal morphological changes in the control rats (Figure 6). However, liver histology of rats intoxicated with $CdCl_2$ confirms the serum indicators of hepatotoxicity. The signs of hepatotoxicity include various degrees of focal necrosis, congestion, and foci of inflammation. On a cellular level, toxicity included hepatocyte swelling, pyknosis, and karyorrhexis. The livers of rats treated with SME alone presented with normal hepatic cells with a well-preserved hepatocyte structure. Interestingly, pre-administration of SME in the $CdCl_2$-induced hepatotoxicity model showed prominent recovery and a normal liver tissue architecture with mild residual degeneration.

To investigate whether the observed hepatoprotective effects of SME were related to the anti-apoptotic and anti-inflammatory activities of SME, the expression levels of *Bcl-2*, *Bax* and *TNF-α* in hepatic tissue were examined. Our findings revealed that the mRNA expression of *Bcl-2* was significantly down-regulated ($p < 0.05$), while the mRNA expression levels of *Bax* and *TNF-α* were up-regulated in $CdCl_2$ intoxicated rats (Figure 7). Rats treated with SME prior to $CdCl_2$ showed significant up-regulation of *Bcl-2* and down-regulation of *Bax* and *TNF-α*.

Consistent with the qRT-PCR findings, liver sections showed increased expression of TNF-α in the $CdCl_2$-intoxicated group (Figure 8b). After pretreatment with SME, TNF-α expression decreased but remained higher than that of the normal group (Figure 8d). Control and SME livers showed weak immunoreactivity for TNF-α in the hepatocytes (Figures 8a and c).

To explore whether the observed hepatoprotective potential of SME was related to the anti-apoptotic activity of SME, the expression level of caspase-3 in hepatic tissue was examined. The results revealed that the expression level of the caspase-3 protein was markedly up-regulated in the $CdCl_2$ treated livers (Figure 9b). However, rats pretreated with SME exhibited obvious down-regulation of caspase-3 protein in the liver (Figure 9d). Control and SME liver sections showed weak immunoreactivity for caspase-3 in the hepatocytes (Figures 9a and c).

Discussion

Studies suggest that consumption of berry fruits, including strawberry (*Fragaria x ananassa*), may have beneficial effects against oxidative stress mediated diseases, such as cancer and diabetes. Strawberries contain many phenolic compounds that are thought to contribute to their biological properties. Moreover, recent reports also suggest that strawberry has antioxidative, antilipidemic and anti-diabetic activities (Ibrahim and Abd El-Maksoud, 2015). However, whether strawberry could alleviate damage from heavy metals is not clear. Thus, in the present study, we investigated the hepatoprotective effects of strawberry methanolic extract on cadmium-induced liver injury in rats. In the present study, our results confirmed the involvement of oxidative stress in cadmium-induced liver injury and showed a significant ameliorative effect of strawberry, as evidenced by decreasing the levels of cadmium concentration, lipid peroxidation and nitric oxide and inhibiting the decrease in antioxidant enzyme activities and the GSH content. The mitigation of cadmium-induced oxidative stress by strawberry was likely due to its ability to restore the balance between the generation and clearance of ROS. Reduced levels of liver function markers towards control values were an indication of the stabilization of the plasma membrane and hepatocyte repair. Moreover, histological examination of the liver sections revealed severe damage in hepatocytes treated with cadmium, while in sections obtained from rats treated with SME and cadmium, hepatocyte damage was reduced. Thus, these results suggested that inhibition of elevated liver function markers and liver damage may participate in the protective effect of SME against cadmium-induced hepatotoxicity.

Many previous reports in several animal species have shown that Cd primarily accumulates in the liver (Haouem and El Hani, 2013, Dkhil *et al.*, 2014). In agreement with these reports, our findings showed that Cd was accumulated and detected in liver tissue after 5 days of administration. Furthermore, the effect of Cd on the relative weight of the liver has been previously reported. Some studies have revealed that this can atrophy the liver (Hwang and Wang, 2001), while others have reported liver enlargement following cadmium exposure (Pari and Murugavel, 2005). The data presented herein show that Cd accumulated in the liver had a deleterious effect on the relative weight of the liver. However, SME pretreatment can blunt or reverse the hepatotoxicity of Cd by reducing the accumulation of Cd in the liver tissue. SME is

rich in flavonoids that act as metal chelators due to the presence of multiple hydroxyl groups that form a coordinate bond with Cd^{2+} (Kumar and Pandey, 2013). However, the mechanism underlying the metal chelation property of SME is not clear and requires further investigation.

