EVALUATION OF MANCOZEB INDUCED SUBACUTE ORAL TOXICITY IN RATS

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VETERINARY PHARMACOLOGY AND TOXICOLOGY
(Minor Subject: Animal Biotechnology)

By

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CERTIFICATE I

This is to certify that the thesis entitled, **"EVALUATION OF MANCOZEB INDUCED SUBACUTE ORAL TOXICITY IN RATS"** submitted for the degree of **M.V.Sc.,** in the subject of **Veterinary Pharmacology and Toxicology** (Minor subject: **Animal Biotechnology**) of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafied research work carried out by **Sunita Kumawat (L-2018-V-80-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

The present study was designed to evaluate the toxic manifestations of subacute oral mancozeb toxicosis as well as to study the endocrine and neurobehavioral effects of subacute oral mancozeb exposure. A total of 12 healthy wistar rats were procured and divided into 2 groups (Group I: Control; Group II: Treatment) having 6 animals in each. Group II animals were administered with mancozeb (500mg/kg bwt) orally for 28 consecutive days whereas, the control group animals were only provided with *adlib* feed and water. Repeated mancozeb exposure, produced alteration in various parameters. Mancozeb exposure produced mild to moderate signs of toxicity, significant ($p<0.05$) elevation in absolute organ weight of liver, kidneys, brain, testes, epididymis and relative weight of liver while, significant ($p<0.05$) reduction in relative weight of heart. Haematological parameter showed, a significant ($p<0.05$) reduction in TEC and TLC count whereas, significant increase in ESR, MCV and neutrophils in mancozeb-exposed animals. In biochemical parameters, a significant elevation in AST, ALT, LDH, GGT, creatinine, BUN, alkaline phosphatase, creatine kinase, HDL and LDL-cholesterol levels and a significant reduction in total plasma protein, blood glucose and triglycerides were observed in mancozeb-exposed animals as compared to control group. A significant increase in TSH while significant decrease in T_3 and T_4 levels were found in mancozeb-exposed rats. Neurobehavioral parameters, the Elevated plus-maze and T-maze test results, showed anxiety-like behaviour in mancozeb-exposed animals. The rota rod activity and grip strength decreased significantly. Reproductive parameters altered significantly, decrease in the live sperm and total sperm count while increase in dead and abnormal sperm count were found in rats due to mancozeb exposure. Histopathological examination showed necrosis and degeneration with cytoplasmic vacuolization in the liver section, congestion and degeneration in the kidney section and vacuolization in the pons area of brain in mancozeb-treated rats. Thus, the results of the present study, suggested that mancozeb fungicide produced a pronounced toxicological insult in the exposed animals at tested dose and might be detrimental to human and animal health.

Keywords: Biochemical, Haematological, Histopathology, Mancozeb, Neurobehavioural, Reproductive, Toxicity, Wistar rats

Signature of Major Advisor **Signature of the student**

CONTENTS

CHAPTER	TOPIC	PAGE NO.
I	INTRODUCTION	1 – 5
II	REVIEW OF LITERATURE	6 – 21
III	MATERIALS AND METHODS	22 – 40
IV	RESULTS AND DISCUSSION	41 – 88
V	SUMMARY AND CONCLUSIONS	89 – 92
	REFERENCES	93 – 109
	VITA	

LIST OF TABLES

Table No.	Title	Page No.
1	Experimental design for in vivo sub-acute toxicity study in male wistar rats	23
2	Following haematological parameters were analysed in the blood collected in EDTA vials at 0 day and 29 day of experiment	30
3	Weekly body weight (g) variations on exposure to mancozeb *per os* in male wistar rats	43
4	Absolute (g) and Relative organ weight (%) variations after exposure to Mancozeb *per os* in male wistar rats	44
5	Haematological variations in parameters including Haemoglobin, Packed cell volume, Erythrocyte sedimentation rate, Total erythrocyte count and Total leucocyte count after sub-acute exposure to mancozeb *per os* in male wistar rats	47
6	Haematological variations in parameters including Neutrophils, Mononuclear cells, Mean corpuscular volume, Mean corpuscular haemoglobin and Mean corpuscular haemoglobin concentration after sub-acute exposure to mancozeb *per os* in male wistar rats	48
7	Blood Biochemistry variations in parameters indicating liver function after sub-acute exposure to mancozeb *per os* in male rats	51
8	Blood Biochemistry variations in parameters indicating renal function after sub-acute exposure to mancozeb *per os* in male rats	59
9	Blood Biochemistry variations in parameters indicating lipid profile after sub-acute exposure to mancozeb *per os* in male rats	64
10	Variations in Endocrine parameters after exposure to Mancozeb *per os* in male rats	68
11	Variations in Elevated Plus Maze parameters after exposure to mancozeb per *os* in male wistar rats	73
12	Variations in T-Maze parameters after exposure to mancozeb per *os* in male wistar rats	75
13	Neurobehavioural assessment of male wistar rats after exposure to mancozeb per *os* in male wistar rats	77
14	Effect of sub-acute oral mancozeb exposure on acetyl choline esterase activity (AChE) in male wistar rats	81
15	Effect of sub-acute oral mancozeb exposure on sensory parameters in male wistar rats	82
16	Effect of sub-acute oral mancozeb exposure on reproductive parameters in male wistar rats	84

LIST OF FIGURES

Fig. No.	Title
1	Structure of mancozeb
2	Body weight measurement of rats during experimentation period on weekly basis
3	Effect of sub-acute exposure of mancozeb *per os* on body weight (gm) and percent body weight gain of male rats at the end of experimentation
4	Effect of sub-acute exposure of mancozeb *per os* on absolute organ weight (mg) of various organs of male rats at the end of experimentation
5	Effect of sub-acute exposure of mancozeb *per os* on relative organ weight (%) of various organs of male rats at the end of experimentation
6	Variation in haematological parameters (Hb, PCV, ESR, TEC, TLC) after sub
7	Variation in haematological parameters (Neutrophils, Mononuclear cells, MCV, MCH, MCHC) after sub
8	Blood Aspartate amino transferase variations after sub-acute exposure to mancozeb *per os* in male wistar rats
9	Blood Alanine amino transferase variations after sub-acute exposure to mancozeb *per os* in male rats
10	Blood Lactate dehydrogenase variations after sub-acute exposure to mancozeb *per os* in male rats
11	Blood Gamma glutamyl transpeptidase variations after sub-acute exposure to mancozeb *per os* in male rats
12	Blood glucose variations after sub-acute exposure to mancozeb *per os* in male rats
13	Blood alkaline phosphatase variations after sub-acute exposure to mancozeb *per os* in male rats
14	Blood creatinine variations after sub-acute exposure to mancozeb *per os* in male rats
15	Blood urea nitrogen variations after sub-acute exposure to mancozeb *per os* in male rats
16	Total plasma protein variations after sub-acute exposure to mancozeb *per os* in male rats
17	Blood creatinine kinase variations after sub-acute exposure to

Fig. No.	Title
	mancozeb *per os* in male rats
18	Blood High density lipoprotein variations after sub-acute exposure to mancozeb *per os* in male rats
19	Blood low density lipoprotein variations after sub-acute exposure to mancozeb *per os* in male rats
20	Blood Triglyceride variations after sub-acute exposure to mancozeb *per os* in male rats
21	Blood Thyroid stimulating hormone (TSH) variations after sub-acute exposure to mancozeb *per os* in male rats
22	Blood Tri-iodothyronine (tT_3) variations after sub-acute exposure to mancozeb *per os* in male rats
23	Blood Thyroxine (tT_4) variations after sub-acute exposure to mancozeb *per os* in male rats
24	Variation in Elevated Plus Maze parameters after sub-acute exposure to mancozeb *per os* in male rats
25	Variation in Elevated Plus Maze parameter (Travelled distance) after sub-acute exposure to mancozeb *per os* in male rats
26	Variation in T-Maze parameters after sub-acute exposure to mancozeb *per os* in male rats
27	Variations in T-Maze parameter (Travelled distance) after sub-acute exposure to mancozeb *per os* in male rats
28	Variation in the grip strength force after sub-acute exposure to mancozeb *per os* in male rats
29	Variation in the Rota rod activity of control and mancozeb group rats
30	Variation in Acetyl choline esterase activity in the blood and brain tissue after mancozeb exposure in control and mancozeb group rats
31	Effect of mancozeb exposure on Eddy's hot plate test in control and mancozeb group rats
32	Effect of mancozeb exposure on reaction time of tail immersion test in control and mancozeb group rats
33	Effect of mancozeb exposure on male reproductive parameters in control and mancozeb group rats
34	Representative photomicrograph of sperms from experimental male wistar rats

Fig. No.	Title
35	Control group: Photomicrograph of the liver showing normal liver parenchyma (H&E, Bar=100 μm)
36	Mancozeb group: Liver showing necrosis and degeneration with cytoplasmic vacuolization with mononuclear cell infiltration (H&E, Bar=100μm)
37	Control group: Kidney showing normal structure of tubules (H&E, Bar= 50μm).
38	Mancozeb group: Kidney showing congestion and degeneration in the Proximal Convulated Tubules. (H&E, Bar=100μm)
39	Control group: Pons showing normal nuclei of pons (H&E, Bar=50 μm)
40	Mancozeb group: Pons showing formation of the vacuoles in the longitudinal sections of the nerve fibres. The vacuoles coalases and formed larger vacuoles. (H&E, Bar=100μm)

ABBREVIATIONS USED

%	:	Percentage
AChE	:	Acetylcholinesterase
ALP	:	Alkaline phosphatase (ALP)
ANOVA	:	Analysis of variance
AST	:	Aspartate aminotransferase
BUN	:	Blood urea nitrogen
BW		Body weight
bwt	:	Body weight
CK	:	Creatine kinase
CMs	:	Ccarbamates
Conc.	:	Concentration
CS_2	:	Carbon disulphide
DDT	:	Dichlorodiphenyltrichloroethane
DMSO	:	Dimethyl sulfoxide
DTC	:	Dithiocarbamate
EBDC	:	Ethylene-bis-dithiocarbamate
EBI	:	Ethylene bisisothiocyanate
EBIS	:	Ethylene bis-isothiocyanate sulfide
EDTA	:	Ethylene diamine tetra acetic acid
EPA	:	Environmental Protection Agency
EPM	:	Elevated plus maze
ETU	:	Ethylenethiourea
EU	:	Enzyme Unit
Fig	:	Figure
G	:	Group
G6PD	:	Glucose-6-phosphate dehydrogenase
GABA	:	Gamma-Aminobutyric acid
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
GGT	:	Gamma-glutamyl transpeptidase
GIT	:	Gastro intestinal tract
GPx	:	Glutathione peroxidase
GR	:	Glutathione Reductase
GSH	:	Reduced glutathione
GST	:	Glutathione-S-transferase
h	:	hour
H&E	:	Haematoxylin and eosin stain
H_2O_2	:	Hydrogen peroxide

Hb	:	Haemoglobin
HDL	:	High density lipoprotein
hrs	:	hours
IAEC	:	Institutional Animal Ethics Committee
IFCC	:	International Federation of Clinical Chemistry
IL	:	Interleukin
IL-6	:	Interleukin-6
LDH	:	Lactate dehydrogenase
LDL	:	Low density lipoprotein
MB	:	Mmaneb
MCH	:	Mean corpuscular haemoglobin
MCV	:	Mean corpuscular volume
MCZ	:	Mancozeb
MZ	:	Mancozeb
OP	:	Organophosphate
Ops	:	Insecticides (organophosphates
ROS	:	Reactive oxygen species
SOD	:	Superoxide dismutase
T	:	Time
T_3	:	Tri-iodothyronin
T_4	:	Thyroxine
TC	:	Total cholesterol
TDPs	:	Tthyroid disrupting pesticides
TEC	:	Total erythrocyte count
TG	:	Triglyceride
TL	:	Total lipid
TLC	:	Total leukocyte count
TPP	:	Total plasma protein
TSH	:	Thyroid stimulating hormone

CHAPTER I

INTRODUCTION

The world population is expected to rise from the current 7.8 billion to 8.5 billion by 2030, thereby putting immense pressure on food supplies and a growing demand for higher crop yields. A wide range of pesticide compounds including insecticides, fungicides, rodenticides, herbicides, nematicides, molluscicides, plant growth regulators and other chemicals are therefore used indiscriminately to cope up with the increased production of crops.

The different synthetic insecticides like organophosphate (OP) insecticides came into use in 1960s, carbamates in 1970s and pyrethroids in 1980s and the introduction of herbicides and fungicides were done in 1970s-1980s, which contributed greatly to pest control and agricultural output. In the process of development of agriculture, pesticides have become an important tool as a plant protection agent to boost up food security, as these chemicals play a significant role by controlling many dreadful diseases. Since pesticides are designed to kill or adversely affect living organisms, by their nature, they pose certain risk to humans and non-target plants and animal population. They not only contaminate the ecosystem but also bio-accumulate in the food chain and can be traced in plant and animal tissues causing serious health hazards (Johan *et al* 2001).

Although the pesticide consumption in India is still very low, there has been a widespread contamination of food commodities with pesticide residues basically due to their indiscriminate and non-judicious application. The need for safe and environment-friendly crop protection chemicals has never been greater. Residues in the food of humans and feed for livestock is repurcussion of direct application of a chemical to the food source, by the presence of pollutants in the environment or by transfer and biomagnification of the chemical along the food chain (Kumar *et al* 2013).

Modern day agriculture greatly relies on the use of different groups of pesticides in order to manage the crop pests and to gain a better crop yield (Quazi *et al* 2011). Fungicides are awning management tools in agricultural practices, as the fungal pathogens are the number one cause of crop loss around the world and can

cause serious damage in agriculture, resulting in critical losses of yield, quality and profit in both plants and animals (Poole and Arnaudin 2014). Fungicides have been used widely to control fungal diseases and increase crop production. The modes of action of fungicides were never well classified and presented, making it difficult to estimate their possible non-target effects (Yang *et al* 2011).

Dithiocarbamates (DTCs) represent an important class of fungicides, widely used in agricultural sector. They are characterized by a broad spectrum of activity against various plant pathogens, low acute mammalian toxicity and low production costs. In combination with modern systemic fungicides, they are also used to manage resistance and to broaden the spectrum of activity. Besides agriculture, DTCs are largely used as slimicides in pulp and paper manufacturing, wood preservatives or vulcanization accelerators in the rubber industry, which may also be a critical source of non-residual contamination for food and feed samples during handling. Furthermore, DTCs can also be used clinically for the treatment of chronic alcoholism and as anticancer and antitoxic drug agents (Crnogorac *et al* 2009).

Mancozeb (MZ) is a fungicide belonging to the subclass of ethylene bisdithiocarbamates pesticides (Srivastava and Singh 2014) having a metal atom coordinated with ethylene-bis-dithiocarbamate (EBDC) (Calviello *et al* 2005). It is a combination of two other dithiocarbamates: maneb and zineb and has a multi-site, protective action upon contact. The mixture controls many fungal diseases in a wide range of field crops, fruits, nuts, vegetables and ornamentals.

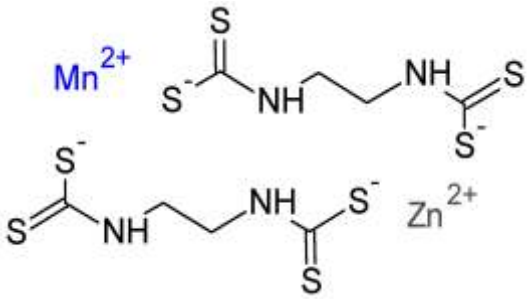


Fig. 1: Structure of mancozeb (Zamora *et al* 2019)

Mancozeb is used as a fungicide against a wide range of fungi including ascomycetes, basidiomycetes and oomycetes which is active over 70 crops and 400 different diseases (Leader *et al* 2008). It inhibits the fungal-spore germination (Szkolnik 1981; Wicks and Lee, 1982; Wong and Wilcox 2001). Mancozeb itself does

not act as a fungicide; rather it is effectively considered as a pro-fungicide. When it is exposed to water, it may degrade to ethylene bis-isothiocyanate sulfide (EBIS) and ethylene bisisothiocyanate (EBI) (Saha *et al* 2016). Both these compounds (EBIS and EBI) act as active toxicants. Mancozeb interferes with enzymes containing sulphydryl groups and disrupts many core enzymatic processes of the fungal cell cytoplasm and mitochondria (Ludwig and Thorn 1960, Sijpesteijn 1982). The half-life of mancozeb is about 1-7 days, but the half-life of its primary metabolite (ethylenethiourea) is 5-10 weeks. The metabolite of mancozeb, ethylenethiourea (ETU) has the potential to contaminate the ground water (Srivastava and Singh 2013). Mancozeb, like other carbamates, may attack the nervous system. It inhibits the function of neurotransmitter acetylcholinesterase (AChE) in the central nervous system of insects by its primary metabolites, ETU and carbon disulphide (CS_2). Ethylenethiourea is also responsible for thyroid dysfunction and carcinogenic effects in various organisms (Srivastava and Singh 2013).

Mancozeb displays the characteristics of a typical multi-site protectant-only fungicide, following application into the target plant, as the compound remains only on the leaf surface and does not penetrate through the cuticle where systemic redistribution can occur. This is clearly important because penetration of a general toxophore such as mancozeb into plant cells would likely cause phytotoxicity. The rate of breakdown of mancozeb into EBIS and EBI can directly affect the residual activity of the compound on plant foliage. Each mancozeb particle consists of a zinc-rich shell surrounding a central nucleus of polymer-structured EBDC. This structure is extremely stable and the low solubility of the zinc shell means EBDC can pass through this layer and be deposited on the leaf surface at a controlled rate. This controlled release helps to regulate the duration of fungicidal efficacy and improves foliar residuality (Gullino *et al* 2010).

The adverse effects of mancozeb in humans and other living organisms have not been widely studied and there are only a few reported studies evaluating the neurotoxic action of mancozeb in experimental models (Domico *et al* 2006). Diverse chronic adverse health effects of mancozeb and other metal ethylene-bis-dithiocarbamate (EBDC) pesticides have been observed, such as structural and functional alterations of gonads (Baligar and Kaliwal 2001). The majority of studies

have focused on adverse health effects of ethylene thiourea (ETU), the main metabolite of mancozeb. This compound has been reported to have carcinogenic, teratogenic and goitrogenic effects (Chhabra *et al* 1992). Although its mutagenic effect is still controversial, it is considered a potent carcinogenetic agent.

Even mild changes in human thyroidal function due to mancozeb exposure in early prenatal life have been known to cause severe consequences in the child's neurological development (Haddow *et al* 1999, Pop *et al* 1999, Kooistra *et al* 2006, Henrichs *et al* 2010, Li *et al* 2010). The degradation product of the dithiocarbamates, ethylene thiourea (ETU), also exerts various toxic effects in rats, including thyroid neoplasms, developmental toxicity and teratogenicity (NTP 1992).

Mancozeb has been shown to cause adverse health effects in both humans and experimental animals. Mancozeb exposure was associated with increased incidence of thyroid disease in female spouses of pesticide applicators (Goldner *et al* 2010), and a moderate association was also seen between mancozeb exposure and neural tube defects in newborns from farmer families (Nordby *et al* 2005). Mancozeb has also been reported to cause decrease in serum thyroxine (T_4) levels, thyroid peroxidase activity and iodine uptake and increase in production of thyroid stimulating hormone (TSH) and thyroid weight, causing hyperplasia and hypertrophy of follicular cells in the thyroid, as well as thyroid cancer (WHO 1988, Trivedi *et al*1993, Kackar *et al* 1997b, Hurley *et al*1998).

Certain reproductive toxicity due to mancozeb exposure has also been observed like decreased weight of ovary, testes and epididymis, disrupted estrus cycle, and histopathological changes in the reproductive organs (Kackar *et al* 1997a, Mahadevaswami *et al* 2000, Baligar and Kaliwal 2001). Higher doses of mancozeb has been known to cause general toxicity as well as neurotoxic effects in rats including dyspnea, salivation, diarrhea and paralysis of the hind limbs followed by death of the animals (Axelstad *et al* 2011).

Although mancozeb has been reported to possess low acute toxicity and scarce persistence in the environment, diverse chronic adverse health effects of mancozeb have also been observed such as increased carcinogenic potential (Chhabra *et al* 1992), endocrine system disruption (Kwon *et al* 2018) and toxic effects on the immune system. Since mancozeb is an important ingredient of pest control programs

and being frequently recommended by State Agriculture Universities including Punjab Agricultural University, Ludhiana (Package and Practices 2015) for various crop practices, it becomes pertinent to understand its potential harmful effects on non-target organisms. Since, relatively little is known about the action of mancozeb in vertebrates, the present investigation aims to accomplish the following objectives:

1. To evaluate the toxic manifestations of subacute oral mancozeb toxicosis.
2. To study the endocrine and neurobehavioral effects of subacute oral mancozeb exposure.

CHAPTER II

REVIEW OF LITERATURE

Pesticides and agricultural chemicals are among the most widely used group of chemicals in modern world and have provided immense benefits to mankind by controlling pests, significantly enhancing food production, and improving health. However, their massive and indiscriminate use in crop protection, food preservation, and insect pest control has led to acute or chronic poisoning incidents in human, domestic animals and wildlife and resulted in widespread ecological adverse effects. Various survey indicated that today herbicides and insecticides (organophosphates [OPs] and carbamates [CMs]) are commonly used in agriculture and industry and around homes/gardens throughout the world. Small animals often encounter poisoning with these insecticides via malicious activity or accidental exposure, while livestock may ingest freshly sprayed crop or contaminated feed. Among other pesticides, rodenticide (warfarin), and fumigant (aluminum phosphide) are the common causes of poisoning malicious/suicide poisonings because of their easy availability (Gupta 2019).

Fungicides are canopy management tools in agricultural practices, as the fungal pathogens are the number one cause of crop loss around the world and can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit in both plants and animals (Poole and Arnaudin 2014). Fungicides have been used widely to control fungal diseases and to increase crop production. The most important class of fungicides for controlling the fungi of agricultural crops is known to be ethylene-bis-dithiocarbamate (EBDC) (Sakr *et al* 2007). They are characterized by a broad spectrum of activity against various plant pathogens, low acute mammalian toxicity and low production costs. In combination with modern systemic fungicides, they are also used to manage resistance and to broaden the spectrum of activity.

2.1 Mancozeb

Mancozeb (MZ), is a polymeric complex of 20 % manganese and 2-5% Zinc salt, which is a contact fungicide in the subclass of carbamate pesticides called dithiocarbamates (Negga *et al* 2012). Mancozeb, manganese ethylene-bis-

dithiocarbamate with zinc salts, is one of the most commonly used fungicides. Increasing evidence has shown a strong association between pesticides and neurodegenerative diseases. It has been shown that mancozeb (MZ) and maneb (MB), both widely used EBDC fungicides, are equipotent neurotoxicants that produce cell loss in mesencephalic dopaminergic and GABAergic cells (Domico *et al* 2006).

2.2 Physical and chemical properties

Mancozeb [[1,2-ethanediylbis]carbamodithioate]](2-)]manganese mixture with [[1,2-ethanediylbis[carbamodithioate]](2-) zinc belongs to the di-thiocarbamate group of fungicides and to be more specific to the class of compounds known as ethylene bis-dithiocarbamates (EBDCs). Mancozeb itself is not fungicidal but can be considered a pro-fungicide which, when exposed to water, breaks down to release ethylene bis-isothiocyanate sulfide (EBIS), which then is converted by the action of UV light into ethylene bisisothiocyanate (EBI). Both EBIS and EBI are considered to be the active toxicants and the fatal disruption of core enzymatic processes inhibit or interfere with at least six different biochemical processes within the fungal cells (Gullino *et al* 2010).

Trade names: Aazimag, Manzate 200, Dithane M-45, Manco-75; Molecular weight: (265.3) + (65.4); Empirical Formula: $(C_4H_6MnN_2S_4)_x$ $(Zn)_y$; Melting Point: 192 – 194^oC; Solubility: Water-6mg/L at 25^oC (Heller and Herner,1990;Kidd and James, 1991); Organic Solvents at 25^oC (R & H company ,1987a), Ethanol : <5mg/L, Dimethylformamide: < 5mg/L.

2.3 Mechanism of action

Mancozeb itself does not act as a fungicide, rather it is effectively considered as a pro-fungicide. When it gets exposed to water, it is converted into ethylene bis-isothiocyanate sulfide (EBIS) and ethylene bisisothiocyanate (EBI), which are active toxicants (Saha *et al* 2016). Mancozeb interferes with enzymes containing sulphydryl groups and disrupts many core enzymatic processes of the fungal cell cytoplasm and mitochondria (Ludwig and Thorn 1960, Sijpestein 1982). Mancozeb, like other carbamates, may attack the nervous system. It inhibits the function of neurotransmitter acetylcholinesterase (AChE) in the central nervous system of insects by its primary metabolites,ethylenethiourea (ETU) and carbon disulphide (CS_2). Ethylenethiourea, inhibits the fungal-spore germination (Szkolnik

1981; Wicks and Lee, 1982; Wong and Wilcox 2001) and is also responsible for thyroid dysfunction and carcinogenic effects in various organisms (Srivastava and Singh 2013). Fungicide exposure is associated with inhibition of mitochondrial respiration, mitochondrial uncoupling and neuronal damage. It has been reported that reactive oxygen species (ROS) produced neuronal damage with mancozeb exposure (Domico *et al* 2007). The production of ROS may occur due to the chemical structure of mancozeb containing coordinated transitional metals which are known to catalyse the formation of ROS through the Fenton reaction (Calviello *et al* 2006). It has been shown that increased free radical production results into inhibition of mitochondrial respiration. This indicates involvement of oxidative stress due to generation of ROS leading to genotoxicity which, in turn, induces apoptosis (Srivastava *et al* 2012).

2.4 Toxicity profile

In several acute oral toxicity studies with rats, the LD_{50} value of mancozeb ranged from 13,030 – 17,250 mg/kg (Kacker *et al* 1997) and for dermal exposure, the LD_{50} value ranges from 4500-11200mg/kg for rats and 5000-15000mg/kg for rabbits (Cecconi *et al* 2007).

2.5 Toxicodynamics and Toxicokinetics

The rate of breakdown of mancozeb into EBIS and EBI can directly affect the residual activity of the compound on plant foliage. Each mancozeb particle consists of a zinc-rich shell surrounding a central nucleus of polymer-structured EBDC. This structure is extremely stable and the low solubility of the zinc shell means EBDC can pass through this layer and be deposited on the leaf surface at a controlled rate. This controlled release helps to regulate the duration of fungicidal efficacy and improves foliar residuality (Gullino *et al* 2010). The toxicological manifestation of dithiocarbamates (DTC) can occur due to their absorption through skin exposure, ingestion and inhalation. Mancozeb after being absorbed into the body from the gastrointestinal tract, gets distributed to various target organs and almost completely gets excreted in 96 hours. The half-life of mancozeb is about 1-7 days, but the half-life of its primary metabolite (ethylenethiourea) is 5-10 weeks. The metabolite of mancozeb, ETU has the potential to contaminate the ground water (Srivastava and Singh 2013).

Dithiocarbamates undergo rapid biotransformation and excretions in humans usually within hours or days following absorption. The lipophilic nature of DTC makes them suitable for their passage across the cell membrane. The absorption of ethylenebisdithiocarbamate from the GIT can be altered by the presence of cations occurring in the food. The majority of studies have focused on adverse health effects of ethylenethiourea, the main metabolite of mancozeb. This compound has been reported to have carcinogenic, teratogenic and goitrogenic effects (Chhabra *et al* 1992).

The metal moiety of ethylenebisdithiocarbamate is eliminated in the metabolic process. Metabolic studies have shown that DTCs (like thiram, disulfiram, mancozeb) undergo detoxification through glucorinidation to different metabolites such as carbon disulphide (CS_2). CS_2 undergoes further metabolism to form thiourea, alkylamines, ethyleneamines and several biotransformation products (Edwards *et al* 1991: US EPA 2001). ETU, the major metabolite, can be detected in urine, thyroid and liver but rarely in blood.

2.6 Toxicological studies

2.6.1 Alterations in haematological, biochemical and histopathological parameters

Yahia *et al* (2015) conducted a study to evaluate the effects of mancozeb fungicide on haematological and biochemical parameters related to function of kidney and liver. Male wistar rats were treated with different doses of mancozeb orally: 500 and 1000 mg/body weight/day for 8 weeks. The results showed an increase in the absolute weight of liver, spleen and kidneys mainly in the group exposed to 1000 mg/ kg. Hematological observations revealed a decrease in red blood cells, haemoglobin and haematocrit values and increase in white blood cells in mancozeb-exposed rats. The biochemical findings showed increase in liver transaminases (AST/ALT), alkaline phosphatase and total serum bilirubin, whereas total protein levels and total lipids decreased significantly in rats treated with mancozeb (1000 mg/kg). Urea and creatinine levels showed significant elevation. From these results, it was concluded that chronic mancozeb exposure produced biochemical and haematological alterations in parameters related to the liver and kidney function.

Adjrah *et al* (2013) studied the potential effect of mancozeb-treated lettuce on the rat liver physiology. Mancozeb-treated lettuce at two doses were administered for

28 days to rats. Plasma concentrations of transaminases, alkaline phosphatase and total bilirubin were increased in rats administered with mancozeb-treated lettuce. The plasmatic concentration of total protein did not decrease significantly.

Zakharov *et al* (2016) reported a case of a 48year old man having 75 kg bwt admitted with symptoms of fever and generalized skin or mucous lesions after fungicide application. There was necrotic desquamation of mucosa of respiratory or gastrointestinal tract, ocular lesion and skin epidermolysis of 90% of body surface. Elevation of inflammatory parameters, hyper-glycaemia, increased urea, creatinine, liver enzymes, hypoalbuminemia and electrolyte disturbances were observed in the laboratory investigation.

Mancozeb when administered orally by gavage at doses of 250 and 500 mg/kg body weight/ day to male albino rats for 30 days, induced various haematological and clinical biochemistry changes. There was a significant decrease in total erythrocyte count (TEC), total leukocyte count (TLC), and significant elevation in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). In addition, results showed some alterations in the biochemical parameters such as elevation in plasma aspartate aminotransferase (AST), alkaline phosphatase (AKP), acetylcholinesterase (AChE) activity, albumin, total lipid (TL), total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL) levels, while a decrease in blood urea nitrogen (BUN), creatinine and triglyceride (TG) levels. There was an increase in the weight of liver, kidney, brain and testes but weight of lungs and heart of intoxicated rats were decreased (Ahmed and Gamila 2017).

The study related to the evaluation of the subacute toxicity of a pesticide Rivanebe® (the non-systemic dithiocarbamate fungicide), widely used in agriculture in the Batna region (Algeria), on the parameters related to liver and thyroid functions in domestic rabbits. The experimental study was performed on 12 mature male rabbits of the genus *Cuniculus lepus,* which were divided into 2 groups (n = 6): a control group (G1) and a treated group (G2) with Rivanebe®80 orally for 15 days. Analysis of the results obtained has shown that treatment with Rivanebe®80 causes harmful effects on the organism, resulting in a significant increase in body mass and consumption of food by treated animals, compared to the control group. In addition, a hepatotoxic effect by a significant increase in the mass of liver and biochemical

parameters was reported including hyperglycemia, hypertriglyceridemia, hypercholesterolemia and an increase in bilirubin and transaminases (Assia *et al* 2020).

