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Hepatotoxicity of Short Term Exposure to Mancozeb Fungicide in Male Wistar Rats

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Abstract

Mancozeb is a dithiocarbamate fungicide used effectively to protect plant products against fungi. The hepatic effects of short term exposure to mancozeb in adult male Wistar rats were investigated in the present study. Twenty-four animals were divided into four equal groups. Two groups were administered mancozeb (60 mg/kg body weight as single dose or 30 mg/kg body weight daily for 10 days, intraperitoneally), and the others, which served as control groups, received normal saline. Liver biochemical parameters in plasma were measured using standard methods. Liver homogenates were analysed for oxidative stress biomarkers and liver histopathology was studied. Single dose and 10 days exposures of mancozeb caused elevation in the activities of Alanine Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), and Gamma Glutamyl Transpeptidase (GGT) in plasma ($p<0.05$-0.001) compared with control. Mancozeb also caused elevation in the plasma level of total bilirubin, and reductions in albumin, total protein, and conjugated bilirubin. In addition, Malondialdehyde (MDA) and Advanced Oxidation Protein Product (AOPP) levels were increased in hepatic tissues ($p<0.001$) of all mancozeb exposed rats. Furthermore, hepatic levels of protein, reduced Glutathione (GSH) and vitamin C were decreased ($p<0.01$), together with the activities of Superoxide Dismutase (SOD), catalase, and Glutathione Peroxidase (GPx) enzymes ($p<0.01$-0.001). Histological analysis showed severe histopathological changes in mancozeb exposed rats. The results demonstrated that single dose intraperitoneal exposure of mancozeb (60 mg/kg body weight) or short term (10 days) daily exposure at 30 mg/kg body weight is capable of causing hepatotoxic effects in rats.

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1. Introduction

Mancozeb is a pesticide used in treatment of fungal plant diseases. It is an ethylene bisdithiocarbamate fungicide effectively used for the control of a wide range of fungal diseases affecting agricultural, horticultural and ornamental crops[1,2]. The fungicide is unstable in aqueous solutions and easily converted to Ethylene Bisisothiocyanate sulphide (EBIS) and Ethylene Bisisothiocyanate (EBI), and both products inhibit spore germination in fungal cell[3]. Being one of the mostly applied fungicides, and also requiring regular application to provide good protection, potential exposure of mancozeb to humans and animals is high. Exposure to mancozeb and other pesticides, which can occur during foliar application, seed treatment or food chain, generally cause long term or short term health effects. Toxic human exposures to mancozeb fungicide (1–100 ppm)[4] can occur via dermal, inhalational and gastrointestinal exposures. The dermal and inhalational routes are more associated to occupational exposure, while the general population is likely to have mancozeb exposure from contaminated food and crops which have been reported as large outbreaks in developing countries[5].

In earlier studies, the chemical has been reported to induce adverse effects on various organs and systems in humans and animals, including the hematopoietic system[6,7], and nervous system[8]. In addition, mancozeb is classified as a carcinogen with evidence of genotoxic effects[9], induces immunotoxicity effects[10], and affects thyroid organ function[11]. Recent studies have equally indicated that mancozeb may play critical role in pathogenesis and progression of chronic diseases in humans especially at high doses, as it altered cell metabolism and induced cell death dose-dependently at concentrations ranging 1-100 ppm[4].

The liver plays central role in detoxification of chemical compounds and drugs, and is also susceptible to many toxic injuries from chemical compounds. Though, some works have indicated hepatotoxic effects of mancozeb[12–14], the objective of the present research was to do a more focused study, looking at the effect of acute or short term exposure in great detail as most of the earlier studies reported longer exposure periods. Toxicokinetics studies have indicated that toxicity outcome of mancozeb varies greatly with the route of exposure, as the chemical is poorly absorbed via the gastrointestinal tract and skin[15–17]. The dose would therefore be a very essential consideration for different routes of exposure in mancozeb toxicity studies. In this light, it has been demonstrated that intraperitoneal administration of mancozeb at 40 mg/kg b.w. is capable of causing bone marrow toxicity in rats[18]. In the present study, we evaluated the potential hepatotoxic effect of acute or short term mancozeb exposure in Wistar rats by giving single dose or 10 days treatment of mancozeb to the animals intraperitoneally, using 60 and 30 mg/kg dose levels, respectively.

2. Materials and Methods

2.1 Chemicals

Mancozeb, a Zn Mn-ethylene bisdithiocarbamate, commercial grade consisting of 80% wettable powder (Sabero Organic Gujarat Ltd., Mumbai, India), diethyl ether (JHD, Guangdang Guaunghua Chemical Factory Co. Ltd., China), potassium chloride (BDH Chemicals Ltd. Poole, England), phosphate buffer (BDH Chemicals Ltd. Poole, England), and formal-saline (BDH Limited Poole, England), were purchased. Mancozeb was dissolved in normal saline for ip administration.

