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The Effect of *Salvia officinalis* Extract on Alleviating Oxidative Stress and Hepatic Dysfunction Induced by Carbon Tetrachloride in Mice

Amina E. Essawy[1,2*], Hawazin A. Lamfon[1], Abeer B. Al Harbi[1], Awatef M. Ali[2] and Nahid A. Lamfon[3]

[1]Biology Department, Faculty of Applied Science, Umm Al- Qura University, Makkah, Saudi Arabia;[2]Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt;[3]Consultant Family and Community Medicine, King Abdullaziz Medical City, National Guard Health Affair, Riyadh, Saudi Arabia

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Abstract

The current study is aimed at evaluating the hepatoprotective effects of the aqueous leaf extract of *Salvia officinalis* against carbon tetrachloride (CCl_4)-induced liver injury in Swiss albino mice. CCl_4 [1.9 mL/kg body weight (b.wt)] was given orally every other day for four weeks. The aqueous suspension of *S. officinalis* (10 mL/kg b.wt.) was administered every other day alternated with CCl_4 for four weeks. The liver marker enzymes (ALT, AST, ALP and LDH) were determined in serum after two and four weeks, while a liver tissue was used for the histopathological and ultrastructure assessment. The liver enzymes were significantly elevated in the animals treated with CCl_4 compared to the control. Histopathological and ultrastructure observations also revealed severe damage in the structure of liver tissue in the animals intoxicated with CCl_4. Animals exposed to the treatment with CCl_4 combined with *S. officinalis* showed a marked improvement in the biochemical, histological as well as the ultrastructure findings. The protective effects of the leaf extract of *S. officinalis* against CCL4-induced liver injury was time dependent.

Keywords: CCl_4, Hepatotoxicity, Histology, Liver enzymes, *Salvia officinalis*

1. Introduction

Liver is one of the highest metabolic organs in the human body, where many bio-molecules are metabolized by diverse enzymes (Meyer and Kulkarni, 2001). Liver cells possess large bio-transforming abilities which make these cells a vulnerable target for adverse side effects that may affect the liver functions and possibly lead to liver fibrosis and liver failure (Cullen, 2005). Carbon tetrachloride (CCl_4) is widely used to induce liver damage in experiments (Yan *et al*., 2006; Essawy *et al*., 2012). Within the body, CCl_4 is broken down by the cytochrome P450 enzyme into a variety of highly toxic free radicals mainly, trichloromethyl (CCl_3) and trichloroethyl peroxyl (CCl_3O_2). These compounds are known to induce liver cell damage (Ohta *et al*., 2000).

Several plant-derived extracts showed antioxidant activity against hepatotoxicity induced by CCl_4 via inhibiting lipid peroxidation and/or enhancing the activity of antioxidant enzymes (Shahjahan *et al*., 2004). *Salvia* is one of the most important members of the family Lamiaceae, with up to 900 species (Longaray -Delamare *et al*., 2007). Several oils and phenolic extracts of *Salvia* have shown antioxidant capacity (Oboh and Henle, 2009; Khudiar and Hussein, 2017), and are currently used as antiviral (Geuenich *et al.,* 2008), antibacterial (Soković *et al*., 2010), anticancer (Jiang *et al*., 2017), anti-inflammatory, and as immune regulatory agents (Elwy and Tabl, 2013). Moreover, *in vitro* and animal studies have shown that several *Salvia* species contain a variety of active compounds that may promote the cognitive activity and protect against the degeneration of neurons (Lopresti, 2017)

Salvia officinalis is an aromatic plant with many biological activities described for its dried leaves' extracts (Lima *et al*., 2006; Mayer *et al*., 2009, Cwikla *et al*., 2010). These biological activities were attributed to some important bioactive compounds found in *S. officinalis* such as the phenolic rosmarinic acid, phenolic diterpenes and other phenolic compounds which possess strong antioxidant activities (Oniga *et al*., 2007; Poeckel *et al*., 2008; Johnson, 2011). These phenolic compounds are considered to be key factors for the several therapeutic actions of medicinal plants.

The current study has been designed to evaluate the protective effects of the leaf extract of *S. officinalis* against CCL_4-induced liver toxicity in mice.

* Corresponding author e-mail: amina_essawy@yahoo.com.

2. Materials and Methods

2.1. Experimental Animals

Ten-week-old laboratory male Swiss albino mice weighing about 25 g each were used for this study. They were kept under observation for about one week before the beginning of the experiment to exclude any underlying infection and to be allowed to acclimatize.