Jacobsen *et al* (2004) investigated the effect of pesticide exposure in six groups of male and female rats administered with different doses like 0, 0.15, 0.006, 0.03, 0.15 or 0.3 mg/kg bw/day chlorpyrifos (groups 1–6) and additional daily doses of 18 mg/kg bw/day of alphacypermethrin (group 3), 30 mg/kg bw/day bromopropylate (group 4), 45 mg/kg bw/day carbendazim (group 5) and 12.5 mg/kg bw/day of mancozeb (group 6) were administered for 28 days. In group 2, 5 and 6 males, plasma acetylcholinesterase was decreased significantly. White blood cell count reduced in females of group 6 while red blood cell count, haematocrit and haemoglobin concentration was decreased significantly in both male and female rats of groups 5 and 6. Alteration in relative liver weight, absolute and relative thyroid gland weight and absolute thymus weight were observed. In liver of group 6 animals, mild centrilobular cell hypertrophy was noticed.

A study was conducted to investigate mancozeb toxicity and its effects on physiological characteristics of common carp. Fish were reared for one week to acclimatize with the experimental conditions. For 96h-LC_{50} determination, the fish were stocked in 16 aquaria at the density of 10 fish per aquarium. The aquaria were exposed to 8 mancozeb concentrations (two aquaria per concentration) for 96 h (0, 0.94, 1.87, 3.75, 7.50, 15, 30 and 60 mg/L). The 96 h-LC_{50} was calculated based on the fish mortality, being 8.03 (4.95-13.2) mg/L. For sub-acute test, the fish were exposed to 0 (control), 1.6, 2.4 and 3.2 mg/L mancozeb (20, 30 and 40% of the 96h-LC_{50}) for one week. Blood samples were taken from each treatment for determination of plasma glucose, total protein, albumin, globulin, calcium, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Mancozeb exposure led to increase in glucose and AST, and decrease in plasma proteins and ALT. Mancozeb exposure causes stress response, health problem and tissue damage in common carp (Simakani et al 2018).

The histo-chemical analysis of the lipid and the biochemical analysis of cholesterol content induced by mancozeb on Swiss albino mice was studied. In this investigation, three groups of mice were taken, where Group I was served as control

and Group II and III were administered with mancozeb at a dose of 4.2 mg/kg BW and 6.7 mg/kg BW respectively for 4 weeks. The histo-chemical analysis of lipid was performed using Sudan Black III stain in liver and kidney tissues, which showed normal lipid content in the control, while increase in the lipid content of mancozeb-treated animals. The biochemical analysis of the total serum cholesterol level also showed increase in the treated groups as compared to the control group. Therefore, the study indicated marked alteration in the lipid content in mancozeb-exposed albino mice (Razia 2015).

Cypermethrin and mancozeb were evaluated for their possible effects on haematopoiesis *in vitro*. Hematopoietic stem or progenitor cells from human cord blood were isolated and then exposed for 14 days to cypermethrin or mancozeb at non-cytotoxic doses (0.9–16µM) and the effect on haematopoiesis was screened via a methylcellulose-based clonogenic assay. Results indicated significant concentration-related decreases in clonogenic potentials of erythroid and granulocyte-macrophage colony formation. The inhibitory concentration (IC_{50}) value with erythroid progenitors for cypermethrin was 8.7 [±0.2µM; mean [± SE]) and for mancozeb 6.2 [±0.2] µM. Similarly, IC_{50} values with granulocyte-macrophage progenitors for cypermethrin and mancozeb were 19.2 [±1.0] and 8.1 [±0.2] µM, respectively. These data suggested that erythroid progenitors are more sensitive to these pesticides (Mandarapu and Prakhya 2015).

2.6.2 Alterations in endocrine parameters

Boberg *et al* (2011) investigated the effects of mancozeb exposure on thyroid hormone levels, reproductive and neurological development of the offspring. They observed that mancozeb exposure induced neurotoxicity in dams at above 150 mg/kg bw/day. The observed changes in maternal T_4 levels did not cause any behavioral effects in the offspring. However, 8 days of mancozeb exposure caused 20 to 40% decreases in T_4 levels in rat dams, which indicates that mancozeb exposure may be a potential contributor to thyroid disruption in human beings.

Kwon *et al* (2018) evaluated mancozeb-induced toxicity on the liver, thyroid and testis of male rats. Mancozeb was orally administered at 800 mg/kg body weight/day for 4 weeks. Histological studies of the testis of male rats treated with mancozeb revealed spermatogenesis inhibition, which was reflected by significant

decreases in the number of spermatogonia, primary spermatocyte, spermatid and sperm. Chronic exposure to mancozeb induced gonadal hormone disturbance. Concentrations of estradiol, progesterone and testosterone in the serum were significantly decreased in mancozeb-treated rats. The volume and histopathology of the thyroid were also distinctly altered. Serum levels of T_3 and T_4 were markedly decreased in the mancozeb diet group. However, liver toxicity was not observed upon chronic mancozeb exposure.

Bano *et al* (2020) tested the anticipation of disruption of immune system in mice on exposure to thyroid disrupting pesticides (TDPs) like mancozeb (MCZ) and fipronil (FPN) during early postnatal days (PND) development aimed to study the cross regulation between neuroendocrine system and immune system during developmental of embryonic/early neonatal stages. They exposed mice to MCZ and FPN separately (0.5% LD_{50} each) and along with mixtures (0.25% and 0.5% LD_{50} each) from PND 31 (initiation phase) till PND 60 (maturation phase) of immune response. From PND 51 to PND 60, thyroxine (T_4) supplementation was given, while assessment was done at PND 61 as well as at PND 91 (adults). Immune-toxicity was exerted by individual pesticide exposure while noticeable immunosuppression was produced by mixture of pesticide exposure. Pesticides mixture produced immunosuppression by synergistic toxicity along with hypothyroidism. The thyroid disruption mediated immune-suppression was hampered by T_4 supplementation.

A study was done by Axelstad *et al* (2011) aimed to assess whether perinatal exposure of mancozeb would cause developmental neurotoxicity in rats since fungicide mancozeb has been known to cause hypothyroxinemia and some other adverse effect on hormonal system of thyroid gland. In human being, hypothyroxinemia in early pregnancy can lead to cognitive function and motor development impairement and adverse effect on nervous system development. In this study, wistar rats were administered with doses 0, 50, 100, or 150 mg mancozeb/kg body wt./day orally by gavage from gestation day (GD) 7 to postnatal day (PND) 16, and during gestation total thyroxine (T_4) levels were measured. On PND 16, in offspring, the organ weight and hormonal levels, while in adult offspring, motor activity, startle response and cognitive function were investigated. In pregnant dams, mancozeb dose of 150 mg/kg/day caused neurotoxicity and was therefore reduced to

dose 100 mg /kg /day in mid study. There was dose-dependent and significant decrease levels of T_4 seen in dams treated with mancozeb on GD 15, whereas T_4 levels, thyroid weight, and histology of offspring was unaffected on PND 16. No effect on behavior and reproductive organ weights were observed. These results together, indicated that during gestation, the moderate maternal hypothyroxinemia did not necessarily led to hyperactivity or reduced special learning abilities in the offspring. Mancozeb exposure reduced T_4 levels in dams which may have led to potential contribution to thyroid disruption and adversely affected the developing brain in human.

2.6.3 Neurotoxicity

In an *in vitro* study, the effect of widely used EBDC fungicides mancozeb (MZ) and maneb (MB) were evaluated, which are equipotent neurotoxicants that produce cell loss in mesencephalic dopaminergic and GABAergic cells after an acute 24 h exposure. Fungicide exposure were associated with inhibition of mitochondrial respiration, mitochondrial uncoupling and neuronal damage. It has been shown that increased free radical production resulted into inhibition of mitochondrial respiration. It was further explored that reactive oxygen species (ROS) produced neuronal damage with mancozeb exposure. Monitoring of ROS production was done by production of hydrogen peroxide (H_2O_2) using Amplex Red which increased in a dose-dependent manner in response to mancozeb (Domico *et al* 2007).

The behavioral damage and differential neuronal vulnerability was studied after mancozeb exposure using *Caenorhabditis elegans*, an important mid-trophic level soil organism that is also a powerful model for studying mechanisms of environmental pollutant-induced neurodegenerative disease. Higher concentrations of mancozeb revealed behavioral dysfunction, notably in the serotonin-mediated egg-laying behavior, but without showing evident serotonergic neuronal structural damage. Thus, measurements of behavioral dysfunction can be a sensitive early marker of fungicide toxicity (Brody *et al* 2013).

Mancozeb (manganese/zinc ethylene bis-dithiocarbamate) is an organometallic fungicide that has been associated with human neurotoxicity and neurodegeneration. Mancozeb, has been found to significantly potentiate $KCNQ_2$ activity. Mancozeb was validated electrophysiologically as a $KCNQ_2$ activator with

an EC_{50} value of 0.92 ± 0.23 μM. Further examination showed that manganese but not zinc ethylene bis-dithiocarbamate is the active component for the positive modulation effects. In addition, the compounds are effective when the metal ions are substituted by iron but lack potentiation activity when the metal ions are substituted by sodium, signifying the importance of the metal ions. However, the iron (Fe^{3+}) alone, organic ligands alone or the mixture of iron with the organic ligand did not show any potentiation effect, suggesting that the active ingredient is a specific complex rather than two separate additive or synergistic components. Thus, the study suggested that potentiation on $KCNQ_2$ potassium channels might be the possible mechanism of mancozeb toxicity in the nervous system (Li *et al* 2013).

A study was conducted to evaluate the acute toxicity of mancozeb in freshwater fish, *Oreochromis mossambicus* (Tilapia). The LC_{50} values of mancozeb to *O. mossambicus* were 14.40, 13.40, 12.34 and 11.68 mg/l at 24, 48, 72 and 96 h respectively. The mortality rate of the treated fish exposed to mancozeb significantly varied over the control at all the concentrations at all the exposure times. Simultaneously mortality rate of the fish also significantly varied at different times of exposure (24, 48 and 72 and 96 h) at different doses. The fish showed excessive mucous secretion and hyper-excitability at the higher concentrations at 24 and 48 h of exposure. The opercular movement of the fish increased significantly compared to the control group with increasing concentrations but it decreased significantly with progress of time at all the treatments (Saha *et al* 2016).

2.6.4 Reproductive toxicity

In a reproductive toxicity study, mancozeb fungicide was administered orally to male wistar rats at a dose 500 mg/kg body wt/day for 30 days. The parameters like sex organ weight analysis, fertility, biochemical enzyme levels and testosterone levels were evaluated. A marked decrease in weight of testis, epididymis, seminal vesicle and prostate were observed. There was significant reduction in sperm count and fertility in exposed rats. A notable increase in the protein and cholesterol content of testis along with decrease in sialic acid and testicular glycogen were noticed. In addition, a significant increase in acid phosphatase enzyme activity whereas, a sharp decline in alkaline phosphatase activity were evaluated, which showed that mancozeb exerts toxic effect on testis and diminishes testosterone level (Joshi *et al* 2005).

Paro *et al* (2012) studied the reproductive toxicity of mancozeb on human and mouse ovarian somatic granulosa cells where parameters like morphology, induction of apoptosis and p53 expression levels were examined. The expression of p53 gene, in terms of mRNA and protein content, decreased significantly along with time and dose-dependent modification in their morphology was observed when compared to unexposed cells. The toxic effect of mancozeb could be attributed due to the metabolite called ethylene thiourea (ETU).

Kackar *et al* (1997) evaluated marked biochemical and pathological alteration in male rat gonads after chronic mancozeb exposure orally administered at doses of 500, 1000 and 1500mg/kg body weight/day for 30, 90, 180 and 360 days. Reduction in body weight and sign of toxicity mortality pattern were observed in a dose-dependent manner. A notable increase in weight of testes and decrease in epididymis weight were related with degeneration in seminiferous and epididymal tubules with sperm loss. The decrease in activity of gonadal acid phosphatase (ACP) and succinic dehydrogenase (SDH) along with increase in alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were noticed with increased serum cholesterol. Dose-dependent reduction in sialic acid and protein content of testis and epididymis were observed.

In another study, mancozeb was administered at different doses of 500, 600, 700 and 800 mg/kg body weight /day orally to wistar rats for 30 consecutive days and body weight and vaginal smear were recorded daily. The rats were sacrificed on 31st day. In mancozeb-treated groups, there was significant reduction in number of estrous cycle, with concomitant increase in diestrus duration compared to control group. Significant decrease in the number of healthy follicles and a significant increase in the atretic follicles were recorded. Reduction in number of corpora lutea and decrease in size of ovary was recorded in high doses. In all mancozeb-treated rats, significant thyroid weight increment was observed except in 500 mg/kg/ day group. Mancozeb treatment at dose 500 mg/kg/day showed increase in total level of lipids in liver whereas, remarkable reduction in glycogen levels and total lipids in the uterus and liver was shown in 600 mg/kg/day mancozeb doses. Rats treated with 700 mg/kg/day mancozeb, showed a notable reduction in ovary protein levels, neutral lipids, total lipids and phospholipids in uterus with increase in phospholipids and neutral lipids

level in ovary and total lipids, phospholipids and neutral lipids in liver. A remarkable decrease in protein and glycogen level of ovary and decrease in protein, glycogen, total lipids, neutral lipids and phospholipids in uterus was seen along with increase in total lipids, phospholipids and neutral lipids in the ovary and liver in 800 mg/kg/day mancozeb-treated group. The recorded effect of mancozeb on estrous cycle, follicle and biochemical component may be due to toxic effect or hormonal imbalance (Baligar *et al* 2001).

Cecconi *et al* (2007) studied that mancozeb when used *in vivo* experiments is known to induce several disturbances on estrus cycle. However, when used *in vitro* at low concentrations, it caused human health injuries, where, the fungicide impaired mouse embryo development and meiotic spindle assembly. The female germ cell (oocyte) is considered to be the specific target of mancozeb, which suggested that the fungicide is a probable inductor of infertility in exposed human populations.

Mancozeb, was orally administered at doses of 200, 400, 600 and 800 mg/kg/ day to male swiss albino mice for 30 days. Daily body weights of the mice were recorded and the mice were sacrificed on the 31st day. Testes weight decreased significantly in all the mancozeb treated mice except 200 mg/kg/ day treated mice. Mice treated with 600 and 800 mg/kg/ day showed significant decrease in the number and diameter of spermatogenic cells and Leydig cells. However, treatment with 600 mg/kg/ day revealed no significant change in the diameter of spermatogonia and primary spermatocytes. Histological studies of the testis of the mice treated with high doses of mancozeb revealed spermatogenesis inhibition reflected by significant decrease in number of spermatogenic cells and sperms. The thyroid effect of mancozeb was revealed by the number of small follicles decrease, whereas the number of medium and large follicles increased significantly with hypertrophy and hyperplasia of follicular cells with loss of colloid in mice treated with high doses of mancozeb when compared with corresponding parameters of control. Mice treated with 800 mg/kg/ day mancozeb showed significant decrease in the weight of prostate and Cowper's glands. Mice treated with 600 and 800 mg/kg/ day mancozeb showed significant decrease in the kidney, spleen and liver weight, whereas thyroid weight increased significantly. However, thymus weight increased significantly in the mice treated with 600 and 800mg/kg/ day mancozeb. Mice treated with 200 and 400 mg/kg/

day mancozeb caused no significant change in the biochemical constituents in testes, liver and kidney. However, treatment with 600 mg/kg/ day mancozeb caused significant decrease in the level of protein in liver and glycogen in the liver and kidney and a significant increase in the level of total lipids in testis. Treatment with 800 mg/kg/ day mancozeb caused significant decrease in the levels of protein and glycogen and significant increase in the level of total lipids in the testis and liver and a significant decrease in the protein, glycogen and total lipids in the kidney. These observed effects of mancozeb on testis, thyroid and biochemical constituents were suggested to be due to hormonal imbalance in any of the stages in the hypothalamo-hypophysial-testicular axis or hypothalamo-hypophysial-thyroid axis (Ksheerasagar and Kaliwal 2010).

Mancozeb (MNZ) impact on oocyte maturation of first-generation mice pups as well as their fertilization rate, embryo development, and implantation along with the preventative effect of vitamins E and C were evaluated. Pregnant mice were randomly divided into six groups: control, vehicle, and MNZ (500 mg/kg body weight (BW)), vitamin E (200 mg/kg BW), MNZ plus vitamin E, MNZ plus vitamin C (100 mg/kg BW) and MNZ plus the two vitamins. All treatments were conducted by oral gavage every 2 days from the second day of gestation until the end of lactation. Vitamin treatment was initiated 30 min before receiving MNZ. After birth, first-generation mice pups were kept until adulthood (8–10 W). Adult female mice pups superovulated and then the collected oocytes were examined for nuclear maturity status. After *in vitro* fertilization of metaphase II oocytes with sperm of the first-generation male mice pups, fertilization rate and embryo development were evaluated over 24 h. Also, the fecundity rate and the number of implanted embryos *in vivo* were studied on the eighth day of pregnancy. MNZ exposure during embryo development and lactation significantly decreased the total number of collected oocytes, oocyte maturation, fertilization rate, implantation rate, fecundity rate and embryo development compared with the control group in the first-generation pups. In contrast, vitamin treatments significantly increased these parameters compared to the MNZ group. Reduction in the quality of oocyte, the rate of fertilization, embryo implantation and development following MNZ exposure decreased female reproductive success, while coadministration of vitamins E and C prevented these complications (Esmaiel *et al* 2019).

Results from *in vitro* studies provide evidence that mancozeb may indirectly disrupt or impair reproduction at the cellular level and should be regarded as a reproductive toxicant. Animal studies suggested that males chronically exposed to mancozeb experience significant changes in physiological, biochemical, and pathological processes that may lead to infertility (Runkle *et al* 2017).

2.6.5 Effect on oxidative stress parameters

The particular study, investigated the effects of 96 h of exposure to 3, 5, or 10 mg L^{-1} of Tattoo (corresponding to 0.9, 1.5, or 3 mg L^{-1} of Mancozeb) on the levels of oxidative stress markers and the antioxidant enzyme system of brain, liver and kidney of goldfish (*Carassius auratus*). In liver, Tattoo exposure resulted in increased activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). The content of protein carbonyls (CP) in liver was also enhanced, indicating extensive damage. Similar increases in CP levels accompanied by reduced glucose-6-phosphate dehydrogenase activity was observed in kidney of fish exposed to Tattoo; however, SOD activity increased in the tissue after treatment with 10 mg L^{-1} Tattoo. In brain, a rise in lipid peroxide level was observed after exposure to 10 mg L^{-1} Tattoo and was accompanied by elevation of high molecular mass thiols. Tattoo exposure also resulted in a concentration-dependent decrease in glutathione reductase activity in brain. The data collectively showed that exposure of goldfish to 3–10 mg L^{-1} of the carbamate fungicide, Tattoo, resulted in the development of mild oxidative stress and activation of antioxidant defense systems in goldfish tissue (Atamaniuk *et al* 2014).

Mancozeb is a manganese/zinc ethylene-bis-dithiocarbamate fungicide that is widely used in agriculture to control a broad variety of fungal infections of both vegetables and ornamental plants. The study was carried out to investigate the possible effect of mancozeb on oxidative stress and some of the biochemical markers in male Wistar rats. In this experiment, adult male rats weighing between 200 and 250 g were treated orally for 4 weeks with two different doses of 800 and 1200 mg/kg per day. Reduced glutathione (GSH) levels were observed in all treated groups compared to control ones. A significant increase in the fresh weight of liver in individuals of both doses. Moreover, mancozeb exposure caused a significant fall in aspartic aminotransferase (AST) and alanine aminotransferase (ALT) in groups treated with

1200 and 800 mg/kg/day. Similarly, alkaline phosphatase (ALP) activity underwent a significant increase in both groups. The obtained observations clearly revealed hepatotoxic effects of mancozeb in rats and therefore constitute an environmental health risks to living organisms (Yahia *et al* 2014).

2.6.6 Genotoxic and carcinogenic effect

A study was designed to evaluate genotoxic markers of mancozeb exposure and withdrawal in colon and liver tissues together with histological changes in the gastrointestinal tract of Sprague Dawley rats. Thirty rats were divided into three equal groups; group I: treatment, 250 mg/kg mancozeb dissolved in corn oil administered twice weekly for 7 weeks; group II: withdrawal, the same treatment as group I after which animals were untreated for 5 weeks; group III: control, administered corn oil on the same schedule as group I for 7 weeks. All administrations were by oral gavage. Serum samples were analyzed for biochemical parameters. The comet assay and histopathological examinations were done on liver and colon specimens. The results demonstrated that mancozeb exposure caused significant increase in triglycerides and total cholesterol accompanied by decrease in glucose levels, with extensive DNA damage in liver and colon together with pathological changes in stomach, colon and liver. Mancozeb withdrawal for 5 weeks improved the lipid and glucose profiles and decreased the degree of DNA damage and changes in the architecture of the stomach, colon, and liver. It was concluded that discontinuing exposure to mancozeb fungicide for 5 weeks could ameliorate the adverse effects induced by 7 weeks of exposure to mancozeb. A longer withdrawal time may further reduce the observed genotoxicity (Yahia *et al* 2019).

Mancozeb is reported to possess carcinogenic and co-carcinogenic activity in various tumor models. Shukla (2001) studied the transplacental carcinogenesis effect of mancozeb in swiss albino mice. In this study, pregnant swiss albino mice were treated with mancozeb intraperitoneally on the 14th day of gestation. The first filial generation (F_1 progeny) was promoted with a well-known tumor promoter 12-*o*-tetradecanoyl phorbol-13-acetate (TPA). The results showed, a significantly high tumor incidence in the F_1 progeny of the animals treated with mancozeb or a well known, carcinogen 7, 12-dimethyl benzanthracene (DMBA) and promoted with TPA, compared to animals that were either from mothers given only the vehicle dimethyl

sulfoxide (DMSO) and promoted with TPA in F_1 progeny or not promoted with TPA in F_1 progeny. Therefore, the results suggested that mancozeb or its metabolites are capable of crossing the placental barrier and can exert DNA damage and cause tumor induction in the fetal cells, after promotion with TPA, and get converted into neoplastic cells.

2.6.7 Effect on muscles

A study was done by Stephenson and Trombetta 2020) to investigate the effect of Dithiocarbamates (DTCs) like mancozeb (MZ) and disulfiram (DS) on striated muscle tissues. Long-Evans rats were treated with either MZ or DS to analyze the muscle biomarkers and metal levels. DS resulted in increased serum lactate dehydrogenase (LDH), cardiac troponin, and myoglobin levels along with decrease in creatine kinase-MB levels. Mancozeb showed an increase in serum LDH only. Both treatments resulted into alteration of metal levels in the myocardium but not in the skeletal muscle. Structural alterations were observed in mitochondria, myofibril and intercalated disc.

CHAPTER – III

MATERIALS AND METHODS

The present study was designed to evaluate Mancozeb-induced sub-acute oral toxicity in male Wistar rats. The research work was conducted in the Division of veterinary Pharmacology and Toxicology, GADVASU, Ludhiana, Punjab (India).

3.1 Experimental animals

A total of 12 male Wistar rats weighing about 120- 150 gm were procured from National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab (India). The animals were caged in the polyacrylic cages in the departmental animal house at a temperature of around 27-30^{0}C along with 12 h of light and 12 h of dark cycle. The animals were provided with ad-libitum rat feed pellets (Ashirwad industries, Mohali, Punjab) and water during the whole experimentation period. The protocol used in the study was approved by Institutional Animal Ethics Committee (IAEC) of College of Veterinary Science, GADVASU, Ludhiana, India (Approval no. GADVASU/2019/IAEC/50/03; dated: 23.07.2019). All experiments have been carried out in accordance with the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

The animals were allowed to get accustomed to the environment for 15 days under ambient conditions before starting the experiment. All animals were weighed after the adaptation period for dose calculation at the start of experiment and from there on weekly basis during experimentation.

3.2 Chemicals and reagents

Mancozeb **(**a manganese/zinc ethylene-bis-dithiocarbamate) fungicide was commercially obtained from the local market, from Ludhiana as 75% WP form with brand name Manco-75. Autopak kits for biochemical parameters and hormonal assay were purchased from ERBA (Transasia bio-medicals Ltd., india in technical collaboration with ERBA diagnostics Mannheim GmbH Mallaustr, Germany). All other chemicals/reagents used for the study were of analytical grade, purchased from reputed companies.

3.3 Experimental design

The investigation was conducted to accomplish the following objectives:

1. To evaluate the toxic manifestations of subacute oral mancozeb toxicosis.

2. To study the endocrine and neurobehavioral effects of subacute oral mancozeb exposure.

3.4 Treatments

Twelve healthy rats (approx. 150-200 grams) were randomly divided into two groups of 6 animals each. Group I served as control and Group II animals were administered mancozeb orally @ 500 mg/kg BW. The drug, mancozeb at a suitable dose was administered orally using gavage needle, for 28 consecutive days according to body weight (Table 1)

Table 1: Experimental design for in vivo sub-acute toxicity study in male wistar rats

Specifications	Control	Treatment
No. of animals	6	6
Mancozeb	-	500 mg/kg BW
Duration	28 days	28 days
Route	-	Oral

3.5 Methodology and observations:

Based on the EPA datasets, the oral acute toxicity dose i.e. LD_{50} value of mancozeb is >5000 mg/kg. Considering the fact and to investigate the induced toxicity in our study, a $1/10^{th}$ level of the LD_{50} i.e, 500 mg/kg/day was selected for group II. Another group viz: group I was kept as healthy control in which no drug was administered. The requisite amount of fungicide was dissolved in 10 ml distilled water and simultaneously drenched to the rats daily basis between 9.00 am to 11.00 am for 28 consecutive days. Blood samples were collected on day 0 and 29^{th} day viz one day before and after experimentation for the analysis of various parameters. EDTA containing blood was used for haematology and heparinized blood for the estimation of various biochemical parameters. Animals were then humanely sacrificed followed by tissue samples collection at the end of experimentation.

3.6 Collection and processing of samples

3.6.1 Blood collection

Blood samples were collected on the 0 day from the retro-orbital sinus of animals before starting the experiment and from the heart of animals on the day of

sacrifice in EDTA and heparinised vials for haematological and biochemical parameters respectively.

3.6.2 Plasma collection

Heparinized blood samples were centrifuged at 3500 rpm for 15 min and plasma was collected from the top most layer. The extracted plasma samples were then stored at -20ºC till further analysis.

3.6.3 Tissue collection

After the termination of treatment period i.e. on 29th day of the experiment, all the rats were sacrificed Viscera samples including liver, kidneys, brain, lungs, heart and testes were collected and smeared with tissue paper, then weighed. Liver, brain, kidney and testes were collected in 10% (w/v) Formalin Buffer Saline for histopathology. Samples of brain were weighed and 10% tissue homogenate was prepared by tissue homogenizer (IKA® T25 digital ultra Turrax®) in ice-cold PBS constituting 8gm/l NaCl, 0.20gm/l KCl, 1.44 gm/l $Na_2HPO_4.2H_2O$ and 0.24gm/l KH_2PO_4. The volume was made upto 1 litre with distilled water. The pH was adjusted to 7.4 using 0.1N NaOH and 0.1N HCl solutions. The homogenates were prepared under cold conditions. The homogenates were centrifuged for 10 min at 3000 rpm at 4 ^{0}C. The supernatant was collected and used for estimation of Acetyl choline esterase (AchE) activity.

3.7 Parameters studied

The following parameters were analyzed at day 0 and day 29th of the experimental study by using suitable technique/equipment.

3.7.1 Toxic Symptoms

All the animals used for the experiment purpose were closely monitored for the appearance of toxic symptoms, if any. The nature, duration and severity of different toxic symptoms and lethality, if any were recorded during the full course of experimental period.

3.7.2 Physiological parameters

3.7.2.1 Body weight and organ weight

Body weight of all rats were recorded one day before the start of experiment for dose calculation using weighing balance and at weekly interval till the completion

of experimentation. The percent weight gain was calculated at the end of experimentation by using the following formula mentioned below.

$$\text{Percent body weight gain} = \frac{\text{Final body weight (g)} - \text{Initial body weight (g)}}{\text{Final body weight (g)}} \times 100$$

At the end of experimentation period i.e, 29th day, all the rats were sacrificed humanely and vital organs including lungs, liver, kidney, brain, testes and epididymis were collected and weighed. The relative organ weight was estimated by using the formula mentioned below:

$$\text{Relative organ weight (per 100 gm b.wt.)} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100$$

3.7.3 Biochemical parameters

Following biochemical parameters were analyzed utilizing the plasma sample separated from heparinized blood by centrifugation at 3500 rpm for 10 minutes in our own lab and then stored at -20°C by using autopak kits procured from ERBA diagnostics Mannheim GmbH, Germany. Before analysis, frozen stored plasma was thawed properly using a series of temperature viz 4°C followed by room temperature (25°C).

3.7.3.1 Aspartate aminotransferase

The level of Aspartate aminotransferase (AST) in plasma was measured using standardized technique of Tietz (1986) international federation of clinical chemistry (IFCC) with ERBA autopak kit on auto analyzer. Briefly, AST catalyzes the transfer of amino group between L-aspartate and α- ketoglutarate to form oxaloacetate and glutamate. The oxaloacetate formed, reacts with NADH in presence of malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD was measured in the form of decrease in absorbance which is directly proportional to the AST activity in the sample. The absorbance was measured at 340 nm auto analyzer. AST levels were expressed as U/L.

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^{+} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}$$

$$\text{Sample pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}$$

Where,

AST = Aspartate aminotransferase

MDH= Malate dehydrogenase

LDH= Lactate dehydrogenase

3.7.3.2 Alanine aminotransferase

Alanine aminotransferase (ALT) level in plasma sample was measured by the pre-described method of Bradley *et al* (1972) international federation of clinical chemistry (IFCC) with ERBA autopak kit on ELISA reader. ALT catalyzes the transfer of amino group between L-alanine and α- ketoglutarate to form pyruvate and glutamate. The pyruvate formed reacts with NADH in presence of lactate dehydrogenase to form NAD. The rate of oxidation of NADH was measured as decrease in absorbance which is proportional to the ALT activity in the sample. The absorbance was measured at 340 nm. ALT level was expressed as U/L.

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}$$

Where,

ALT= Alanine aminotransferase

LDH= Lactate dehydrogenase

3.7.3.3 Lactate dehydrogenase

Lactate dehydrogenase (LDH) estimation in plasma was made by optimized kinetic method of Deutsche Gesselschaft fur KlinischeChemie (DGKC 1972) with ERBA Autopak kit on ELISA reader. Lactate dehydrogenase catalyzes the conversion of pyruvate to lactate and NADH to NAD. LDH activity is directly proportional to the rate of decrease in the absorbance of NADH at 340 nm and expressed as U/L.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^+$$

3.7.3.4 Gamma- glutamyl transpeptidase

Gamma-glutamyl transpeptidase (GGT) acts as catalyst in the the transfer of the gamma-glutamyl group from the substrate gamma-glutamyl para-nitranilide to glycylglycine releasing free p-nitroaniline which absorbs light at 405 nm. Plasma estimation of gamma-glutamyl transpepetidase was done using ERBA Autopak kits by kinetic method of international federation of clinical chemistry described by

Kachmar *et al* (1976). The rate of formation of 5-amino-2-nitrobenzoate is directly proportional to the activity of GGT present in sample and can be measured kinetically at 405 nm.