2.2 Experimental Animals and Design

The study was carried out on adult male Wistar rats aged about 3 months, weighing 180-200 g obtained from the Animal House of our institution. They were housed in plastic cages and maintained at constant temperature of 27±5°C, 12 h light and dark cycle and the rats were allowed free access to commercial standard pellet diet, and tap water. Animal handling was in compliance with the International Guidelines for Animal Care[19], and experimental procedures were approved by our local Research Ethics Committee (UPH/CHREC/APP/032/2019).

The rats (total number of 24) were randomly distributed into four different groups (Groups I, II, III and IV) with six rats in each group. Group I rats were given mancozeb solution (60 mg/kg b.w. single dose for acute study), while Group II rats received mancozeb solution (30 mg/kg b.w. once a day for 10 consecutive days for short term study) by ip route. Groups III and IV served as control groups and were given normal saline following the same regimes as Groups I and II, respectively. The doses used in the present study were selected based on

previous studies[15,18]. The animals were anesthetized at the end of the scheduled treatment periods (24 h after the last dose of treatment), weighed, operated, and blood was collected into lithium heparin tube. Animals' livers were excised, cleared of adhering tissues and weighed. Blood sample was centrifuged at 3000 rpm for 15 min, plasma was drawn and stored at -80°C for analysis of biochemical parameters. Some parts of the livers were rinsed in ice cold 1.15% KCl solution, homogenized in phosphate buffer, and centrifuged. Thereafter, the supernatants obtained were maintained at -80°C for biochemical assays. The remaining parts were fixed in 10% buffered formalin solution and embedded in paraffin for histopathological study. Relative liver weight (organ/body weight ratio) was determined.

2.3 Biochemical Analysis

2.3.1 Biochemical Parameters in Plasma

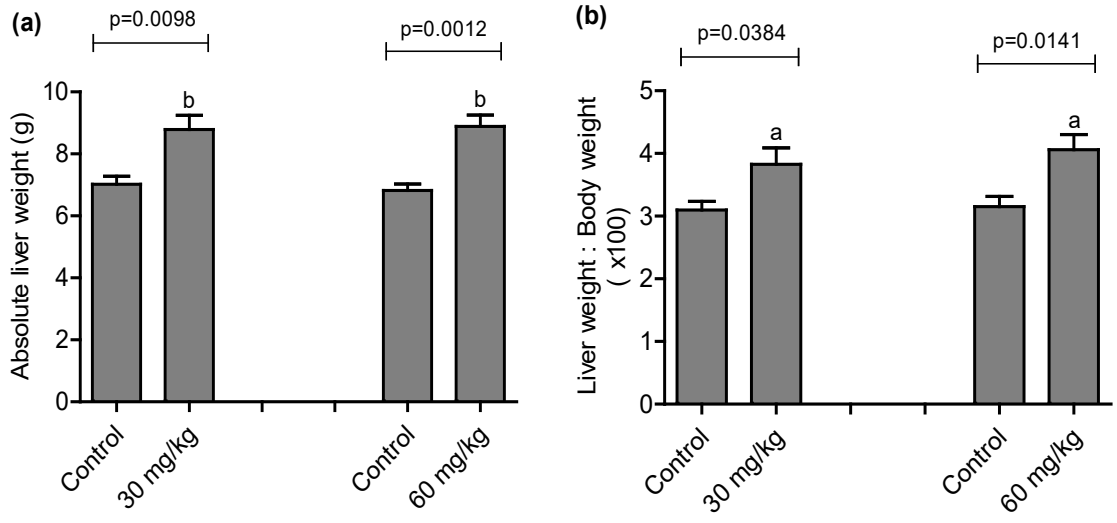
The levels of total protein, albumin, conjugated and total bilirubin in plasma and the activities of liver transaminase enzymes (alanine transaminase, ALT and aspartate transaminase, AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), and lactate dehydrogenase (LDH), were assayed spectrophotometrically using commercially available diagnostic kits (Randox Laboratory Kits, England) following standard procedures[20–24].

2.3.2 Biochemical Parameters in Liver Homogenates Oxidation Stress Biomarkers

Malondialdehyde (MDA) content in liver, lipid peroxidation marker was measured spectrophotometrically at 532 nm according to the method of Buege and Aust[25], and the values were expressed as micromole per litre (μmol/l). Liver advanced oxidation protein product (AOPP) level measurement was performed using the method described by Witko-Sarsat *et al.*[26] Total protein content in liver homogenates was estimated according to the method of Lowry *et al.*[20]

2.3.3 Enzymatic Antioxidants

Catalase (CAT) activity in liver tissue was assayed colorimetrically at 480 nm and expressed as units per millilitre (U/ml), following the method of Aebi[27]. Superoxide dismutase (SOD) activity was estimated using the method of Sun and Zigman[28]. Enzyme activity was calculated by measuring the change in absorbance at 480 nm and the values were expressed as units per millilitre



Values are means ± SEM for six rats per group.
[a]p<0.05, and [b]p<0.01: compared with control.