The animals were kept in rodent cages in the laboratory under constant conditions of temperature (23±2°C) with a reveres natural dark-light cycle 12/12 h. Animals were maintained on a standard rodent diet, and water was available *ad libitum*. The maintenance of animals and experimental procedures were approved by the animal ethical committee in accordance with the guide for care and use of laboratory animals.

2.2. Chemicals Used

CCl_4 (98.8 % purity) was purchased from El-Nasr Pharmaceutical Chemical Company (Egypt).

Olive oil (Laboratory grade) was obtained from Sigma Chemical Co. (St. Louis, MO). It had been used as a vehicle for CCl_4.

2.3. Preparation of the Aqueous Extract of Salvia officinalis

Dried leaves of *S. officinalis* were purchased from a local herb grocery (Makkah, Saudi Arabia). 10 gm of powdered leaves were boiled in 1000 ml of distilled water and heated for thirty minutes. The extract was filtered, cooled, and given orally to the mice at a volume of 10 mL of the extract /kg body weight (Farhoudi *et al*., 2011).

2.4. Experimental Design

The animals were randomly divided into five groups of twenty mice each and were treated as follows:

Group I: Animals in this group received 10 mL/kg b.wt. of normal saline (0.9 % NaCl) every other day for four weeks and group I served as the negative control.

Group II: Each animal in this group was orally given olive oil at a dose level of 10 mL/kg b.wt. (Essawy *et al*., 2010) every other day for four weeks and group II served as the positive control (vehicle).

Group III: Each mouse orally received Salvia extract at a dose level of 10 mL/kg b.wt. (Farhoudi *et al*., 2011) every other day for four successive weeks.

Group IV: Each animal was orally given CCl_4 dissolved in olive oil at a dose level of 1.9 mL/kg b.wt. (¼ LD50, Essawy *et al*., 2010) every other day for four weeks.

Group V: Each mouse orally received *Saliva* extract at a dose level of 10mL/kg b.wt. every other day alternated with CCl_4 at a dose level of 1.9 mL/kg b.wt (¼ LD50) for four successive weeks.

2.5. Physiological Study

After two and four weeks of treatment, blood samples were collected in clean and dry tubes each via a cardiac puncture method and were allowed to clot. The serum was rapidly separated by centrifuging the clotted blood at 3000g for ten minutes in a Beckman Model T-6 refrigerated centrifuge. The level of liver enzymes, Aspartate aminotransferase (AST), Alanine amino-transferase (ALT), Alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH) were determined using commercially available diagnostic kits (Biomerieux SA, France).

2.6. Histological and Ultrastructural Studies

After four weeks of treatment, the samples of liver from the control and experimental mice groups were removed for histopathological and ultrastructure studies. For the histopathological investigation, pieces of the liver were fixed in buffered formalin, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Four micron thick sections were stained with haematoxylin and eosin, and were examined with light microscope (Lillie, 1965).

For the transmission electron microscopy, small slices of the liver were immediately fixed in 4F1G in phosphate buffer (pH7.2) and post-fixed in 2 % OsO_4 in the same buffer. The specimens were dehydrated through graded series of ethanol, embedded in epon-araldite mixture, and were polymerized at 60°C. Ultrathin (50 nm) sections were cut, double stained with uranyl acetate and lead citrate, and were examined by Jeol 100CX electron microscope (Cheville and Stasko, 2014).

2.7. Statistical Analysis

The data are expressed as Mean ± standard error for each group (n = 5). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by least significance difference(LSD) post-hoc test using Statistical Package for Social Sciences (SPSS) software version 25.0 for Windows. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Physiological Results

Table 1 represents the effects of CCl_4 and *S. officinalis* on the activity of liver enzymes in the serum of albino mice. The administration of CCL_4 for two and four weeks significantly ($P<0.05$) increased the activity of AST (201.2 % and 258.6 %), ALT (131.4 % and 156.6 %), ALP (145 % and 178.2 %), and LDH (67% and 88%) compared to the normal control groups.

