Studies on cartap toxicity in male rats and its amelioration by α-tocopherol



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OF

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IN

VETERINARY PHARMACOLOGY & TOXICOLOGY

BY

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(2022)

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This is to certify that thesis entitled, **"Studies on cartap toxicity in male rats and its amelioration by α-tocopherol"** submitted by **Dr. Sugam Thakur, Enrolment No. V-2109/19** in partial fulfillment of the requirements for the award of the **Master of Veterinary Science** in **Veterinary Pharmacology and Toxicology** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura (UP),** India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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Dated..... 17/01/2022

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%	:	Percent (age)
<	:	Less than
>	:	Greater than
°C	:	Degree Celsius
µ	:	Micro
µM	:	Micromolar
ACP	:	Acid phosphatase
b.wt.	:	Body weight
BSA	:	Bovine Serum Albumin
CAT	:	Catalase
cDNA	:	Complimentary deoxyribonucleic acid
CDNB	:	1-Chloro-2,4-dinitrobenzene
DEPC	:	Diethyl pyro carbonate
DF	:	Dilution Factor
DMSO	:	Dimethyl Sulfoxide
DNTP	:	Deoxy nucleoside triphosphate
DTNB	:	5,5-dithiobis-2-nitrobenzoic acid
EC	:	Extinction Coefficient
et al.	:	et alia
FITC-PSA	:	Fluorescein isothiocyanate pisum sativum agglutinin
g	:	Gram (s)
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GGT	:	Gamma glutamyl transferase
GSH	:	Reduced Glutathione
GST	:	Glutathione-S-Transferase
H_2O_2	:	Hydrogen Peroxide
HEPES	:	4-(2-hydroxyethyl)-1-piperzineethanesulfonic acid.
HOST	:	Hypo Osmotic Swelling Test
HSP-70	:	Heat Shock Protein 70
i.e.,	:	That is
LDH	:	Lactate dehydrogenase

LPO	:	Lipid Peroxidation
M	:	Molar
MDA	:	Malondialdehyde
mg	:	Milligram
min	:	Minute (s)
mM	:	Millimolar
mOsmol	:	Milli osmolar
MTT	:	Methylthiazolyldiphenyl-tetrazolium bromide
n	:	Number of observations
NADP	:	Nicotinamide adenine dinucleotide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NFW	:	Nuclease free water
NSS	:	Normal Saline Solution
PBS	:	Phosphate Buffer Saline
RNA	:	Ribonucleic acid
ROS	:	Reactive Oxygen Species
RT-PCR	:	Real Time polymerase chain reaction.
S.E.	:	Standard Error
SDH	:	Sorbitol dehydrogenase
sec	:	Second
SOD	:	Superoxide Dismutase
SP-TALP	:	Sperm Tyrode’s albumin lactate pyruvate
TAE	:	Tris acetate EDTA

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ABSTRACT

Present study was undertaken to evaluate the ameliorative effect of α-tocopherol (100 mg/kg) against cartap (16.25 mg/kg and 32.5 mg/kg) induced toxicity following 28 days oral exposure in adult male Wistar rats. Cartap exposure did not produce any clinical signs of toxicity in rats at both the doses. Weekly Average body weight gain was significantly reduced following cartap (32.5 mg/kg). Both absolute and relative organ weights did not differ significantly between different treatment groups. Hematological parameters did not differ significantly in any of the treatment groups. Plasma Glucose, Total Cholesterol, Triglycerides, AST, ALT, BUN levels were found to be markedly increased in cartap alone treatment groups. Plasma calcium levels showed irregular but non-significant change in response to cartap doses. α-tocopherol co-treatment with cartap produced non-significant decrease in the plasma levels of Glucose, AST and ALT but the levels of Total Cholesterol Triglycerides and Calcium were found to be significantly decreased in both the co-treated groups. BUN levels remained unaffected following α-tocopherol co-treatment. Plasma Total Protein, Albumin, Globulin, LDL, HDL, Bilirubin and Creatinine levels showed non-significant change following exposure to cartap at both the doses. A significant decrease in Total Protein and increase in MDA levels was observed in testes and liver at both the doses of cartap. CAT, SOD, and GST activities were reduced markedly in liver and testes of cartap alone treatment groups. GSH levels showed non-significant change in testes but significant reduction in liver at cartap dose of 32.5 mg/kg. Following co-treatment with α-tocopherol Total protein levels were significantly increased in testes and liver but MDA level was decreased in Liver only. Rest of the oxidative stress parameters showed non-significant changes. Testicular ACP, LDH and GGT levels were significantly increased at cartap dose of 32.5 mg/kg but non-significant change was observed in SDH level. Moderate decrease in ACP, LDH and GGT levels was observed in co-treated groups. Plasma testosterone levels were significantly reduced in cartap alone treatment groups. Non-significant change in plasma testosterone levels was observed in both co-treated groups. Gross examination of organs revealed swelling and congestion in lungs of cartap alone treatment groups. A visible decrease in severity of gross changes in lungs was observed in cartap and α-tocopherol co-treated groups. Histopathological examination revealed mostly degeneration and congestion in lungs, liver, kidneys spleen, heart, testes and trachea in cartap alone treatment groups. α-tocopherol co-treatment revealed a visible decrease in severity of pathological changes in both co-treated groups. Significant upregulation of testicular HSP-70 gene was observed at both the doses of cartap. Sperm concentration, % live spermatozoa and % HOS+ve spermatozoa were found to be significantly reduced in cartap dose of 32.5 mg/kg. A marked increase in % live and % HOS+ve spermatozoa was observed following co-treatment with α-tocopherol in both co-treated groups. The present study showed that α-tocopherol was able to suppress some of the negative effects of cartap exposure to a certain degree but could not produce complete amelioration in most of the studied parameters. Based on the above results, cartap seem to exert toxic effects on lungs, liver, kidney, spleen, heart and male reproductive system and α-tocopherol possesses moderate restorative potential against Cartap toxicity.