Upon clinical examination, the increased ALT and AST levels in the blood are considered to be a biomarker of liver damage (Abdel Moneim, 2016). When exposed to Cd, the levels of ALT and AST usually increased, indicating liver dysfunction. The present investigation showed that SME pretreatment resulted in marked improvement in liver function markers. The results indicated that SMA may stabilize the cellular membrane of hepatocytes and maintain its function by reducing oxidative stress in Cd-treated rats. Again, this effect may arise from the ability of SME to reduce Cd accumulation in liver tissue. Our findings also suggested that the reduction in increased lipid peroxidation due to Cd hepatotoxicity may be an important factor in the action of SME. Hamed et al. (2016) studied the preventive effect of strawberry juice on carbon tetrachloride (CCl_4)-induced toxicity in male Wistar rat livers. The authors showed that the levels of serum ALT, AST and ALP that were increased by CCl_4 were reversed by strawberry treatment. They concluded that strawberry juice has the potential to protect rat liver tissue against toxicant-induced hepatotoxicity. Indeed, it appears that the potent hepatoprotective activity of SME may be attributed to its high polyphenol and flavonoid contents.

Generation of ROS during Cd-induced liver injury is the major factor underlying liver damage (Dkhil *et al.*, 2014). To address this hypothesis, experiments were performed to measure lipid peroxidation, nitric oxide, GSH content and antioxidant enzyme activity in the liver tissue of the control and experimental rats. LPO and NO were significantly elevated by 1.6- and 1.4-fold, respectively, in $CdCl_2$-treated tissues compared to controls. The abnormal formation of ROS induced by $CdCl_2$ exposure may rapidly deplete GSH and significantly decrease the GSH content in the liver (Zhai *et al.*, 2013). Consistent with these observations, $CdCl_2$-treated liver tissue had 1.7-fold less GSH compared to untreated rats (Figure 4). The drastic elevation in ROS and formation of metallothioneins may also exceed the antioxidative capacity of antioxidant enzymes, causing the exhaustion of these enzymes and a reduction in their activities (Thijssen *et al.*, 2007). However, pretreatment with SME enhanced the activities of antioxidant enzymes (SOD, CAT, GPx and GR), increased the antioxidant content (GSH), and diminished the amount of LPO and NO following $CdCl_2$-induced hepatotoxicity in these rats, suggesting that the activity

of antioxidants and the decreased production of LPO and NO may play a role in the hepatoprotective mechanism of strawberry.

Tumor-necrosis factor (TNF), a cytokine produced by activated macrophages in response to pathogens and other injurious stimuli, is a necessary and sufficient mediator of local and systemic inflammation. TNF-α amplifies and prolongs the inflammatory response by triggering other cells to release both cytokines, such as IL-1, and mediators, such as eicosanoids, nitric oxide and reactive oxygen species, all of which promote further inflammation and tissue injury (Olszowski *et al.,* 2012). Yazihan et al. (2011) demonstrated that chronic exposure of Wistar rats to 15 ppm Cd caused a marked increase in the TNF-α level in heart tissue. Moreover, a 7-week exposure of rats to 100 ppm cadmium in their drinking water resulted in a 470% increase in their TNF-α plasma levels compared to controls (Afolabi *et al.,* 2012). Stosic et al. (2010) demonstrated that i.p. injection of Cd in rats caused a higher TNF-α level in lung homogenates. Furthermore, it has been suggested that TNF-α is important in the development of $CdCl_2$-induced hepatotoxicity (Olszowski *et al.,* 2012). Because TNF-α is produced predominantly by the monocyte macrophage lineage and the major population of this lineage in the liver is Kupffer cells (Decker *et al.,* 1989), increased production of TNF-α by activated Kupffer cells may be responsible for hepatotoxicity. In our study, the effects of TNF-α in the damaged liver was evaluated by immunohistochemistry. Compared to the control group, treatment with cadmium up-regulated the expression of *TNF-α*, while pretreatment with SME down-regulated the expression of *TNF-α* compared to the $CdCl_2$-intoxicated group.