Figure 1. Changes in **(a)** absolute weight and **(b)** relative weight in rat liver after treatment with mancozeb at 30 mg/kg body weight, given once daily for 10 days; or 60 mg/kg body weight, given as single dose

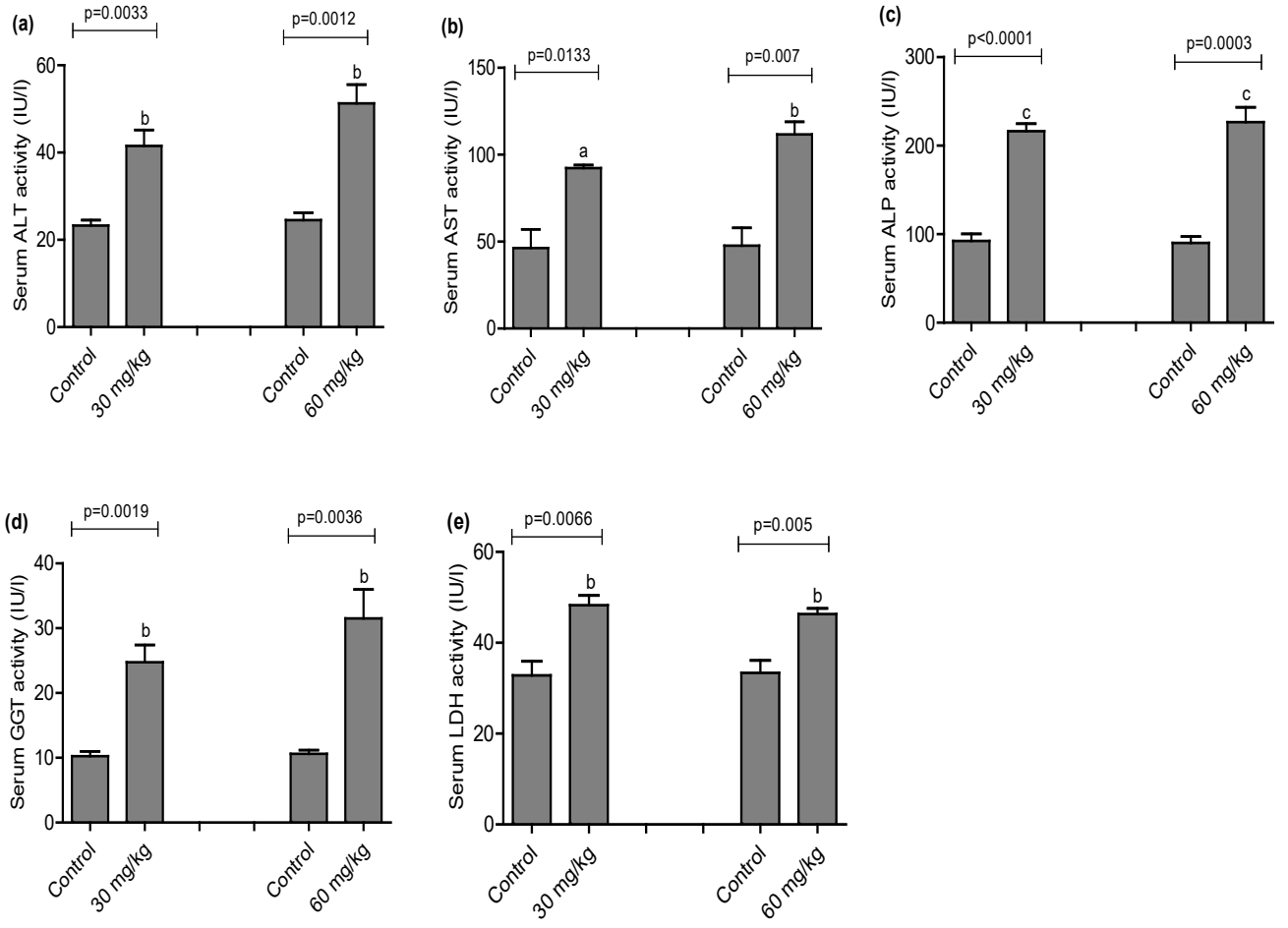
(U/ml). Glutathione peroxidase (GPx) activity was measured according to the method described by Flohe and Gunzler[29]. Enzyme activity was expressed as units per millilitre (U/ml).

2.3.4 Nonenzymatic Antioxidants

Reduced glutathione (GSH) content in liver homogenates was quantified at 412 nm according to the method of Brehe and Burch[30] with slight modification. The GSH concentration was calculated using the extinction coefficient of 1.34×10^4 M^{-1} cm^{-1} and the values were expressed as micromole per litre (µmol/l). Liver ascorbic acid (vitamin C) content was determined using the dinitrophenylhydrazine method described by Angirekula *et al.*[31] The final sample product was read at 520 nm data were expressed as milligram per decilitre (mg/dl).

2.4 Histological Studies

The liver specimens were removed from the 10% buffered formalin solution, washed and dehydrated through series of graded ethanol solutions. They were then embedded



Values are means ± SEM for six rats per group.
[a]p<0.05, and [b]p<0.01: compared with control.