Keywords: Cartap, α-tocopherol, LPO, LDH, Testosterone, HSP-70, Sperm



Introduction

CHAPTER-1

INTRODUCTION

The term “pesticide” represents a broad range of chemicals and other agents that control pests or produce other biological effects. The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 1996 defines the term “pesticide” as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest or intended for use as a plant regulator, defoliant, or desiccant (EPA, 2021). Documented history of pesticides began in ancient Rome, but their extensive use began after the second world war with the introduction of dichlorodiphenyltrichloroethane (DDT), beta-hexachlorocyclohexane (BHC), aldrin, dieldrin, endrin, and 2,4-dichlorophenoxyacetic acid (2,4-D) (Maksymiv, 2015). Pesticides can be classified on the basis of target organism (e.g., insecticides, herbicides, fungicides), chemical structure (organochlorines, organo-phosphates, carbamates, phenoxy acids), and physical state (solid, liquid, aerosol) (Bohmont, 1997). To use wide range of chemicals to destroy pests and weeds is an important aspect of agricultural practice in both developed and developing countries. Undoubtedly, this has increased crop yield and reduced postharvest losses. However, the expanded use of such pesticides expectedly results in residues in foods, which has led to widespread concern over the potential adverse effects of these chemicals on human health (Al-Saleh,1994). Pesticides are found in soil, water, air, plants, food and feed and their residues enter plants and animal products and accumulate in humans and animals by the food chain (Hashimi et al., 2021). Acute pesticide poisonings of wildlife and domestic animals can occur due to accidents with substances approved for several uses, and due to deliberate and illegal attempt to poison animals even with baits, and by secondary poisonings (Grilo et al., 2021). Pesticides are known for their high persistence and pervasiveness in the environment, and along with products of their biotransformation, they may remain in and interact with the environment and living organisms in multiple ways, according to their nature and chemical structure, dose and targets (Lushchak, 2018). Over 98 % of sprayed insecticides and 95 % of herbicides reach a destination other than their target species, including non-target species, air, water and soil (Miller, 2004). Pesticide toxicity can result from ingestion, inhalation or dermal absorption. Residues of pesticides can be found in a great variety

of everyday foods and beverages, including for instance cooked meals, water, wine, fruit juices, refreshments, and animal feeds (McGill and Robinson, 1968; Chourasiya et al., 2015). Furthermore, it should be noted that washing and peeling cannot completely remove the residues (Reiler et al., 2015). In the majority of cases, the concentrations do not exceed the legislatively determined safe levels (Lorenzin, 2007). However, these "safe limits" may underestimate the real health risk as in the case of simultaneous exposure to two or more chemical substances, which occurs in real-life conditions and may have synergistic effects (Kortenkamp, 2007). Pesticides residues have also been detected in human breast milk samples, and there are concerns about prenatal exposure and health effects in children (Lu et al., 2015). Continued exposure to these chemicals for a long period may result in various diseases including neurological, psychological and behavioral dysfunctions, hormonal imbalances, immune system dysfunction, reproductive system defects, cancers, genotoxicity and blood disorders (Maksymiv, 2015). India is presently the 4th largest manufacturer of basic pesticides in the world (Nayak and Solanki, 2021). Currently, organophosphates and carbamates are being used to control the insects and pests (Gupta et al., 2019). Cartap hydrochloride (Padan^{R}) is a commonly used thiocarbamate pesticide in India. Due to its high insecticidal activity, cartap is widely used all over the world (Nagawa et al., 1971). It is an analogue of nereistoxin, a neurotoxic substance isolated from the marine annelid *Lumbriconereis heteropoda* (Kumar et al., 2011). Cartap is commonly used to control weevils and caterpillars. and is predominantly applied in paddy fields of India, at a recommended dose of 10 μg ml^{-1} to kill chewing and sucking insect pests of rice crop (Kaur and Goyal, 2021). It was recently reported to be very effective against *Opisina arenosella* i.e., the coconut black headed caterpillar (Sharma et al., 2021). It is moderately hazardous insecticide that is increasingly used for deliberate self-harm in India and can cause neuromuscular weakness resulting in respiratory failure (Kalyaniwala et al., 2016). Cartap toxicity has been studied in both rat and mice animal models. Sublethal exposure of cartap resulted in significant increase in serum triglyceride level and decrease in HDL level in adult male wistar rats (Rai et al., 2009). Sublethal exposure in adult male mice resulted in increase in serum biomarkers (ALT, AST and BUN), tissue oxidants and decrease in antioxidants along with histopathological findings in liver, kidney and brain tissues (Sharma et al., 2021). Cartap was found to increase methemoglobin production in red blood cells of adult male wistar rats exposed to

sublethal dosage of cartap (Emadi et al., 2019). Sublethal exposure to cartap was also found to significantly increase hepatic oxidant (ROS), decrease antioxidant enzyme levels and resulted in mild congestion in the liver of adult male wistar rats (Gupta et al., 2019). Cartap causes tonic contraction of diaphragm in adult male mice (Liao et al., 2000). Various forms of nuclear destruction like pyknosis, prominent yellowish-brown pigment and cytoplasmic degeneration were observed in liver of *Labeo rohita* fingerlings exposed to sublethal concentration of cartap (Vivek et al., 2016). Studies on the effect of cartap on reproductive system are limited however some early studies in pregnant female rats have suggested some affinity of the compound for the reproductive system. Weaning survival index was lower in the progeny of pregnant female rats exposed to sublethal dosage of cartap (Olson and Busey, 1972). In a similar study Skeletal abnormalities were observed in the progeny of female rats exposed to sublethal dosage of cartap (Mizutani et al., 1971.) Effect of cartap on male reproductive system particularly testes is yet to be reported. Thus the literature on cartap toxicity as well its amelioration is limited and its systemic effects are also not well understood. Also, the effect of cartap on male reproductive system needs to be explored. Therefore, the following study was designed to investigate the toxicological effects of cartap hydrochloride and the role of alpha tocopherol as a potential ameliorant with the following objectives:

2. Objectives:

1) To investigate the toxic effect of cartap in male rats with special reference to testes; and
2) To evaluate the ameliorative effect of α- tocopherol against cartap-induced toxicity in male rats.



Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

Pesticides are chemical compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants (weeds) (WHO, 2004). The uniqueness of their chemical structure, and/or their interactions with the environment characterize the nature of pesticides (Rajmohan et al., 2020). Based on chemical composition, pesticides are classified into four main groups namely; organochlorines, organophosphorus, carbamates and pyrethrin and pyrethroids. (Kaur et al., 2019). The chemical-based classification of pesticides is rather complex. In general, modern pesticides are organic chemicals and they include pesticides of both synthetic and plant origin (Damalas and Eleftherohorinos, 2011). The industrialization of the agricultural sector and increased demand of food around the world has led to increased usage of chemical pesticides in crop fields (Stamati et al., 2016). Pesticides are designed to kill and because their mode of action is not specific to one species, they often kill or harm organisms other than pests, including humans. The WHO estimates that there are 3 million cases of pesticide poisoning each year and up to 0.22 million deaths, primarily in developing countries (Schaffer et al., 2019). Various incidents of pesticide poisonings throughout the history around the world have been reported. In 1962, Rachel Carson published the book Silent Spring, an impassioned denouncement of the consequences of chemical contamination of the environment, with particular emphasis on the bioaccumulation of DDT and its effects on bird reproduction during the late 50s (Costa, 1987). Outbreaks of poisoning in Iraq due to alkylmercury fungicides in 1971-1972 were reported extensively (Abubakar et al., 2020). In 1986 Over 1000 tons of pesticides were spilled in the Rhine river in Germany resulting in mass mortality of aquatic life (Hassan and Nimir, 2020). The Bhopal disaster, also referred to as the Bhopal gas tragedy, was a gas leak incident on the night of 2 December 1984 at the Union Carbide India Limited (UCIL) pesticide plant in Bhopal, Madhya Pradesh, India. Methyl isocyanate is an intermediate chemical in the production of carbamate pesticides (such as carbaryl, carbofuran, methomyl, and aldicarb). Over 500,000 people were exposed and 3,787 victims were killed (Mishra et al., 2009). Aerial spraying of Endosulfan in

the cashew estates of kasaragod, kerala, over a period of 20 years, caused severe health hazards and deaths. The kerala state department of health and family welfare classified 6,728 persons with different types of diseases as "endosulfan generated," and 779 deaths were recorded as due to endosulfan poisoning (Sreekumar and Prathapan, 2013).

Impact of pesticide exposure on reproduction has been reported in both male and female reproductive systems. Infertility among men exposed to the fumigant dibromochloropropane (DBCP) provided a dramatic example in the 1970s of men's vulnerability to testicular toxicants. Workers who handled DBCP had low sperm concentrations, and most of the affected men had azoospermia or severe oligospermia Potashnik and Porath, 1995). Reduced fecundity was noted among men who were exposed to pyrethroids, organophosphates and carbamates in greenhouses (Sallmen et al., 2001). Studies on farm families in Minnesota and Ontario revealed increased incidence of miscarriages in women. Most miscarriages occurred in spring (when herbicides were used), and risk was found to be highest if the husband did not wear protective equipment when applying pesticides (Arbuckle et al., 2001). A cross-sectional study to identify health problems related to organophosphate pesticide (OP) exposure in groundnut farmers revealed alterations in seminal parameters (pH, viscosity, motility, morphology, and sperm count) and in a reproductive hormonal assay (follicle-stimulating hormone and testosterone) (Lwin et al., 2018). Reproductive toxicity following pesticide exposure particularly OP's, OC's, carbamates and pyrethroids has been extensively studied in rat/mice model. Alterations in seminal parameters and foetal abnormalities (abortions and birth defects) have been well reported in male and female animals respectively (Arican, 2020).

Thiocarbamates are the semi-sulfur analogues of carbamates characterized by the presence of OSCN group (Nomura and Casida, 2010). At room temperature, thiocarbamates are liquids or solids with a low melting point. As they are usually N, N -dialkyl substituted and have a sulfur atom in place of oxygen, they are less polar than methylcarbamates and are miscible with most organic solvents (Vallejos et al. 2011). Thiocarbamates are sensitive to light and photo degradation depends on state of compound, hence there is minimum effect on solid state due to poor penetration of UV light through the compound (Demarco and Hayes, 1979). Absorption of UV light

breaks the carbonyl bond (C-S bond) and produces free radicals which combine with the protons present in solvent giving formamide and mercaptans. Formamide is further degraded by UV light to dialkylamine which is converted into carbon dioxide (CO_2) that is eliminated in environment or used by plants (Demarco and Hayes, 1979). Thiocarbamates are metabolized into the body by solfoxidation followed by conjugation with glutathione then acetylated and eliminated as S-carbamoyl mercapturic acid through urine (98%) (Hubbell and Casida, 1977; Chen and Casida, 1978). Some of the commonly used thiocarbamates include cartap, EPTC, Triallate and prothiocarb (Thind and Hollomon, 2017) and these pesticides have been reported to produce low to moderate toxicity in rats and do not pose acute hazards (Gupta et al., 2019).