L-glutamyl-3-carboxy-4-nitroanilide + Glycylglycine

↓

L-glutamylglycylglycine + 5-amono-2-nitrobenzoate

3.7.3.5 Creatinine

Plasma creatine level was estimated by Jaffe's detection method using ERBA Autopak kits on ELISA reader. Succinctly, creatinine reacts with alkaline picrate to produce an orange-yellow color (the Jaffe's reaction). Specificity of the assay had been improved by the introduction of an initial rate method. The absorbance of the orange-yellow color formed is directly proportional to creatinine concentration and is measured at 500-520 nm and expressed as mg/dl.

3.7.3.6 Blood urea nitrogen

Blood urea nitrogen (BUN) estimation was done by GLDH-Urease method as described by Tietz (1976) using ERBA Autopak kits on ELISA reader. Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced combines with α-ketoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD. The rate of decrease in absorbance is monitored at 340 nm and is directly proportional to urea concentration in the sample, expressed as mg/dl.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{-KG} + \text{NADH} \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}$$

Where,

α-KG = α- Ketoglutarate

GLDH= Glutamate dehydrogenase

3.7.3.7 Total plasma protein

Total plasma protein (TPP) estimation was done using ERBA Autopak kits by Biuret End Point method on Auto analyzer. The peptide bonds of protein react with copper II ions in alkaline solution to form blue- violet complex (Biuret reaction), each copper ion complexing with 5 or 6 pepetide bonds. Tartarate is added as a stabilizer

while iodine is used to prevent auto-reduction of the alkaline copper complex. The color formed is proportional to the protein concentration and is measured at 546 nm (520-560 nm), expressed in unit g/dl.

3.7.3.8 Alkaline phosphatase

Plasma alkaline phosphatase (ALP) was estimated by the kinetic method recommended by International Federation of Clinical Chemistry. It utilizes 4-nitrophenyl phosphate as the substrate. Under optimized conditions ALP present in the sample catalyses the following reaction.

$$AMP + 4\text{-}NPP + H_2O \xrightarrow[Mg^{2+}/\text{ Alkaline PH}]{ALP} 4\text{-nitrophenol} + \text{phosphate}$$

At the optimum pH of the reaction, 4-nitrophenol has an intense yellow colour. The reagent also contains a metal ion buffer system to ensure that optimal concentration of zinc and magnesium are maintained. The metal ion buffer can also chelate other potentially inhibitory ions which may be present. The reaction was monitored by measuring the rate of increase in absorbance at 405 or 415 nm which is proportional to the activity of ALP in the plasma and has been expressed as IU/L.

3.7.3.9 Creatine kinase

Creatine kinase (CK) in the plasma was estimated by the method recommended by International Federation of Clinical Chemistry (IFCC). Creatine kinase in the sample catalyses the following reaction. The rate of absorbance change at 340 nm is directly proportional to creatine kinase activity and is expressed as IU/L.

$$\text{Creatine phosphate} + ADP \xrightarrow{\text{Creatine kinase}} \text{Creatine} + ATP$$

$$\text{D-Glucose} + ATP \xrightarrow{\text{Hexokinase}} ADP + \text{Glucose -6-Phosphate}$$

$$\text{Glucocose-6-Phosphate} + NADP^+ \xrightarrow{G6PDH} \text{6-Phospho Gluconate} + NADPH + H^+$$

G6PDH= Glucose -6- Phosphate dehydrogenase

3.7.3.10 Glucose

The blood glucose level was measured using ERBA autopak kits and absorbance was measured by auto analyzer.

3.7.3.11 HDL-Cholesterol

Plasma HDL-cholesterol estimation was done by Phosphotungstic Acid End Point Method described by Burstein *et al* (1970) using ERBA Autopak kits on ELISA reader. In this method Chylomicrons, LDL and VLDL (low and very lowdensity

lipoproteins) are precipitated from plasma by phosphotungstate in the presence of divalent cations such as magnesium. The HDL cholesterol remains unaffected in the supernatant and is estimated using ERBA cholesterol reagent. The absorbance was measured at 505 nm or 505/670 nm and was expressed as mg/dl.

$$\text{Serum / Plasma} \xrightarrow[Mg2^{+}]{\text{Phosphotungstate}} \underset{\text{(supernatant)}}{\text{HDL}} + \underset{\text{(precipitate)}}{\text{(LDL +VLDL + Chylomicrons)}}$$

3.7.3.12 LDL-Cholesterol

Plasma LDL-Cholesterol estimation was done using ERBA Autopak kits on ELISA reader at 600 nm wavelength. The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene–glycol methyl ether (PEGME) coupled classic precipitation method with the improvement in using optimized quantities of PVS/PEGME and selected detergents. LDL, VLDL and chylomicrones (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER), whereas HDL reacts with the enzymes. Addition of R2 containing a specific detergent releases LDL from the PVS/PEGME complex. The released LDL reacts with enzymes to produce H_2O_2 which is quantified by the Trinder reaction.

$$\text{HDL + LDL + VLDL + CM} \xrightarrow{\text{PVS, PEGME}} \text{HDL + (LDL +VLDL+CM).}$$

$$\text{HDL} \xrightarrow{\text{CHOD, CHER}} \text{Fatty acid + } H_2O_2$$

$$\text{(LDL+VLDL+CM). PVS/PEGME} \xrightarrow{\text{Detergent}} \text{LDL+(VLDL+CM). PVS/PEGME}$$

$$\text{LDL} \xrightarrow{\text{CHOD, CHER}} \text{Fatty acid + } H_2O_2$$

$$2H_2O_2 + \text{4-AA+TODB} \xrightarrow{\text{Peroxidase}} \text{Quinone + } 5H_2O$$

3.7.7.13 Triglycerides

Triglycerides in plasma was estimated by GPO-Trinder end point method of Wako and the modifications by McGowan *et al* (1983) and Fossati *et al* (1969) using ERBA Autopak kits.

$$\text{Triglycerides + } H_2O \xrightarrow{\text{LPL}} \text{Glycerol + Free fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow[Mg^{2+}]{\text{GK}} \text{Glycerol-3-Phosphate + ADP}$$

$$\text{Glycerol-3-Phosphate + } O_2 \xrightarrow{\text{GPO}} \text{DAP + } H_2O_2$$

$$H_2O_2 + \text{4AAP +3, 5-DHBS} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye + } 2H_2O$$

Where,

LPL- Lipoprotein Lipase

GK- Glycerol Kinase

GPO- Glycerol Phosphate Oxidase

DAP- Dihydroxyacetone phosphate

ATP- Adenosine triphosphate

4-APP- 4-Aminoantipyrine

DHBS- 3, 5-Dichloro-2-hydroxybenzene sulfonate

The intensity of chromogen (quinoneimine) formed is proportional to the triglycerides concentration in the sample when measured at 505nm (500-540nm)

3.7.4 Haematological studies

Table 2: **Following haematological parameters were analysed in the blood collected in EDTA vials at 0 day and 29 day of experiment.**

Parameters	**Units**	**Reference /Method**
Haemoglobin (Hb)	g/dl	Benjamin (1985)
Packed cell volume (PCV)	%	Benjamin (1985)
Erythrocyte sedimentation rate (ESR)	mm/24 hrs	Benjamin (1985)
Total leucocyte count (TLC)	X 10^3/µl	Benjamin (1985)
Differential leucocyte count (DLC)	%	Benjamin (1985)
Total erythrocyte count (TEC)	X 10^6/µl	Benjamin (1985)
Mean corpuscular volume (MCV)	Fl	Benjamin (1985)
Mean corpuscular haemoglobin (MCH)	Pg/dl	Benjamin (1985)
Mean corpuscular haemoglobin concentration (MCHC)	g/dl	Benjamin (1985)

The erythrocyte indices

a. Mean corpuscular volume (MCV) expresses the average volume of individual erythrocyte and is calculated from the formula:

$$\text{MCV (fl)} = \frac{\text{Packed cell volume (\%)}}{\text{Total erythrocyte count (million /µl)}} \times 10$$

b. Mean corpuscular haemoglobin (MCH) is the amount of haemoglobin in the average erythrocyte and is calculated from the formula:

$$\text{MCH (pg/dl)} = \frac{\text{Haemoglobin (g/dl)}}{\text{Total erythrocyte count (million /µl)}} \times 10$$

c. Mean corpuscular Haemoglobin concentration (MCHC) is the concentration of haemoglobin in the average erythrocyte or ratio of weight of haemoglobin to volume in which it is contained and is calculated from the formula:

$$\text{MCHC (g/dl)} = \frac{\text{Haemoglobin (g/dl)}}{\text{Packed cell volume (\%)}} \times 100$$

3.7.5 Endocrine parameters

Following hormones were analyzed in plasma at day 0 and 29 of experiment by using commercially available kits purchased from ERBA Mannheim (Transasia bio-medicals Ltd., Ringanwada, Daman, India).

1. Thyroid stimulating hormone (TSH)
2. Tri-iodothyronin (T_3)
3. Thyroxin (T_4)

3.7.5.1 Thyroid stimulating hormone (TSH) Assay

ErbaLisa® Thyroid stimulating hormone (TSH) is a solid phase sandwich ELISA method. The samples and anti-TSH-HRP/Biotin conjugate are added to the wells coated with streptavidin. TSH in sample forms a sandwich between two specific antibodies to TSH. Unbound protein and HRP conjugate are washed off by wash buffer. Upon the addition of the substrate, the intensity of color is proportional to the concentration of TSH in the sample. A standard curve is prepared relating color intensity to the concentration of TSH. The absorbance was taken at 450 nm in the ELISA reader.

3.7.5.2 Total triiodothyronine (tT_3) Assay

ErbaLisa® Total triiodothyronine (tT_3) is a solid phase competitive ELISA. The sample, assay buffer and T_3 enzyme conjugate are added to the wells coated with anti-T_3 monoclonal antibody. T_3 in the serum competes with the T3 enzyme conjugate for binding sites. Unbound T_3 and T_3 enzyme conjugate is washed off by washing

buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of T_3 in the samples. Using 6 standards, a curve is plotted, relating to the concentration of the T_3. The absorbance/ OD was taken at 450 nm in the ELISA reader.

3.7.5.3 Total thyroxine (tT_4) Assay

ErbaLisa® Total thyroxin (tT_4) is a solid phase competitive ELISA. The samples, working T4-HRP conjugate and anti-T4-Biotin solution are added to the wells coated with streptavidin. T4 in serum competes with the T4 enzyme (HRP) conjugate for binding sites. Unbound T4 and T4 enzyme conjugate is washed off by washing buffer. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of tT_4 in the samples. Using 6 standards, a curve is plotted, relating color intensity to the concentration of T4. The absorbance was taken at 450 nm in the ELISA reader.

3.7.6 Neurobehavioral Parameters

3.7.6.1 Elevated Plus Maze (EPM)

This procedure was carried out using Orchid Scientific Plus Maze and the software Tracking Param Converter and Viewer 2.1. The maze used in this study was made of wood, consisted of 2 open arms and 2 close arms which had three side walls. This maze was elevated 50 cm from the floor and had a webcam above the maze. All the observations were recorded by the camera attached to the software (All Maze Software). Elevated plus maze is the most simplest apparatus to study neuroprotective effect (Latha *et al* 2015) and anxiolytic response produced by test drug. It is used to test almost all types of anxiolytic agents. Exposure of animals to novel maze alley evokes an approach-avoidance conflict which is stronger in open arm as compared to enclosed arm. Rodents have an aversion for high and open space and prefer enclosed arm, therefore, spend a greater amount of time in enclosed arm. When animals enter open arm, they freeze, become immobile, defecate and show fear-like movements (Pellow *et al* 1985). Major advantage of this test procedure are: (a) it is simple, fast, and less time consuming, (b) no prior training or noxious stimuli (sound or light) is required, and (c) it is predictable and reliable procedure for studying anxiety response as well as anxiolytic action of drug. In the EPM, anxiety is expressed by the animal spending more time in the enclosed arm.

The experiment began by transferring the rats to the test room, where they were allowed to rest for 30 min and then placed individually in the centre of the plus-maze facing an open arm, and allowed 5 min of free exploration (Sanchez-Amate *et al* 2001). The maze was cleaned with ethanol (10%, v/v) to avoid possible bias due to odors and/or residues left by rats tested earlier. For the purpose of analysis, total number of entries into and time spent in open and enclosed arms; the total number of arm entries; sleeping, open-arm activity was quantified as the time spent on the open arms relative to the total time spent in both arms (open/total × 100), and number of entries into open arms relative to the total number of entries into any arm (open/total × 100). In addition, the total number of entries as well as the closed arm entries were used as an index of locomotor activity (Hogg 1996). The procedure was conducted in sound attenuated room. The unit of expression is in time (seconds).

3.7.6.2 Elevated T-maze

This procedure was carried out using Orchid Scientific Plus Maze and the software Tracking Param Converter and Viewer 2.1. It is shaped like letter T, providing the subject, typically a rodent, with a straight forward choice. The apparatus consists of three elevated arms, one enclosed and two open arms and used to study how the rodents function with memory and spatial learning through applying various stimuli. The concept is used to assess rodent behavior. The different tasks, such as left- right discrimination and forced alteration, are mainly used with rodents to test reference and working memory. The procedure was adapted from the one validated by (Zangrossi and Graeff 1997, Graeff *et al* 1998). The experiment began with transfer of the rats to the test room, where they were allowed to rest for 30 min. For the inhibitory avoidance task, each animal was placed at the distal end of the closed arm, facing the intersection of the arms. The time taken to leave this arm with all four paws (Baseline Latency) was recorded by a trained observer. This procedure was repeated in two subsequent trials (Avoidance 1 and 2) with a 30-s interval. Learning is indicated by the increase of the withdrawal latency along the trials. Baseline latency (time to first withdrawal from the closed arm) was used as a secondary index of locomotor activity (Texeira *et al* 2000). Thirty seconds after the last trial of inhibitory avoidance, the rats were placed at the end of one open arm and the latency to enter the

enclosed arm (one-way escape) was recorded. A cut off time of 300 s was established for inhibitory avoidance and one-way escape. After each trial the maze was cleaned with ethanol (10%, v/v) to avoid possible bias due to odors and/or residues left by rats tested earlier.

Inhibitory avoidance-representing learned fear was measured by recording the time taken to leave the enclosed arm in three consecutive trials. Unconditioned fear was evaluated by recording the time to escape from the open arm. Restraining the animals at the end of the enclosed arm for 30 s did not change the first (baseline) withdrawal latency, indicating that rats were not escaping from the experimenter's hand. In addition, rats trained in a T-maze with the three arms enclosed did not show the usual increase in withdrawal latency over the three consecutive trials. These results indicated that open arm experience, not handling, motivated inhibitory avoidance-learning. The same experiment also showed that the latency to leave the open arm did not undergo habituation over five consecutive trials, thereby providing evidence of an aversive motivation for this response.

3.7.6.3 Grip Strength

The grip strength of limbs or muscular strength was measured using Grip Strength Meter (Ugo Basile, Milan, Italy). In this triangular shaped metallic grip was attached to the transducer that measured the force which in turn was connected to a computer to digital signal. The Grip-Strength Meter automatically measures grip-strength (i.e. peak force and time resistance) of fore limbs and also via the optional grid of hind limbs in rats. The effects of drugs, toxins, muscle relaxants, disease, ageing or neural damage on muscle strength may be assessed.

After adequate training the animals to hold the grip, animal was pulled back at increasing force holding the abdomen and tail, and the rats ability to hold on triangular holder was measured in GF (Grip force) unit. The trial was repeated thrice in order to avoid the false reading and the highest force measured was considered for further evaluation.

3.7.6.7 Rota-rod activity

The rotarod performance test is a performance test based on a rotating rod with forced motor activity being applied, usually by a rodent. The rotarod consists of

a circular rod turning at a constant or increasing speed Bohlen *et al* (2009). The test measures parameters such as riding time (seconds), endurance and motor coordination especially in testing the effect of experimental drugs or after traumatic brain injury. The rotarod test is widely used to generally assess motor performance in rats and mice (Zausinger *et al* 2000; Luesse *et al* 2001; Jeong *et al* 2003; Karl *et al* 2003). In the test, a rodent is placed on a horizontally oriented, rotating cylinder (rod) suspended above a cage floor, which is low enough not to injure the animal, but high enough to induce avoidance of fall. Rodents naturally try to stay on the rotating cylinder, or rotarod, and avoid falling to the ground. The length of time that a given animal stays on this rotating rod is a measure of their balance, coordination, physical condition, and motor-planning. The speed of the rotarod is mechanically driven, and may either be held constant, or accelerated. The rotarod test (Dunham and Miya 1957) is one of the oldest tests used in assessing the effects of a drug on animal behavior. It provides a rapid and simple first estimation of neuromuscular coordination. Animals placed on the rotating rod try to remain on it rather than fall onto a platform just below. Drugs known to alter neuromuscular coordination, such as the benzodiazepines, reduce the time the animals are able to remain on the rod Rustay *et al* (2003). This test is easily automated with several animals being tested simultaneously on the same rod. Vertical barriers are used to separate the animals from one another. The test measures a rat's ability to maintain itself on a rod that turns at accelerating speeds. The rotarod test has gained an extremely wide dynamic range, and is very sensitive to detecting graded improvements or graded deterioration of motor function after different types of interventions. Rats were allowed to run after setting rota-rod speed at 15 rpm, and were tested three times. Individual animals were put on the rotating rod and the total time spent on the revolving rod was measured on the horizontal plane within five minutes (Dunham and Miya 1957).

3.7.6.4 Acoustic or Auditory Startle response

It consisted of a series of rapid movements beginning at the head and moving caudally involving contraction and extension of major muscle groups in response to auditory stimuli with a rapid onset, or rise time. Responses were not graded but mere presence or absence and development of habituation and/or sensitization are recorded (Davis 1982).

3.7.6.5 Righting Reflex

In order to study the righting reflex, a rat was placed on its back on a tabletop and the time taken for the animal to right itself through 180° was measured. All animals were assessed for the righting reflex (Ferguson *et al* 2010). In order to study the righting reflex, the animal was positioned with one hand holding the tail and the other holding the dorsal neck skin. The animal was inverted and placed immediately on a flat table, and the time required for the animal to right itself through 180° was measured.

3.7.6.6 Corneal Reflex

The cornea of the rat was touched with fine thread and the response to close the eye was checked. The mere presence or absence of reflex was taken into consideration.

3.7.7 Acetyl-cholinesterase (AChE) Activity

AChE was measured by spectrophotometric assay, as described by Ellman et al (1961), in blood plasma and tissues (brain homogenate 10%). The esterase activity was calculated with an artificial substrate, acetylthiocholine (ATC), being given. Thiocholine produced as a result of AChE's cleavage of ATC was allowed to react with the -SH reagent 5'5'-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced to thionitrobenzoic acid, a yellow-colored anion with maximum absorption of 412 nm. The thionitrobenzoic acid extinction coefficient was 1.36 ~104/M/cm. The thionitrobenzoic acid concentration measured using a UV spectrophotometer was then taken as a direct approximation of the activity of the AChE.

Reagents

1. Phosphate buffer (150 mM, pH 8.0): 23.4 g $NaHPO_4.2H_2O$ was dissolved in 750 ml distilled water, pH was adjusted to 8.0 with 1N NaOH solution and made the volume up to 1 litre with distilled water.
2. Magnesium chloride (13.0 mM): 165.3 mg of $MgCl_2$ was dissolved in 100 ml distilled water.
3. Buffered Ellman’s reagent (DTNB): 3.96 mg of 5,5’-dithiobis (2-nitrobenzoic acid) was dissolved in 10 ml phosphate buffer (150 mM, pH 8.0) and to this 15 mg of sodium bicarbonate was added. The solution was prepared freshly.

4. Acetylthiocholine iodide solution: It was prepared freshly by dissolving 28.9 mg acetylcholine iodide in 10 ml distilled water.

Procedure

2.6ml of phosphate buffer was taken in a test tube and to it, 0.2ml of $MgCl_2$ solution, 0.02ml of acetylcholine iodide solution and 0.1ml DTND solution (Ellman's reagent) were added. Further, 10µl of plasma/tissue homogenate was added and mixed thoroughly and incubated at 37°C for 30 min. Absorbance was measured at 412nm in spectrophotometer. When absorbance reached a stable value, it was recorded as the basal reading. 20µl substrate (i.e. acetythiocholine iodide) was added and change in absorbance recorded for the next five minutes at an interval of 1 min. Change in the absorbance per minute was thus determined.

AChE (µM/min/ml) = (OD/ EC) x (total volume/ volume of sample) x (1/time x DF)

Where: EC= 1.36 x 104 /M/cm; DF= dilution factor

3.7.8 Sensory Parameters

3.7.8.1 Eddy's hot plate test

The hot plate test involves higher brain function, and is considered to be a supraspinally organized response (Eddy and Leimbach, 1953). This test consists of introducing a rat or mouse into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode or a boiling liquid (MacDonald and Woolfe 1944, O'Callaghan and Holzman, 1975). Many authorities have used the hot plate procedure and it is appropriate for central (but not peripherally) functioning hyper-algesia assessment. A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered to be supraspinally integrated responses. Adult, male wistar rats were assessed for analgesic activity using the rat paw-hot plate assay. The latency hot plate paw withdrawal was measured by a Hot Plate Analgesia Meter (MAC, MSW-737). The temperature of the hot plate was set and calibrated at 56°C±1^{o}C and the rats were removed from the heat stimulus by 36 seconds after placement. Paw licking, jumping or hopping are known to be indicators of the end point level. These parameters were recorded for individual animals and, the recording time was 5 minutes.

3.7.8.2 Tail Immersion Test test

Increase in body temperature results into emotional reactions due to thermoregulatory core activation and consequently the HPA axis. Abrupt temperature changes using either hot or cold water or congeler compartment have also been used to cause discomfort in animals. Water temperature was maintained at precisely 55°C±1°C during the entire process. The lower 5 cm part of the rat tail was labeled and submerged in a cup of hot water at temperature 55°C±1°C. The reaction time was measured in second(s) i.e. efforts made to eliminate the tail or tail removal. The reaction time was measured in second(s) by stopwatch to eliminate the tail. The immersion cut-off time was 15 seconds. Untreated animals had a withdrawal period of between 1 and 5.5 sec. Therefore, a withdrawal period of more than 6 sec was deemed a good response.

3.7.9 Male Reproductive Parameters

3.7.9.1 Epididymal sperm isolation

Male mice under anesthesia were sacrificed at end of the exposure period and the scrotal area was filled with 70 percent ethanol and dissected to reveal the full epididymis. Thorough dissection of epididididymis was done to remove adventitious tissues. Cauda and caput epididididymis were aseptically minced in sterile petri dishes containing 1 ml of Dulbecco's PBS (HiMedia) pre-warmed (35 °C) and screened through a thin layer of cotton mesh. The filtrate was used to determine the parameters of the epididymal sperm.

3.7.9.2 Epididymal total sperm count

The epididymal sperms were collected as described in the earlier section, incubated at 35 °C which is the optimal temperature for mice epididymal sperm. The epididymal fluid was then diluted in Dulbecco’s pre-warmed (35 °C) PBS to a volume of 5 ml. The spermatozoa were counted by hemocytometer with improved Neubaur's (Deep 1/10 mm. LABART, Germany) chamber as described by Pant and Srivastava (2003) using semen diluting fluid (0.1g eosin, 5 gm sodium bicarbonate, 1 ml of formalin and 99 ml of distilled water).

Total viable cells/ml = (Average number of sperm per chamber) $\times 10^3 \times$ (Dilution Factor)

3.7.9.3 Viable Sperm Count

Per cent live sperm was measured using Eosin-Nigrosin stain (EN) differential staining technique. These slides were also observed morphologically to measure the proportion of irregular sperm based on visible sperm area head, back, mid-piece and tail anomalies. On a cool, grease-free preheated glass slip, a drop of epididymal semen was taken and 2-3 drops of Eosin-Nigrosine stain (1% Eosin and 5% Nigrosine in a 3 % sodium citrate dehydrate solution; 37°C) added. Then gently mixed epididymal semen and stain using a blunt fine glass rod. A thin smear was made over a smooth, grease-free glass slide within a period of 0.5 to 1 minute. All the slides were immediately dried on heating plates at 37^0C. Finally, smears were observed under light microscope (Nikon DSRi1, Japan). Within a slide, a total 200 spermatozoa were counted, and differentially counted side by side for pink or partly stained spermatozoa i.e. dead sperm.

3.7.9.4 Sperm Morphology

Two hundred spermatozoa (heads only or intact sperm) per rat were evaluated for head and/or flagellar/tail defects by microscopy. Classifications of individual spermatozoa into a) normal, b) normally shaped head separated from flagellum, c) misshapen head separated from flagellum, d) misshapen head with normal flagellum, e) misshapen head with abnormal flagellum, f) degenerative flagellar defect(s) with a normal head, and g) other flagellar defects(s) with a normal head.

3.7.10 Histopathology

After thorough gross examination, small representative pieces (approximately 0.5 cm thickness) of different tissues like liver, kidney, brain and testes were collected from sacrificed animals and fixed in 10% neutral buffered formalin. After proper fixation for 3-4 days, the tissues were cut into thinner sections (1-2 mm thick). The tissues were then washed in running water for 7- 8 h, dehydrated in ascending grades of ethyl alcohol, cleared in benzene and embedded with paraffin wax. The paraffin blocks were prepared and the sections were cut at 4-5 µ thickness with a hand operated microtome. The paraffin-embedded sections were then affixed to microscope slides and passed through sequential steps of deparaffinization in xylene, rehydration through descending grades of ethyl alcohol to running water and coloured with one or more stains to increase the visibility of various cellular and intracellular components. Sections were then stained by routine staining as mentioned below:

Routine Hematoxylin and Eosin (H&E) staining

Hematoxylin and Eosin stain was used principally in which staining of nucleus with hematoxylin gives blue colour, which is followed by counterstaining with an aqueous or alcoholic solution of eosin, which colours eosinophilic structures in various shades of red, pink and orange (Kiernan 2008).

3.7.11 Statistical analysis

All the values were expressed as mean ± SE (n=6). All the values were analysed by using two way analysis of variance (ANOVA) with fixed factors groups (Treatment and Control groups) and days (0 day, 29th day) using SPSS 20.0 version software with the following model:

$Y_{ij} = \mu + G_i + T_j + G_i.T_j + e_{ijk}$ where

Y_{ij} = each parameter

μ = overall mean

G_i = effect of i^{th} group (control/treatment)

T_j = effect of j^{th} days/time (0, 29)

$G_i.T_j$ = effect of interaction between group and time.

e_{ijk} = residual error

Tukey's *b* test was used for the post-hoc comparison of different treatment means and statistical differences were considered significant at $P<0.05$ (Snedecor and Cochran 1989).

CHAPTER IV

RESULTS AND DISCUSSION

In the present investigation, evaluation of oral sub-acute mancozeb toxicity was undertaken in male wistar rats and various parameters including physiological, biochemical, haematological, endocrine and neuro-behavioural were analysed for toxic manifestations induced by mancozeb.

Mancozeb (MZ) is a fungicide belonging to the subclass of ethylene bisdithiocarbamates pesticides (Srivastava and Singh 2014) having a metal atom coordinated with ethylene-bis-dithiocarbamate (EBDC) (Calviello *et al* 2005). It is a combination of two other dithiocarbamates: maneb and zineb and has a multi-site, protective action on contact. The mixture controls many fungal diseases in a wide range of field crops, fruits, nuts, vegetables and ornamentals.

Mancozeb itself does not act as a fungicide, rather it is effectively considered as a pro-fungicide. When it is exposed to water it may degrade to ethylene bis-isothiocyanate sulfide (EBIS) and ethylene bisisothiocyanate (EBI) (Saha *et al* 2016). Both of these compounds (EBIS and EBI) act as active toxicants.

The adverse effects of mancozeb in humans and other living organisms have not been widely studied and there are only a few reported studies evaluating the neurotoxic action of mancozeb in experimental models (Domico *et al* 2006). Diverse chronic adverse health effects of mancozeb and other metal ethylene-bis-dithiocarbamate (EBDC) pesticides have been observed, such as structural and functional alterations of gonads (Baligar and Kaliwal 2001). Majority of studies have focused on adverse health effects of ethylene thiourea (ETU), the main metabolite of mancozeb. This compound has been reported to have carcinogenic, teratogenic and goitrogenic effects (Chhabra *et al* 1992). Although its mutagenic effect is still controversial, it is considered a potent carcinogenetic agent.

In the present investigation, mancozeb was administered orally at the dose rate of 500 mg/kg for 28 consecutive days to wistar rats. Twelve healthy male wistar rats were divided into two groups of six animals each. Group I animals served as control in which no drug was administered, whereas the group II animals were administered with mancozeb at the dose rate of 500mg/kg ($1/10^{th}$ of LD_{50} i.e; 5000mg/kg) to induce

toxicity. All animals exposed to mancozeb were closely observed for neuro-behavioral activities and for the appearance of toxic effects and nature, duration and severity of toxicity and lethality, if any.

Experiment: Oral sub-acute toxicity studies of mancozeb in rats.

4.1 Effect of sub-acute mancozeb exposure on physiological status of male wistar rats.

To evaluate the physiological status of animals, following parameters were taken into consideration.

4.1.1 Toxic Signs

Daily oral administration of mancozeb at the dose rate of 500mg/kg body weight to rats for 28 consecutive days produced mild signs of toxicity like moderate degree of anorexia, listlessness, lacrimation, nasal bleeding, sluggish movement and mild degree of hind limb paralysis when compared with control group animals. These signs were observed in milder form after 10-15 days of drug administration and then resolved gradually. However, no lethality was observed in treatment as well as in control group animals. Our findings were in agreement with those of (Shukla *et al* 1990), who observed anorexia and sluggish movement in mice treated with mancozeb. Administration of mancozeb at different doses to male rats for 360 days, produced signs of toxicity including diarrhoea, salivation, nasal bleeding, dyspnoea, hind limb paralysis and death during the course of experimentation in a dose dependent manner (Kackar *et al* 1997).

4.1.2 Body weight measurement on weekly basis

All animals were weighed after the adaptation period for dose calculation at the start of experiment and from than onwards on weekly basis. No significant alteration in body weight gain was observed on repeated mancozeb exposure when compared to control group (Table 3; Fig. 2). Moderate decrease in body weight was observed in animals during first week of experimentation followed by increase in body weight in subsequent weeks. There was increase in body weight and percent body weight gain in animals after 28 days of mancozeb exposure in treatment group. Kackar *et al* (1997) reported similar increase in body weight after mancozeb exposure. Stephenson and Trombetta (2020) showed no significant difference in weight gain in mancozeb treated animals when compared to controls. In contrast to

our study (Kackar *et al* 1999) observed a decrease in body weight gain on mancozeb exposure.