Figure 2. Plasma activities of liver enzymes after treatment with mancozeb at 30 mg/kg body weight, given once daily for 10 days; and 60 mg/kg body weight, given as single dose.

in paraffin, blocked out and thereafter sectioned at a thickness of 5 µm and stained with hematoxylin and eosin. Liver sections were viewed under the light microscope and histopathological changes were noted.

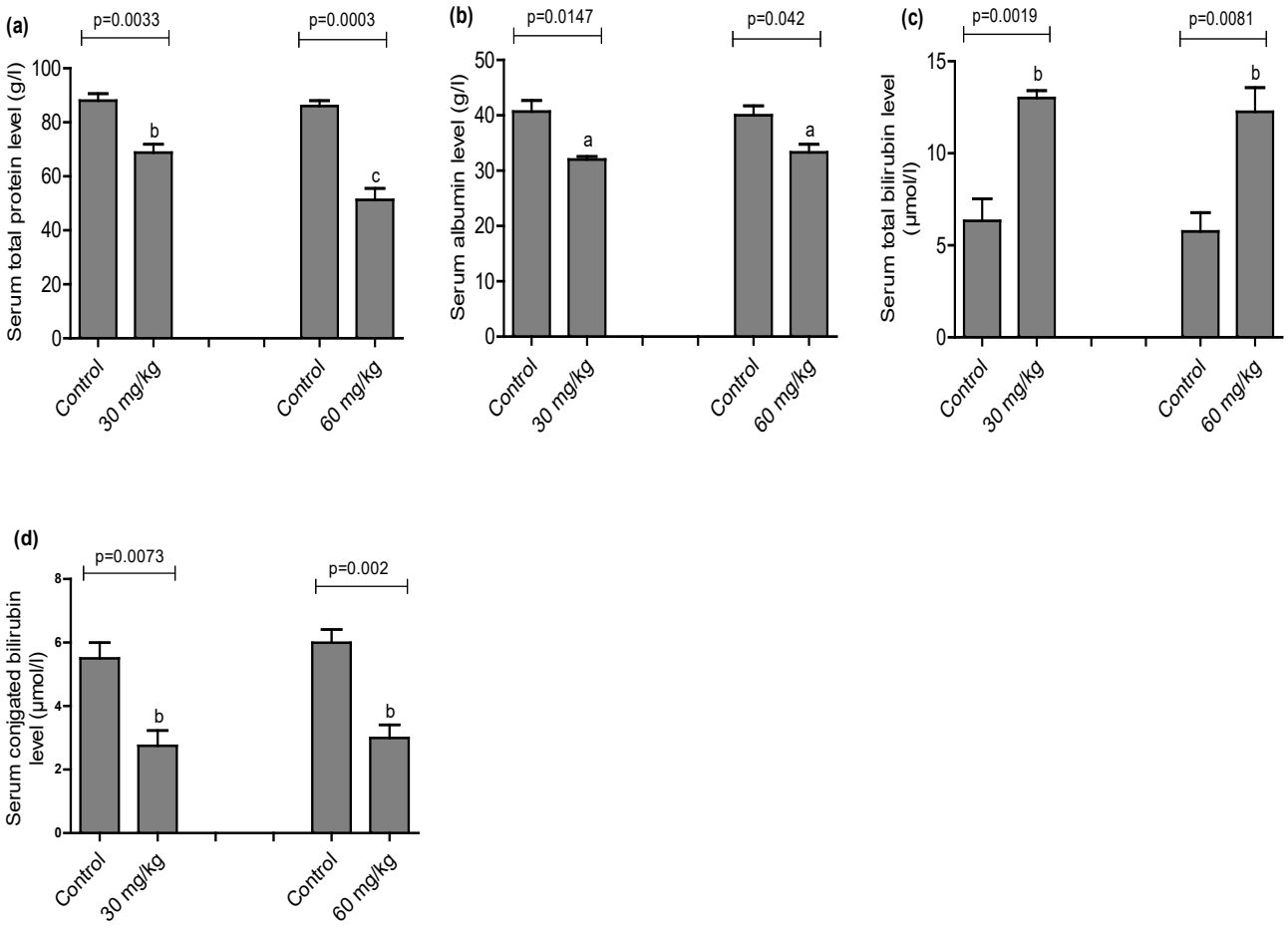
2.5 Statistical Analysis

The data of the present work were analysed using the GraphPad Prism 5 Software (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was performed using student unpaired t-test to compare between control and test groups. All values were expressed as means+SEM, and differences in the values were considered statistically significant at $p<0.05$.

3. Results

3.1 Liver Weight

Absolute and relative weights of liver in rats that received 30 mg/kg body weight mancozeb daily for 10 days or



Values are means ± SEM for six rats per group.
[a]$p<0.05$, and [b]$p<0.01$: compared with control.