2.1 Cartap hydrochloride

Cartap is a thiocarbamate is recognized as an analogue of the neurotoxic substance isolated from the marine annelid *Lumbriconereis heteropoda* (Ray and Laws, 1991). Nereistoxin was first isolated by Nitta in 1934, the structure was elucidated by Okaichi and Hashimoto (1962) and synthesized by Hagiwara and Numata (1968).Cartap was first synthesized in Japan in 1967as an organonitrogenous thiocarbamate insecticide and recognized as a nereistoxin analog (Ray and laws, 1991). Cartap and its metabolite, nereistoxin, are nicotinergic acetylcholine blockers, causing paralysis by blocking cholinergic transmission in central nervous system of insects (Emadi et al., 2019). Cartap was first introduced into the market in Japan in 1967. In India two formulations are available, one is 50% soluble powder and another is 4% granular form (Abbasi and Krishnan, 1993).Cartap is essentially a contact and stomach poison (Boorugu and Chrispal, 2016). It is used for the control of chewing and sucking pests and results in insect paralysis (Ray and laws, 1991). This pesticide is largely applied for wide range of insects, for example, Lepidoptera, Coleoptera, Diptera, and Hemiptera in rice and sugarcane fruit trees, vegetable yield, and tea plantations (Emadi et al., 2019). It has been categorized as a high-effectiveness, low-toxicity, and low-residue pesticide used in rice and sugarcane fields (Boorugu and Chrispal, 2016). WHO (2004) categorized cartap as a moderately toxic substance (Toxicity class-II). Inadvertent use of it could result in exposure to animals and human beings. Cartap toxicity has been considered to be minimal, but a number of animal models have shown significant neuromuscular toxicity resulting in respiratory

failure (Boorugu and Chrispal, 2016). However, not much information is available about its impact on the redox status in various organs and tissues of the mammalian systems (Gupta et al., 2019). The excessive use of cartap can lead to hazardous levels of residues in various foods which results in a risk to human health, since cartap causes significant neuromuscular toxicity and ultimately results in a multisystem organ failure (Emadi et al., 2019). The main routes of exposure are eye, ingestion, and skin contact (Kumar et al., 2011). Despite its wide usage, there are few reports on human exposure to cartap resulting in clinical toxicology (Kurisaki et al., 2010).

2.2 Chemical structure of cartaphydrochloride

Chemically cartap is S, S-[2-(dimethylamino)-1,3-propanediyl] dicarbamothioate and is normally used as a hydrochloride (Abbasi and Krishnan, 1993). Chemical structure of cartap hydrochloride is as follows:

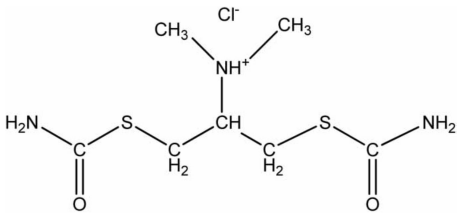


Fig.1 Structure of cartap hydrochloride

2.3 Toxicokinetics of Cartap hydrochloride

Cartap hydrochloride is rapidly absorbed, metabolized and excreted in urine both in rat and mice. There are no apparent differences in absorption and excretion of the compound in the two rodent species. In urine, following a single acute oral administered dose, 94 % and 89 % is excreted within 24 hours in rats and mice, respectively (Fujita et al., 1971). There was no storage of cartap or its metabolites in the body as might be expected of predominantly water-soluble nature of the compound. It is stable in acidic media but hydrolysed in alkaline and neutral medium solution. In rat orally administered cartap hydrochloride is primarily metabolized by hydrolysis of carbonyl carbon and oxidation of sulphur atom to sulfoxide (SO), sulfone (SO_2), and ultimately the sulfonate (SO_3H) (Kamesaki et al., 1976). In mammals, nereistoxin is methylated and oxidized at the sulfur atom as well

undergoing a series of oxidative demethylation reactions at the dimethylamino moiety.

2.4 Toxicodynamics of cartap hydrochloride

Nereistoxin inhibits post synaptic nicotinic acetyl choline receptor (nAChR) ion channels (El defrawi et al., 1980). By using single patch clamp technique on pheocytochroma cells of rat it was shown that cartap hydrochloride acts on nicotinic acetylcholine receptor (nAChR) in open state and causes burst of sub conductance of action potential (Nagata et al., 1997). As a result, it was generally stated that similar to neriestoxin, cartapalso produces its acute toxicity by neuromuscular blockade, leading to respiratory failure (Ray and laws, 1991). It was also suggested that the effect of cartap hydrochloride could be myogenic in nature because alpha-bungarotoxin and tetrodotoxin (both nAChR blockers) were unable to abolish cartap induced muscle contractions (O' malley et al., 1990). Rather than neuromuscular blockage, it was later demonstrated that cartap causes marked irreversible contracture in the isolated mouse phrenic-nerve diaphragm, and that this contracture is Ca^{2+} dependent (Liao et al., 2000). It is hypothesized that the primary effect of Cartap hydrochloride is through inhibition of the [H]-ryanodine binding to the Ca^{2+} release channel in the sarcoplasmic reticulum in a dose-dependent manner and promotion of extracellular Ca^{2+}influx and induction of internal Ca^{2+} release. This results in tonic diaphragmatic contraction rather than paralysis (Liao et al., 2000).

