

Effect of zinc sulphate and Essentiale® Forte in managing carbon tetrachloride-induced hepatotoxicity in adult Wistar rats

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Abstract

Objectives: The aim of this study was to assess and compare the ameliorative effect of zinc sulphate and Essentiale® Forte on the histomorphology and lipid histochemistry of carbon tetrachloride (CCl₄)-induced hepatic injury in adult Wistar rats.

Methods: Twenty-five adult Wistar rats, weighing between 150 g and 170 g, were used for the study. The animals were divided into five groups A, B, C, D and E (n=5). Group A received 0.7 ml/kg of olive oil orally. Groups B, C, D and E were administered CCl₄ (0.7 ml/kg, orally) for 1 week in 1:1 dilution with olive oil. After CCl₄ administration, Group C was treated with Essentiale® Forte (4.5 mg/kg/bw, orally) for four weeks. Group D was treated with zinc sulphate (7 mg/kg/bw, orally) daily for four weeks. Group E received zinc sulphate (7 mg/kg/bw, orally) and Essentiale® Forte (4.5 mg/kg/bw, orally) for a period of 4 weeks, while Group B was left untreated. Animals were left for another one week and subsequently sacrificed under ether anaesthesia. The liver was harvested, weighed and divided into two parts for histological and histochemical studies.

Results: Histological analysis showed that treatment of liver with CCl₄ caused hepatotoxicity as marked by presence of inflammatory cells and distortion in the connective tissue fibers, as compared to the ones treated with zinc sulphate and or Essentiale® Forte which restored the hepatic histoarchitecture and protected the liver tissue from fatty and degenerative changes.

Conclusion: This study showed that combination of Essentiale® Forte and zinc supplement offered better ameliorative effects on the liver of Wistar rats following CCl₄-induced hepatotoxicity compared with separate administration of either Essentiale® Forte or zinc sulphate.

Keywords: carbon tetrachloride; Essentiale® Forte; hepatotoxicity; liver; zinc sulphate

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Introduction

Liver is highly vulnerable to a wide variety of metabolic, toxic, microbial, circulatory and neoplastic insults. Liver diseases may be primary, but more often hepatic involvement is secondary. The liver has enormous functional reserves, such that early liver impairment is clinically masked and the progression of the deranged liver function makes the condition life threatening.^[1] Morphologically, liver responds to injurious events in five different ways, irrespective of the cause, degeneration and intracellular

accumulation, necrosis and apoptosis, inflammation, regeneration, fibrosis.^[1]

Liver injury may follow the inhalation, ingestion or parenteral administration of a number of pharmacologic and chemical agents. These include industrial toxins *e.g.* carbon tetrachloride (CCl₄), trichloroethylene and yellow phosphorus, heat-stable toxic bi-cyclic octapeptides of certain species of *Amanitia* and *Galerina* (hepatotoxic mushroom poisoning), and more commonly, pharmacologic agents used in medical therapy. Hepatotoxic drugs can injure the hepatocyte directly, *e.g. via* a free radical or

metabolic intermediate that causes peroxidation of membrane lipids and that results in liver cell injury.^[2]

CCl₄ is a colourless liquid, ether-like in odour with a density of 1.6 g/cm³ melting point is 22.9°C, boiling point is 76.7°C and soluble in water at 0.08 g/100 ml (25°C). It is also soluble in alcohol, ether, chloroform, benzene, naphtha and carbon sulfide. The vapour pressure is 11.9 kPa at 20°C and refractive index is 1.5. CCl₄ has a crystal structure with tetrahedral shape. It is not flammable, its auto-ignition temperature is 982°C and LD₅₀ is 2350 mg/kg.^[3] It has been reported to produce free radicals which affect the cellular permeability of the liver cells, leading to altered level of serum biochemistry and liver enzymes.^[4] In time series, CCl₄ was found to have an atmospheric life time of 85 years and liver damage inflicted by it has lethal consequences.^[4]