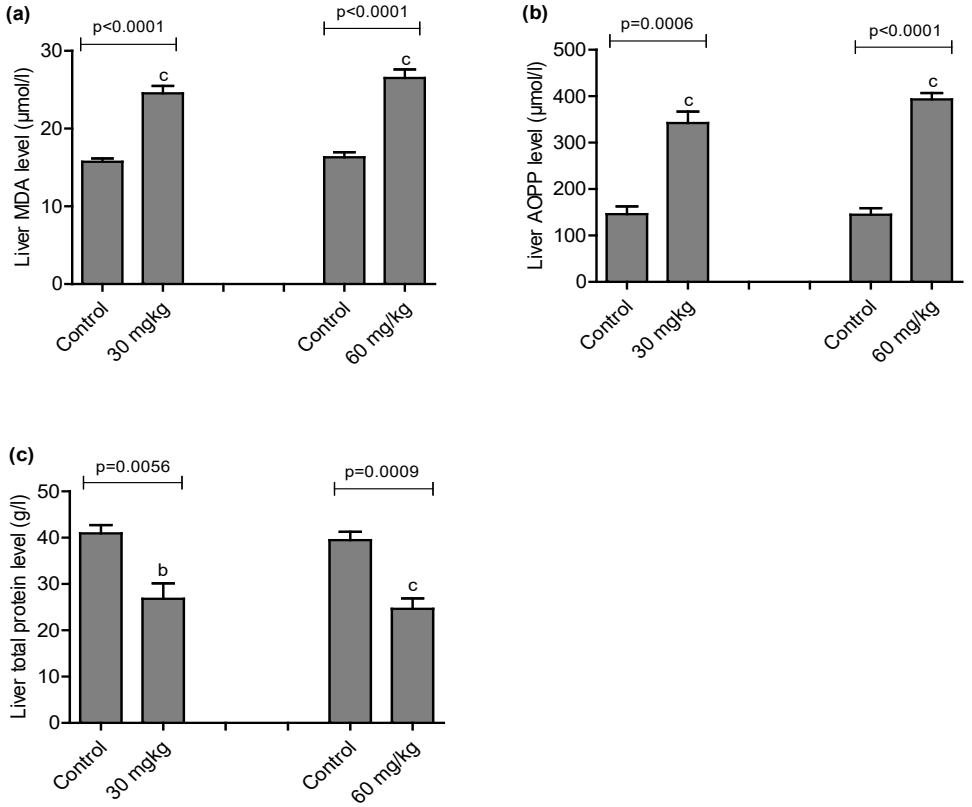
Figure 3. Plasma levels of **(a)** total protein, **(b)** albumin, **(c)** total bilirubin and **(d)** conjugated bilirubin after treatment with mancozeb at 30 mg/kg body weight, given once daily for 10 days; and 60 mg/kg body weight, given as single dose

single dose 60 mg/kg b.w. were significantly increased when compared to control rats (p<0.05-0.01). The increase in absolute weight corresponded to about 25 and 30%, respectively, while that of the relative weight was increased by about 24 and 29%, respectively in the rats (Figures 1a and 1b).

3.2 Plasma Biochemical Parameters Assay

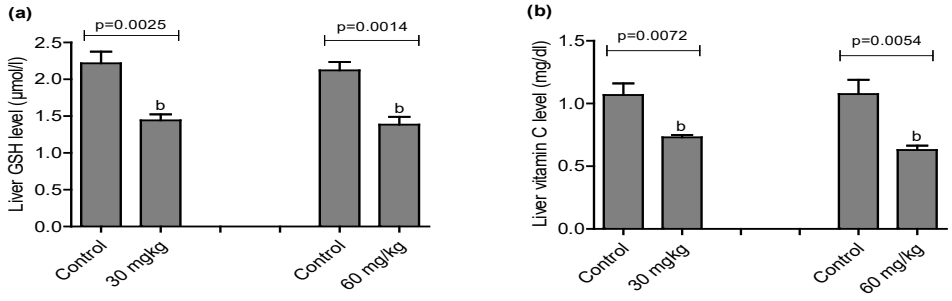
The results obtained for the activities or concentrations of biochemical markers evaluated in plasma of control and mancozeb exposed rats are presented in Figures 2 and 3. ALT, AST and ALP activities in all rats that received single or multiple doses of mancozeb were increased (p<0.05-0.001) compared to the control (Figures 2a, 2b and 2c). GGT and LDH activities in 30 or 60 mg/kg b.w. mancozeb treated rats were also elevated (p<0.01). GGT increase in the 30 or 60 mg/kg b.w. mancozeb treated rats was over twofold (i.e., >100%), while LDH increase was about 40%, compared to the control (Figures 2d and 2e). In addition, total protein, albumin, and conjugated bilirubin were reduced (p<0.05-0.001), while total bilirubin level was elevated (p<0.01) in all mancozeb injected rats (Figures 3b, 3c and 3d).

ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, gamma glutamyl transpeptidase; LDH, lactate dehydrogenase.