Table 3: Weekly body weight (g) variations on exposure to mancozeb *per os* in male wistar rats (n=6)

Groups	0th Day	7th Day	14th Day	21st Day	28th Day
Control	165.3^{a}±4.52	162±8.60	171.8±10.25	180.3±7.13	188.2^{b}±6.90
Treatment	185.5^{a}±3.17	179.2±9.86	177.5±13.68	201.3±9.39	208.5^{b}±5.97

Means bearing superscripts $^{a, b}$ differ significantly in a row.

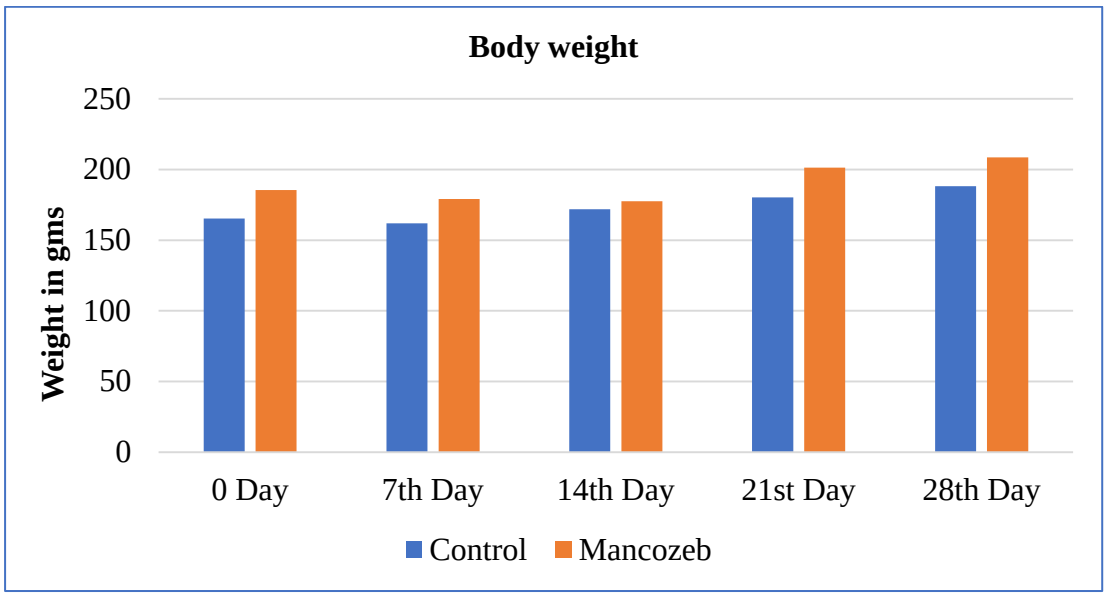


Figure 2: Body weight measurement of rats during experimentation period on weekly basis (n=6)

4.1.3 Organ weight measurement

At the end of experimentation i.e, 29th day, all animals were sacrificed humanely and various organs including liver, kidneys, lung, heart, brain, testes and epididymis were removed by transverse abdominal incision and weighted for absolute as well as for relative weight measurement. The analysis of relative organ weight in toxicological studies is an important criterion for identification of potentially harmful effects of chemicals (Bailey *et al* 2004). Significant elevation in absolute organ weight of liver, kidneys, brain, testes and epididymis were observed (Table 4; Fig. 3,4,5).

Table 4: Absolute (g) and Relative organ weight (%) variations after exposure to Mancozeb *per os* in male wistar rats (n=6)

Parameters	Control Group	Treatment Group	P value
Initial bwt.	165.33^{a}±4.52	185.5^{b}±3.17	0.001
Terminal bwt.	188.17^{a}±6.90	208.5^{b}±5.97	0.001
% bwt. Gain	11.93±1.46	10.82±1.85	0.649
Liver	6.24^{a}±0.27	7.73^{b}±0.39	0.012
Liver %	3.31^{a}±0.05	3.68^{b}±0.12	0.018
Kidney	1.44^{a}±0.04	1.68^{b}±0.05	0.005
Kidney %	0.77±0.03	0.806±0.01	0.369
Lung	1.40±0.068	1.55±0.067	0.136
Lung %	0.74±0.02	0.75±0.04	0.881
Heart	0.98±0.08	0.811±0.04	0.092
Heart %	0.52^{b}±0.04	0.388^{a}±0.01	0.010
Brain	1.63^{a}±0.03	1.91^{b}±0.08	0.009
Brain %	0.87±0.01	0.916±0.02	0.192
Testes + epididymis	2.67^{a}±0.08	3.23^{b}±0.11	0.002
Testes+epididymis%	1.42±0.06	1.55±0.07	0.213

Means bearing superscripts^{a,b} differ significantly ($p \leq 0.05$) in a row.

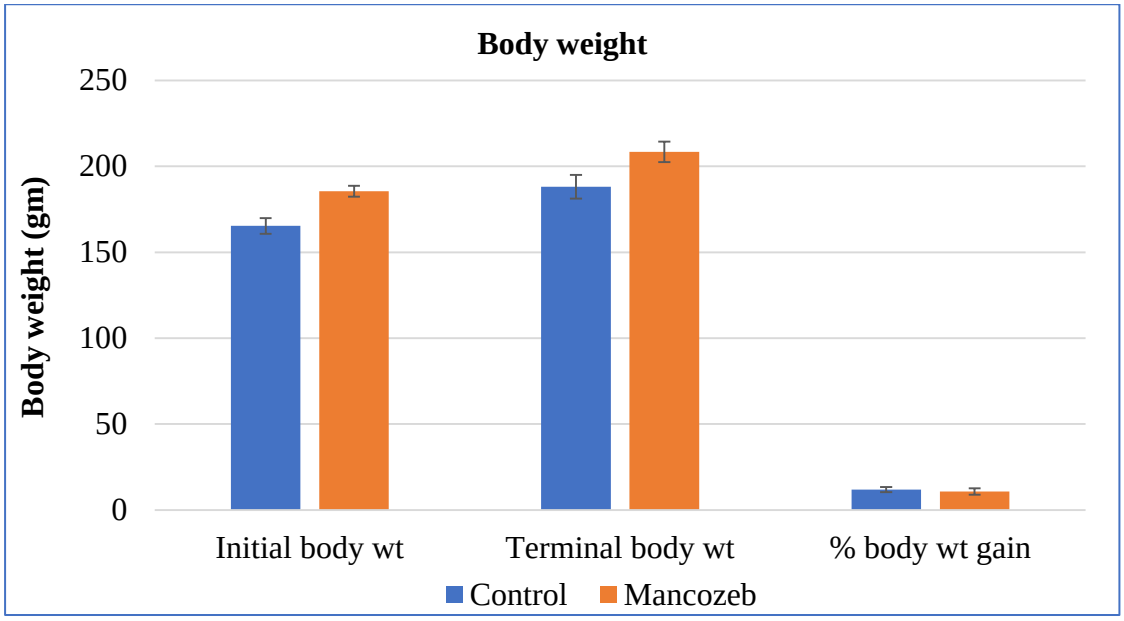


Figure 3: Effect of sub-acute exposure of mancozeb *per os* on body weight (gm) and percent body weight gain of male rats at the end of experimentation (n=6)

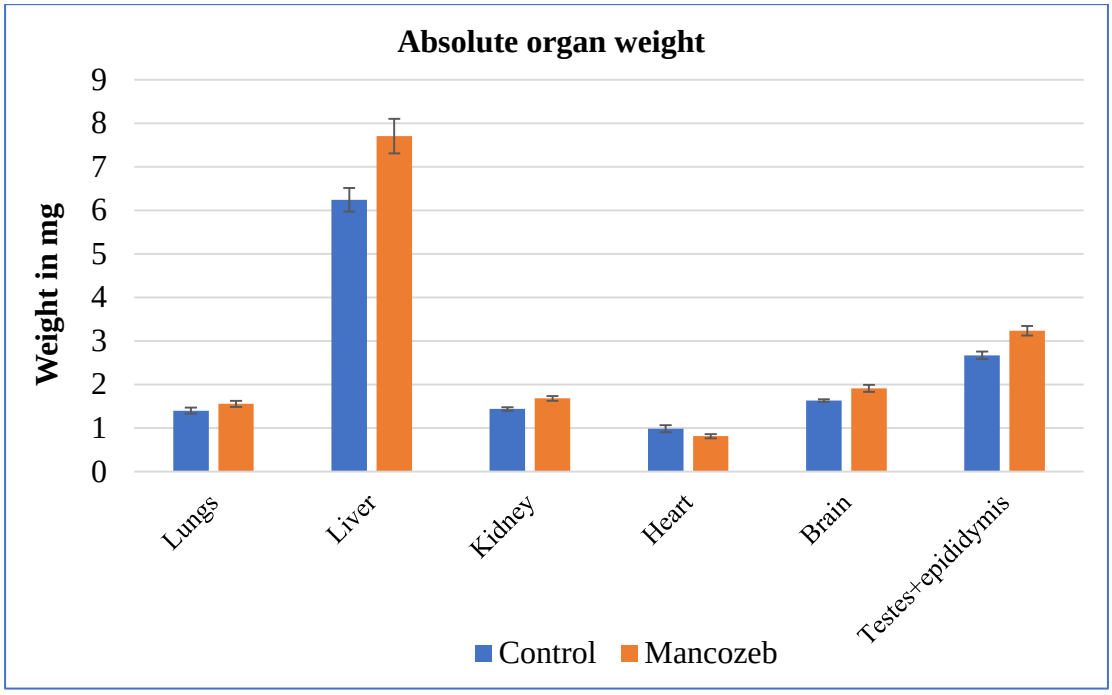


Figure 4: Effect of sub-acute exposure of mancozeb *per os* on absolute organ weight (mg) of various organs of male rats at the end of experimentation (n=6).

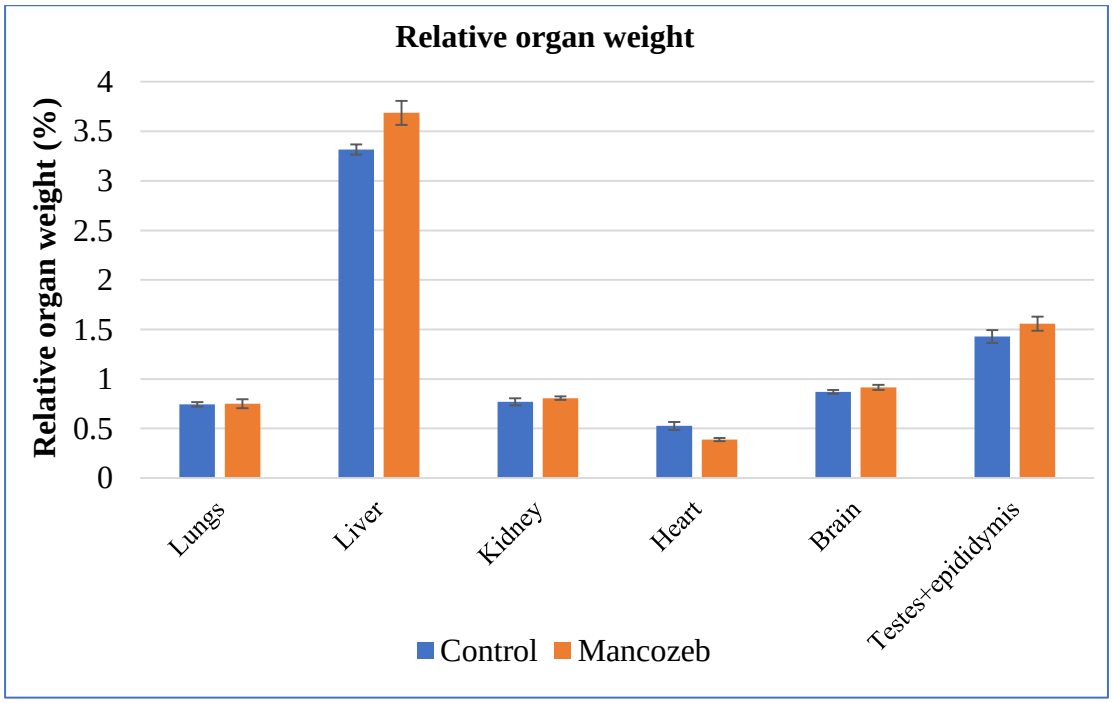


Figure 5: Effect of sub-acute exposure of mancozeb *per os* on relative organ weight (%) of various organs of male rats at the end of experimentation (n=6).

Ahmed and Gamila (2017), also reported significant increase in absolute weight of liver, kidneys, brain and testes due to mancozeb exposure. Organ weight of lungs and heart were not altered significantly. No significant alteration in percent body weight gain was recorded in mancozeb-exposed animals. Relative weight of liver increased significantly while relative weight of heart decreased significantly. In agreement to our study, a significant increase in the fresh weight of liver in individuals after mancozeb exposure was observed (Yahia *et al* 2014) and oral administration of mancozeb for 8 weeks led to a significant increase in the absolute weight of the liver, kidneys and spleen (Yahia *et al* 2015). Our results are similar to those obtained by El-Sayed *et al* (2012), who found that exposure to low dose equivalent to (1 / 20 LD_{50}) and high dose equivalent to (1 / 10 LD_{50}) of dimethoate, carbofuran and carbendazim administered for 30 days increased the relative weight of liver, and kidney. Also, El-Neweshy *et al* (2013) noticed a significant increase in the absolute and relative weight of liver and kidney of rats in the group treated with 400 mg / kg BW of Solignum®. In addition, El-Tawil and Abdel-rahman (2001) noted an increase in the relative weights of lungs, liver, kidneys and brain of both sexes rats fed on poisoned diet containing chlorpyrifos (at 200 ppm), or Chlorpyrifos-methyl (at 2000 ppm) for 12 weeks followed by 3 weeks of recovery period compared to those of controls. Furthermore, changes in kidney weight may reflect renal toxicity, tubular hypertrophy or chronic progressive nephropathy and alteration in brain weights were associated with neurotoxicity (Greaves 2000). Creasy and Foster (2002) suggested that changes in testes weights may reflect changes in seminiferous tubules or interstitial oedema and therefore usually contribute little further understanding of toxicity.

4.2 Effect of sub-acute mancozeb exposure on haematological parameters in rats.

Blood samples were collected on day zero and 29th day of experimentation from orbital sinus and heart of animals, respectively for analysis of various haematological parameters. Significant decrease in TLC, TEC and mononuclear cells were observed while ESR, MCV and neutrophils increased significantly in mancozeb-exposed animals when compared to control group animals. Whereas, slight decrease in haemoglobin and PCV was noticed. No significant alterations were recorded in other haematological parameters (Table 5,6; Fig. 6,7).

Table 5: Haematological variations in parameters including Haemoglobin, Packed cell volume, Erythrocyte sedimentation rate, Total erythrocyte count and Total leucocyte count after sub-acute exposure to mancozeb *per os* in male wistar rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
Haemoglobin (g/dl)					
Control	11.57±0.26	12.8±0.45	0.063	0.698	0.010
Treatment	11.9±0.46	10.97±0.31			
Packed cell volume (%)					
Control	41.5±1.26	43±1.53	0.692	0.147	0.026
Treatment	44.83±1.79	38.33±1.09			
Erythrocyte sedimentation rate (mm/hr)					
Control	2.02±0.14	2.2^{A}±0.26	0.021	0.013	0.069
Treatment	2.15^{a}±0.21	3.2^{Bb}±0.26			
Total erythrocyte count (10^6/µl)					
Control	6.55±0.14	6.73^{B}±0.09	0.001	0.001	<0.001
Treatment	6.68^{b}±0.18	4.97^{Aa}±0.31			
Total leucocyte count (10^3/µl)					
Control	12.70±0.17	13.17^{B}±0.29	0.020	0.049	0.001
Treatment	13.05^{b}±0.22	11.45^{Aa}±0.36			

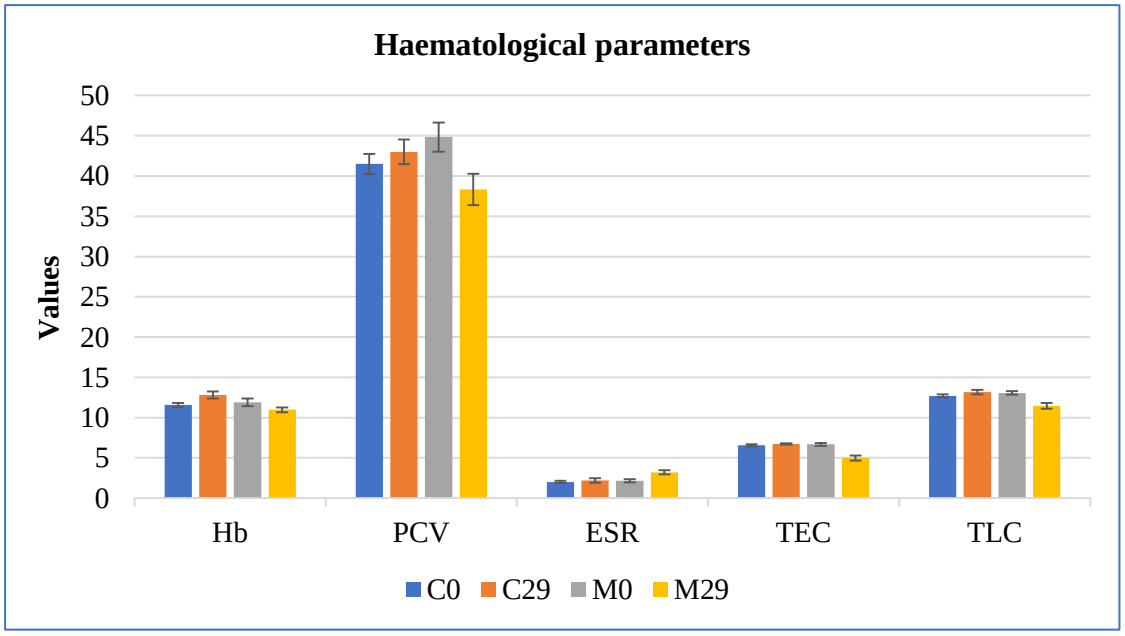


Figure 6: Variation in haematological parameters (Hb, PCV, ESR, TEC, TLC) after sub-acute exposure of mancozeb *per os* in male rats (n=6).

Table 6: Haematological variations in parameters including Neutrophils, Mononuclear cells, Mean corpuscular volume, Mean corpuscular haemoglobin and Mean corpuscular haemoglobin concentration after sub-acute exposure to mancozeb *per os* in male wistar rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
Differential leucocyte count (Neutrophils)					
Control	27.83±1.74	29.00^{A}±1.12	0.034	0.069	0.160
Treatment	28.50^{a}±0.99	33.83^{Bb}±1.70			
Differential leucocyte count (Mononuclear cells)					
Control	72.16±1.74	71.00^{B}±1.12	0.034	0.069	0.160
Treatment	71.50^{b}±0.99	66.17^{Aa}±1.70			
Mean corpuscular volume (fl)					
Control	63.45±2.29	63.94^{A}±2.26	0.018	0.115	0.147
Treatment	67.24^{a}±2.83	78.31^{Bb}±5.54			
Mean corpuscular haemoglobin (pg/dl)					
Control	17.68±0.48	19.05±0.74	0.100	0.010	0.131
Treatment	17.84±0.69	22.61±1.83			
Mean corpuscular haemoglobin concentration (g/dl)					
Control	27.96±0.83	29.81±0.69	0.235	0.040	0.839
Treatment	26.63±0.87	28.86±1.24			

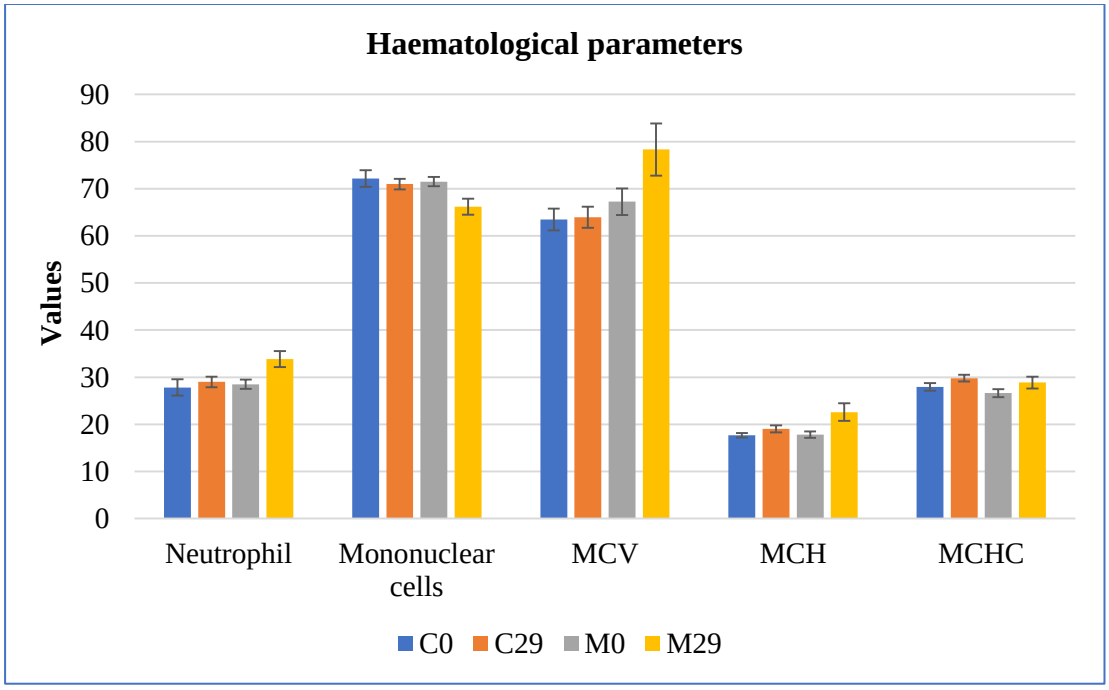


Figure 7: Variation in haematological parameters (Neutrophils, Mononuclear cells, MCV, MCH, MCHC) after sub-acute exposure of mancozeb *per os* in male rats (n=6)

For monitoring stress responses, prediction of systematic relationships and the physiological adaptations of animals, haematological parameters have been used for the assessment of the general health of animals (Akhtarunnessa and Masudul 2012). Haematological parameters can be used to assess the extent of the adverse effects of foreign substances on the blood constituents of an animal (Friday *et al* 2012). Minimum haematological indices should include packed cell volume (PCV), haemoglobin (Hb), total erythrocyte counts (TEC), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), as these parameters are frequently used in toxicological investigations (Shah and Altindag 2004).

Blood or haematological parameter changes are the most rapid and used to detect alterations during stress condition and are acting as fuel to assess various health conditions (Hymavathi and Rao 2000). Results of our present investigation are in agreement to the study conducted by (Ahmed and Gamila 2017), where after mancozeb administration, RBC count was decreased. Negga (2012) also reported a significant decrease in the RBC count on metalaxyl treatment to mice for 2 and 4 weeks. The lowering of RBC count might be due to the damaging action of pesticides on peripheral red cells due to which viability of the cells were affected (Felix and Saradhamani 2015). Witeska (2004) suggested that the decrease in WBC count can be the result of autolysis caused due to leakage of haemolytic enzymes from the cells under toxicant stress. The increase in MCV and MCH values were also reported after exposure to deltamethrin, which indicated that a reduced RBC count could be due to the destruction of erythrocytes or their decreased synthesis in bone marrow (Rauf and Arian 2013).

Patil and Govindwar (2003) have explained that many steps of the heme biosynthesis are inhibited by the pesticide exposure. Poisoning by pesticides residues may induce anaemia caused due to interference of haemoglobin biosynthesis and shortening of the lifespan of circulating red blood cells. Administration of carbosulfan caused certain changes in haematological constituents by altering the production of red blood cells with a significant decrease of haemoglobin in all the treated groups. Low haemoglobin level could be probably due to the decrease in oxygenation capacity or disruption of iron metabolism. Our results are also in agreement with

those of Venees *et al* (2011) where lower rates in haemoglobin synthesis after diazinon treatment for three weeks were observed. The decline of haematocrit values in pesticide treated rats, can be due to the synergistic link between blood parameters in all vertebrates (Köprücü *et al* 2006). We also observed a significant decrease in white blood cells due to increased utilization of the body's immune system as a defense mechanism. Whereas in contrast to our findings, Yahia (2015) observed a significant increase in white blood cells which may indicate an activation of the animal's immune system (Kalender *et al* 2006).

Basir *et al* (2011) reported that rabbits treated with lambda-cyhalothrin revealed a significant decrease in red blood cell and white blood cell counts, haemoglobin concentration and lymphocytes, while the mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), neutrophils, monocytes and eosinophils had increased. Mehadevaswami *et al* (2001) reported that anaemia can occur due to accelerated red blood cell destruction by haemolytic agents or rapid cell removal from an abnormality of cell shape or over-activity of the spleen or due to quantitative decrease in blood formation due to quantitative decrease in red marrow from aplasia or a quantitative decrease in marrow activity from a deficiency in the substances necessary for normal bone marrow activity.

Contrary to our findings, Atamanalp and Yanik (2003) reported that in Rainbow Trout (*Oncorhynchus mykiss*), a significant decrease in Hb levels after exposure to a sublethal dose of mancozeb, which may impair oxygen supply to various tissues, causing slow metabolic rate and low energy production (Ahmad *et al* 1995). Similarly, in freshwater catfish (*Heteropneustes* fossilis), the Hb level was also decreased after 30 days exposure to deltamethrin (Kumar *et al* 1999). In agreement to our findings, the decrease in PCV observed by Ahmad (1995), may show the extent of the shrinking cell size due to insecticide intoxication.

4.3 Effect of sub-acute mancozeb exposure on biochemical parameters in rats

Analysis of serum biochemical parameters are especially useful to identify target organs of toxicity as well as the general health status of animals and is advocated to provide early signs of critical modifications in stressed organisms (Jacobson and Keller 2001). This study investigated the changes in haematological as

well as biochemical status related to liver and kidney functions and organ weight changes after mancozeb exposer to male wistar rats using 1/10 LD_{50} dose of mancozeb. Liver and kidney are important vital organs in the animal body as they are the sites of detoxification and elimination of toxic materials. A foreign body in the form of a chemical stress is sufficient enough to cause severe hepatic and renal dysfunction (Waggas 2013).

Table 7: Blood Biochemistry variations in parameters indicating liver function after sub-acute exposure to mancozeb *per os* in male rats (n=6)

Groups	0 day	29th day	P value		
			G	T	G*T
Aspartate aminotransferase (IU/L)					
Control	2.44±0.34	3.09^{A} ±0.36	<0.001	<0.001	<0.001
Treatment	2.85^{a} ±0.38	8.92^{Bb} ±0.98			
Alanine aminotransferase (IU/L)					
Control	3.18±0.33	3.80^{A} ±0.38	<0.001	<0.001	<0.001
Treatment	2.92^{a} ±0.32	9.16^{Bb} ±0.60			
Lactate dehydrogenase (IU/µl)					
Control	0.14±0.01	0.15^{A} ±0.01	<0.001	<0.001	<0.001
Treatment	0.15^{a} ±0.01	0.53^{Bb}±0.04			
Gamma-glutamyl transpeptidase (IU/L)					
Control	0.51±0.09	0.73^{A} ±0.18	<0.001	<0.001	<0.001
Treatment	0.52^{a} ±0.09	2.31^{Bb}±0.30			
Glucose (mg/dl)					
Control	112.8±4.19	106.4^{A}±3.03	0.011	0.002	0.067
Treatment	109.4^{b}±3.69	87.31^{Ba}±4.90			
Alkaline phosphatase (IU/L)					
Control	31.56±2.20	32.79^{A}±3.10	0.025	0.003	0.007
Treatment	32.34^{a}±2.78	50.67^{Bb}±4.37			

Means bearing superscripts $^{a, b}$ differ significantly in a row. Means bearing superscripts $^{A, B}$ differ significantly in a column.

For evaluation of toxic manifestation of mancozeb exposure, various biochemical parameters were analyzed on day 0 and 29th day of experimentation. The biochemical findings in mancozeb-treated male wistar rats reflecting liver and kidney function and lipid profile have been shown in tables 7, 8 and 9, respectively. Repeated oral administration of mancozeb produced a significant elevation in the biochemical parameters like AST, ALT, LDH, GGT, alkaline phosphatase, creatinine, BUN, creatine kinase, HDL and LDL cholesterol levels when compared to control group as well as day 0 levels of treatment group. A significant reduction in total plasma protein, blood glucose and triglycerides were seen in mancozeb-exposed group indicating liver and kidney damage at tested dose.

4.3.1 Aspartate amino-transferase (AST)

Aspartate transaminases, catalyze the reductive transfer of an amino group from aspartate to α-ketoglutarate to yield oxaloacetate and glutamate. Besides liver, it is also found in other organs like heart, muscle, brain and kidney. Injury to any of these tissues can cause an elevated blood level of this enzyme (Nathwani *et al* 2005). Restoration of activity of serum aspartate transferases occurs with the healing of hepatic parenchyma and regeneration of hepatocytes (Jia *et al* 2014). Repeated oral administration of mancozeb produced a significant elevation in AST levels when compared to control group as well as day 0 ALT activity of treatment group, indicating damaged to liver at tested dose (Table 7, Fig. 8). In agreement to our study, elevation in AST activity was also reported by Simakani *et al* (2018), who concluded that mancozeb exposure causes stress response, health problem and tissue damage in common carp. Similarly, increase in AST level has also been reported in male wistar rats after mancozeb exposure (Yahia *et al* 2015). In another study done by Ahmed and Gamila (2017), elevated level of AST in mancozeb treated male albino rats. Talha *et al* (2006) also showed significant alteration in liver enzymes due to mancozeb treatment in male wistar rats. Serum aminotransferase elevations are due to direct liver injury or liver injury that is a secondary consequence of rhabdomyolysis. The enzymatic alterations are found to be associated with pathomorphological changes in the liver, kidney and brain. Certain soluble enzymes are used as diagnostic marker for tissue damage particularly of liver. Hepatocellular damage such as necrosis is

associated with tissue and serum enzymes. Similarly, escalation of AST enzyme activity following exposure to Carbamate pesticides has been reported (Djeffal *et al* 2015; Dhouib *et al* 2015; Apaydin *et al* 2017; El-Bieh *et al* 2017). Hamdi *et al* (2019) also suggested disruption of liver ALT in wistar rats treated with a triazole fungicide (Epoxiconazole).

In support of our findings, Sakr and Saber (2007) stated an insignificant elevation in AST in the serum of animals treated with mancozeb for 2 weeks which was followed by a significant increase after 4 and 6 weeks of treatment.