2.5 Acute, sub-acute and chronic toxicity studies of cartap hydrochloride

Cartap hydrochloride has an oral LD_{50} of 325 mg/kg bwt in rats and dermal LD_{50}>1000 mg/kg (WHO, 2004). It has an acceptable oral intake (ADI) of 0.1 mg/kg body weight and a no observed effect level (NOAEL) of 10 mg/kg in rats (WHO, 2004). In a study, male mice were given three different dose levels of cartap, i.e., 5 mg/kg, 7.5 mg/kg and 15 mg/kg body weight respectively, for 28 days orally. Water was used as vehicle to dissolve cartap. Oral administration of cartap caused significant increase in serum biomarkers, tissue oxidants and decrease in antioxidants along with histopathological findings in liver, kidney and brain tissues (Sharma et al., 2021). Cartap was fed @ 0,15,45 and 135 mg body weight for a period of 3 months to a group of rats (8-9 animals per group) of each sex and only slight reduction in body weight gain and food consumption in male animals was observed at highest dose

level (Tsubura et al., 1976). In 80 weeks long term study on two groups of rats of different sex (40 animals in each group); cartap was fed @ 0, 10, 20, and 40 mg kg b. wt. in diet and only slight reduction in body weight gain during first 52 weeks at 20 mg kg body weight and after 80 weeks additionally, reduced activity of erythrocyte cholinesterase and plasma cholinesterase in male and female animals respectively at highest dose level were observed (Hunter et al., 1975). *Labeo rohita* fingerlings exposed to sub-lethal concentration of cartap for 24, 48, 72 and 96 h, showed various degrees of histopathological changes in the liver along with alterations in oxidative stress parameters (Vivek et al., 2016). Histological alterations along with changes in oxidative stress parameters were found in the liver of rats exposed to cartap (10% LD_{50}) for a period of 15 days (Gupta et al., 2019).

2.6. Effect of cartap on liver

Cartap (10% LD_{50}) was found to significantly perturb the redox status of liver in the exposed wistar rats by increasing the production of free radicals (high MDA value) and decreasing the levels of GSH, SOD, CAT, and GST along with mild congestion and disorganization of hepatic cords (Gupta et al., 2019). Cartap (5mg/kg) produced mild congestion of portal area, while at 7.5 mg/kg and 15 mg/kg produced vacuolar degeneration and degenerative necrotic changes in hepatocytes respectively (Sharma et al., 2021). In the liver tissues of *Labeo rohita* fingerlings exposed to sub-lethal concentration of cartap for 24, 48,72 and 96 h, various forms of nuclear destruction like pyknosis, prominent yellowish-brown pigment (48 h), cytoplasmic degeneration and vacuoles (72 h and 96 h), disappearance of hepatic cells and change in the shape of the hepatocytes, karyolysis were observed. Karyolysis and karyorrhexis were found to have increased in the sub-lethal concentration (0.03851mg/L) of cartap (Vivek et al., 2016).

2.7 Effect on cartap on Excretory and Respiratory system

Following sublethal exposure to cartap in adult male mice, histopathological examination revealed degenerative changes around the glomerular area and in proximal renal tubule and thickening of alveolar septa/wall along with neutrophilic infiltration (Sharma et al., 2019, 2021).

2.8 Effect of cartap on Hematopoietic system

Cartap hydrochloride depredates the heme structure and adversely affects oxygen affinity and disturbs oxygen transport, which causes Hb to lose its normal function. Met-Hb is increased in the presence of cartap and this situation could lead to methemoglobinemia. Hydrogen bonds and hydrophobic interactions play a critical role in cartap interaction with Hb which can denature the protein structure (Emadi et al., 2019).

2.9 Effect of Cartap on Reproductive system

In two generations reproductive study, a group of 10 male and 20 female rats were given cartap in feed @ 0, 100, 1000 ppm for 9 weeks prior to mating. At 1000 ppm F1A generation showed reduction in body weight, feed consumption and impairment of fertility (Olson and Busey, 1972). Weaning survival index was lower in F1b generation than F1A generation. Only slight incomplete ossification was seen in F2b group of progeny at 1000 ppm. In an experiment group of mice and rats having 20, 12, 14 and 21, 12, 17 pregnant female animals in each group respectively were administered cartap by stomach tube @ 0,50,100 mg/kg body weight from day 8to day 13 of mice and from day 9 to day 15 of rat gestation period respectively. Mice and rats were sacrificed at $19^{th}$and 21^{st} days of pregnancy respectively and fetus were delivered by caesarean section. Only slight skeletal abnormality was seen (fusion of10 vertebrae) at 50 mg/kg (1.8 %) and 100 mg (1.7 %) in mice and 14.4% of bilateral twin thoracic vertebral centrae at 100 mg kg b. wt. in rats (Mizutani et al., 1971).

2.10 Effect of Cartap on Sensory systems

2.10.1 Dermal toxicity

In a study cartap was applied on intact as well as abraded skin of five groups of rabbit (five animals in each group) at dose level 0, 15, 60 and 240 mg/kg body weight/day and mortality was observed at highest dose level (240 mg/kg) within a week. Treatment continued on 15 and 60 mg kg bwt for 3 consecutive weeks and there was only mild dermal irritation in second week and reduced body weight gain, food consumption and incoordination of hind limb at 60 mg/kg body weight especially in third week of treatment (Davies et al., 1974).