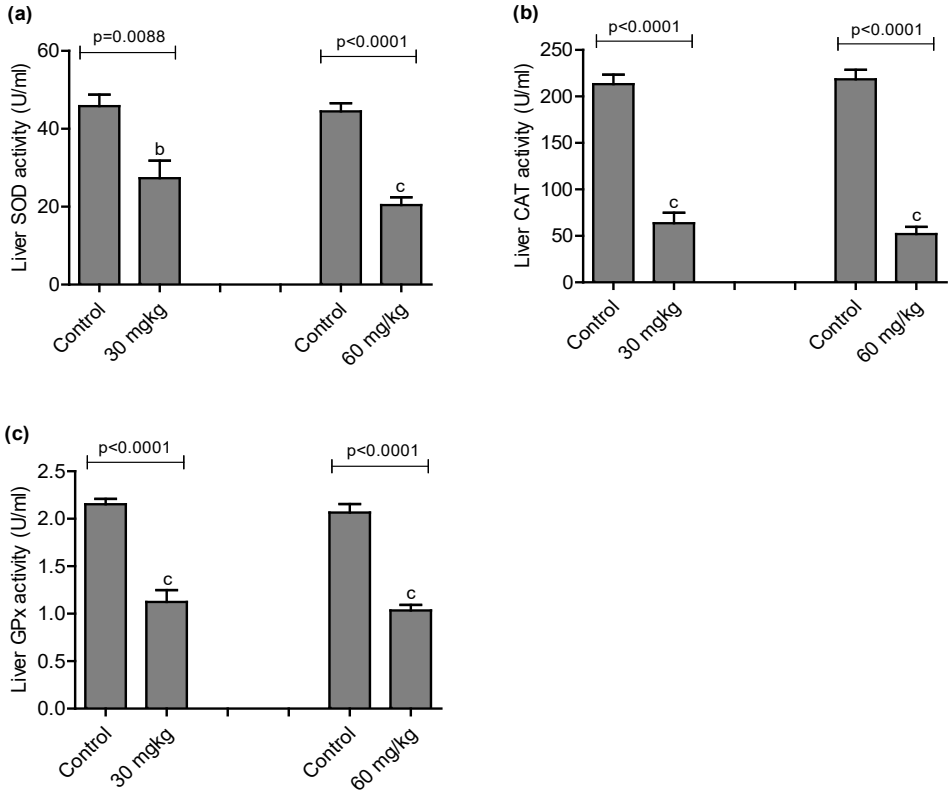
Values are means ± SEM for six rats per group.
[b]p<0.01, and [c]p<0.001: compared with control.
MDA, malondialdehyde; AOPP, advanced oxidation protein product

Figure 4. Changes in **(a)** MDA, **(b)** AOPP, and **(c)** total protein concentrations in rat liver homogenate after mancozeb at 30 mg/kg body weight, given once daily for 10 days; or 60 mg/kg body weight, given as single dose.



Values are means ± SEM for six rats per group.
[b]p<0.01: compared with control.
GSH, reduced glutathione

Figure 5. Changes in **(a)** GSH and **(b)** vitamin C concentrations in rat liver homogenate after treatment with mancozeb at 30 mg/kg body weight, given once daily for 10 days; or 60 mg/kg body weight, given as single dose



Values are means ± SEM for six rats per group.
[b]p<0.01, and [c]p<0.001: compared with control.
SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

Figure 6. Changes in **(a)** SOD **(b)** CAT, and **(c)** GPx activities in rat liver homogenate after treatment with mancozeb at 30 mg/kg body weight, given once daily for 10 days; or 60 mg/kg body weight, given as single dose

3.3 Liver Biochemical Parameters Assay

3.3.1 Oxidative stress biomarkers (MDA, AOPP, and protein)

Liver levels of MDA and AOPP in rats that received 30 or 60 mg/kg b.w. mancozeb were increased ($p<0.001$) compared to control rats, whereas protein was decreased ($p<0.01$-0.001) as presented in Figures 4a and 4b.

3.3.2 Nonenzymatic Antioxidant Biomarkers (GSH and vitamin C)

Liver contents of GSH and vitamin C of mancozeb-treated rats in rats that received 30 or 60 mg/kg b.w. mancozeb were decreased ($p<0.01$) when compared to control rats (Figures 5a and 5b).

3.3.3 Enzymatic Antioxidant Biomarkers (SOD, CAT, and GPx)

The activities of SOD, CAT and GPx enzymes decreased ($p<0.01$-0.001) in the livers of rats that received 30 or 60 mg/kg b.w. mancozeb (Figures 6a, 6b and 6c).

4. Liver Histopathology

Light microscopic examination of liver sections from control rats showed normal histo-architecture and histological features, like hepatic artery, hepatic vein, bile duct, well distributed hepatocytes, and sinusoidal lining (Figure 7a). Histopathological changes were observed in rats that received mancozeb when compared with control rats. Liver sections of rats that received 30 mg/kg b.w. mancozeb daily for 10 days indicated vascular congestion,