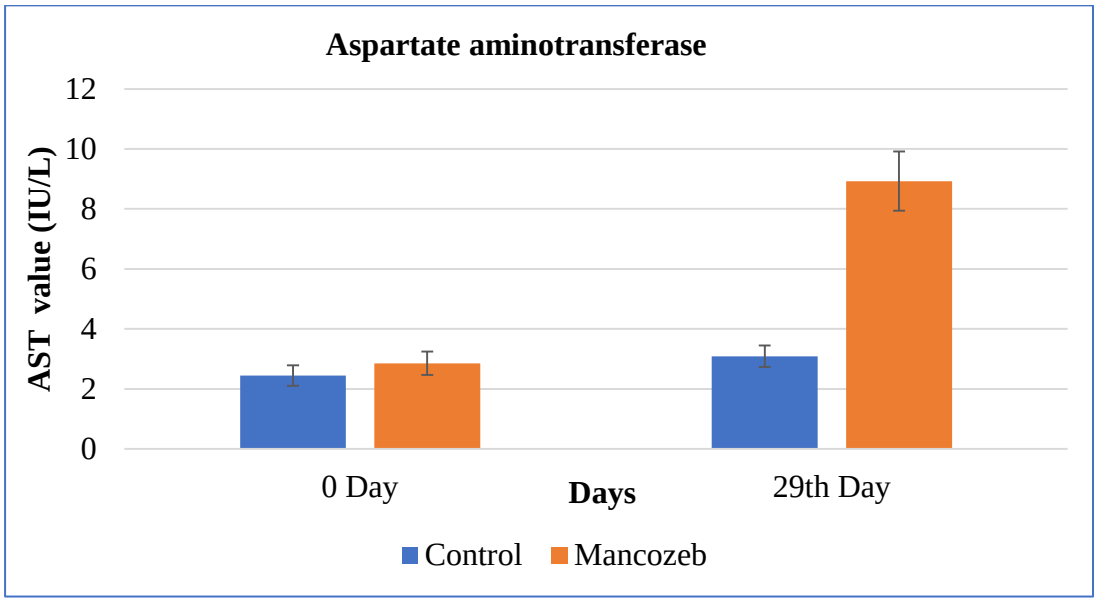


Figure 8: Blood Aspartate amino transferase variations after sub-acute exposure to mancozeb *per os* in male wistar rats (n=6)

4.3.2 Alanine amino-transferase (ALT)

A significant increase in the activity of ALT level was observed in male wistar rats exposed to mancozeb consecutively for 28 days when compared to control group as well as day 0 ALT activity of treatment group, indicating damaged to liver at tested dose (Table 7, Fig. 9). Estimation of alanine aminotransferase (ALT) activity is the most frequently relied biomarker of hepatotoxicity. The estimation of this enzyme is a more specific test for diagnosing hepatic damage, since it is primarily found in the liver (Dufour *et al* 2001; Amacher 2002). The increase in ALT activity in the present study might be due its leakage from damaged hepatocytes by production of oxidative radicals in excess, which subsequently resulted in release of ALT in blood circulation.

In agreement to our study, escalation of ALT enzyme activity following exposure to carbamate pesticides has been documented in various studies (Djeffal *et al* 2015; Dhouib *et al* 2015; Apaydin *et al* 2017; El-Bieh *et al* 2017). The increase of enzyme activities might be caused due to the adverse effects of mancozeb on hepatic cells causing leakage of enzymes in blood, as also evidenced by the histopathological examination. Similar to our finding, Yahia *et al* (2014), revealed hepatotoxic effects of mancozeb in rats, therefore, an environmental health risks to living organisms. Elevation in alanine aminotransferase (ALT) levels is widely viewed as a specific indicator of liver necrosis.

In support of our findings, Sakr and Saber (2007) stated an insignificant elevation in ALT after the second week of mancozeb treatment followed by a significant increase after 4 and 6 weeks of treatment. Similarly, increase in ALT level has also been reported in male wistar rats after mancozeb exposure (Yahia *et al* 2015). In another study done by Ahmed and Gamila (2017), elevated level of ALT in mancozeb treated male albino rats. Talha *et al* (2006) also showed significant alteration in liver enzymes due to mancozeb treatment in male wistar rats. In contrast to our findings, Simikani *et al* (2018) reported decrease in ALT activity due to mancozeb exposure in common carp. Hamdi *et al* (2019) also suggested disruption of liver ALT in wistar rats treated with a triazole fungicide (Epoxiconazole).

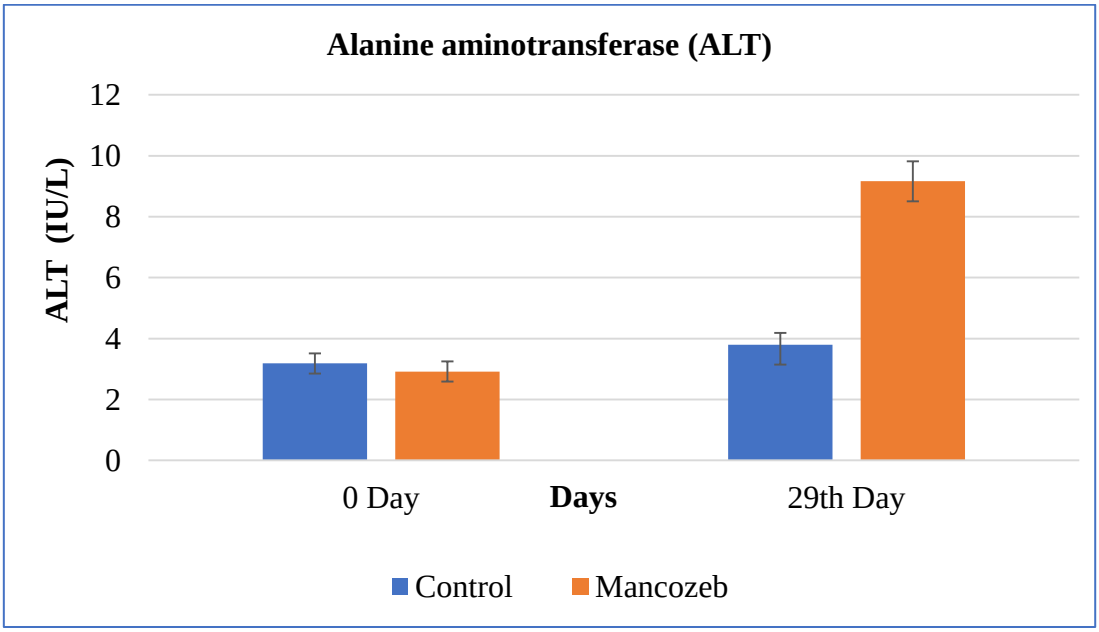


Figure 9: Blood Alanine amino transferase variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.3.3 Lactate dehydrogenase (LDH)

LDH is an intracellular enzyme which catalyses the conversion of lactate to pyruvate using NAD^+ as coenzyme of NAD (Burtis and Ashwood 1986). LDH is localised in cytoplasm of cells and is extruded into the serum when cells are damaged and is disproportionately elevated after an ischemic liver injury (Gitlin and serio 1992). It is sensitive intracellular enzyme which increases in serum indicating liver damage. A significant increase in LDH activity in present investigation, due to repeated mancozeb exposure in treatment group when compared with control group as well as day 0 LDH activity of treatment group, indicated damage to liver in male wistar rats (Table 7, Fig. 10). Our results of present study were in agreement with the the study Stephenson and Trombetta (2020), who reported increase in LDH activity after Dithiocarbamates (DTCs) like mancozeb (MZ) and disulfiram (DS) exposure in Long-Evans rats. Kacker *et al* (1997) also observed increase in LDH activity in male rats with mancozeb exposure. Increased level of LDH was also observed by (Das *et al* 2003).

The LDH level may be increased whenever there is cell injury or during neoplastic proliferation of cells (Dar 2011). The increase in plasma LDH activity in present investigation reflects damage to a range of tissues including skeletal or cardiac muscles, kidney and liver. Metabolites of mancozeb affect several enzymatic pathways. LDH may indicate changes and hypofunction of liver under the toxicants effects on the hepatocytes are in the form of tissue damage in which cellular enzymes are released from the cell into the blood serum.

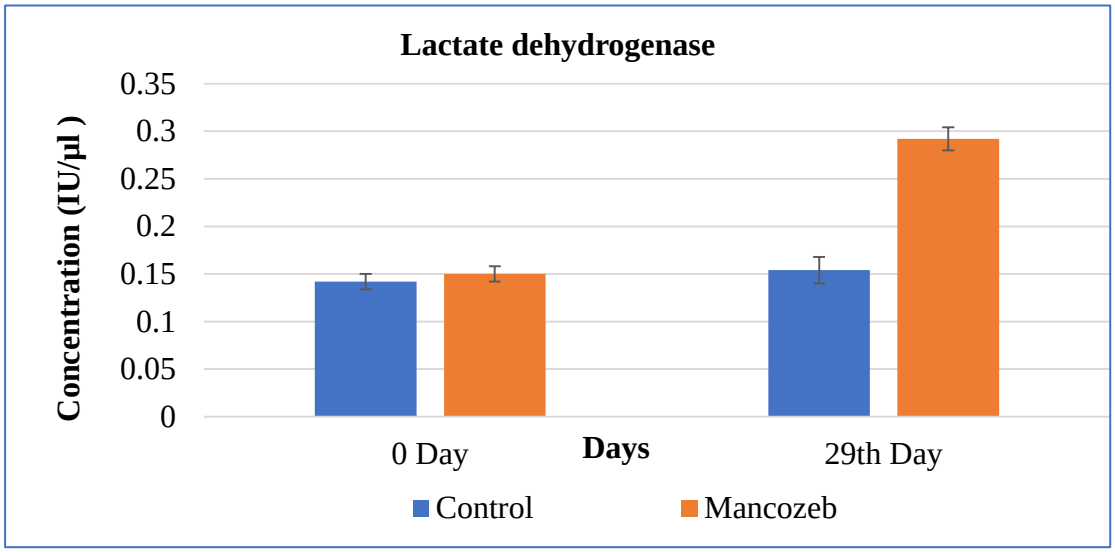


Figure 10: Blood Lactate dehydrogenase variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.3.4 Gamma-glutamyl transpeptidase (GGT)

Repeated oral administration of mancozeb for 28 consecutive days, produced a significant elevation in GGT levels when compared to control group as well as day 0 GGT activity of treatment group, indicating damaged to liver at tested dose (Table 7, Fig. 11). GGT is a microsomal enzyme, which is widely distributed in tissues including liver. Since, GGT is a hepatic microsomal enzyme, its activity is elevated in all forms of hepatic dysfunctions (Chander *et al* 1994). The elevation of serum GGT concentration is regarded as one of the most sensitive indices of hepatic damage and its elevation is highest in cases of intrahepatic and post hepatic billiary obstruction. It is more sensitive than ALP, leucine amino peptidase and transaminases in detection of obstructive jaundice and cholangitis. GGT arises earlier than all other enzymes and persists longer. Yahia *et al* (2014), revealed hepatotoxic effects of mancozeb in rats, therefore, an environmental health risks to living organisms. Talha *et al* (2006) also showed significant alteration in liver enzymes due to mancozeb treatment in male wistar rats. Similarly, hepatotoxic effect of mancozeb exposure in male rats also suggested (Kwon *et al* 2018).

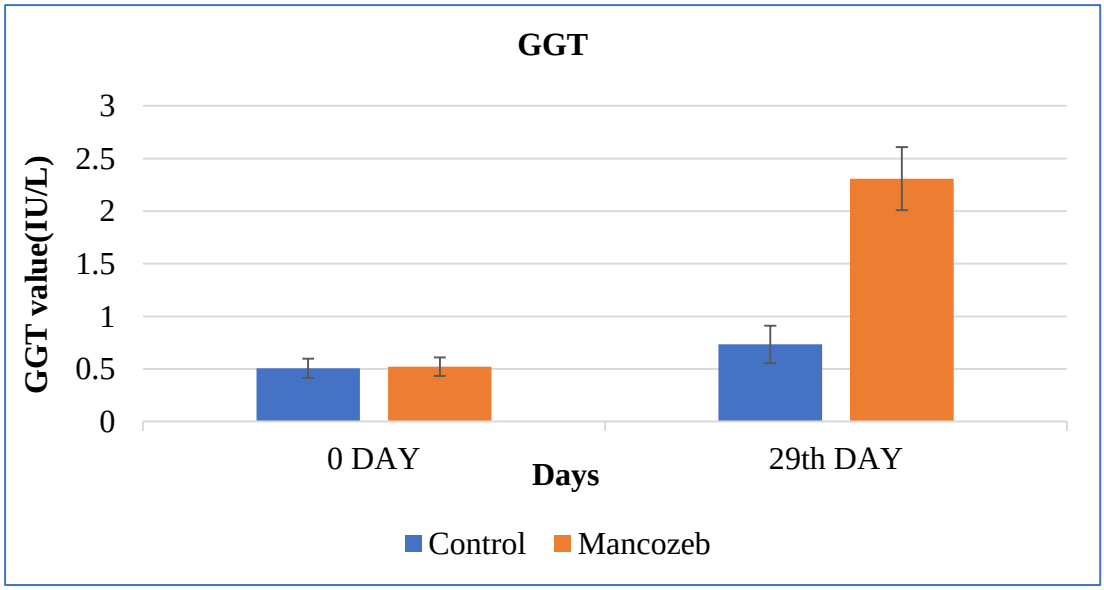


Figure 11: Blood Gamma glutamyl transpeptidase variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.3.5 Glucose

Glucose is formed by digestion of carbohydrates and the conversion to glycogen in the liver is the main source of energy for most of the body cells. It is

regulated by insulin, glucagon, liver enzymes, thyroid hormone and adrenal hormones. It is elevated in diabetes, liver disease, obesity, pancreatitis, steroids, stress or diet. Low levels may be indicative of liver disease, overproduction of insulin, hypothyroidism and alcoholism. Repeated oral administration of mancozeb for 28 consecutive days, produced a significant decrease in blood glucose levels when compared to control group as well as day 0 glucose levels of treatment group (Table 7, Fig. 12). Yahia *et al* (2019) also reported similar decrease in glucose level due to mancozeb exposure in Sprague Dawley rats. In contrast to our finding, increase in glucose was observed in common carp by Simakani et al (2018). The metabolites of mancozeb in ETU, EU and natural products may cause depletion of glycogen in the body. During exposure to sub lethal concentration of mancozeb, animals may have come under stress condition and needed more energy to cope up with the toxicant. Depletion of glycogen in the liver and tissue may be due to increament in glycolysis pathway. During stress condition the glycogen reserve depletes to meet the energy demand (Srivastava and Singh 2013). In support of present results, Ksheerasagar and Kaliwal (2010) reported a significant decrease in the levels of blood glucose and globulin in mancozeb-treated rats, due to low thyroxine level as a result of impaired thyroid function.

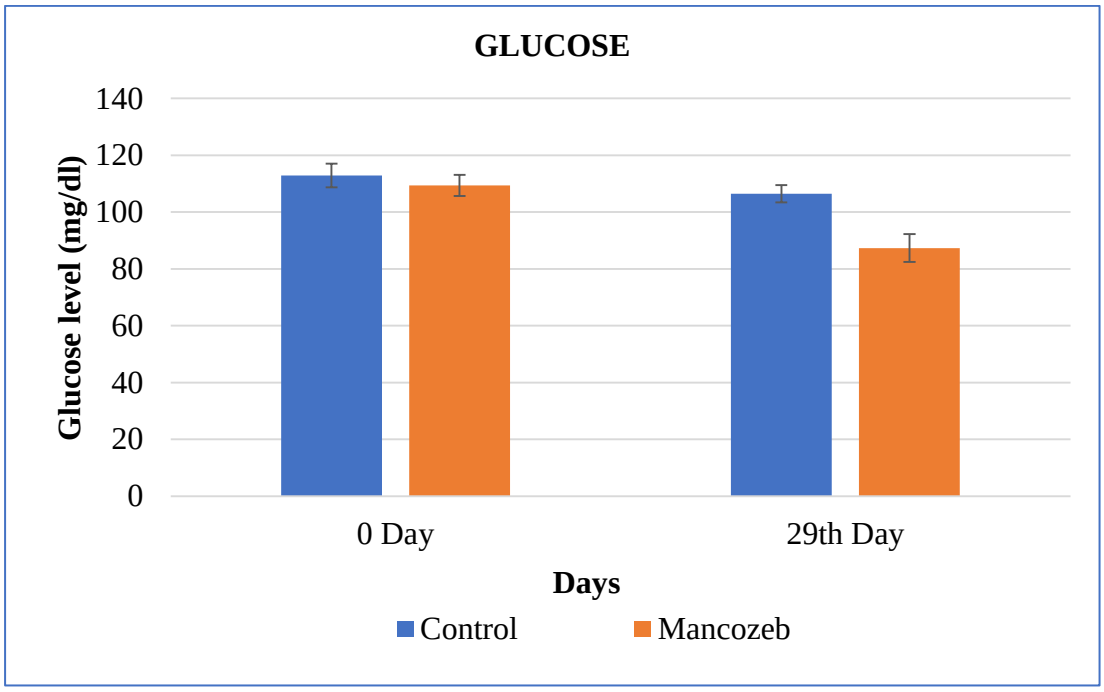


Figure 12: Blood glucose variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.3.6 Alkaline phosphatase (ALP)

A significant increase in the activity of ALP was observed in male rats exposed to mancozeb at dose rate of 500 mg/kg for 28 consecutive days when compared with control group and zero day ALP activity of treatment group (Table 7, Fig. 13).

Alkaline phosphatase is a hydrolase membrane bound enzyme that is secreted into the bile. It hydrolyzes monophosphates at an alkaline pH. It is particularly present in the cells which line the biliary ducts of the liver. ALP is excreted normally via bile in the liver. Apart from liver, it is also found in other organs including bone, placenta, kidney and intestine (Gaw *et al* 1998). Elevation of ALP indicates the disturbed excretory function of liver, cholestasis, biliary cirrhosis, fatty liver etc (Pagana and Pagana 1998). Our results are in agreement with previous studies (Djeffal *et al* 2015; Dhouib *et al* 2015; Apaydin *et al* 2017; El-Bieh *et al* 2017) undertaken in male Wistar rats. Similarly, alkaline phosphatase (ALP) activity increased significantly on mancozeb exposure (Yahia *et al* 2014). The rise in the liver and serum levels of ALP indicates possible disturbance taking place in hepatic dysfunction of animals. Elevated activity of ALP in the liver and serum may be considered to be associated as a cholestatic injury. Yahia *et al* (2015) reported that serum level of alkaline phosphatase (ALP) was increased significantly in mancozeb exposed male wistar rats at 1000 mg/kg. Kacker *et al* (1997) also observed increase in ALP activity in male rats with mancozeb exposure.

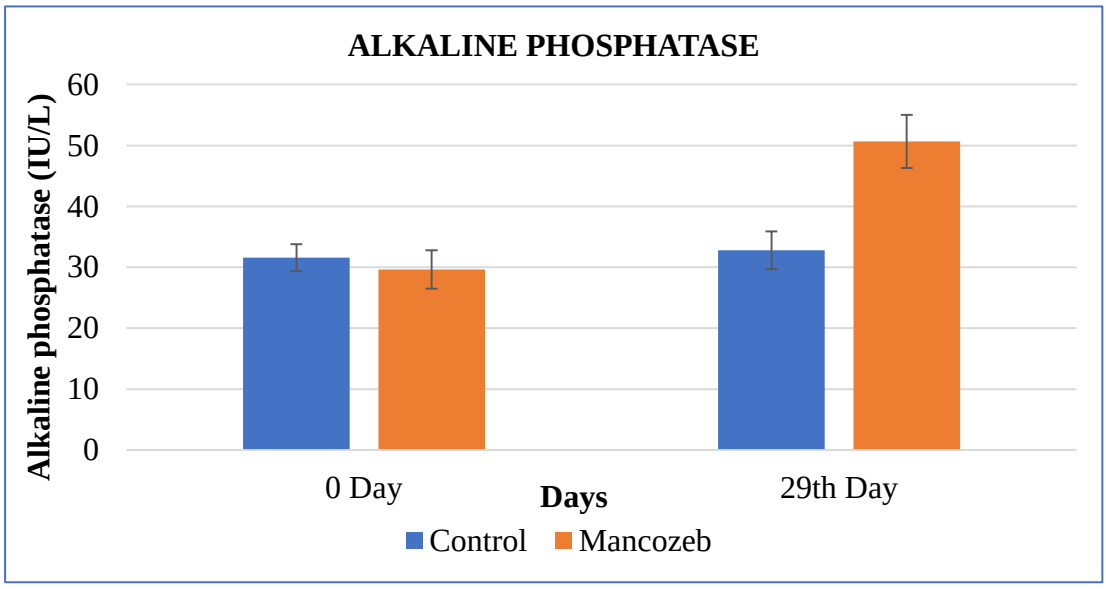


Figure 13: Blood alkaline phosphatase variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

Table 8: Blood Biochemistry variations in parameters indicating renal function after sub-acute exposure to mancozeb *per os* in male rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
Creatinine level (mg/dl)					
Control	1.79±0.33	2.14^{A} ±0.35	<0.001	<0.001	<0.001
Treatment	1.87^{a}±0.27	6.25^{Bb}±0.49			
Blood urea nitrogen (mg/dl)					
Control	5.91±0.48	6.53^{A}±0.70	<0.001	<0.001	<0.001
Treatment	6.01^{a}±0.48	27.41^{Bb}±1.67			
Total plasma protein (g/dl)					
Control	9.31±0.84	8.97^{A}±0.89	0.011	0.009	0.024
Treatment	9.04^{b}±0.92	4.74^{Ba}±0.50			
Creatine kinase (IU/L)					
Control	2.13±0.61	3.23^{A} ±0.74	<0.001	<0.001	<0.001
Treatment	2.54^{a}±0.50	9.77^{Bb}±0.85			

Means bearing superscripts $^{a, b}$ differ significantly in a row. Means bearing superscripts $^{A, B}$ differ significantly in a column.

4.3.7 Creatinine level

Creatinine is a waste product formed in muscle from the high energy storage compound, creatine phosphate. The amount of creatinine produced is fairly constant (unlike urea) and is primarily a function of muscle mass. It is removed from plasma by glomerular filtration and then excreted in urine without any appreciable reabsorption by the tubules. Creatinine is an useful indicator of renal function.

A significant increase was observed in creatinine level in the present investigation, due to repeated mancozeb exposure in treatment group when compared with control group as well as day 0 creatinine level of treatment group, indicating kidney impairment (Table 8, Fig. 14). The finding reported in the present investigation is in congruence to the findings of Zakharov *et al* (2016) and Hamdi *et al* (2019) who reported elevation of creatinine in a 48year old man having 75kg bwt and wistar rat, respectively, with a history of fungicide exposure. Yahia *et al* (2015) also reported a significant increase in creatinine levels after decrease in total protein

values. In contrast to our findings, Ahmed and Gamila (2017) observed decrease in creatinine levels in mancozeb exposed rats. Talha *et al* (2006) also showed significant alteration in creatinine levels due to mancozeb treatment in male wistar rats.

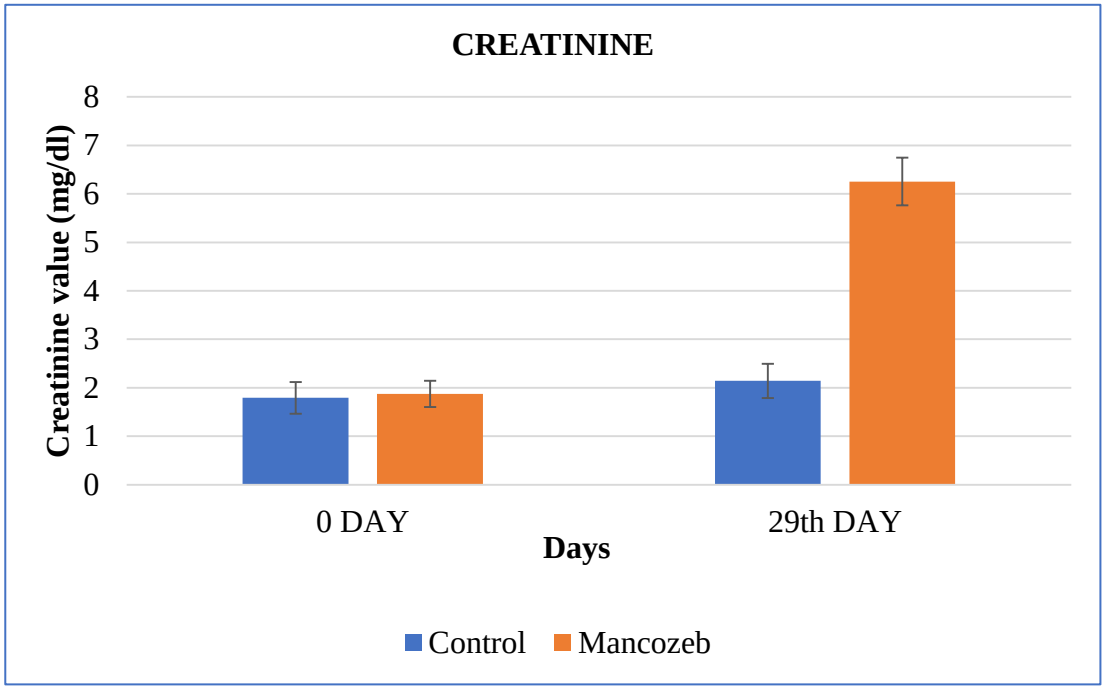


Figure 14: Blood creatinine variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.3.8 Blood urea nitrogen (BUN)

The level of BUN increased significantly in mancozeb exposed male rats as compared to control group (Table 8, Fig. 15). Urea is the major end product of protein metabolism and constitutes the largest fraction of the non-protein nitrogen component of the blood. Urea is produced in the liver and excreted through the kidneys in the urine. Elevated serum urea concentrations are observed in impaired kidney function, liver diseases and diseases which impair kidney function. Escalation of urea concentration following exposure of carbamates in male Wistar rats has also been reported in previous studies (El-Bieh *et al* 2017 and Djeffal *et al* 2015). Yahia *et al* (2015) also reported a significant increase in BUN after decrease in total protein values. In contrast to our findings, Ahmed and Gamila (2017) observed decrease in BUN in mancozeb exposed rats. Talha *et al* (2006) also showed significant alteration in BUN due to mancozeb treatment in male wistar rats.

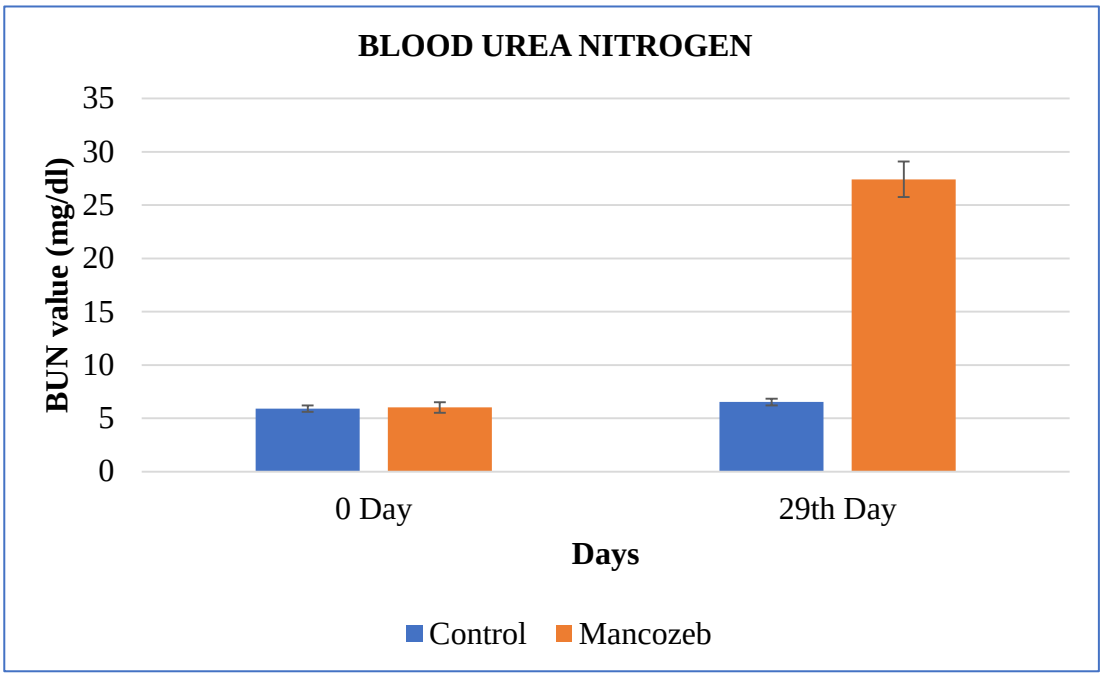


Figure 15: Blood urea nitrogen variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.3.9 Total plasma protein (TPP)

Total protein is useful for monitoring gross changes in protein levels caused by various disease states. Decrease in levels of total protein is seen in subjects with renal dysfunction. Albumin is a major plasma protein, synthesized in the liver from amino acids which are absorbed from ileum. Its function includes regulation and distribution of extracellular fluid, transportation of various hormones, vitamins and trace metals. A significant reduction in total protein level was noticed in the present investigation, due to repeated mancozeb exposure in treatment group when compared with control group as well as day 0 total protein level of treatment group (Table 8, Fig. 16).

In agreement to our study, decrease in plasma protein was also reported by (Simakani *et al* 2018), who concluded that mancozeb exposure causes stress response, health problem and tissue damage in common carp. Decrease in the level of albumin is seen in cases of kidney damage causing excessive protein loss and decreased synthesis due to dietary deficiency. Stephenson and Trombetta (2020), who reported decrease in total protein in rats after mancozeb exposure. Similar to our findings, Ksheerasagar and Kaliwal (2010) reported that mice treated with 800 mg/kg/ day

mancozeb, it caused significant decrease in the level of protein in testis, liver and in kidney. The changes in the levels of protein with mancozeb treatment may suggest either an increased catabolism of the biomolecules to meet the enhanced energy demand of animals under stress or their reduced synthesis due to impaired tissue function. Yahia *et al* (2015) also reported a significant decrease in total protein values after mancozeb exposure in male wistar rats. Talha *et al* (2006) also showed significant alteration in total protein due to mancozeb treatment in male wistar rats.

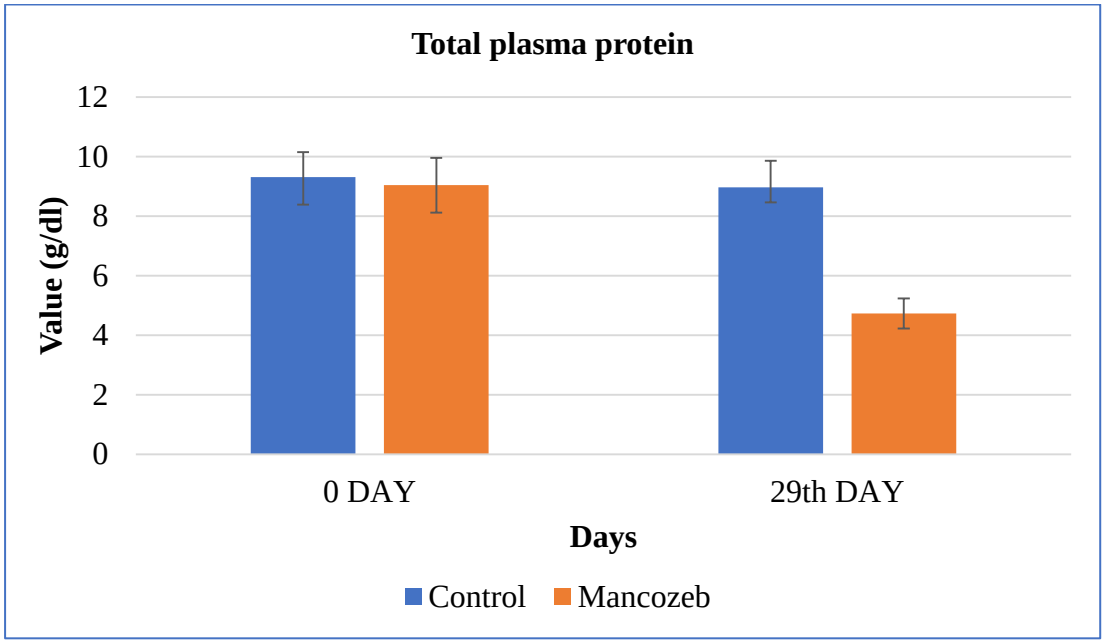


Figure 16: Total plasma protein variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

The changed biochemical constituents in the present study may be due to increased catabolism of biochemicals to meet the enhanced energy demand of animals under stress or due to an effect on synthesis resulting from impaired tissue function (Baligar and Kaliwal 2004). The administration of the pesticide has been suggested to decrease the protein and glycogen content of heart muscle, pancreatic tissue, and hepatic tissue (Umadevi 1976, Siddiqui *et al* 1987). Mancozeb is known to produce it biological effects through metabolites like ethylenethiourea and carbon disulphide, thereby altering biochemical constituents in normal and hemicastrated rats (Thorn and Ludwing 1962).