Over the years, chronic liver disease (CLD) is one of the major health challenges, and despite research and development of various new drugs, morbidity and mortality accompanying hepatic pathology still remained high.^[5] Prolonged exposure of liver to CCl₄ has been researched with consequent spectrum of chronic liver disease, such as fatty liver, hepatitis, liver cirrhosis, hepatoma.^[5] Liver damage can occur after 24 hours of exposure to CCl₄, and in serious cases this can result in painful swollen liver, ascites, hemorrhages, hepatic coma and death.^[3]

Essentiale® Forte has been for a long time indicated and used in the cases and management of CLD. It is a 300 mg hard gel capsule that contains de-oiled enriched phospholipids from neutral soya beans. It works by replacing and regenerating the phospholipids in the liver cell membranes that have been damaged by various means especially through hepatotoxin. It is mainly made up of poly-ethylphosphatidylcholine from natural soya beans with vitamins B1, B2, B3, B6, and B12. The pharmaceutical excipients are chiefly ethanol (96%), hard fat, hydrogenated castor oil, ethyl vanillin, 4-methoxyl phenyl ethanone, alpha tocopherol, gelatin, colouring agents, sodium lauryl sulfate, and purified water.

Zinc is known as an essential trace element necessary for protein metabolism, as well as membrane integrity and also involved in the structure and function of over 300 metalloenzymes. It has important functions in skin and connective tissue, metabolism as well as in wound healing.^[6] It exerts its antioxidant effects indirectly by maintaining membrane structures, involving in the structure of SOD, increasing the metallothionein concentrations and, competing with redox reactive metals, iron and cuprous for critical binding sites.^[7] It is shown that hepatic and serum zinc levels of patients in liver disease decreased depending on the degree of liver damage.^[8]

This study was thus designed to investigate the effect of zinc in form of zinc sulphate (ZnSO₄) when used separately or in combination with the long standing known drug Essentiale® Forte on CCl₄-induced hepatotoxicity in adult Wistar rats.

Materials and Methods

Twenty-five adult Wistar rats, weighing between 150 g and 170 g (6–10 weeks old) obtained from Animal Holding of International Institute of Tropical Agriculture Ibadan, Oyo State, Nigeria were used in this study. The animals were housed in plastic cages in a clean environment under standard laboratory conditions of light, temperature and humidity, in the animal holding of the Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. They were fed on standard laboratory rat pellets and given water *ad libitum*. Ethical clearance for the study was obtained from the Health Research Ethical Committee, Institute of Public Health, Obafemi Awolowo University, Ile-Ife, Nigeria (Approval number: IPHOAU/12/416). The animals were given humane care according to the guidelines of HREC and IPHOAU.

2.5 liters of CCl₄ was obtained from the central research laboratory of Obafemi Awolowo University. 60 ml of CCl₄ was diluted with 60 ml of olive oil in 1:1 equivalent, and this was administered orally at a dose of 0.7 ml/kg. Three tablets of ZnSO₄ (20 mg each, Chi Pharmaceuticals Ltd, Lagos, Nigeria) were dissolved in 60 ml of water and this was administered at a dose of 7 mg/kg. 300 mg Essentiale® Forte capsule (Sanofi Aventis, manufactured by Nattermann & Cie GmbH, Cologne, Germany) was prepared by dissolving a capsule in 60 ml of water, and was administered at a dose of 4.5 mg/kg all being freshly prepared on each day of administration.

The rats were randomly divided into five groups: A, B, C, D and E (n=5, each). Group A: negative control, received oral administration of olive oil only; Group B: positive control, received daily administration of CCl₄ (0.7 ml/kg p.o) for 1 week in 1:1 dilution with olive oil without treatment; Group C received Essentiale® Forte 4.5 mg/kg/day for four weeks after the administration of CCl₄; Group D received ZnSO₄ (7 mg/kg/day, orally) daily for four weeks after the administration of CCl₄; Group E received ZnSO₄ 7 mg/kg/day for four weeks and Essentiale® Forte (4.5 mg/kg/day p.o) concurrently for four weeks, after CCl₄ administration. All administrations were via oral route and the animals were then monitored for another one week for possible recovery.