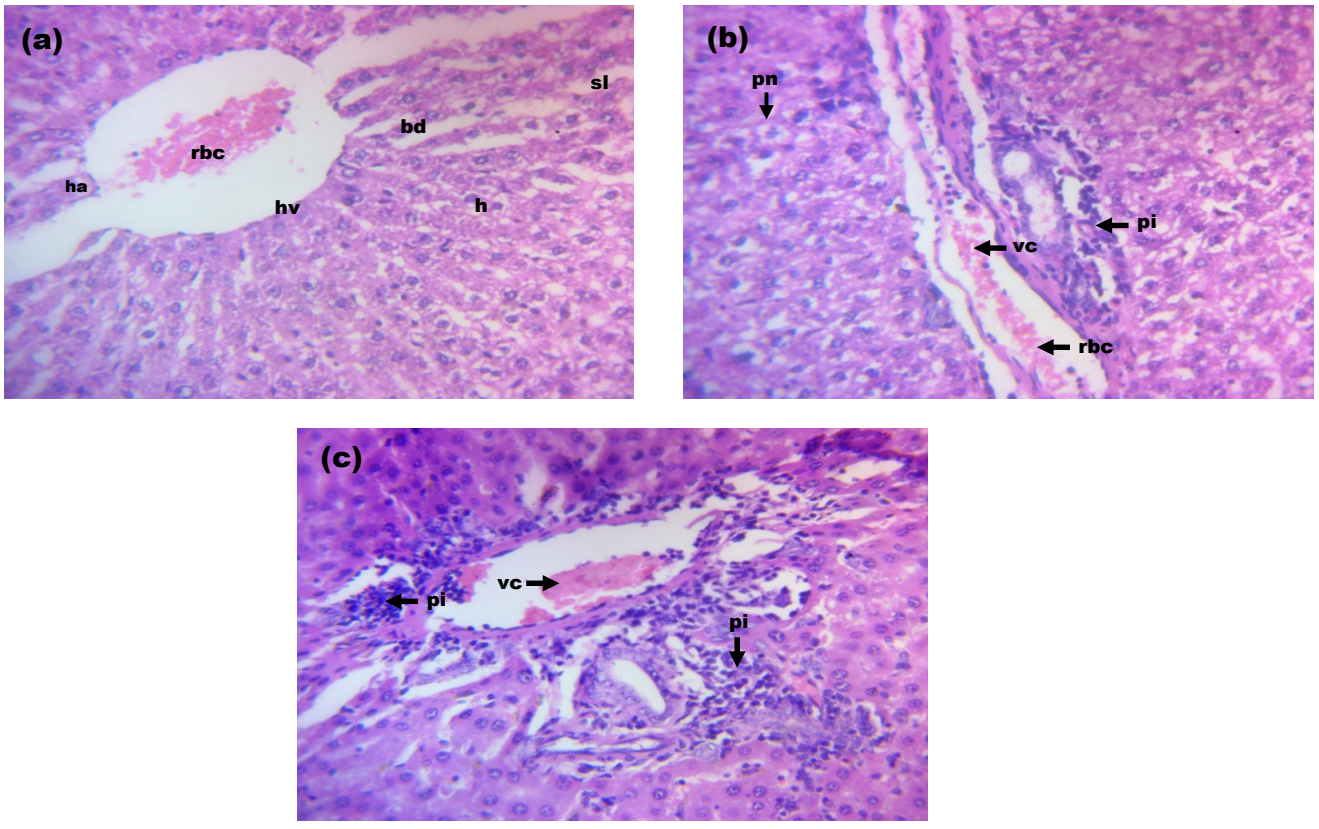


Figure 7. Photomicrographs of H&E sections of rat liver after treatment with mancozeb fungicide. Tissues were obtained from **(a)** control rats; **(b)** rats that received 30 mg/kg body weight, given once daily for 10 days; and **(c)** rats that received 60 mg/kg body weight, given as single dose bd, bile duct; h, hepatocyte; ha, hepatic artery; hv, hepatic vein; pi, peri-portal inflammation; pn, pyknotic nuclei; rbc, red blood cells; sl, sinusoidal lining; vc, vascular congestion.

periportal inflammation as well as presence of pyknotic nuclei in hepatocytes (Figure 7b). Liver sections of rats that received single dose 60 mg/kg b.w. mancozeb indicated histopathological changes that were similar to the animals that were exposed to multiple doses of 30 mg/kg b.w. mancozeb, but with more pronounced periportal inflammation (Figure 7c).

5. Discussion

Besides carrying out essential physiological functions, the liver plays central roles in detoxification of xenobiotics, and drug metabolism. This also makes the organ vulnerable to toxicity. Mancozeb is an important fungicide frequently used in agriculture. Since humans and animals could be easily exposed to the fungicide because of its wide utilization, evaluation of its hepatotoxic profile was considered in this study. Some reports are available on the oral administration of mancozeb and its toxicity in the liver[12,13,32], but the subject has not been comprehensively studied. The present study is important because it focused on the effect of acute and short term exposure in great detail, while most of the earlier studies reported longer exposure periods.