The oral administration of the aqueous leaf extract of *S. officinalis* at a dose of 10 mL/kg b.wt significantly decreased the level of ALT, AST, ALP and LDH compared with CCl_4- treated animals

Table 1. Effect of CCl_4 and/or *Salvia officinalis* on the level of liver enzymes in albino mice

	Time	Control	Olive oil	*Salvia officinalis*	CCL_4	CCL_4+ *Salvia officinalis*
AST	2wks	$51.60^{a} \pm 5.33$	$56.80^{a} \pm 9.19$	$58.0^{a} \pm 5.39$	$155.40^{b} \pm 3.39$	$104.40^{c} \pm 6.98$
(IU/L)	4wks	$49.80^{a} \pm 2.24$	$54.0^{a} \pm 8.73$	$58.80^{a} \pm 3.43$	$178.60^{b} \pm 3.14$	$128.80^{c} \pm 4.45$
p		0.764	0.831	0.903	0.001^{*}	0.018^{*}
ALT	2wks	$47.20^{a} \pm 3.89$	$54.60^{ac} \pm 9.08$	$50.20^{a} \pm 6.18$	$109.20^{b} \pm 2.50$	$67.60^{c} \pm 3.49$
(IU/L)	4wks	$49.20^{a} \pm 4.14$	$48.60^{a} \pm 6.49$	$52.40^{a} \pm 7.33$	$126.20^{b} \pm 1.24$	$73.80^{c} \pm 3.15$
p		0.734	0.605	0.824	$<0.001^{*}$	0.224
ALP	2wks	$47.20^{a} \pm 2.35$	$43.20^{a} \pm 6.29$	$52.20^{ac} \pm 5.27$	$115.60^{b} \pm 1.83$	$77.80^{c} \pm 4.12$
(IU/L)	4wks	$50.40^{a} \pm 2.75$	$47.40^{a} \pm 4.62$	$45.60^{a} \pm 3.03$	$140.20^{b} \pm 1.85$	$80.20^{c} \pm 4.68$
p		0.402	0.605	0.309	$<0.001^{*}$	0.710
LDH	2wks	$932.20^{a} \pm 48.41$	$1064.20^{ac} \pm 40.49$	$1051.0^{ac} \pm 108.06$	$1556.40^{b} \pm 35.71$	$1154.40^{c} \pm 47.83$
(U/L)	4wks	$988.40^{a} \pm 96.36$	$1044.0^{a} \pm 26.41$	$1063.0^{a} \pm 94.03$	$1857.60^{b} \pm 44.95$	$1445.60^{c} \pm 41.37$
p		0.616	0.687	0.935	0.001^{*}	0.002^{*}

AST indicates aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.
Data represented as mean ± SE. (n = 5), *P*: *P* value for student t-test between the two weeks and four weeks in each group.
Different superscripts within each row indicate the statistical significant difference among groups at 0.05 assessed using ANOVA test (LSD). *: Statistically significant at $P \leq 0.05$.

3.2. Histological observations

Liver sections from control, olive oil, and *S. officinalis*-treated mice showed hepatocytes with a normal structure indicated as well-preserved cytoplasm, prominent nuclei, nucleoli, central vein and compact arrangement of hepatocytes (Figure 1a-c). The liver cells are arranged in cords radiating from the central veins. The hepatic sinusoids appear as narrow spaces in between the hepatic cords.

In contrast, mice receiving CCl_4 showed a variety of pathological changes, including normal hepatic structure with deformed hepatocytes, cellular infiltration, dilated portal veins, damaged blood sinusoids with dense Kupffer cells and an elevated number of binucleated hepatocytes (Figure 1d and e). The hepatocytes appeared with cytoplasmic vacuolization especially at the vicinity of the nuclear envelope.

Animals treated with CCl_4 combined with the aqueous extract of *S. officinalis* for four weeks revealed remarkable signs of recovery. Hepatocytes were observed with more or less defined morphology, improved nuclei, and less vacuolated cytoplasm (Figure 1f). Normal blood sinusoids and central veins and small foci of inflammatory cells were also observed.