At the end of the experimental procedures, all animals were sacrificed by cervical dislocation. A midline

incision was made along the anterior abdominal wall blood were taken by cardiac puncture, the liver specimens were perfused with isotonic saline, excised, blotted dry and weighed. Liver tissues were fixed in 10% formal saline processed for routine paraffin embedding a semi-quantitative method described by Pilette et al.^[9]

Sectioned tissues were stained with Haematoxylin and Eosin for demonstration of the liver architecture, Masson's trichrome stain for demonstration of collagen fibers, Gordon and Sweet's silver impregnation method for demonstration of reticular fibers, and Sudan black B for the demonstration of lipid histochemistry. Conventional morphological evaluation of the liver tissue was made. Stained sections were viewed under a Leica DM 750 microscope (Leica Microsystems, Wetzlar Germany) and digital photomicrographs were taken using an attached Leica ICC50 camera. The degree of hepatic necrosis and fibrosis were determined by a semi-quantitative method.^[9]

One-way ANOVA was used to analyze the data, followed by Student Newman-Keuls test for multiple comparisons. Graph Pad Prism 5 (Version 5.03; Graph Pad Inc., San Diego, CA, USA) was the statistical package used for data analysis. Significant difference was set at $p < 0.05$.

Results

There was no significant weight gain in Group B, but a relative increase in organ weight compared to Group A. Significant weight gain was observed in all groups except Group B (Table 1).

The histological layout of the liver in the control group stained with Hematoxylin and Eosin, with the cytoplasm stained in pink and the nucleus stained in purple, showed parenchymal cells arranged in lobules with the characteristic central vein at the center and portal canals located peripherally (Figure 1a). The liver cells were arranged in a branching pattern within which were the sinusoidal plates. The portal triads contained the bile duct, hepatic vein and hepatic artery. In the untreated CCl₄ group, the

hepatocytes showed features of inflammation with the presence of inflamed and inflammatory liver cells as seen in Figures 1b–d; there was also disruption of the normal histoarchitecture of the of the liver lobules. There were vacuolations, sinusoidal congestion, inflammatory cell infiltration within the sinusoids and aggregating around the central vein in the group treated with CCl₄ and Essentiale® Forte (Figure 1c). However, in the group treated with CCl₄ and ZnSO₄, there were areas of sinusoidal congestion which were not as congested as Group B, the inflammatory cells were less numerous, there was gradual restoration of the hepatic histoarchitecture, though the cytoplasmic boundaries were not distinctly demarcated (Figure 1d). In the group treated with CCl₄, Essentiale® Forte and ZnSO₄, there were prominent nucleoli within the hepatocyte nucleus surrounding the central vein, and the inflammatory liver cells were reduced (Figure 1e).

In Masson's trichrome stainings, the negative control group presented normal hepatic histoarchitecture as maintained with radially arranged sinusoids. In addition, there was scanty distribution of the collagen fibers around the central vein as shown in Figure 2a. Collagen fibers were found aggregated around the sinusoids, close to the central vein of the untreated group (Figure 2b). There were also collagen fibers around the central vein and within the sinusoids of animals in Group C (Figure 2c). Collagen fibers in the hepatic tissues of animals in Group D revealed increased density within the liver sinusoids around the inflammatory cells (Figure 2d). In Group E, deposition of collagen fibers around the hepatic vessel and sinusoids were minimally evident (Figure 2e).

In Gordon and Sweet's silver impregnation stainings, regular distribution of the reticular fibers around the central vein and within the sinusoids of the hepatic tissue were observed (Figure 3a). The reticular fibers were observed to be scanty in the untreated group as indicated by the arrowed area (Figure 3b). In Group C, the reticular fibers were sparsely distributed as represented by the arrowed area in Figure 3c compared to the nor-

Table 1
Absolute organ weight and difference in body weight (bw) in control and treatment groups (mean±SEM; n=5 for each group).