In a study reported by Kubrak *et al* (2012) the total blood protein concentration was reduced by in fish exposed to the lowest concentration of

mancozeb (3 mg/ L) whereas, at higher exposure levels this parameter did not differ from control group values, which suggested that low and high pesticide concentrations differentially affect blood protein content. The decrease in total protein and increase in free amino acids level in both tissue and liver suggest the high protein hydrolytic activity due to elevation of protease activity. Increase in free amino acid level may be due to breakdown of protein for energy requirements and impaired incorporation of amino acids in protein synthesis and decline in nucleic acid levels (Srivastava and Singh 2013). Mahadevaswami *et al* (2000) reported that the levels of protein of the liver, uterus and ovary of the sham-operated rats were significantly decreased when compared with hemicastrated (HC) control rats.

4.3.10 Creatine kinase (CK)

A significant increase in CK activity was seen in male rats exposed to mancozeb for 28 days in comparison to non-treated group as well as zero day CK activity of treatment group (Table 8, Fig. 16).

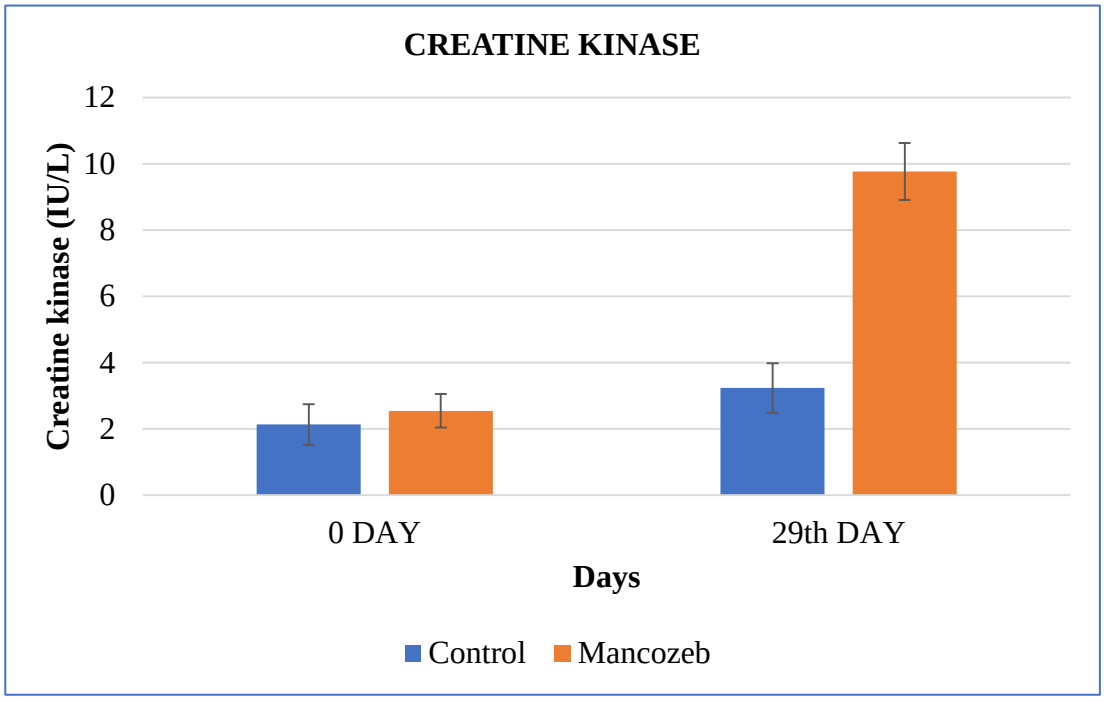


Figure 17: Blood creatinine kinase variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

CK is found in cardiac, skeletal and cerebral muscle tissues, CK allows energy storage as creatine phosphate when demand is low, but when energy is needed for muscle contraction, CK catalyzes the transfer of high energy phosphate from creatine

phosphate to ADP from ATP. CK activity is in greatest concentration in skeletal muscle followed by heart muscle, diaphragm and smooth muscle and then brain (Keller 1981). CK activity is mainly used as a marker of skeletal muscle injury associated with trauma, nutritional myopathies, exercise induced muscle injury and congenital myopathies. The significant increase in CK activity in our study may be correlated with the adverse effects observed in cardiac muscles during histopathological examination. In contrast to our findings, Stephenson and Trombetta (2020) reported decrease in creatine kinase levels due to dithiocarbamates exposure in Long-Evans rats on striated muscle tissues.

Table 9: Blood Biochemistry variations in parameters indicating lipid profile after sub-acute exposure to mancozeb *per os* in male rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
HDL-Cholesterol (mg/dl)					
Control	249.9±20.24	258.6^{A}±24.12	0.002	<0.001	0.001
Treatment	242.3^{a}±24.01	445.1^{Bb}±31.78			
LDL- Cholesterol (mg/dl)					
Control	62.09±1.68	66.36^{A}±1.71	0.014	<0.001	<0.001
Treatment	58.50^{a}±1.38	81.16^{Bb}±3.11			
Triglycerides (mg/dl)					
Control	1504.7±111.5	1497.6^{A}±115.2	<0.001	<0.001	<0.001
Treatment	1513.8^{b}±110.96	636.5^{Ba}±55.55			

Means bearing superscripts $^{a,\ b}$ differ significantly in a row. Means bearing superscripts $^{A,\ B}$ differ significantly in a column.

4.3.11 HDL and LDL cholesterol

Liver is the major site for cholesterol synthesis and metabolism. Hepatic cholesterol homeostasis is maintained by equilibrium between the activities of the hydroxyl methyl glutryl Coenzyme A reductase and acyl co enzyme A cholesterol acyl transferase. Low density lipoproteins (LDL) are synthesized in the liver by the action of various lipolytic enzymes on triglyceride rich very low density lipoproteins

(VLDL). Specific LDL receptors exist to facilitate the elimination of LDL from plasma by liver parenchymal cells. It has been shown that most of the cholesterol stored in atherosclerotic plaques originates from LDL. For this reason the LDL cholesterol concentration is considered to be the most important clinical predictor, of all single parameters, with respect to coronary atherosclerosis. LDL is also termed as bad cholesterol. The increase in LDL values seen in mancozeb exposed group indicates that the rats exposed to mancozeb is likely to develop high risk for heart diseases. High-density lipoproteins (HDL) compose one of the major classes of plasma lipoproteins. They are synthesized in liver as complexes of apolipoprotein and phospholipid and are capable of picking up cholesterol and carrying it from arteries to the liver, where the cholesterol is converted to bile acids and excreted into the intestine. HDL is also referred to as good cholesterol. A significantly higher level of HDL and LDL cholesterol were observed in male rats of mancozeb-treated group, as compared to rats of control and zero day mancozeb group (Table 9, Fig. 18,19). In agreement to our findings, hypercholesterolemia was observed due to pesticide exposure in rabbits of genus *Cuniculus lepus* (Assia *et al* 2020). Ahmed and Gamila (2017), reported significant increase in HDL and LDL-cholesterol along with significant decrease in triglycerides after mancozeb exposure, similar to our findings. Joshi *et al* (2005) also reported significant increase in testicular cholesterol. Ksheerasagar and Kaliwal (2010) reported that mice treated with 600 mg/kg/ day mancozeb showed a significant increase in the level of total lipids in liver and a significant decrease in glycogen and total lipids in kidney. Again, in mice treated with 800 mg/kg/ day mancozeb, it caused a significant increase in the level of total lipids in testis. The changes in the levels of total lipids with mancozeb treatment may suggest either an increased catabolism of the biomolecules to meet the enhanced energy demand of animals under stress or their reduced synthesis due to impaired tissue function. Kacker *et al* (1997) also observed increase in serum cholesterol in male rats with mancozeb exposure.

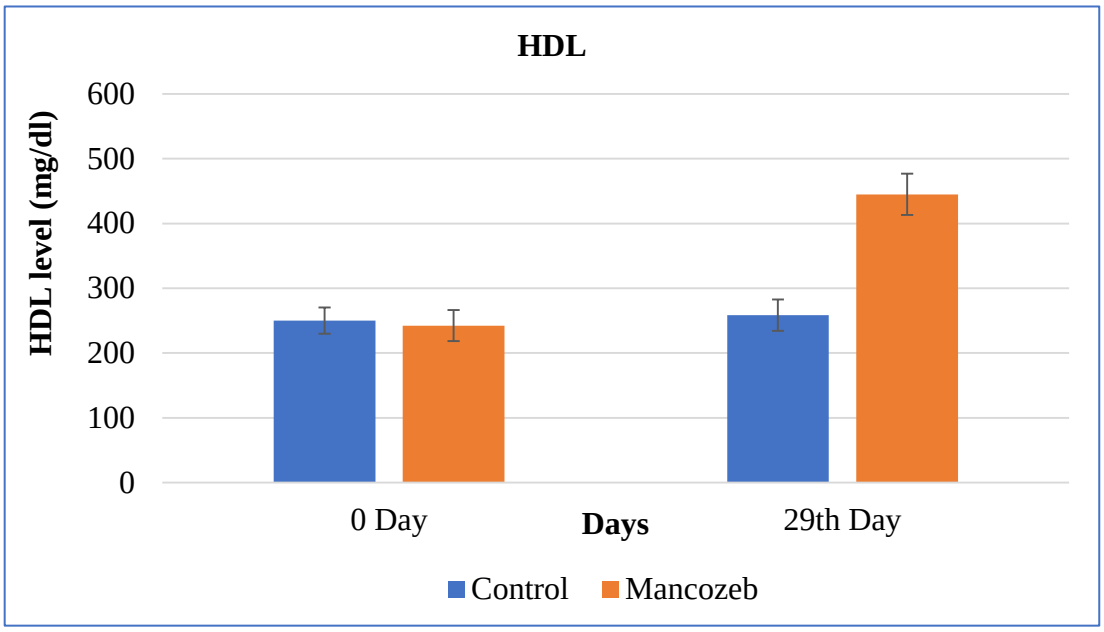


Figure 18: Blood High density lipoprotein variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

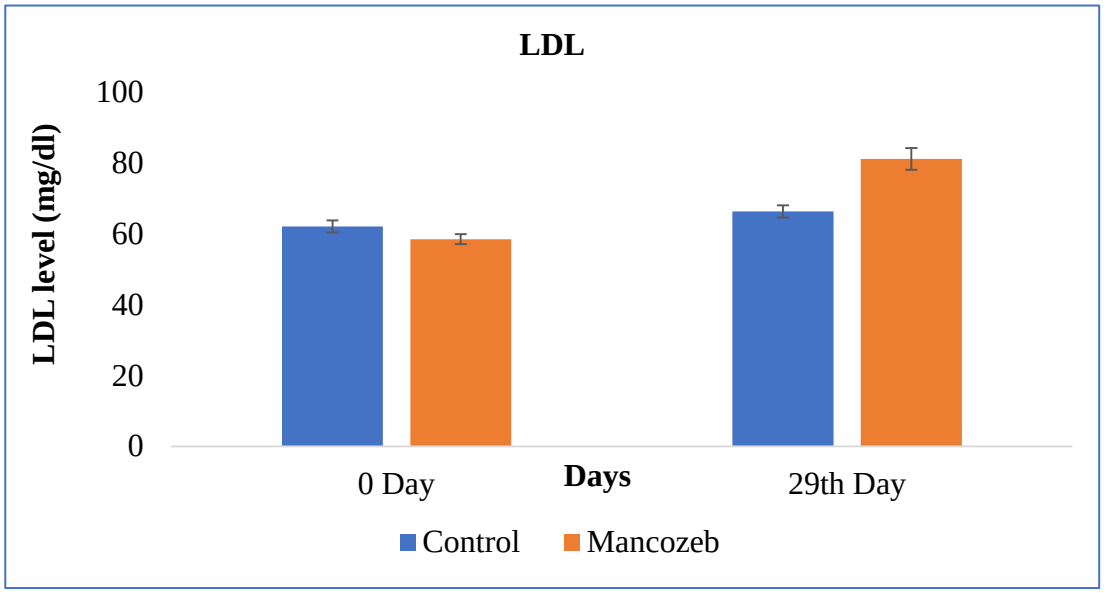


Figure 19: Blood low density lipoprotein variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.3.12 Triglycerides

Triglycerides are the family of lipids absorbed from diet and produced endogenously from carbohydrates. Measurement of triglycerides is important in the diagnosis and management of hyperlipidemias. These diseases can be genetic or secondary to other disorders including nephrosis, diabetes mellitus and endocrine

disturbances. Repeated mancozeb exposure produced a significant reduction in triglycerides level when compared with control group as well as zero day treatment group (Table 9, Fig. 20). Similarly, decrease in triglyceride levels were reported due to mancozeb exposure in rats by Ahmed and Gamila (2017). On the contrary, Yahia *et al* (2019) observed elevation in triglyceride concentration in Sprague Dawley rats after mancozeb exposure. Mahadevaswami *et al* (2000) reported that the levels phospholipids and neutral lipids of the liver, uterus, and ovary and uterine total lipids of the sham-operated rats were significantly decreased when compared with hemicastrated (HC) control rats. As there was a significant decrease noticed in the levels of phospholipids and neutral lipids in the liver, uterus, and ovary and uterine total lipids of HC rats treated with 600, 700, and 800 mg/kg/day of mancozeb when compared with HC control rats. These observed effects of mancozeb on biochemical constituents may be due to hormonal imbalance in any of the stages in the hypothalamo – hypophysial –thyroid axis. Mancozeb though have acute mammalian toxicity exhibit significant toxicological effects after repeated exposure.

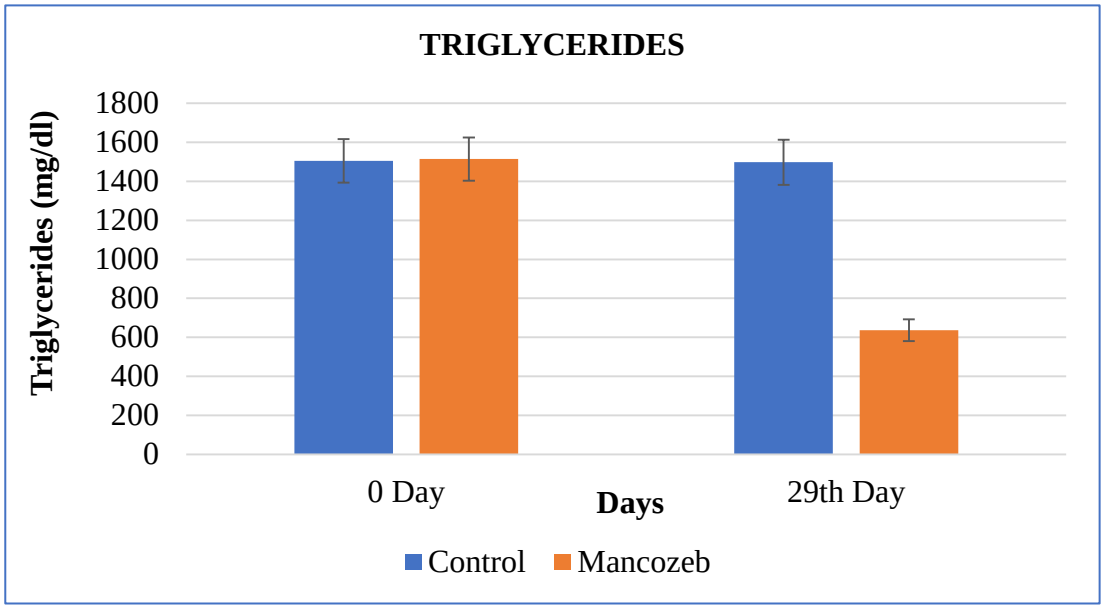


Figure 20: Blood Triglyceride variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.4 Effect of sub-acute mancozeb exposure on endocrine parameters in rats

For evaluation of toxic manifestation of mancozeb exposure on hormone status, following endocrine parameters were analyzed on day 0 and 29th day of experimentation.

Table 10: Variations in Endocrine parameters after exposure to Mancozeb *per os* in male rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
Thyroid Stimulating Hormone (µIU/ml)					
Control	0.153±0.03	0.155^{A}±0.04	0.001	0.001	0.001
Treatment	0.157^{a} ±0.04	0.458^{Bb}±0.04			
Tri-iodothyronine (ng/ml)					
Control	3.12±0.68	2.56^{A}±0.44	0.204	0.016	0.129
Treatment	3.26^{b}±0.57	1.01^{Ba}±0.37			
Thyroxine (µg/dl)					
Control	19.6±2.05	17.6^{A}±2.64	0.006	0.003	0.030
Treatment	18.03^{b}±2.09	6.06^{Ba}±1.51			

Means bearing superscripts $^{a, b}$ differ significantly in a row.
Means bearing superscripts $^{A, B}$ differ significantly in a column.

4.4.1 Thyroid stimulating hormone (TSH)

Thyroid stimulating hormone is a glycoprotein hormone secreted by the pituitary gland and regulates the synthesis or release of T_3 and T_4 by thyroid gland. TSH has two subunits, namely alpha and beta. The alpha subunit is similar to alpha subunit found in LH, FSH and hCG glycoprotein hormones. However, beta subunit is specific and differs from hormone to hormone. The serum TSH measurement is one of the most important tools in the diagnosis of thyroid disorders. Increased serum TSH is an early and sensitive indicator of decreased thyroid reserve and overt primary hypothyroidism. Decreased TSH level is an indicator of TSH-dependent hyperthyroidism (Graves disease). Repeated mancozeb exposure to wistar rats for 28 days produced a significant elevation in TSH concentration when compared with control group as well as zero day treatment group (Table 10, Fig. 21). In agreement to our study, Srivastava and Singh (2013) also reported the thyroid disrupting potential of ethylenethiourea which is main metabolite of mancozeb. Pandey and Mohanty (2015) reported that the plasma TSH was increased in response to decrease of T_4 on mancozeb exposure showing responsiveness of the hypothalamic–pituitary–thyroid

(HPT) axis to feedback regulation to elucidated the thyroid disrupting potential of a neonicotinoid imidacloprid and a dithiocarbamate mancozeb in a seasonally breeding wildlife bird, Red Munia (*Amandava amandava*). The results are consistent with the finding of (WHO 1988, Trivedi *et al*1993, Kackar *et al* 1997, Hurley *et al*1998), increase in production of thyroid stimulating hormone (TSH) and thyroid weight, causing hyperplasia and hypertrophy of follicular cells in the thyroid, as well as thyroid cancer due to mancozeb exposure.

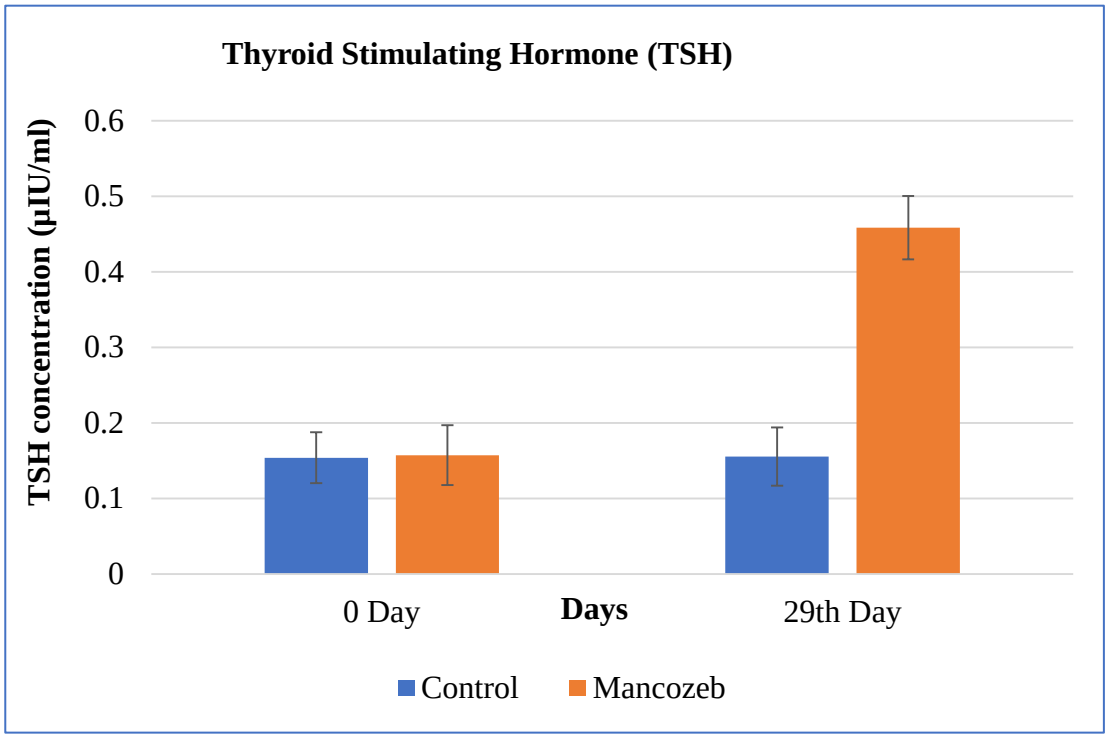


Figure 21: Blood Thyroid stimulating hormone (TSH) variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.4.2 Total tri-iodothyronine (tT_3)

Total tri-iodothyronine is a useful marker for the diagnosis of hypothyroidism and hyperthyroidism. The level of tT_3 is decreased in hypothyroid patient and is increased in hyperthyroid patients (Graves disease). A significant depreciation in tT_3 concentration in serum was observed in mancozeb administered group rats as compared to control group and zero day treatment group (Table 10, Fig. 22). In support to our results, Kwon *et al* (2018) also showed the decrease in serum levels of T_3 in rats with mancozeb diet.

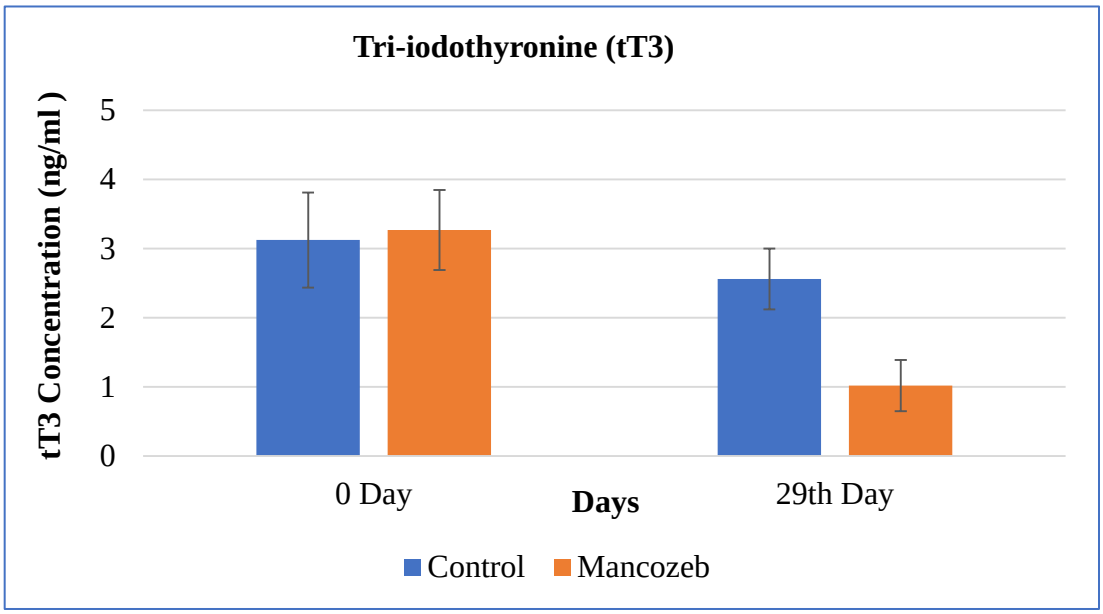


Figure 22: Blood Tri-iodothyronine (tT_3) variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.4.3 Total thyroxine (T_4)

Total thyroxine is a useful marker for the diagnosis of hypothyroidism and hyperthyroidism. The levels of total T_4 is decreased in hypothyroid patients increased in hyperthyroid patients and are normal in euthyroid individuals. Repeated mancozeb exposure to wistar rats resulted into a significant decrease in T_4 concentration when compared with control group as well as zero day treatment group (Table 10, Fig. 23). In agreement to our study Axelstad *et al* (2011) reported that mancozeb exposure reduced T_4 levels in dams which may have led to potential contribution to thyroid disruption and adversely affected the developing brain in human. Pandey and Mohanty (2015) elucidated the thyroid disrupting potential of a neonicotinoid imidacloprid and a dithiocarbamate mancozeb in a seasonally breeding wildlife bird, Red Munia (*Amandava amandava*) and reported that the plasma TSH was increased in response to decrease of T_4 on mancozeb exposure showing responsiveness of the hypothalamic–pituitary–thyroid (HPT) axis to feedback regulation. Our results are further in agreement with Goldner *et al* (2010) who reported association of increased incidence of thyroid disease with mancozeb exposure in female spouses of pesticide applicators. The results are consistent with the finding of (WHO 1988, Trivedi *et al*1993, Kackar *et al* 1997b, Hurley *et al*1998), who reported decrease in serum thyroxine (T_4) levels, thyroid peroxidase activity and iodine uptake, and increase in production of thyroid stimulating hormone (TSH) and thyroid weight, causing

hyperplasia and hypertrophy of follicular cells in the thyroid, as well as thyroid cancer due to mancozeb exposure. Boberg *et al* (2011) also reported decrease levels of T_4 in rat dams, which indicated that mancozeb exposure may be a potential contributor to thyroid disruption in humans. Moreover, Bano *et al* (2020) also reported thyroid disrupting potential of mancozeb which was evident by hypothyroidism. Adverse effect of mancozeb on thyroid gland was also reported by Ksheerasagar and Kaliwal (2010). Kwon *et al* (2018) also showed the decrease in serum levels of T_4 in rats with mancozeb diet.

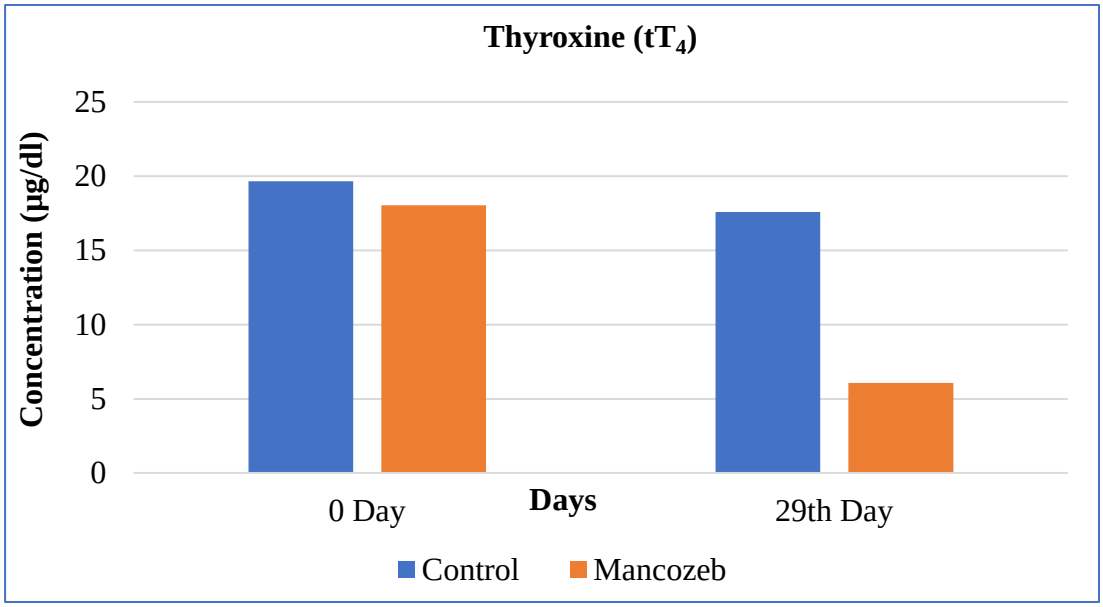


Figure 23: Blood Thyroxine (tT_4) variations after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.5 Assessment of neurobehavioral parameters in male wistar rats after sub-acute mancozeb exposure.

Following neuro- behavioral acivities were analysed on day 0 and 29th day of experimentation for evaluation of toxic manifestation of repeated mancozeb exposure for 28 consecutive days.

4.5.1 Elevated Plus Maze (EPM)

The Elevated plus-maze test is commonly used to study anxiety-like behaviour in laboratory animals (Rodents). Handley and Mithani (1984) first suggested the Elevated plus maze (EPM), the most common animal model of anxiety/depression assessment under clinical practice (File et al 1990). The test is based on the concept that rodents have a strong propensity to discover new habitats and inherent avoidance of vulnerable light, and elevated areas (represented by the open arms). The Maze consists

of two open (stressful) and two enclosed (protecting) elevated arms that form a "plus". The security is provided by the closed arms of plus Maze whereas the open arms offers exploratory value. EPM is an established model for assessment of anxiety. All rats were exposed to EPM for 5 minutes and behavior was recorded with the help of camera attached to software (Orchid All maze software). Elevated plus maze is the most simplest apparatus to study neuroprotective effect (Latha *et al* 2015) and anxiolytic response produced by test drug. It is used to test almost all types of anxiolytic agents. Exposure of animals to novel maze alley evokes an approach-avoidance conflict which is stronger in open arm as compared to enclosed arm. Rodents have an aversion for high and open space and prefer enclosed arm, therefore, spend a greater amount of time in enclosed arm. When animals enter open arm, they freeze, become immobile, defecate and show fear-like movements (Pellow *et al* 1985).