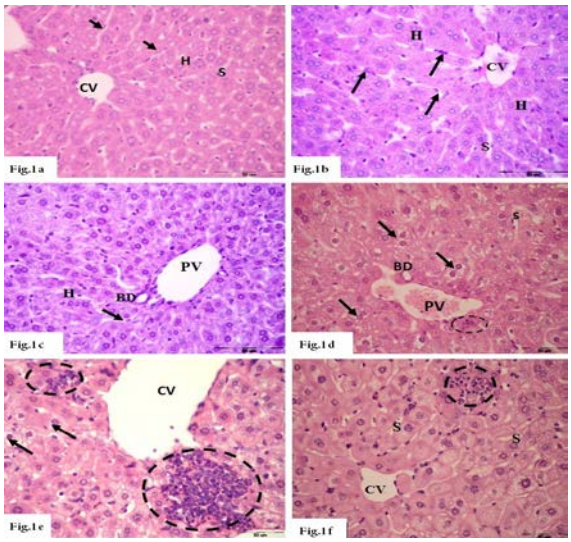

Figure 1. Light photomicrographs of liver sections (H&E stain) from control untreated mice **(a)** mouse that received olive oil (**b**) mouse that received *Salvia* (**c**), illustrating normal hepatic architecture, normal hepatocytes, central vein, blood sinusoids with Kupffer cells (arrows) and portal vein. (**d)** section from a mouse treated with CCl_4 illustrating: deformed hepatocytes, binucleated hepatocytes (arrows), damaged sinusoids with denser Kupffer cells, congested portal vein and leucocytic infiltration (dashed circle) . **(e)** Enlarged part from Fig.(d) illustrating, deformed hepatocytes with atrophied densely stained nuclei (arrows), narrow blood sinusoids, dilated central vein and intense lymphocytic infiltration (dashed circles). (**f)** section from a mouse treated with CCl4 +*Salvia* illustrating: hepatic tissue with normal central vein and well-organized hepatic strands separated by blood sinusoids. Dashed circle demonstrates the area of lymphocytic infiltration. BD: bile ductule, CV: central vein, H: hepatocytes, PV: portal vein, S: blood sinusoids.

3.3. Electron Microscopical Observations

The ultrastructure observation of liver thin sections of control, olive oil and *S. officinalis*- treated mice (Figure 2a-d) showed normal structure of hepatocytes with more or less rounded nuclei surrounded by a regular nuclear envelope. The cytoplasm possesses stalks of rough endoplasmic reticulum, polyribosomes and a large number of round and oval mitochondria (Figure 2 a,c and d). Bile canaliculi with short microvilli are present in between the liver cells (Figure 2a). In addition to hepatocytes, a number of large Kupffer cells with prominent triangular nuclei, marginated heterochromatin, and distinct nuclear envelope were also observed (Figure2b).

In contrast, the hepatocytes of the liver sections from the animals treated with CCl_4 showed hepatocytes with several alterations including shrunken and pyknotic nuclei and irregular nuclear envelope (Figure 3a-c). The mitochondria appeared atrophied and deformed with a lytic membrane and disturbed cristae (Figure 3c and d). The cisternae of rough endoplasmic reticulum in some hepatocytes were highly fragmented (Figure3b). Also, some hepatocyte appeared with dilated rough endoplasmic reticulum (Figure 3c and d). Accumulation of lipid droplets, cytoplasmic vacuolization, random dispersion of free ribosomes and1ry and 2ry lysosomes were observed (Fig.3a-d). Blood sinusoids with deformed endothelial cells were found and included hypertrophoid abnormal Kupffer cells (Figure 3e). The microvilli of bile canaliculi appeared degenerated and fragmented (Figure 3f). On the other hand, most of the aforementioned alterations in hepatocytes were markedly attenuated in animals treated with CCl_4 plus *S. officinalis* (Figure 4a-c).

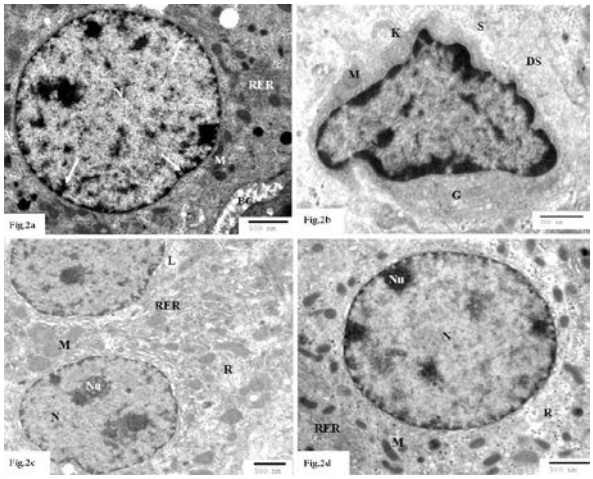

Figure 2. Electron photomicrographs of liver sections from control untreated mice (a and b) mouse that received olive oil (c) and mouse that received *Salvia* (d), illustrating normal hepatocytes in (a,c and d) with euchromatic nuclei (N) having marginated heterochromatin (arrows), numerous mitochondria (M), rough endoplasmic reticulum (RER) , free ribosomes (R), few lipid droplets (L), and bile canaliculus (BC) between two cells. b) Normal Kupffer cell (K) with prominent triangular nucleus (N), marginated heterochromatin, Golgi vesicles (G), and Disse space (DS).