Groups	Initial bw (g)	Final bw (g)	Change in bw (g)	Absolute liver weight (g)	Relative liver weight (%)
Group A (Olive oil)	161.2±3.14	165.6±2.42	4.4±2.29	3.35±0.16 [†]	2.10±0.09
Group B (CCl ₄)	160.4±1.97	160.8±3.06	0.6±1.36**	4.68±0.17**	2.92±0.12*
Group C (CCl ₄ +EPL)	162.2±3.15	170.6±3.63	8.4±1.29 [†]	3.93±0.1	2.42±0.1
Group D (CCl ₄ +ZnSO ₄)	162±2.49	166.2±2.4	4.2±0.80 [†]	4.16±0.09*	2.56±0.17*
Group E (CCl ₄ +ZnSO ₄ +EPL)	161.8±1.99	167.4±1.72	5.6±0.81 [†]	4.04±0.10*	2.50±0.16*

*Significantly different from normal control at $p < 0.05$, [†]Significantly different from toxic control at $p < 0.05$, **Significantly different from C, D and E at $p < 0.05$. EPL: Essentiale® Forte

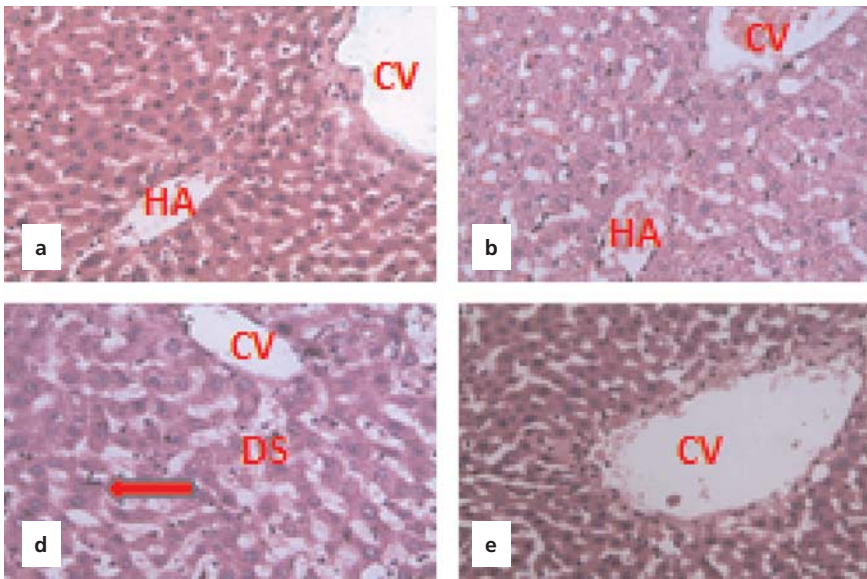


Figure 1. Photomicrographs of the liver with Hematoxylin and Eosin stain (x400). (a) Negative control, (b) Positive control, (c) CCl₄+ Essentiale® Forte, (d) CCl₄+ZnSO₄, (e) CCl₄+ Essentiale® Forte+ZnSO₄. CV: central vein; DS: dilated sinusoids, H: Hepatic cell; HE: Hepatic artery. Arrows: inflammatory liver cell. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

mal control Group A. Also, there was very scanty distribution of reticular fibers along the central vein and lining of the sinusoids (Figure 3d). The reticular fibers of the liver tissue in Group E were completely effaced compared to that in the normal control group (Figure 3e).

In Sudan black B staining (Figure 4a), liver histoarchitecture revealed varying degrees of darkly stained blue-black areas where the cells appeared to have clumped together. These are lipid globule depositions in the CCl₄ treated Group B as can be seen in the area marked with arrow and area labeled F in Figure 4b. In contrast, in the CCl₄+ZnSO₄ group, the lipid granules were not evident as shown in Figure 4c. Also, in the other tissue sections

(Figures 4d and e), the lipid granules were not evident either when compared to the untreated group (Figure 4b).