Acute and short-term effects of the chemical were evaluated using single dose (60 mg/kg b.w.) and 10 days daily intraperitoneal exposure (30 mg/kg b.w.), respectively. The doses used were deliberately selected to be lower than the oral doses of the above cited previous studies (which ranged from 500–1500 mg/kg b.w.) because the kinetics of mancozeb vary with the administration route. The LD_{50} of mancozeb via the ip route is 380 mg/kg b.w[15]. This was considered in choosing the doses used for this study to ensure that lethal doses were not administered. The dose selection was also guided by the earlier study of Goldoni *et al.*[18], who reported that intraperitoneal exposure of 40 mg/kg b.w. of mancozeb for 18 days may cause genotoxic effect on bone marrow of rats and may adversely affect DNA integrity of exposed organisms.

From the results, the ten days multiple dose (30 mg/kg b.w.) and single dose (60 mg/kg b.w.) treatments with mancozeb increased absolute and relative liver weight of rats. Alteration in organ weight after xenobiotic treatment is an indication of organ toxicity, with the implication that mancozeb may cause liver toxicity. This agrees with the study of Yahia *et al.*[13] who also reported that mancozeb caused increase in weight of rat liver after 8 weeks oral daily exposure of 1000 mg/kg b.w.

ALT, AST and ALP were elevated by the single dose and ten days mancozeb treatments. The elevation in plasma levels of these enzymes is indicative of hepatic cell injury, enzymes leaking out from damaged cell membrane into the circulation[33,34]. Kackar *et al.*[12] and Yahia *et al.*[13] had reported similarly that mancozeb caused a rise in liver ALT, AST and ALP enzymes but at much higher oral dose levels (500–1500 mg/kg/day b.w.). Elevation in plasma LDH and GGT activities by mancozeb that was observed in these groups of rats provides more evidence of organ membrane integrity damage, and elevated ALT and GGT levels support liver tissue damage[35,36]. This was evident from the histopathological examination where livers of the rats that received single or multiple doses of mancozeb exhibited severe histomorphological changes in the liver. Similar observation has been reported for maneb[37], but this is the first study reporting this result for mancozeb. Added to other possible factors, the pronounced inflammatory changes that occurred in these treated rats may contribute to the earlier reported increase in liver weight that was induced. It is expected that the damage to liver cell will inhibit its physiological functions. Thus, the reduction of proteins, and conjugated bilirubin, and elevation of total bilirubin that were observed in plasma after mancozeb treatment may not be surprising. It simply indicates that protein synthesis capacity and bilirubin excretory functions of the liver were compromised[36,38]. Earlier studies indicated that mancozeb produced similar hepatotoxic effects but at much higher (>250 mg/kg body weight) dose levels following long term oral exposure[12,15,32]. This suggests that gastrointestinal absorption of mancozeb is substantially prevented in the rat, but more studies would be necessary using lower oral doses to make a good conclusion and correctly assess the administration route-toxicity relationship. The data equally indicate that mancozeb possesses high intraperitoneal acute liver toxicity but may have low oral acute liver toxicity, thus demonstrating the fact that administration route immensely affect toxicokinetics of the fungicide.

In the present study, biomarkers of lipid peroxidation and antioxidant activities in the hepatic tissue homogenates were measured separately to determine the oxidative

stress status in the organ. Our findings showed that the morphological changes and physiological impairment observed in livers of the rats that received multiple doses of 30 mg/kg or single dose of 60 mg/kg b.w. of the fungicide could have resulted from increased oxidative stress. MDA (the index of lipid peroxidation) was elevated in liver tissues of the mancozeb exposed rats. The levels of free radicals scavenging non-enzymatic antioxidants (GSH and vitamin C) were also decreased. GSH contains thiol group which readily interacts with free radicals, especially the hydroxyl radical (.OH), and provides protection against oxidative free radical damage by neutralizing ROS[39,40]. Vitamin C mops up free radicals in aqueous phase and protects cell membranes and structures against lipid peroxidation[41]. Reduction of these antioxidants will result in elevation of lipid peroxidation activity which explains the increased level of the lipid peroxidation product. In addition, SOD, CAT, and GPx enzymes activities were decreased in liver tissues of all the rats that were exposed to mancozeb. In humans, superoxide anion O_2 is the most commonly produced ROS and precursor for other free radicals capable for causing cell injury. SOD catalyses the detoxification (dismutation) of superoxide anion to form water and hydrogen peroxide (H2O2)[42]. This is the primary step of the defense mechanism in the antioxidant system against oxidative stress[43]. Hydrogen peroxide is later on broken down by the coordinated actions of CAT and GPx, hence these enzymes eliminate ROS in the cell and prevent cell damage[44,45]. Reduction in antioxidants activities and increased free radical activities (e.g., lipid peroxidation) would shift the redox balance of the cell towards oxidative stress and induce oxidative cell damage. This is consistent with the mechanism of toxicity of mancozeb and other dithiocarbamate fungicides that have been well documented[10,37,46].

In conclusion, the results of the present study demonstrated that single intraperitoneal dose of mancozeb (up to 60 mg/kg b.w.) is capable of causing hepatic damage in rats. Continuous short term (10 days) exposure to mancozeb at 30 mg/kg b.w. would also exhibit hepatotoxic effect. Hepatotoxicity of mancozeb involves increase in oxidative stress.

6. Acknowledgement

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7. Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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