2.10.2 Ocular toxicity

Cartap hydrochloride is not irritant to eye, no irritation of palpebral or bulbar conjunctiva was observed even 48 hours of instillation of 10 % solution (0.2 ml or 15 mg) into the eye of rabbit (Aramaki, 1972).

2.11 Oxidative stress markers

The term 'oxidative stress' means an imbalance between production of oxidants and antioxidant defences that may result in damage to biological systems (Forman and Zhang, 2021).It is caused by an imbalance between production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products. ROS can play several physiological roles (i.e., cell signaling), and they are normally generated as by-products of oxygen metabolism (Pizzino et al., 2017). Environmental stressors (i.e., UV, ionizing radiations, pollutants, and heavy metals) and xenobiotics (i.e., antiblastic drugs) contribute to greatly increase ROS production, therefore causing the imbalance that leads to cell and tissue damage (oxidative stress) (Burton 2011).

2.11.1 Lipid peroxidation

Lipids are susceptible targets of oxidation because of their molecular structure abundant with reactive double bonds. Lipid peroxidation can be described generally as a process occurring in toxic conditions under which oxidants such as free radicals or non-radical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid per oxyl radicals and hydroperoxides (Ayala et al., 2014). In response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death (Yin et al., 2011). The main primary products of lipid peroxidation are lipid hydroperoxides (LOOH) and among the many different aldehydes which can be formed a secondary products during lipid peroxidation, malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) have been extensively studied by Esterbauer and hiscolleagues in the 80s (Gaschler and Stockwell, 2017). They are all derived from polyunsaturated fatty acids (PUFAs) either by enzyme-catalyzed reactions (physiological) or by non-enzyme reactions (pathological) (Mas-Bargues et al., 2021). Several pesticide groups such as

organochlorines, organophosphates, carbamates, and herbicides can contribute to the process of membrane peroxidation by increased production of reactive oxygen species. DDT (40 mg/kg) was found to significantly increase hepatic lipid peroxidation in male rats (Hassoum et al., 1993). Endosulfan (1.5 mg/kg) produced a significant increase in lipid peroxidation in brain tissue in male wistar rats (Zervos et al., 2011). Lindane (100 mg/kg) induced significant increase in lipid peroxidation in heart tissue of wistar rats (Padma et al., 2013). Carbofuran at the concentration of 0.5, 1.0, 2.5 and 5.0 μM caused significant increase in lipid peroxidation in rat liver (Jaiswal et al., 2017).

2.11.2 Catalase

A catalase is one of the most important antioxidant enzymes. It is present in almost all aerobic organisms. Catalase breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water in a two-step reaction (Glorieux and Calderon, 2017). Among the reactive species, hydrogen peroxide is freely diffusible and is relatively long-lived (Grigoras, 2017). It acts as a weak oxidizing as well as reducing agent; however, it is not very reactive, but it is the progenitor of many other reactive oxygen species (ROS) (Nandi et al., 2019). In most cellular injuries, this molecule is known to play an indirect role. Catalase is a key enzyme which uses hydrogen peroxide, a non-radical ROS, as its substrate (Tehrani and Movahedi, 2018). This enzyme is responsible for neutralization through decomposition of hydrogen peroxide, thereby maintaining an optimum level of the molecule in the cell which is also essential for cellular signaling processes (Prieto et al., 2009). Without catalase, excessive H_2O_2, can react with metal ions to form the highly damaging hydroxyl radical (Galasso et al., 2021). Endosulfan (0.5, 1 and 1.5 mg/kg body weight) was found to significantly decrease the levels of catalase in heart and liver of male wistar rats (Alva et al., 2012). A significant decrease in catalase level was found in testicular tissue of rats exposed to DDT (Marouani et al., 2017).

2.11.3 Superoxide dismutase

Superoxide dismutases are enzymes that function to catalytically convert superoxide radical to oxygen and hydrogen peroxide (Abreu and Cabelli, 2009).They are localized in the cytosol and the mitochondrial intermembrane, the mitochondrial matrix and inner membrane (Rosa et al., 2021). Superoxide is produced as a by-

product of oxygen metabolism and, if not regulated, causes cellular damage (Hayyan et al., 2016). The toxicity of several xenobiotics including pesticides has been suggested to be mediated by formation of free radicals derived from reduction of molecular oxygen, forming superoxide anion and hydroxyl radical (Bus and Gibson, 1982). A significant decrease in the activity of SOD was found in the brain of rats fed with diet containing permethrin at three different levels of contamination i.e., 1 %, 5 % and 10 % (w\w) (Otitoju et al., 2008). Methomyl at a dose rat 2.03 mg/kg bw (Oral) produced a significant decrease in plasma SOD activity in male rats (Mansour et al., 2017).

2.11.4 Reduced Glutathione (GSH)

Glutathione is a substance produced naturally by the liver and is also found in fruits, vegetables, and meats (Amores-Sanchez et al., 1999). Reduced glutathione (GSH) is synthesized in a controlled manner intra cellularly in all mammalian cells and is involved in detoxification, antioxidant defense, and modulation of cell proliferation (Lu, 2009).

GSH protects cells by neutralising (i.e., reducing) reactive oxygen species responsible for oxidative damage (Wu et al., 2004). Adequate level of glutathione is essential in mitochondria, where ROS are constantly being formed as a minor by-product of oxidative respiration and in response to some drugs or metabolites that interfere with the mitochondrial respiratory chain (Mares et al., 2021). Glutathione levels are directly affected by toxic xenobiotics.DDT at two different dosages i.e., 50 and 100 mg/kg (Oral) produced a significant decrease in GSH levels in kidneys in adult male rats (Marouani et al., 2017). Carbofuran also produced a significant decrease in GSH in the hearts of adult male wistar rats (Jaiswal et al., 2013).