Discussion

In this study, data showed that CCl₄ toxicity led to a minimum increase in organ weight in Group B, while there was significant weight gain observed in all other groups similar to the findings of Raza et al.^[10] and Teo et al.^[11] Body weight change is one of the parameters used as indicator of adverse effects of drugs and chemicals.^[12] When animals are anorexic, weight loss is bound to ensue due to disturbances in carbohydrate, protein or fat metabolism.^[13]

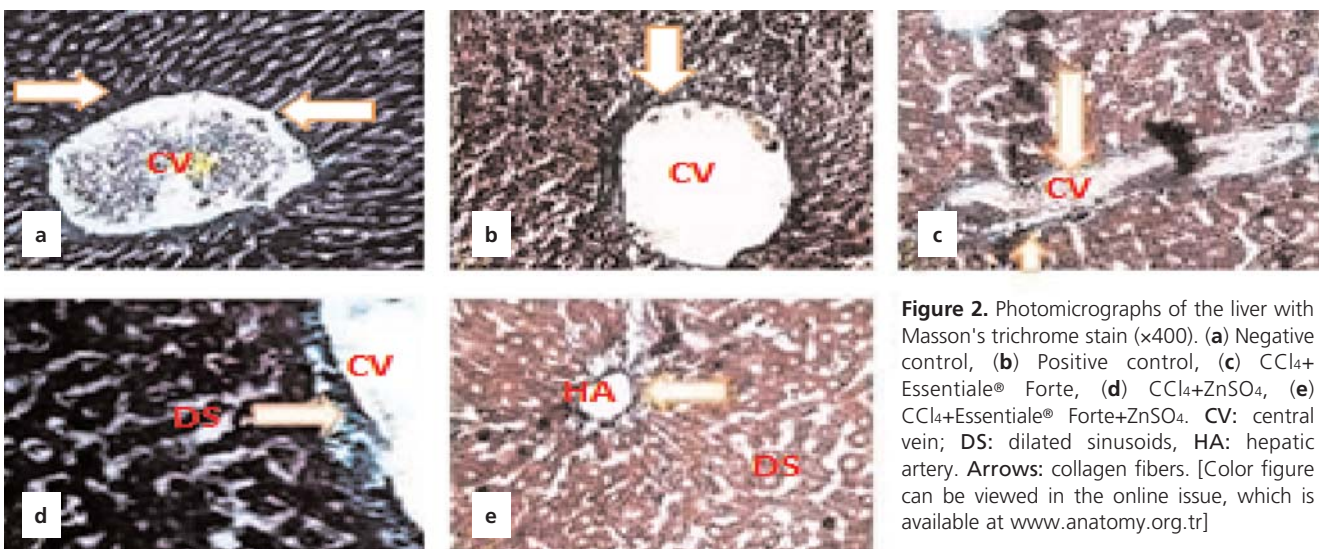


Figure 2. Photomicrographs of the liver with Masson's trichrome stain (x400). (a) Negative control, (b) Positive control, (c) CCl₄+ Essentiale® Forte, (d) CCl₄+ZnSO₄, (e) CCl₄+Essentiale® Forte+ZnSO₄. CV: central vein; DS: dilated sinusoids, HA: hepatic artery. Arrows: collagen fibers. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