Our results revealed that the protein expression levels of *caspase-3* and mRNA expression level of *Bax* were significantly upregulated in the liver of rats treated with cadmium, while the mRNA expression level of *Bcl-2* was significantly downregulated. Chang et al. (2013) demonstrated that Cd induced pancreatic β-cell death through the mitochondria-dependent apoptosis pathway, where mitochondrial dysfunction (loss of mitochondrial membrane potential, elevated cytochrome *c* release, and down-regulation of anti-apoptotic markers and up-regulation in pro-apoptotic parameters) increased poly (ADP-ribose) polymerase (PARP) cleavage and activation of caspase-3/-7-/9, critical events for apoptosis. Wang et al. (2015) demonstrated that the activation of the extracellular signal-regulated kinase (ERK) pathway is involved in the putative mechanisms of apoptosis in Cd-exposed hepatocytes. Furthermore, Ben et al. (2015) reported that an elevated ROS level increased calmodulin (CaM) function, induced [Ca^{2+}] intercellular

elevation, and activated the mitogen-activated protein kinases (MAPK) and mammalian target of rapamycin (mTOR) pathways, thereby leading to caspase-dependent apoptosis of hepatic cells. Conversely, SME pretreatment was effective in preventing apoptosis in hepatic tissue. The hepatoprotective activity of SME is related to the suppression of apoptosis based on the ability of polyphenols and flavonoids to protect against stress-induced apoptosis in addition to the ability of SME to reduced Cd accumulation in the liver tissue.

In conclusion, the present findings have demonstrated that the methanolic extract of *Fragaria ananassa* displays significant protection against cadmium-induced hepatotoxicity in rats and this protective activity can be attributed to (1) SME chelation ability which in turn may enhance the excretion of Cd from the body; and (2) SME has antioxidant and anti-apoptotic activities against Cd and can reverse Cd-induced hepatotoxicity. These findings may be attributed to the numerous effects of the different polyphenols and flavonoids present in the strawberry. However, further studies should be conducted in future to determine how much fresh strawberry should consume by human to attenuate cadmium toxicity.

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Legends

Figure 1: HPLC-DAD phenolics and flavonoids fingerprint for the *Fragaria ananassa* methanolic extract (SME) recorded at 280 nm**.**

Figure 2: Preventive effects of *Fragaria ananassa* methanolic extract (SME) on the final body weight and relative liver weight of rats exposed to cadmium chloride ($CdCl_2$) for 5 days.

Data are expressed as the mean ± SEM (n=8). $^{a}p < 0.05$, significant change with respect to **Control**; $^{b}p < 0.05$, significant change with respect to **$CdCl_2$** using Duncan's post hoc test.

Figure 3: Hepatoprotective effects of *Fragaria ananassa* methanolic extract (SME) on cadmium accumulation (µg/g wet tissue) in the liver tissue of rats treated with cadmium chloride ($CdCl_2$) for 5 days.

Data are expressed as the mean ± SEM (n=8). $^{a}p < 0.05$, significant change with respect to **Control**; $^{b}p < 0.05$, significant change with respect to **$CdCl_2$** using Duncan's post hoc test.

Figure 4: Mitigating effects following the pre-administration of *Fragaria ananassa* methanolic extract (SME) on oxidative stress parameters levels in rats exposed to cadmium chloride ($CdCl_2$) for 5 days.

Data are expressed as the mean ± SEM (n=8). $^{a}p < 0.05$, significant change with respect to **Control**; $^{b}p < 0.05$, significant change with respect to **$CdCl_2$** using Duncan's post hoc test.

Figure 5: Ameliorative effects following the pre-administration of *Fragaria ananassa* methanolic extract (SME) on antioxidant enzyme activities and their corresponding mRNA levels in the liver of rats exposed to cadmium chloride ($CdCl_2$) for 5 days.

Data of antioxidant enzyme activities are expressed as the mean ± SEM (n=8) while the results of the mRNA levels (mean ± SEM of three assays) were normalized to the *GAPDH* RNA level and are shown as the fold induction (in log2 scale) relative to the mRNA level in controls. $^{a}p < 0.05$, significant change with respect to **Control**; $^{b}p < 0.05$, significant change with respect to **$CdCl_2$** using Duncan's post hoc test.