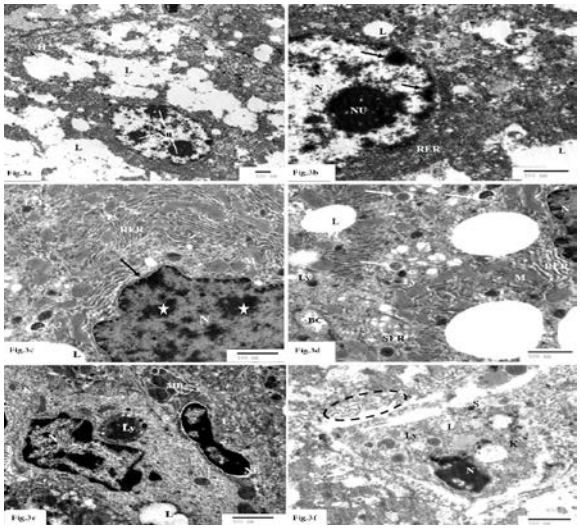

Figure 3. Electron photomicrographs of liver sections from male mice treated with CCL_4 : (**a**) section illustrating, completely deformed hepatocyte (H), multi-nucleolei (Nu) and accumulation of lipid droplets (L). **(b)** Hepatocyte with karyolitic nucleus (N), marginated heterochromatin (arrows), dense nucleolus (Nu), lysosomes (Ly), fragmented RER and lipid droplets (L). (**c)** Part of the nucleus (N) with irregular nuclear envelope (arrow) and heterochromatin masses (stars), proliferated RER, lipid droplets (L), dense 1^{ry} lysosome (Ly1),2^{ry} lysosome (Ly2) and damaged small mitochondria (M) . (**d)** Enlarged part of hepatocyte with a small part of the nucleus (N), hypertrophied SER, deformed mitochondria (M), dilated RER, lipid droplets (L), many dense 1^{ry} lysosome (Ly) and 2^{ry} lysosome (arrows) with internal content. Note: bile canaliculus (BC) between two hepatocytes. (**e)** Damaged blood sinusoid with deformed endothelial cell (E) and dilated nuclear envelope (NE). Note, microbody (MB) with internal cristae, Kupffer cell (K) with abnormal shaped nucleus (N), fat droplets (L) and secondary lysosomes (Ly).**(f)** Damaged blood sinusoid (S) with necrotic Kupffer cell (K), dense pyknotic nucleus (N), fatty infiltration (L) and lysosome (Ly). Note: dilated Diss space with fragmented microvilli (dashed circle).

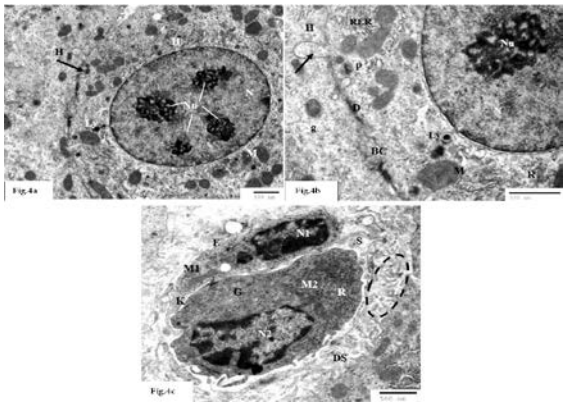

Figure 4. Electron photomicrographs (a and b) of hepatocytes (H) from mice treated with CCL_4+ *Salvia* extract illustrating, intact cell membrane (arrow), euchromatic nucleus (N) with multi- nucleoli (Nu), normal intact mitochondria (M), lysosome (Ly), short stalks of granular endoplasmic reticulum (RER), pale glycogen deposits (g), few lysosomes (Ly), dense ribosome (R), peroxisomes (P) and bile canaliculus (BC) with intact microvilli. Arrow points at evaginated plasma membrane. (c) Blood sinusoid (S) lined with flat endothelial cell (E) containing dense nucleus (N1) and mitochondria (M1), Kupffer cells (K) with a large nucleus (N2), Golgi vesicles (G), small mitochondria (M2) and ribosomes (R). Note, Disse space (DS) filled with microvilli (dashed circle)**.**

4. Discussion

Salvia officinalis is an herbaceous plant that has been widely used in traditional folk medicine.