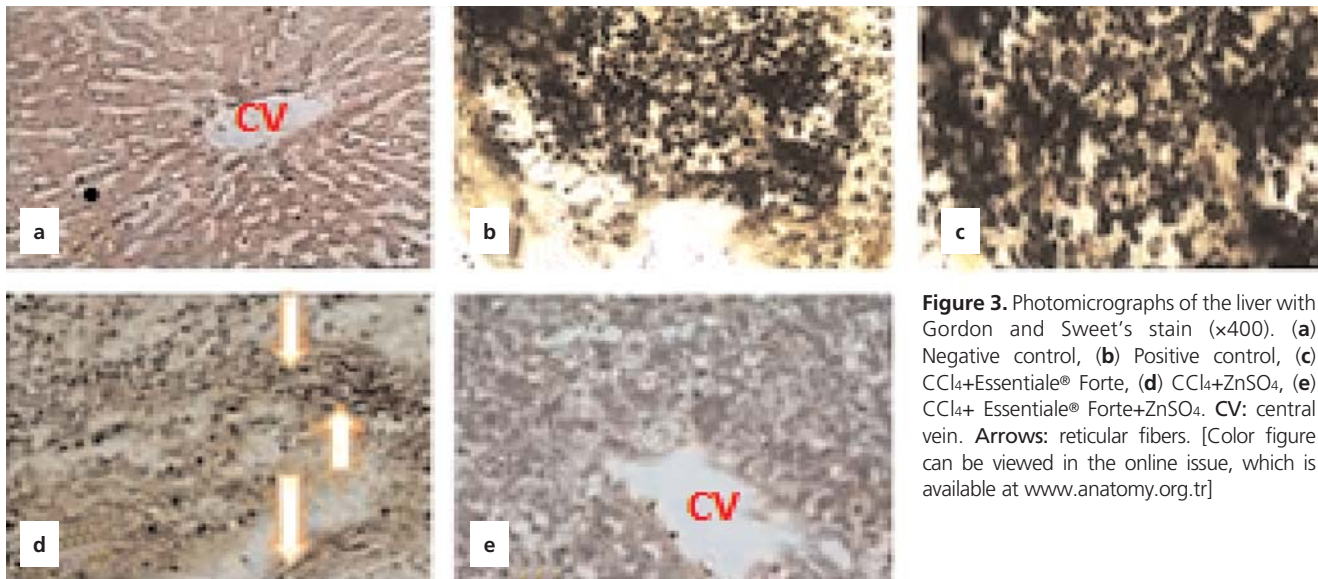


Figure 3. Photomicrographs of the liver with Gordon and Sweet's stain ($\times 400$). (a) Negative control, (b) Positive control, (c) CCl_4 +Essentiale® Forte, (d) CCl_4 + ZnSO_4 , (e) CCl_4 +Essentiale® Forte+ ZnSO_4 . CV: central vein. Arrows: reticular fibers. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

There was also a significant increase in relative liver weight in group B and this is in agreement with previous study where there was mild to moderate liver effects where there was increased liver weight and fatty degeneration following intermittent exposure of mice to CCl_4 .^[14]

Treatment with ZnSO_4 and Essentiale® Forte produced regenerative changes and absence of centrilobular necrosis, indicating that these agents possess hepatoprotective property by restoring the hepatic architecture and protecting the liver tissue from fatty and degenerative changes. These changes are achievable by prevention of

toxic chemical reaction, lipid peroxidation, micro and macro vesicular fatty changes, ultimately preventing necrosis. Hence, the hepatoregenerative effect of ZnSO_4 and Essentiale® Forte may have a role in the process of regeneration and prevention of fibrosis in the long-term use of Essentiale® Forte. In the CCl_4 +Essentiale® Forte group, although the histoarchitecture of liver seemed to be normal, minimal dilatation in sinusoids were observed. Changes in the CCl_4 +Essentiale® Forte+ ZnSO_4 group were minimal, almost close to the structures in the normal control group. This effect may be due to membrane stabiliza-