Figure 6: Light micrographs of the liver. **a** The normal histological structure of the liver in the control group. **b** Various degrees of focal necrosis, congestion, foci of inflammation and extensive degeneration, cytoplasmic vacuolation, and fat deposition (black arrow) in the $CdCl_2$-treated group, the inset in figure 6b shows focal neutrophil mobilization (green arrow) accompanied with hepatocyte necrosis (white arrow). **c** The normal histological structure of hepatocytes in the SME group. **d** The normal histological structure of hepatocytes in the $CdCl_2$-

treated group pre-administered with SME. However, some vacuolated areas are seen (H & E). (X 400)

Figure 7: Mitigating effects following the pre-administration of *Fragaria ananassa* methanolic extract (SME) on the mRNA levels of *Bcl-2*, *Bax* and *TNF-α* in the liver of rats exposed to cadmium chloride ($CdCl_2$) for 5 days.

Data are expressed as the mean ± SEM (n=8). $^{a}p < 0.05$, significant change with respect to **Control**; $^{b}p < 0.05$, significant change with respect to **$CdCl_2$** using Duncan's post hoc test.

Figure 8: TNF-α immunostained sections of hepatocytes in (**a**) control, (**b**) $CdCl_2$-, (**c**) SME-, and (**d**) SME+$CdCl_2$-treated rats. In the control and SME groups, inflamed hepatocytes, i.e., those stained with TNF-α, were sparse and weakly stained. In the $CdCl_2$ group, many hepatocytes were inflamed. In the SME+$CdCl_2$ group, the number of inflamed hepatocytes was decreased. (X 400)

Figure 9: Caspase-3 immunostained sections of hepatocytes in (**a**) control, (**b**) $CdCl_2$-, (**c**) SME-, and (**d**) SME+$CdCl_2$-treated rats. In the control and SME groups, apoptotic hepatocytes, i.e., those stained with caspase-3, were sparse and weakly stained. In the $CdCl_2$ group, many hepatocytes were apoptotic. In the SME+$CdCl_2$ group, the number of apoptotic hepatocytes was decreased. (X 400)

Table 1. Primer sequences of genes analyzed in real time PCR

Name	Accession number	Sense (5'---3')	Antisense (5'---3')
GAPDH	NM_017008.4	GCATCTTCTTGTGCAGTGCC	GATGGTGATGGGTTTCCCGT
SOD2	NM_001270850.1	AGCTGCACCACAGCAAGCAC	TCCACCACCCTTAGGGCTCA
CAT	NM_012520.2	TCCGGGATCTTTTTAACGCCATTG	TCGAGCACGGTAGGGACAGTTCAC
GPx	NM_017006.2	CGGTTTCCCGTGCAATCAGT	ACACCGGGGACCAAATGATG
GR	NM_053906.2	TGCACTTCCCGGTAGGAAAC	GATCGCAACTGGGGTGAGAA
Bcl-2	NM_016993.1	CTGGTGGACAACATCGCTCTG	GGTCTGCTGACCTCACTTGTG
Bax	NM_017059.2	GGCGAATTGGCGATGAACTG	ATGGTTCTGATCAGCTCGGG
TNF-α	XM_008772775.2	AGAACTCAGCGAGGACACCAA	GCTTGGTGGTTTGCTACGAC

The abbreviations of the genes; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; SOD2: Manganese-dependent superoxide dismutase (MnSOD); CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-like protein 4; TNF-α: Tumor necrosis factor.

Table 2: Effect of strawberry methanolic extract (SME) on liver enzyme biomarkers pre-administrated to $CdCl_2$.

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	Total bilirubin (mg/dL)
Control	49.3 ± 3.2	57.2 ± 4.4	152.8 ± 8.6	1.7 ± 0.2
6.5 mg/kg $CdCl_2$	102.3 ± 8.3[a]	96.1 ± 7.8[a]	293.2 ± 15.3[a]	2.9 ± 0.3[a]
250 mg/kg SME	52.1 ± 4.5	53.1 ± 3.9	162.1 ± 11.2	1.6 ± 0.2
SME+$CdCl_2$	73.3 ± 6.2[ab]	68.3 ± 5.8[b]	216.3 ± 14.7[ab]	1.9 ± 0.2[ab]

Data are expressed as the mean ± SEM (n=8). [a]$p < 0.05$, significant change with respect to **Control**; [b]$p < 0.05$, significant change with respect to **$CdCl_2$** using Duncan's post hoc test