2.11.5 Glutathione s transferase (GST)

Glutathione-S-transferases (GST) are detoxifying enzymes that catalyze the conjugation of variety of electrophilic substrates including free radicals to thiol group of GSH, producing less toxic forms (Finkel, 2003). GST form the second phase enzyme detoxification system that protects the cells against endogenous oxidative stress and exogenous toxins (Prysyazhnyuk et al., 2021). GSTs catalyse the conjugation of GSH-via a sulfhydryl group—to electrophilic centers on a wide variety of substrates in order to make the compounds more water-soluble (Oakley, 2011).

This activity detoxifies endogenous compounds such as peroxidised lipids and enables the breakdown of xenobiotics. GSTs may also bind toxins and function as transport proteins, which gave rise to the early term for GSTs, ligandin (Leaver and George, 1998). Decrease in GST activity reflects inadequate detoxification or accumulations of ROS, resulting in oxidative damage and indicates a compromised cellular defence against xenobiotics (Hayes and Pulford, 1995). A significant decrease in GST activity was observed in brain tissue of male rats exposed to fenarimol and Carbofuran (Ozcelebi et al., 2021; Jaiswal, 2016).

2.11.6 Cartap hydrochloride and oxidative damage

Cartap induced oxidative damage is mostly due to generation of ROS (Liao et al., 2006). Reactive oxygen species (ROS), although are required for certain physiological responses, when produced in excess amounts are known to be important tissue damage causing agents, especially concomitant with elevated cytosolic Ca^{2+} (Gordeeva and Labas, 2003; Suzuki and Mittler, 2006). A significant dose and time-dependent increase in the amount of total intracellular ROS was seen in cartap-treated C_2C_{12} cells which is believed to be Ca^{2+} dependent (Liao et al., 2006). Accumulated evidence has shown that there is a close relation between stress-induced cytosolic and/or mitochondrial Ca^{2+} influx and ROS production (Goldman and Popel, 1999; Gordeeva and Labas, 2003; Supinski et al., 1999; Tan et al., 1998). The stimulation of ROS generation is through the activation of certain Ca^{2+} -dependent ROS-generating enzymes such as NADPH-oxidase and phospholipase A_2 (Gordeeva and labas, 2003; Supinski et al., 1999). Cartap (10% LD_{50}) was found to significantly perturb the redox status of liver in the exposed wistar rats by increasing the production of free radicals (high MDA value) and decreasing the levels of GSH, SOD, CAT, and GST (Gupta et al., 2019). Similarly, a significant decrease in the levels of GSH, SOD, CAT, and GST along with significant increase in MDA (LPO) was found in kidneys and brain of mice at three different dose rates i.e., 5, 7.5 and 15 mg/kg body weight (Sharma et al., 2021). A time dependent increase in SOD activity was found in brain, liver and gills of Labeo Rohita fingerlings exposed to cartap @ $1/10^{th}$ LC_{50} i.e., 0.03851 mg/L (Vivek et al., 2016).

2.12 Testicular injury biomarkers

2.12.1 Sorbitol dehydrogenase (SDH)

Sorbitol Dehydrogenase (SDH) is a cytosolic enzyme in polyol pathway and catalyzes the conversion of sorbitol to fructose with NAD+ as the coenzyme (Kabbani et al, 2004).Together with aldose reductase, it provides a way for the body to produce fructose from glucose without using ATP (Bekhit and Gorski, 2021). SDH is mainly located in the cytoplasm and in the mitochondria of liver, kidney and seminal vesicles (Gerlach and Walter, 1974). In testes the activity of SDH is associated with the maturation of germinal epithelial layer of seminiferous tubule (Mills and Means, 1972). Rounded immature germinal epithelial cells undergo successive mitotic and meiotic divisions and a metamorphic change to produce spermatozoa (Cheng and Mruk, 2010). The activity of SDH is increased markedly during the maturation of germinal epithelium and is an indicator of testicular maturation (Jana et al., 2006). Arrested development of spermatogonia cells of germinal epithelium is correlated to fall in SDH activity (Shen and Lee, 1984). Exposure to Copper (33 mg/kg) or flubendiamide (200 mg/kg) alone and both of these in combination resulted in no significant change in testicular SDH in wistar rats (Mandil et al., 2016). Imidacloprid @ 45 mg/kg bwt and 90 mg/kg bwt produced a significant increase in testicular SDH in adult male wistar rats (Lonare et al., 2014).

2.12.2 Lactate dehydrogenase (LDH)

LDH is an important enzyme of the anaerobic metabolic pathway. Its function is to catalyze the reversible conversion of lactate to pyruvate and this enzyme is ubiquitously present in all tissues and serves as an important checkpoint of gluconeogenesis and DNA metabolism (Klein et al., 2020). The increase in LDH activity suggests deterioration of germinal epithelium (Teng et al., 2016). A significant increase in plasma LDH activity was observed in adult lactating female rats on oral administration of chlorpyrifos (Mansour and Mossa, 2010). Carbofuran (1.6 mg/kg bwt) produced a significant increase in LDH activity in the brain of adult male wistar rats (Jaiswal et al., 2018).

2.12.3 Gamma glutamyl transpeptidase (GGT)

Gamma-glutamyl transpeptidase (GGT) is a transferase type of enzyme that catalyzes the transfer of gamma-glutamyl functional groups to an acceptor that

may be an amino acid, or a short chain peptide (Tate and Meister, 1985; Whitfield, 2001). GGT is present in the cell membranes of many tissues, including the kidneys, bile duct, pancreas, gallbladder, spleen, heart, brain, and seminal vesicles (Goldberg, 1980). It plays a major role in amino acid transport for the synthesis of specific proteins known to be secreted by sertoli cells (Tate and Meister 1981). Increase in its activity is due to impaired function of sertoli cells (Hodgen and Sherins, 1976). Lambda cyhalothrin and thiodicarb produced a significant increase in plasma GGT activity in adult male wistar rats (Omayma et al., 2019).