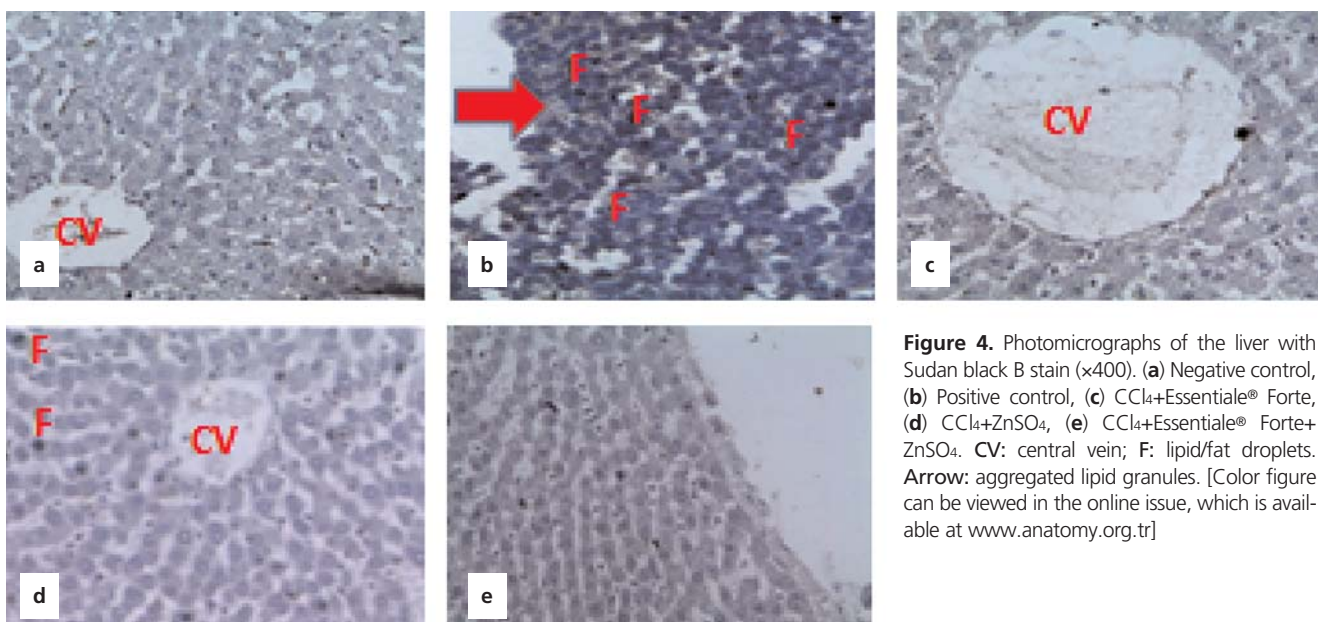


Figure 4. Photomicrographs of the liver with Sudan black B stain ($\times 400$). (a) Negative control, (b) Positive control, (c) CCl_4 +Essentiale® Forte, (d) CCl_4 + ZnSO_4 , (e) CCl_4 +Essentiale® Forte+ ZnSO_4 . CV: central vein; F: lipid/fat droplets. Arrow: aggregated lipid granules. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

tion activity of ZnSO₄ in combination with Essentiale® Forte. Results obtained in this study are in agreement with earlier finding of Yadrick et al.^[7] who reported that zinc exerts its antioxidant effects indirectly by maintaining membrane structures, involving in the structure of SOD, increasing the metallothionein concentrations and, competing with redox reactive metals for critical binding sites.

Histological observations depict that ZnSO₄ along with Essentiale® Forte showed significant recovery over either ZnSO₄ or Essentiale® Forte alone treated groups. In this study, combined supplementation of ZnSO₄ and Essentiale® Forte to CCl₄-treated rats was found to ameliorate hepatic toxicity when compared with the group treated with either ZnSO₄ alone or Essentiale® Forte only. Essentiale® Forte pretreatment in rats protects hepatocytes against lipid peroxidation.^[15] Similar results were found by Guiliano et al.^[16] with even a positive influence on the survival rate. It was also shown that hepatic and serum zinc levels of patients in liver disease decreased depending on the degree of liver damage.^[8]

Therefore, it is assumed that administration of both ZnSO₄ and Essentiale® Forte synergistically act on injury induced by CCl₄. Essentiale® Forte alone also shows a positive effect, however it is less efficacious compared with ZnSO₄ and Essentiale® Forte combination.

Conclusion

Combined administration of ZnSO₄ and Essentiale® Forte may be considered more potentially and synergistically therapeutic through preclusion of cellular leakage mechanism and thereby inhibiting liver toxicity induced by CCl₄. ZnSO₄ and Essentiale® Forte have hepatoprotective activity against CCl₄ induced liver damage, this activity could be due to the presence of flavonoids in Essentiale® Forte and membrane stabilization ability in both, thereby preventing cellular leakage.

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