Rats have an innate fear of height, and when placed at the point of intersection on plus maze, they tend to spend most of the time in closed (hide) area. When they become anxious, the natural tendency of rodents is to prefer closed dark spaces rather than opened brightly light spaces. In this test, anxiety-related behaviour is measured by the degree to which the rodent avoids the closed arms of the maze. Plus maze is also used to study various behaviours of animal like number of entries into the zones, Rearing, Stretching, Curled Up, Time spent in particular zone, Sleeping, Head movements, Total Rotation, Time spent in immobility, Time spent moving slowly and Time spent moving fast.

In male rats exposed to mancozeb, a significantly higher open arm and sleeping time were seen, as compared to control group. A significant reduction was observed in locomotion, hide arm activity, curled up and travelled distance in mancozeb exposed group as compared to control group, indicating anxiolytic effect of mancozeb exposure in male wistar rats (Table 11, Fig. 24,25). In agreement to our study, increasing evidence has shown a strong association between pesticides and neurodegenerative diseases. Domico *et al* (2006) reported that mancozeb (MZ) and maneb (MB), both widely used EBDC fungicides, are equipotent neurotoxicants that produce cell loss in mesencephalic dopaminergic and GABAergic cells. The finding reported in the present investigation is in congruence to the findings of Boberg *et al* (2011) who investigated the effect of mancozeb exposure on neurological development of the offspring and observed that mancozeb exposure induced neurotoxicity in dams.

Table 11: Variations in Elevated Plus Maze parameters after exposure to mancozeb per *os* in male wistar rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
Locomotion (sec)					
Control	69.36±15.8	73.8^{B}±13.97	0.030	0.118	0.063
Treatment	64.56^{b}±14.22	16.93^{Aa}±7.11			
Hide (sec)					
Control	286.13±5.87	287.03^{B}±4.54	<0.001	<0.001	<0.001
Treatment	281.53^{b}±8.51	109.03^{Aa}±6.48			
Head out (sec)					
Control	45.03±16.85	51.53^{B}±13.36	0.265	0.335	0.163
Treatment	49.3^{b}±17.17	15.03^{Aa}±5.82			
Open area (sec)					
Control	22.9±3.78	27.76^{A}±3.94	<0.001	<0.001	<0.001
Treatment	24.7^{a}±4.51	176^{Bb}±18.04			
Sleeping (sec)					
Control	115.3±18.8	136.76^{A}±13.52	0.014	0.002	0.031
Treatment	121.86^{a}±12.79	223.13^{Bb}±22.07			
Curled-up(sec)					
Control	55.46±9.30	61.43^{B}±11.26	0.037	0.393	0.144
Treatment	48.83^{b}±7.15	26.73^{Aa}±8.74			
Travelled distance(mm)					
Control	4127.7±688.3	3953.7^{B}±636.4	0.048	0.018	0.033
Treatment	4236.9^{b}±750.11	1291^{Aa}±145.34			

Means bearing superscripts $^{a,\ b}$ differ significantly in a row. Means bearing superscripts $^{A,\ B}$ differ significantly in a column.

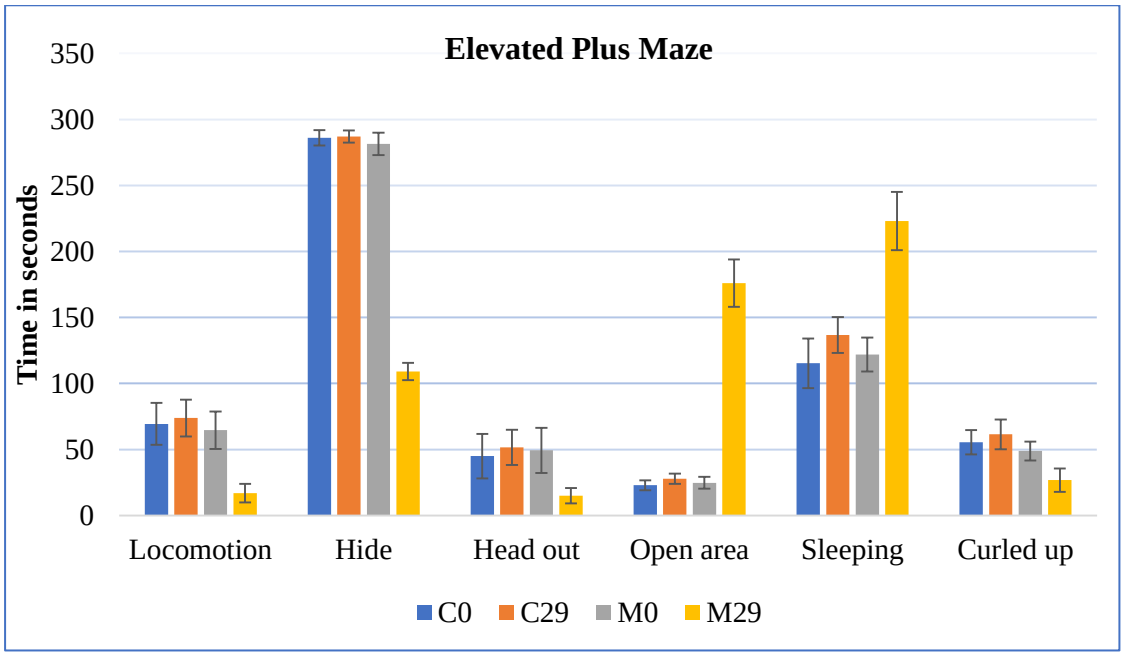


Figure 24: Variation in Elevated Plus Maze parameters after sub-acute exposure to mancozeb *per os* in male rats (n=6)

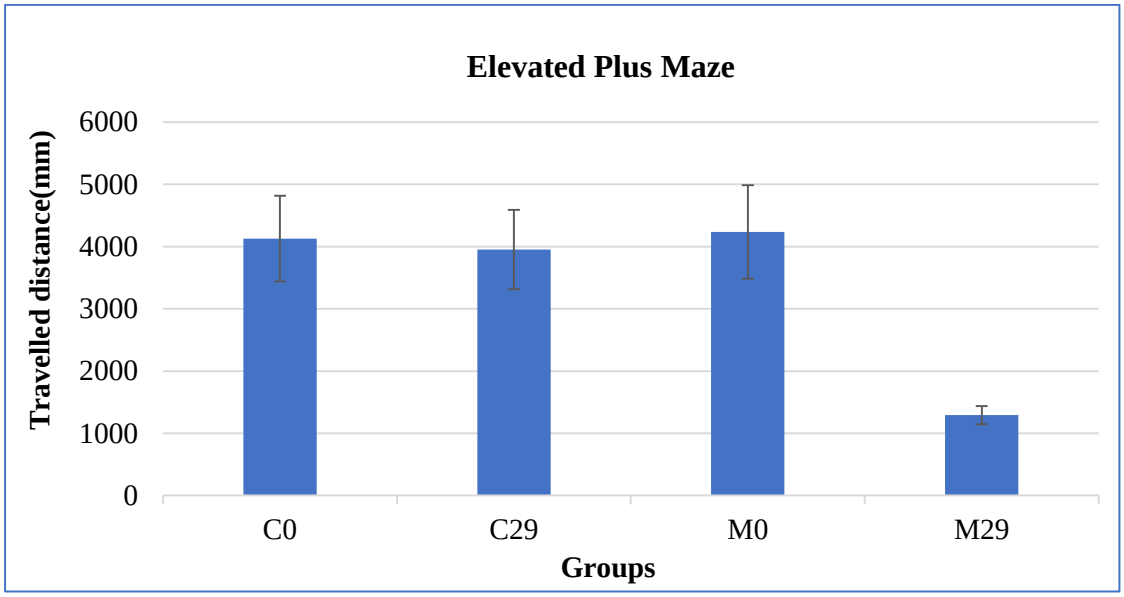


Figure 25: Variation in Elevated Plus Maze parameter (Travelled distance) after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.5.2 T-Maze

The T maze is commonly used for analysing spatial working memory and curiosity in rats and mice, especially for delayed alternation tasks. Using this maze one can observe different activities of animals such as side preference, approach to food or rewards, memory strength, immobility, number of head outs from one zone to other, time spent in each zone, stretch out, curled up, head movements,

number of contact to reward. A significant reduction in locomotion time, stretch out time and travelled distance was recorded in rats exposed to mancozeb as compare to control group. No significant alterations were recorded in immobility, curled up, head out and hide arm (Table 12, Fig. 26,27).

Table 12: Variations in T-Maze parameters after exposure to mancozeb per *os* in male wistar rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
Locomotion (sec)					
Control	82.5±7.25	78.4^{B}±8.44	0.001	0.002	0.007
Treatment	75.3^{b}±10.8	19.4^{Aa}±7.67			
Immobility (sec)					
Control	81.03±10.8	90.63±10.04	0.093	0.063	0.163
Treatment	86.8±23.3	149.5±24.16			
Curled-up (sec)					
Control	175.4±8.63	144.1±15.73	0.172	0.022	0.725
Treatment	159.7±13.9	117.9±18.9			
Stretch out (sec)					
Control	121.7±8.39	156.9^{A}±12.71	0.043	0.002	0.287
Treatment	137.3^{a}±14.24	204.7^{Bb}±20.65			
Head out 1 (sec)					
Control	11.2±2.71	15.8±3.98	0.159	0.055	0.120
Treatment	9.46±2.43	49.86±21.39			
Hide 1(sec)					
Control	209.2±36.12	164.3^{B}±24.2	0.054	0.023	0.256
Treatment	179^{b}±50.14	53.5^{Aa}±18.57			
Travelled distance (mm)					
Control	5404.6±670.16	4204.3^{B}±440.9	0.001	<0.001	0.020
Treatment	4709.4^{b}±563.3	960.1^{Aa}±232.5			

Means bearing superscripts $^{a,\ b}$ differ significantly in a row. Means bearing superscripts $^{A,\ B}$ differ significantly in a column.

Axelstad *et al* (2011) also reported the neurotoxic potential of mancozeb in rat dams. In another study it was suggested that the Mancozeb has been associated with human neurotoxicity and neurodegeneration and the possible mechanism of mancozeb toxicity in the nervous system might be the potentiation of $KCNQ_2$ potassium channels (Li *et al* 2013).

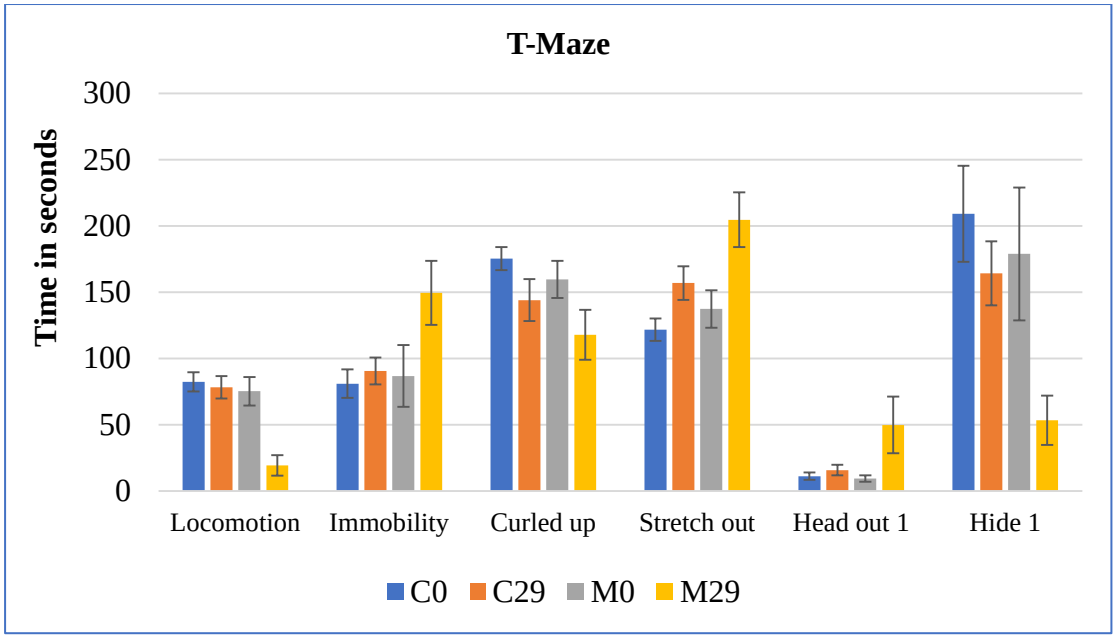


Figure 26: Variation in T-Maze parameters after sub-acute exposure to mancozeb *per os* in male rats (n=6)

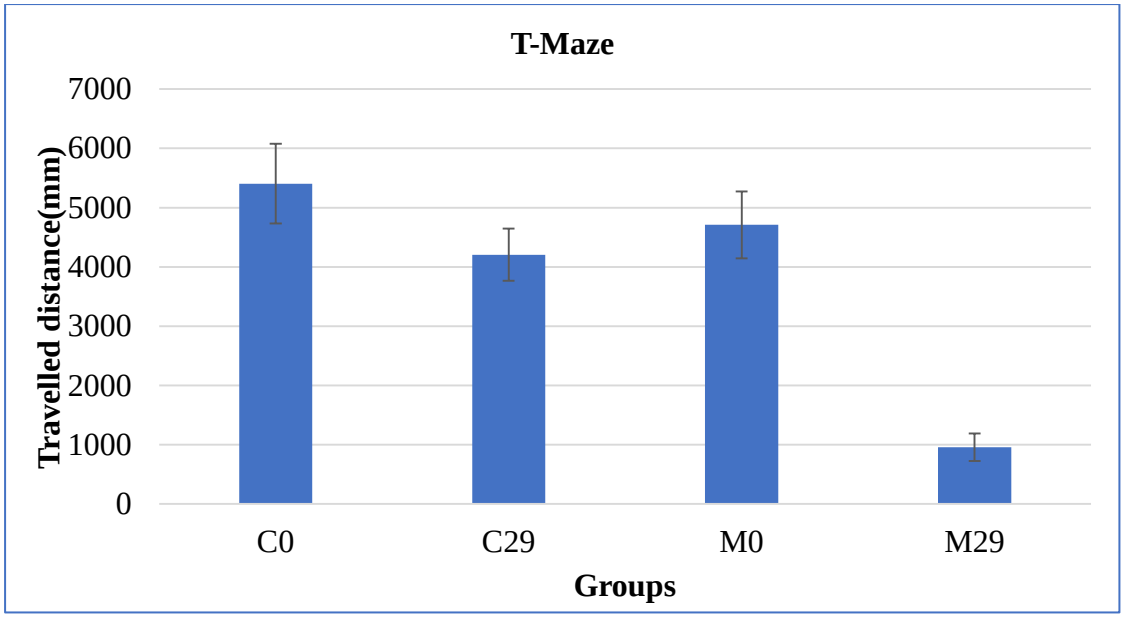


Figure 27: Variations in T-Maze parameter (Travelled distance) after sub-acute exposure to mancozeb *per os* in male rats (n=6)

4.5.3 Grip strength

Repeated mancozeb exposure led to significant reduction in grip strength in rats exposed to mancozeb as compared to control group (Table 13, Fig. 28). Dunham and Miya (1957) first identified the "moving rotor" as a method for assessing rodent neurological defects and used to access medication and genetic influences on rodent motor control (Crabbe et al 1999). The technique was developed decades earlier, to check the rodent griping power as a putative indicator of muscle strength and evidence of motor neurotoxicity (Maurissen *et al* 2003).

Table 13: Neurobehavioural assessment of male wistar rats after exposure to mancozeb per *os* in male wistar rats (n=6)

Groups	0 Day	29th Day	P value		
			G	T	G*T
Grip strength (Force, gf)					
Control	338.4^{a}±9.29	516.1^{Bb}±7.78	<0.001	<0.001	<0.001
Treatment	341.8^{a}±7.75	430.4^{Ab}±9.04			
Rota rod activity (sec)					
Control	20.50^{a}±1.65	15.33^{Bb}±1.25	0.006	<0.001	0.003
Treatment	20.83^{b}±1.90	5.83^{Aa}±0.94			
Acoustic/Auditory startle response					
Control	1.00±0.00	1.00±0.00	-	-	-
Treatment	1.00±0.00	1.00±0.00			
Corneal reflex					
Control	1.00±0.00	1.00±0.00	-	-	-
Treatment	1.00±0.00	1.00±0.00			
Righting reflex					
Control	1.00±0.00	1.00±0.00	-	-	-
Treatment	1.00±0.00	1.00±0.00			

Means bearing superscripts $^{a, b}$ differ significantly in a row. Means bearing superscripts $^{A, B}$ differ significantly in a column (values Mean±SE; n=6)

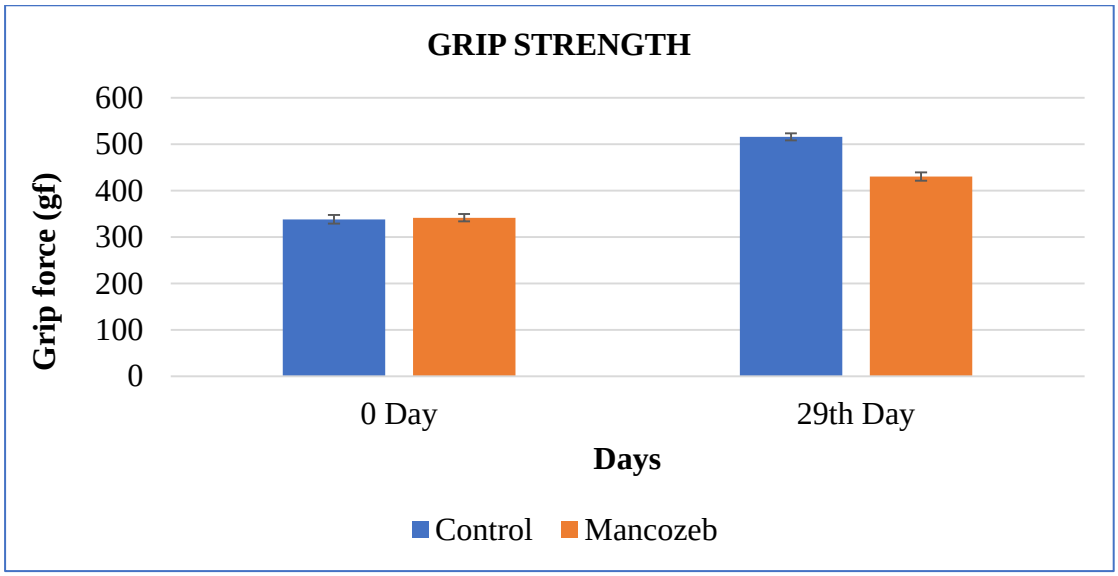


Figure 28: Variation in the grip strength force after sub-acute exposure to mancozeb *per os* in male rats (n=6)

Earlier experiments have shown that concentrations of pesticides and their metabolites in plasma and brain are usually associated with the extent of toxicity and the neurotoxicity symptom have been shown to increase with concentrations of pesticides in the brain (Sheets 1994, Nagata et al 1996). Transient reduced motor function with subchronic dietary exposure to chlorpyrifos and tebupirimphos (Mattson et al 1996, Sheets et al 1997), while, short-term oral parathion exposure induced specific neuromotor and a cognitive defect (Schulz et al 1990) have been reported. Sub-acute chlorpyrifos exposure via subcutaneous route reduced hind limb grip intensity. In addition, a decrease forelimb grip strength was recorded in wistar rats following exposure to carbendazim (Jacobsen et al 2004). Bhardwaj et al (2010) observed that IM exposure caused necrosis of Purkinje cells with loss of dendrites and granules in the cerebellum granular layer in rats. Abdel-Rahman et al (2001) reported neuronal failure in the cerebral cortex, and hippocampal development of adult rat cerebellum after subchronic dermal permethrin exposure. Zhu et al (2001) recorded declines in spontaneous locomotive behavior and neuromuscular control of repeated methyl parathione administration to rats. Adaptation of the skeletal muscle relies on the complex interplay between improvements in production and degradation of muscle proteins. Ubiquitin-proteasome and autophagic-lysosome processes are active in skeletal muscle protein degradation (Solomon and Goldberg 1996). Therefore, repeated mancozeb exposure might have accelerated protein degradation across both

the ubiquitin-proteasome and autophagy-lysosome processes, thus decreasing skeletal muscle mass and decreasing grip strength power.

4.5.4 Rota rod activity

A significant reduction in rota rod activity was observed in mancozeb-exposed rats as compared to control group rats, indicating adverse effect of mancozeb on the muscle coordination and muscle activity (Table 13, Fig. 29). Our results of present study was in congruence with the study reported by (Stephenson and Trombetta 2020) who suggested that dithiocarbamate compounds induce striated muscle toxicity in Long-Evans rats and Cardiac muscle architecturally appears more heavily affected than skeletal muscle after mancozeb treatment.

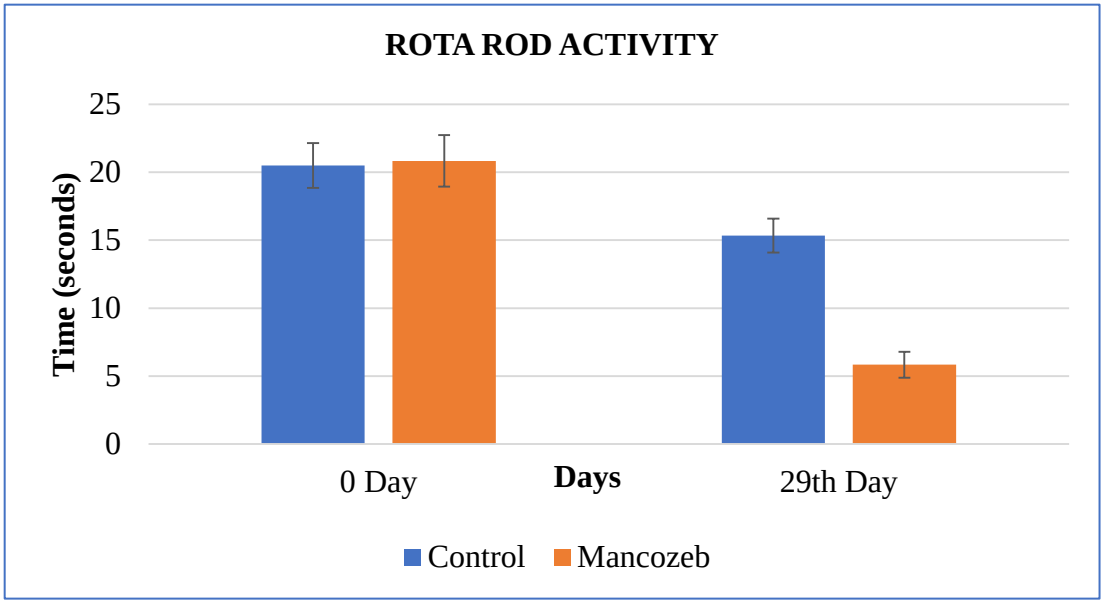


Figure 29: Variation in the Rota rod activity of control and mancozeb group rats (n=6)

4.5.5 Acoustic or auditory startle response

No significant alteration was observed in acoustic or auditory startle response on repeated mancozeb exposure in rats (Table 13). In agreement to our study Vemu (2015) and Gupta (2018), also observed no significant alteration in acoustic or auditory startle response in rats exposed to flubendiamide and thiodicarb, respectively.

4.5.6 Corneal reflex

In rats, sub-acute mancozeb exposure did not cause any significant alteration in corneal reflex when compared with control group (Table 13). In agreement to our

study Vemu (2015) and Gupta (2018), also observed no significant alteration in corneal reflex in rats exposed to flubendiamide and thiodicarb, respectively.

4.5.7 Righting reflex

There was no significant alteration in righting reflex noticed in rats exposed to mancozeb as compared to control group (Table 13). In agreement to our study Vemu (2015) and Gupta (2018), also observed no significant alteration in righting reflex in rats exposed to flubendiamide and thiodicarb, respectively.

4.6 Assessment of Acetyl choline esterase activity (AChE) in male wistar rats after sub-acute mancozeb exposure.

Acetyl choline esterase (AChE) activity was analyzed in the blood as well as in brain tissue to evaluate the adverse effect of repeated oral administration of mancozeb exposure in male wistar rats. Repeated oral administration of mancozeb resulted into inhibition of AChE activity in blood as well as in brain tissue of rats as compared to control group animals (Table 14; Fig. 30). Dithiocarbamates (DTC) may inhibit acetylcholine esterase (AChE) and this is responsible for degradation of the neurotransmitter acetylcholine, causing neuropathy (Edwards *et al* 1991). Administration of mancozeb has significantly inhibited the activity of AChE in the plasma and tissues. Our findings were supported by Dalvi (1992), who reported decrease in the activity of serum cholinesterase of exposed group animals which may have been due to slow enzyme synthesis in the damaged liver. Chemishanska (1982) has also reported disturbance in liver, kidney and central nervous system of rats exposed to higher concentration of mancozeb, mineb and zineb. In another study cholinesterase was markedly depressed to a different degree in plasma of mancozeb-treated rats (Ahmed and Gamila 2017). These findings also were confirmed by Wielgomas and Krechniak (2007), who noticed that cholinesterase was markedly depressed to a different degree in plasma and brain of animals receiving chlorpyrifos alone or in combination daily in rapeseed oil for 14 and 28 days. Also, Jacobsen *et al* (2004) reported that in a repeated dose 28 - day oral study, significant inhibition in plasma AChE in male rats exposed to chlorpyrifos at doses of 0.15 and 0.3 mg/kgbwt/day, was found. Mancozeb metabolites, Carbon disulphide and ethylene thiourea are involved in the dysfunction of the nervous system. Increased production of reactive oxygen species by the actions of mancozeb and zineb is also implicated in their neuronal toxicities (Domico *et al* 2007).

Cholinesterase enzymes (AChE) are essential for breakdown the acetylcholine (AChE) neurotransmitter into choline and acetic acid by the process of hydrolysis. This reaction is necessary to allow a cholinergic neuron to return to its resting state after depolarization. AChE is mostly found in many nerves, muscle, central and peripheral tissues, motor and sensory fibers and cholinergic and noncholinergic fibers. The activity of AChE is higher in motor neurons than in sensory neurons (Koelle *et al* 1954 and Massoulie *et al* 1993). In the present study, mancozeb treatment significantly lowered the AChE activities in plasma and brain. Similarly, Siddiqui *et al* (2007) reported that oral imidacloprid administration cockerels for 28 days caused an insignificant inhibition of AChE activity in plasma. Bhardwaj *et al* (2010) also observed that the exposure of IMI to female rats for 90 days significantly decreased the activity of AChE in serum and brain. Kapoor *et al* (2011) noticed imidacloprid (20 mg/kg, orally; at 6, 12, 24 and 48 h) administration to female rats inhibited AChE in the brain and RBC. Exposure to thiamethoxam, a neonicotinoide altered behavioral and biochemical processes related to the cholinergic systems in rats. AChE activity was measured in the different brain regions (hippocampus, striatum and cortex) and it was found that thiamethoxam reduced spontaneous motor activity with decreased AChE activity in the hippocampus, cortex and striatum. The long-standing inhibition of AChE activity was accompanied by deficits in behavioral performance (Rodrigues et al 2010). Further, Akhtar *et al* (2009) has reported dose-dependent inhibition of AChE activity in RBC and brain in a carbamate toxicity study.

Table 14: Effect of sub-acute oral mancozeb exposure on acetyl choline esterase activity (AChE) in male wistar rats (n=6)

Group	**Blood (0 day)**	**Blood (29th day)**	**Brain (29th day)**
Control	3.92±0.08	3.95^{B}±0.08	13.38^{B}±0.34
Treatment	3.98^{b}±0.08	1.92^{Aa}±0.04	8.56^{A}±0.23

Means bearing superscripts $^{a, b}$ differ significantly ($p<0.05$) in a row. Means bearing superscripts $^{A, B}$ differ significantly($p<0.05$) in a column (values Mean±SE; n=6)
Unit: (µM/ min/ ml or g) of blood/ tissue.

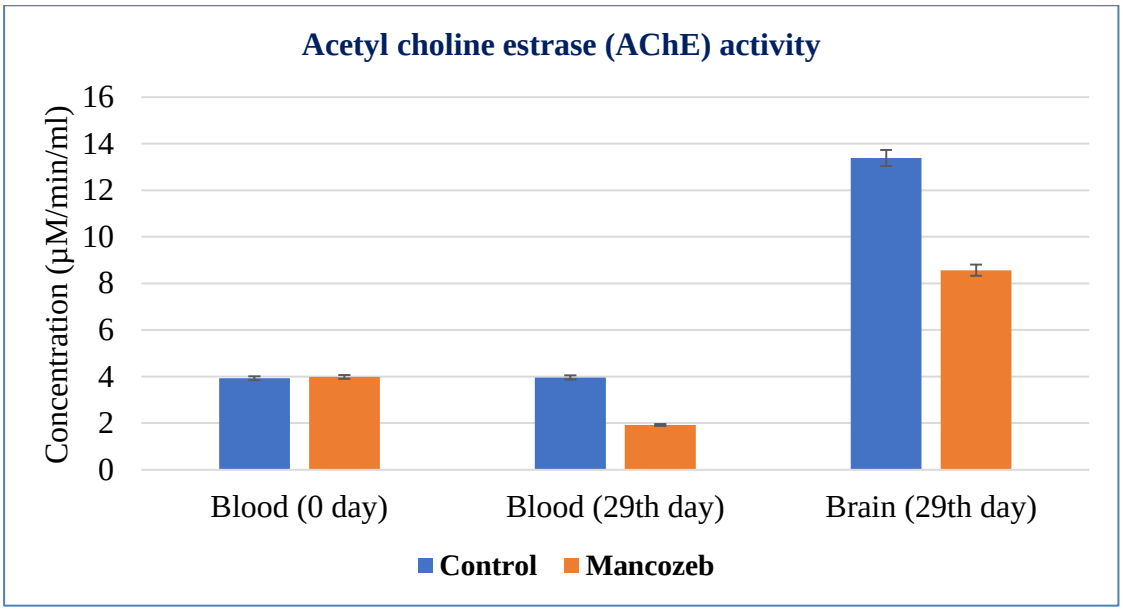


Figure 30: Variation in Acetyl choline esterase activity in the blood and brain tissue after mancozeb exposure in control and mancozeb group rats (n=6)

4.7 Effect of sub-acute oral mancozeb exposure on sensory parameters in male wistar rats

The following sensory parameters were undertaken to analyze the effect of mancozeb exposure at the end of experimentation i.e; 29th day.

Table 15: Effect of sub-acute oral mancozeb exposure on sensory parameters in male wistar rats (n=6).

Parameters	Control	Treatment	P value
Eddy's hot plate test (no. of jumps)	6.17^{a}±0.48	10^{b}±0.58	P<0.001
Tail immersion test (reaction time in sec.)	3.94^{b}±0.23	2.13^{a}±0.15	P<0.001

Means bearing superscripts $^{a, b}$ differ significantly in a row (values: Mean±SE; n=6).