The present work was undertaken to demonstrate the protective ability of the aqueous extract of

Salvia officinalis leaves against CCl_4-induced liver toxicity in mice. In this work, damage of the liver caused by CCl_4 was observed as alterations in serum marker enzymes beside histopathological and ultrastructure changes in liver tissue.

The administration of CCl_4 significantly elevated the serum levels of liver enzymes (AST, ALT, ALP) and LDH, which may indicate dage and leakage of enzymes from hepatocytes (Rajesh and Latha, 2004 ; Bashandy and Al Wasel, 2011; Essawy *et al*., 2012). Ravikumar *et al*. (2005) reported that hepatocellular damage leads to increased ALT activity, and is usually accompanied by a rise in AST and ALP. The elevation of serum liver enzymes can be attributed to the increased levels of free radicals produced by CCl_4 (Kumar *et al*., 2009). According to Zeashan *et al*. (2008), the hepatotoxicity induced by CCl_4 was attributed to its derivative CCl_3, a free radical that is able to attack polyunsaturated fatty acids to produce lipid peroxides. The induction of lipid peroxidation may lead to biological changes in cellular membranes which may result in a serious injury of the liver cells (Balahoroglu *et al.*, 2008).

In the present study, treatment with *S. officinalis* blocked the elevation of liver enzymes induced by CCl_4 in mice. Evidence from previous studies suggests that *S. officinalis* has potent antioxidant activities. Horvathova *et al* (2016) reported that enriching the drinking water of rats with the *S. officinalis* extract increases resistance of rat hepatocytes against oxidative stress. It protects hepatocytes against dimethoxy naphthoquinone- and hydrogen peroxide-induced DNA damage through the elevation of glutathione peroxidase activity (Kozics *et al*., 2013). Zupko *et al*. (2001) attributed this effect to the mixture of antioxidant compounds (salvianolic acid, rosmarinic acid and phenolic glycosides) found in the *Salvia* extract.

The examination of the liver sections from mice which received CCl_4 revealed a clear disturbance of hepatic architecture with deformed hepatocytes, cytoplasmic vacuolization, pyknotic nuclei, leukocyte infiltration, dilatation and congestion of blood vessels, and damaged blood sinusoids with dense Kupffer cells. Sherlock and Dooly (2002) reported that cytoplasmic swelling and vacuolization are the most important responses to cell injury. Increased permeability of cell membranes elevates the intracellular water content which produces cytoplasmic vacuolization. CCl_4 – induced liver damage was also reported by Kumar *et al*. (2009). They reported extensive areas of patchy and confluent liver-cell necrosis, lobular inflammation, and sinusoidal spaces flooded with inflammatory cells. Moreover, previous results showed that CCl_4 induced massive fatty changes, necrosis, infiltration of lymphocytes, and loss of cell boundaries in the liver of treated mice (jin *et al*., 2011; Zowail *et al*., 2012).

In the current study, the examination of electron micrographs of the liver sections from the mice treated with CCl_4 revealed serious ultrastructural alterations. The most frequently alterations were the accumulation of lipid droplets, nuclear deformation, nuclear pyknosis, nucleolar margination, the fragmentation and dilatation of rough endoplasmic reticulum cisternae, degeneration of mitochondria and increase in the number of lysosomes. Similar alterations were reported by other researchers (Cai *et al*., 2010; Zowail *et al*.,2012).The CCl_4-induced hepatotoxicity has been referred to the excessive free radical formation formed during its detoxification in the liver.

Interestingly, the oral administration of the aqueous extract of *S. officinalis* improved most of the histological and ultrastructural adverse effects induced in the liver after the CCl_4 treatment, where most of the hepatocytes nuclei and cytoplasm restored their normal structure. Similarly, Amin and Hamza (2005) reported that animals pretreated with the *S. officinalis* extract did not show neither hepatic necrosis nor infiltration of inflammatory cells in the liver after the azathioprine administration.

5. Conclusion

The present biochemical, histological, and ultrastructure results prove that the aqueous extract of *S. officinalis* possesses a potential protective effect against oxidative damages induced by CCl_4 in the liver tissue. These improving effects of *S. officinalis* can be attributed to the bioactive constituents that alleviated the deleterious effect of CCl_4 either by the well-known free radical scavenging potential or by their potent antioxidant properties.

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