4.7.1 Eddy's hot plate test

In the Eddy's hot plate test, the total number of jumps done by animal during one minute of time period were recorded and paw licking response shown by the animal was observed. There was significant increase in the number of jumps, done by animal in mancozeb-treated group as compared to control group animals (Table 15; fig. 31), indicating increase in sensitivity to pain. The hot plate test is similar to the

tail flick test, a test of the pain response in animals. It is used in basic pain research and in the testing of analgesic efficacy by observing the heat-caused pain reaction response and was suggested by Eddy and Leimbach (1953). Many investigators have used the hot plate test and it was been found to be suitable for central but not peripherally acting analgesic activity assessment. This is one of the oldest and most commonly employed forms of testing to test nociception in rats and mice (Liu et al 2011). A strong connection tends to be between medications that induce anti-nociception on the hot plate check and medicines that are used commonly to relieve pain (Taber 1973). In addition to stimuli amplitude and nociceptive responsiveness, a variety of other variables can affect latency at the hot plate. Numerous studies have shown that latency on hot plate decreases with repeated testing (Plone et al 1996).

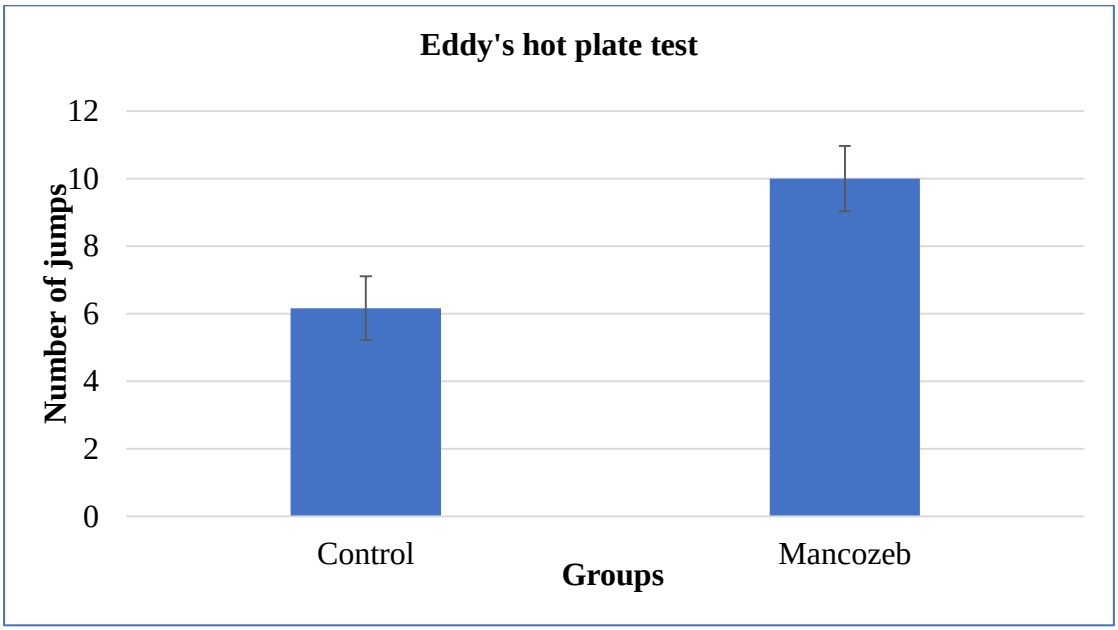


Figure 31: Effect of mancozeb exposure on Eddy's hot plate test in control and mancozeb group rats (n=6)

4.7.2 Tail immersion test

In the present study, tail immersion test was performed to see the sensitivity against hot water (56±1^{o}C). The mancozeb treated animals showed a significant decrease in the reaction time as compared to the control group animals, indicating increase in sensitivity to pain response (Table 15; Fig.32). Similarly, prenatal exposure of carbendazim to rat was reported to cause brain malformations (Sitarek 2001) and brain dysplasia in aquatic organisms (Yoon et al 2008).

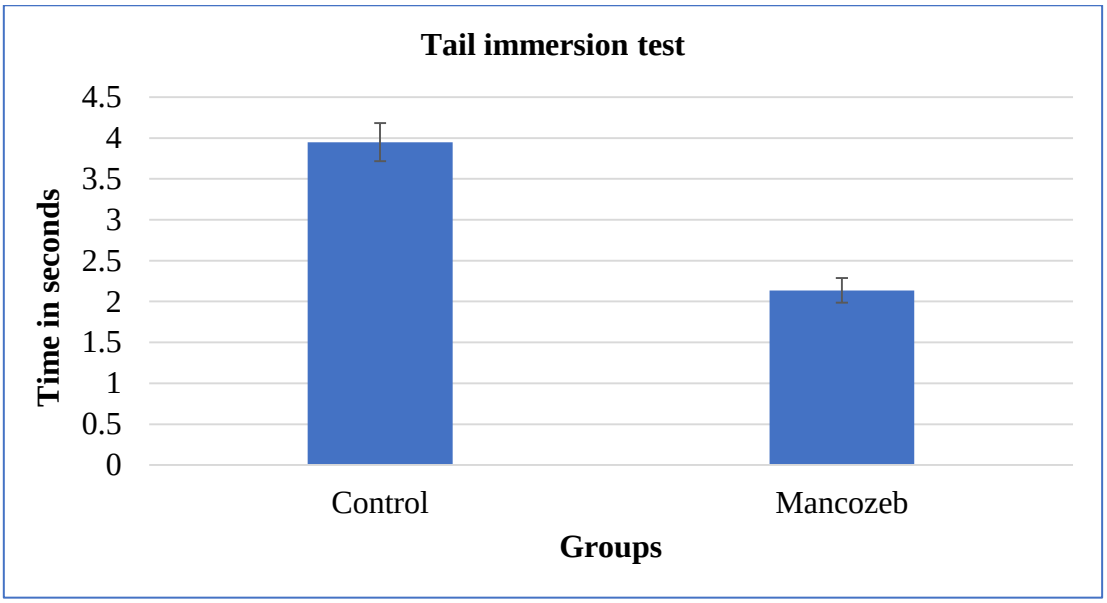


Figure 32: Effect of mancozeb exposure on reaction time of tail immersion test in control and mancozeb group rats (n=6)

4.8 Effect of sub-acute oral mancozeb exposure on reproductive parameters in male wistar rats

Effect of repeated oral sub-acute mancozeb exposure on male reproductive parameters such as live and dead sperm count, sperm abnormalities and total sperm count was analyzed at end of 28 days (Table 15 and Fig. 33 and 34a-f).

Table 15: Effect of sub-acute oral mancozeb exposure on reproductive parameters in male wistar rats (n=6).

Group/Parameters	Control	Treatment
Live sperm (%)	80.33^{b}±2.11	62^{a}±2.13
Dead sperm (%)	19.67^{a}±2.11	38^{b}±2.13
Abnormal sperm (%)	5.33^{a}±0.49	17.17^{b}±0.74
Total sperm count (*10^{7}sperm/epididymis)	46.17^{b}±0.99	33.32^{a}±0.70

Means bearing superscripts^{a,b} differ significantly ($p<0.05$) in a row (values: Mean±SE; n=6).

Exposure of mancozeb resulted into significant decrease in the live sperm count and increase in dead sperm count in mancozeb-treated rats when compared with control group. Mancozeb-treated rats showed significantly higher level of abnormal

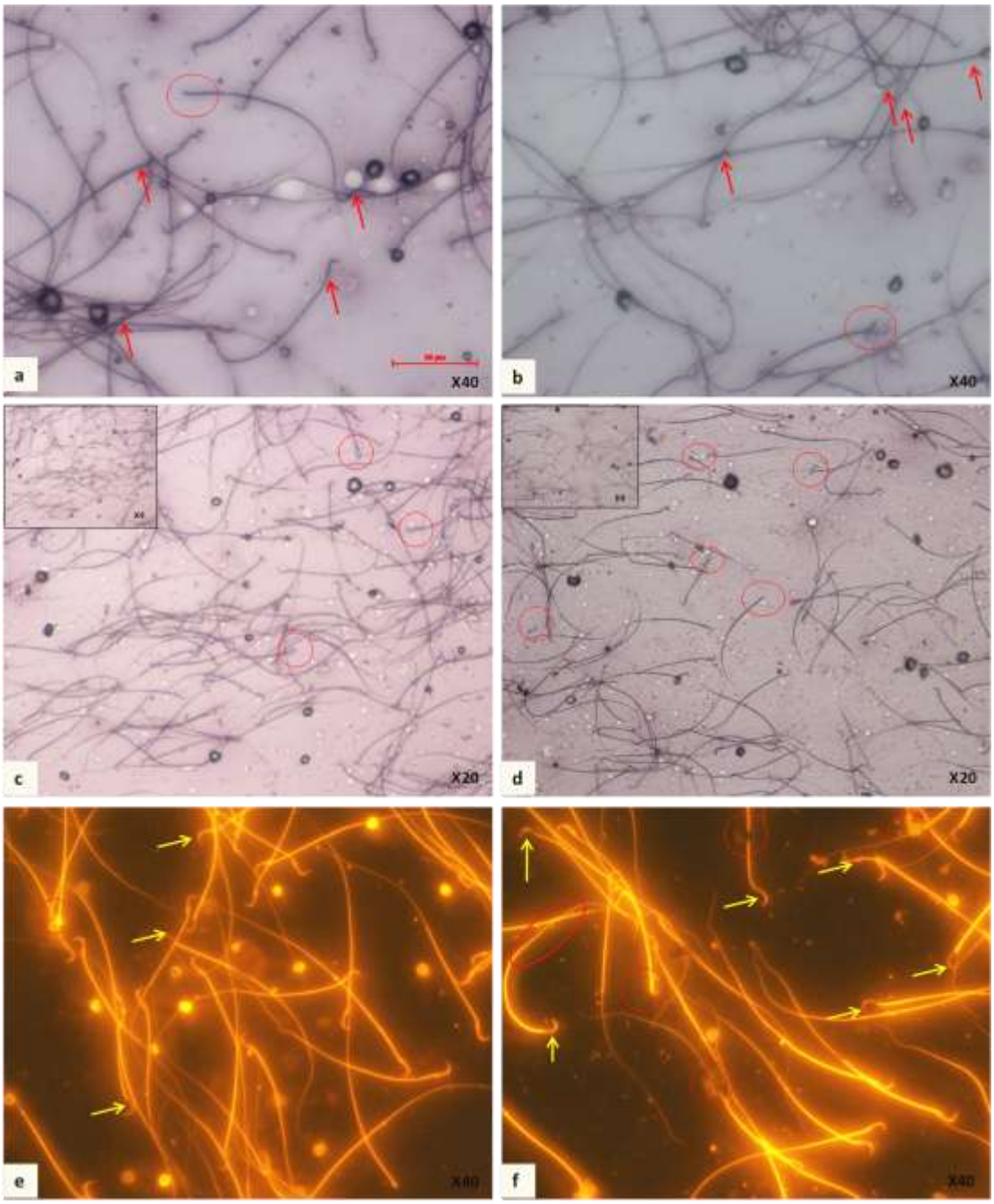


Fig 34(a-f): Representative photomicrograph of sperms from experimental male wistar rats.

Where; a, c and e: Micrograph from control group.
b, d & f: Micrograph from treatment (mancozeb) group.
a-d: Under bright field,
e-f: fluorescent field.

Arrow: dead sperm; Circle: sperm defects broken head, detached tail, damaged head Eosin-nigrosin staining; original magnification: a, b, e and f: x40; c and d: x20.

sperm count including detached head, coiled tail, damaged head etc. as compared to control group rats. In the present study, there was significant decreased in the total sperm count of mancozeb-treated male rats at end of the exposure period as compared to control group (Table 15; Fig. 33 and 34a-f).

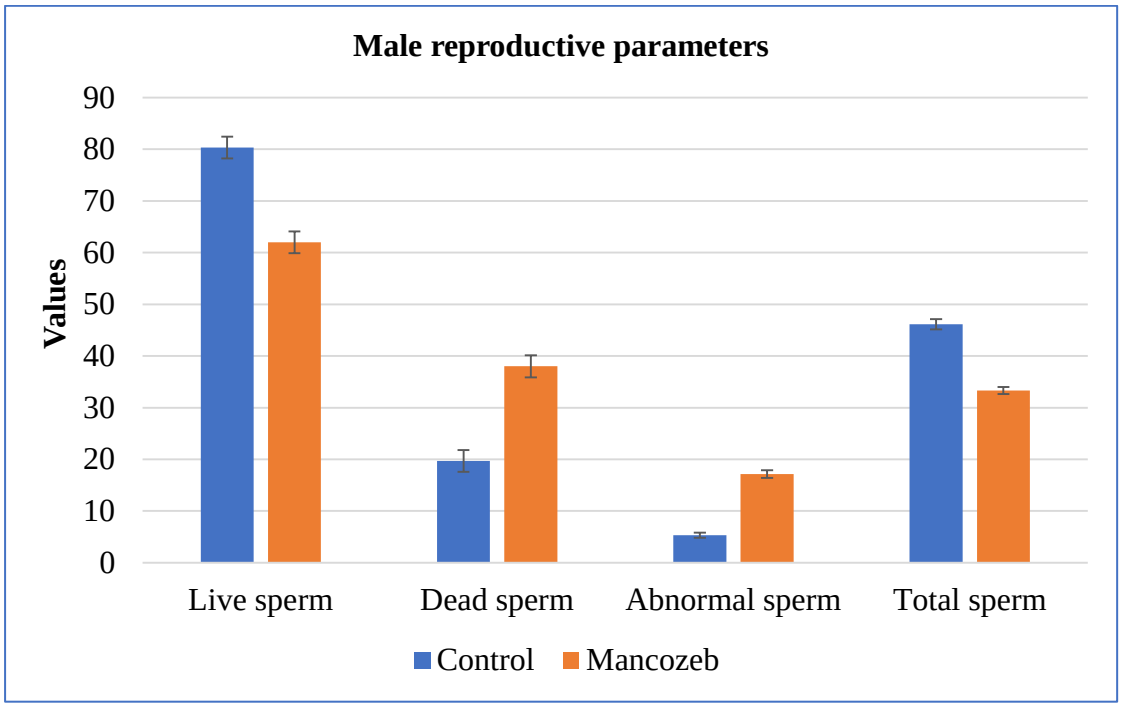


Figure 33: Effect of mancozeb exposure on male reproductive parameters in control and mancozeb group rats (n=6)

Many exogenous factors (chemicals, drugs, etc.) that act in many different ways that can interfere with testicular steroidogenesis by the complex physiological mechanism that regulates the Leydig cell function (Cooke 1998). Consistent to our results, Joshi *et al* (2005) reported, damaged seminiferous tubules, with complete spermatogenic arrest in mancozeb-treated rats and the lumen contained cellular debris, degenerated sertoli cells and was devoid of sperms.

Male infertility may result from impaired Leydig cell activity (Payne et al 1980). Spermatogenesis is a mechanism that includes LH and FSH gonadotropin production under hypophysial hormonal regulation and operates Leydig and Sertoli cells, respectively (Steinberger 1971). Interactions between Leydig cells-Sertoli cells are also needed for the normal development of intratesticular testosterone (Skinner 1991). Changes in the metabolism of testosterone may be associated with several specific physiological conditions (Dufau *et al* 1979). The

amounts of intratesticular testosterone supporting natural spermatogenesis are 100 times greater than those of blood plasma level reached by regular development of the Leydig and Sertoli cells (Dufau et al 1979, Saez *et al* 1987, Russell *et al* 1998). Many studies support the findings of present study. Imidacloprid treatment has been shown to increase cell death, as well as increased immature sperms and decrease the immotile sperm velocity with substantial decrease in serum testosterone level in rats (Najafi *et al* 2010), and responsible for induction of sperm deformities in earthworms have been recorded (Zang *et al* 2000). In fact, lower sperm counts in the epididymis, increased teratozoospermia and sperm nuclei chromatin defects in mice following treatment with Parathion (Guillermino *et al* 1998). Mancozeb is known to have toxic and adverse effect on reproductive organs and might have toxic effect on testicular cells which could be responsible for loss of membrane structure and testicular function leading to altered functionality of testes. Similarly, male rats received carbendazim orally resulted in reduce fertility index and severe induce seminiferous tubular atrophy (Carter *et al* 1987, Lu *et al* 2004). Gray *et al* (1990) reported reduced reproductive potential of the rats treated with carbendazim due to inhibition on sperm production and foetal viability, altered sperm morphology, testicular and epididymal weights, sperm count and change in testicular histology. Barlas *et al* (2002) reported decrease in the total serum testosterone levels and dihydrotestosterone levels, detrimental effects in the testicular tissue such as vacuolization, disorganization, necrosis in germinal epithelium and multinucleated giant cells in the lumen of the seminiferous tubules in carbendazim-treated rats. Carbendazim was reported to be capable of causing prolonged infertility in Japanese quails with decrease in testicular weights and seminiferous tubular diameter (Aire 2005). Sakr and shalaby (2014) reported degeneration of seminiferous tubules, loss of spermatogenic cells, apoptosis and decreased height of epithelium in carbendazim treated animals. Carbendazim known to induce testicular toxicity in mammalian cells by inhibition of microtubule assembly formation and direct alteration of testicular function through germ cell depletion and altering Sertoli and Leydig cell functions leading to sloughing of immature spermatids (Parvinen and Kormano 1974, Gray *et al* 1989, Hess *et al* 1991, Lim and Miller 1997b)

4.9 Histopathological observations

Macroscopic observations

No significant gross changes were observed in any of the organs from all the groups.

Microscopic observations

The histopathological alterations in liver, kidneys and brain tissue observed in rats due to sub-acute oral mancozeb exposure in male wistar rats are demonstrated in Fig. 35-40. Histopathological examination of the liver section of control group animals showed a normal feature of liver parenchyma (Fig. 35) while liver tissue from mancozeb-exposed rats revealed necrosis and degeneration along with formation of vacuolization in the cytoplasm and mononuclear cell infiltration (Fig. 36), indicating hepatotoxic effect of mancozeb treatment. In kidney tissue, histopathological examination of the control group rats showed normal tubular and glomeruli architecture (Fig. 37). However, congestion and degeneration of the proximal convoluted tubules were observed in the kidneys of mancozeb-treated animals (Fig. 38). In brain, mancozeb-exposed rats, showed microscopic changes in cerebellum along with presence of vacuolization in the white matter in pons area of the brain (Fig. 40) as compared to control group animals (Fig. 39).

Liver

In congruence to our findings, Kacker *et al* (1999) observed focal necrosis, fatty infiltration as well as enlarged sinusoidal space of liver due to high concentration of mancozeb (MZ) erposure. In brain, the purkinje cells were necrosed, irregularly arranged and were devoid of axons while the kidney tissue showed tubular necrosis and alterations in the epithelial lining of the PCT. Sakr *et al* (2007) observed that 14 days mancozeb treatment in rats impaired the normal structural architecture of the hepatic lobules with congested central and portal veins, whereas, mancozeb treatment for 28 days to animals revealed a considerable damage to number of hepatic cells and loss in their characteristic appearance and cytoplasmic vacuolization along with congestion and inflammatory leucocytic infiltrations of the intrahepatic blood vessels. By 6 weeks, there were degenerated hepatocytes with fatty changes.

Yahia *et al* (2019) also reported that oral administration of MZ induced congestion and infiltration of inflammatory cells in the portal area of the liver. In

another study, histopathological examination of liver sections of paracetamol treated rats showed hepatic necrosis along with hepatic cell destruction (Wagh 2010). Salahdeen *et al* (2017) reported that rats treated with N-nitro-L-arginine methyl ester (L-NAME), showed mild portal congestion and peritubular cellular infiltration in the liver tissue.

Joshi *et al* (2005) reported, damaged seminiferous tubules, with complete spermatogenic arrest in mancozeb treated rats and the lumen contained cellular debris, degenerated sertoli cells and was devoid of sperms. In agreement to our finding, histopathological examination of the liver section of the Isoniazide and rifampicin treated rats revealed a centrilobular necrosis and cytoplasmic vacuolization as an evidence of liver injury (Sagheer *et al* 2018).

Gürol *et al* (2020) demonstrated the effects on histopathological changes in Zebrafish testes due to mancozeb exposure reflected by degenerative spermatogenic cells, hypertrophy of spermatocytes, fibrosis and disorganizations of seminiferous tubule. Wagha and Shinde (2018), in histopathological examination, demonstrated development of centrilobular necrosis, inflammation and accumulation around the terminal hepatic venule when compared to control group which showed normal cellular architecture with well defined centrilobular venule. In support to our findings Pirozzi *et al* (2016) found that the hepatic toxicity of the mancozeb fungicide exacerbated fatty acid-induced steatosis, as manifested by an increase in intracellular lipid droplet accumulation, studied in an *in vitro* model of hepatic damage, namely fatty acid-induced non-alcoholic fatty liver disease in the HepG2 cell line.

Histological examination of liver and kidney showed several histopathogenic abnormalities in tissues of rats exposed to mancozeb (Talha *et al* 2006).

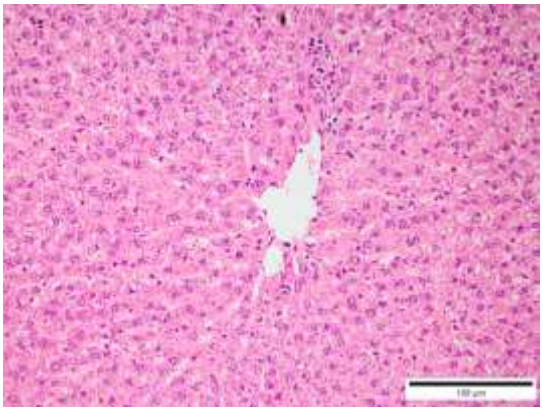


Fig. 35: Control group: Photomicrograph of the liver showing normal liver parenchyma (H&E, Bar=100 µm)

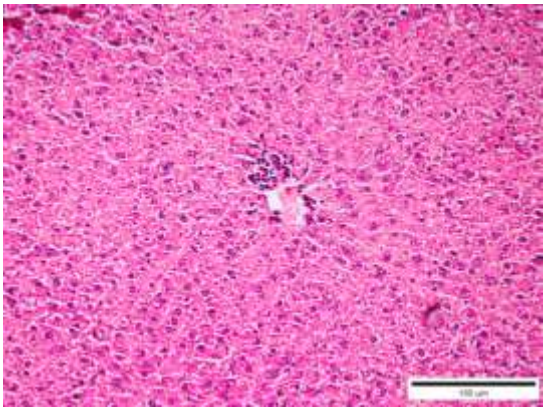


Fig. 36: Mancozeb group: Liver showing necrosis and degeneration with cytoplasmic vacuolization with mononuclear cell infiltration (H&E, Bar=100µm)

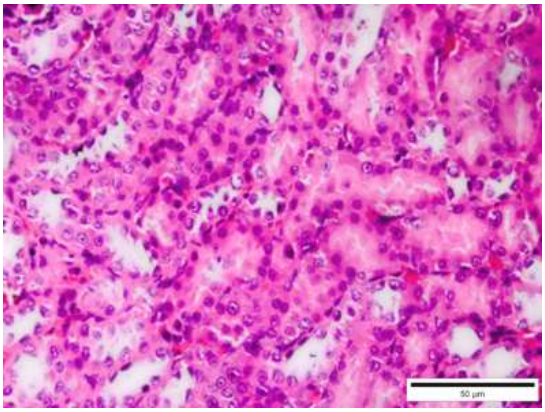


Fig. 37: Control group: Kidney showing normal structure of tubules (H&E, Bar= 50µm)

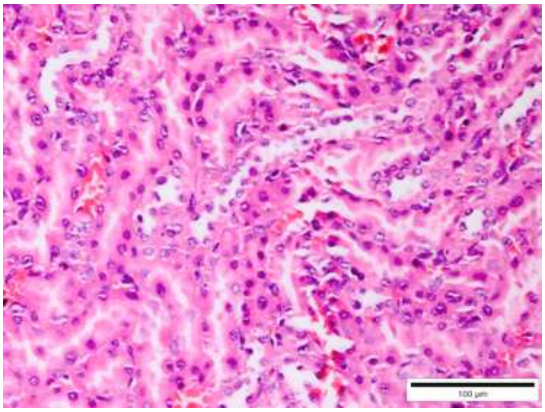


Fig. 38: Mancozeb group: Kidney showing congestion and degeneration in the Proximal Convulated Tubules (H&E, Bar=100µm)

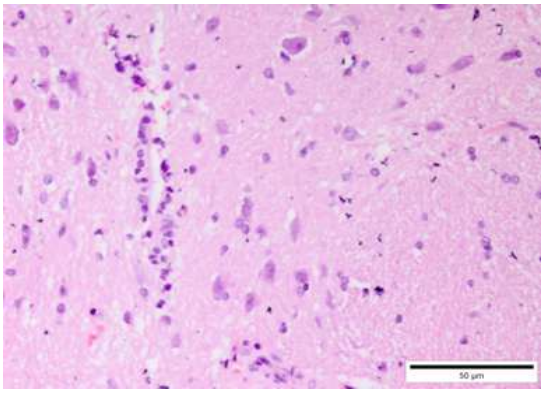


Fig. 39: Control group: Pons showing normal nuclei of pons (H&E, Bar=50 µm)

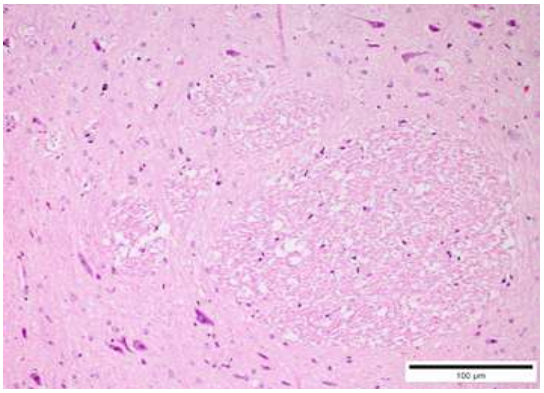


Fig. 40: Mancozeb group: Pons showing formation of the vacuoles in the longitudinal sections of the nerve fibres. The vacuoles coalases and formed larger vacuoles (H&E, Bar=100µm)

CHAPTER V

SUMMARY AND CONCLUSION

The present study was designed for investigation and evaluation of sub-acute oral mancozeb-induced toxicity in rats. The objective of our study was to evaluate the toxic manifestations of subacute oral mancozeb toxicosis.

Dithio-carbamate fungicide mancozeb was administered orally at the dose rate of 500 mg/kg ($1/10^{th}$ of LD_{50} i.e; 5000 mg/kg) for 28 consecutive days to Wistar rats. Twelve healthy male rats were divided into two groups of six animals each. Group I animals served as control in which no drug was administered, whereas the group II animals were administered with mancozeb at the dose rate of 500 mg/kg to induce toxicity. This investigation was undertaken by assessing various parameters including physiological, haematological, biochemical, endocrine, reproductive and neuro-behavioral, for toxic manifestations induced by mancozeb.

At the end of experimentation i.e, 29^{th} day, all animals were sacrificed humanely and measurement of absolute and relative weight of various organs including liver, kidneys, lung, heart, brain, testes and epididymis were taken.

The findings of the conducted experiment are summarized below:

A. Effect of sub-acute mancozeb exposure on physiological status of male Wistar rats.

I. Toxic Signs

Daily oral administration of mancozeb at the dose rate of 500 mg/kg body weight to rats for 28 consecutive days produced mild signs of toxicity like moderate degree of anorexia, listlessness, lacrimation, nasal bleeding, sluggish movement and mild degree of hind limb paralysis when compared with control group animals. However, no lethality was observed in treatment as well as in control group animals.

II. Body weight measurement on weekly basis

Mancozeb exposure resulted into moderate decrease in body weight in animals during first week of experimentation followed by increase in body weight in subsequent weeks. There was overall increase in body weight and per cent body weight gain in animals after 28 days of mancozeb exposure in treatment group as compared to control group.

III. Organ weight measurement

Significant elevation in absolute organ weight of liver, kidneys, brain, testes and epididymis were observed after sub-acute mancozeb exposure. No significant alteration in per cent body weight gain was recorded in mancozeb-exposed animals. Relative weight of liver increased significantly while relative weight of heart decreased significantly.

B. Effect of sub-acute oral mancozeb exposure on haematological parameters in rats.

Significant decrease in TLC, TEC and mononuclear cells were observed while ESR, MCV and neutrophils increased significantly in mancozeb-exposed animals when compared to control group animals. Whereas, slight decrease in haemoglobin and PCV was noticed. No significant alterations were recorded in other haematological parameters.

C. Effect of sub-acute oral mancozeb exposure on biochemical parameters in rats.

Repeated oral administration of mancozeb (500 mg/kg), produced a significant elevation in the biochemical parameters like AST, ALT, LDH, GGT, creatinine, BUN, alkaline phosphatase, creatine kinase, HDL and LDL-cholesterol levels when compared to control group. A significant reduction in total plasma protein, blood glucose and triglycerides were seen in mancozeb-exposed group indicating liver and kidney damage at tested dose.

D. Effect of sub-acute oral mancozeb exposure on endocrine parameters in wistar rats.

Repeated oral administration of mancozeb (500 mg/kg), produced a significant increase in Thyroid Stimulating Hormone (TSH) while significant decrease was noted in Tri-iodothyronine (T_3) and Thyroxine (T_4) levels when compared to control group animals.

E. Effect of sub-acute oral mancozeb exposure on neurobehavioral parameters in rats.

The Elevated plus-maze and T-maze test results, showed anxiety-like behaviour in mancozeb-exposed animals. In the Elevated plus-maze test, a significant reduction

was observed in locomotion, hide arm activity, curled up and travelled distance in mancozeb exposed group as compared to control group, indicating anxiolytic effect of mancozeb in male rats. In T-maze test, a significant reduction in locomotion time, stretch out time and travelled distance was recorded in rats exposed to mancozeb as compare to control group.

Repeated mancozeb exposure led to significant reduction in grip strength and rota rod activity, while no significant alteration was observed in acoustic/auditory startle response, righting reflex and corneal response in mancozeb-exposed rats.

E. Effect of sub-acute oral mancozeb exposure on Acetyl choline esterase (AChE) activity in rats.

Repeated mancozeb exposure produced a significant inhibition of Acetyl choline esterase (AchE) activity in blood and brain tissue of rats.

F. Effect of sub-acute oral mancozeb exposure on sensory parameters in wistar rats.

The sensory parameter test including Eddy's hot plate and Tail immersion test results, indicated a significant increase in sensitivity to pain in mancozeb-exposed animals.

G. Effect of sub-acute oral mancozeb exposure on reproductive parameters in male wistar rats.

Exposure of mancozeb resulted into significant decrease in the live sperm and total sperm count while increase in dead and abnormal sperm count in rats.

H. Histopathological observations

Histopathological alterations were observed in liver, kidneys and brain tissue of exposed to mancozeb. Liver tissue revealed necrosis and degeneration as well as vacuolization in the cytoplasm along with mononuclear cell infiltration while congestion and degeneration of the proximal convoluted tubules were observed in the kidneys of mancozeb-treated animals. The brain of mancozeb-exposed rats, showed microscopic changes in cerebellum along with vacuolization in the white matter in pons of the brain as compared to control group.

Conclusion

From the present study, it can be concluded that mancozeb fungicide produced a pronounced toxicological insult in the exposed animals which was evident by marked alteration in physiological, haematological, biochemical, endocrine, reproductive and neurobehavioral parameters.

Although, mancozeb is widely used in agricultural practices as fungicide, our study revealed that mancozeb exposure for 28 consecutive days produced certain toxicological manifestations in the exposed rats.

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