2.12.4 Acid Phosphatase

Acid phosphatase (ACP) is an enzyme that catalyzes the hydrolysis of phosphate esters in an acidic environment (Bull et al., 2002). This chemical rection is essential to different metabolic processes. It is a lysosomal enzyme which is primarily present in bone, prostate, platelets, erythrocytes, testes and spleen (Garnero et al., 2008). Increase in ACP activity in testes couldbe due to release of this enzyme from lysosomes of degenerating cells, rapid catabolism of injured germ cells, enhanced cell membrane permeability with disturbance in transphosphorylation (Hodgen and Sherins, 1973). Insecticide Vertimec (0.11 and 1.1 mg/kg, PO) produced a significant elevation in plasma acid phosphatase in adult male rats (Eissa and Zidan, 2010). Dimethoate (3.66, 5.50, and 11 mg/kg) produced a significant decrease in testicular acid phosphatase in adult male wistar rats (Ngoula et al., 2014).

2.13 Testosterone

Testosterone is a pleiotropic hormone that plays an important role in males. In male humans, testosterone plays a key role in the development of male reproductive tissues such as testes and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair (Mooradian et al., 1987). Testosterone is the main androgen secreted by the interstitial cells (Leydig cells) of the testes in response to anterior pituitary luteinizing hormone (Bain, 2007). Androgen synthesis in the gonads of males is regulated by secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus stimulating the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (Schally et al., 1971). LH stimulates synthesis of testosterone in testicular tissue. There is feedback inhibition of GnRH

and LH release from the hypothalamus and pituitary, respectively, by testosterone (Schally et al., 1971). Haider and Servos (1998) suggested that decrease in testosterone level is due to reduced testicular steroidogenesis in leydig cells and suggested that endocrine disrupting environmental contaminants have a direct inhibitory effect on testosterone production. Kong et al. (2017) pointed that xenobiotic induced fall in testosterone levels might be indicative of ROS mediated Leydig cell damage. Alterations of reproductive hormone levels may lead to a decline in semen quality (i.e., concentration, motility, and morphology) and sperm DNA damage/fragmentation (Panuwet et al., 2017). These alterations may not only impede fertility, but may also contribute to developmental disorders in offspring (Meeker, 2010). Pesticides are responsible for decreasing testosterone concentration either by inhibiting release of FSH or LH (Slimani et al., 2011). Pesticides are also responsible for the apoptosis of leydig cells and thus decreasing overall production of testosterone (Kaur et al., 2014). Insecticides and pesticides have been shown to act as reproductive toxicants in male rats and histologically induce severe focal necrosis of the germinal cells in seminiferous tubules associated with tubular atrophy (Bustos and Gonzalez, 2003). Chronic exposure to copper (33 mg/kg) or flubendiamide (200 mg/kg) alone and both these in combination resulted in significant decrease in serum testosterone levels in wistar rats (Mandil et al., 2016). Acetaprimid also produced a significant decrease in plasma testosterone levels in male Sprague Dawley rats (Arican et al., 2020).

2.14 Heat Shock Protein-70 (HSP-70)

70-KDa Heat shock proteins (HSP-70s) are central components of the cellular network of molecular chaperones and folding catalysts (Mayer and Bukau, 2004). Intracellularly localized HSP-70s are important part of the cell's machinery for protein folding, performing chaperoning functions, and helping to protect cells from adverse effects of physiological stresses (Tavaria et al., 1996; Morano, 2007). HSP-70 release has been demonstrated in cultured glial cells, human peripheral blood mononuclear cells (PBMCs), human macrophages, human epithelial cells, and human tumor cells (Luo et al., 2008). Several studies have shown that HSP-70 is actively released from viable cells via a mechanism involving lipid rafts (Broquet et al., 2003). HSP-70 expression is the first-tier bio-indicator of chemical induced toxicity since this gene was found to be the first inducible gene in the organism after

chemical stress (Gupta et al., 2005). Stress induced HSPs act by aiding the refolding of denatured proteins, thereby preventing adverse metabolic effects arising from accumulation of misfolded proteins and proteotoxic cell death (Vos et al., 2008). Pesticides when ingested by non-target organisms, generate reactive oxygen species and oxidative stress which catalyzes unfolding and aggregation of native proteins. In response, HSP 70 is synthesized within cells and block protein degradation (Rajak and Roy, 2018). Sublethal Paraquat intoxication in adult male rats (80 mg/kg) resulted in a significant upregulation of HSP-70 gene in lungs of adult male wistar rats (Zhang et al., 2010). Similarly, a significant upregulation of HSP-70 gene was observed in testes of adult male wistar rats exposed to sublethal (5 mg/kg) dosage of deltamethrin (Ismail and Mohamed, 2013).

2.15 Sperm parameters studies

2.15.1Rat spermatozoa

Rat spermatozoa have a falciform – shaped head that is composed of four major parts: the nucleus, the acrosomic system, the perinuclear theca and the plasma membrane (Clermont et al., 1993; Eddy, 2006; Protopapas et al., 2019). The nucleus takes up most of the head volume and contains the haploid genome tightly packed with protamines (Lin et al., 2013). The acrosomic system consists of two distinct regions, the acrosome cap (also known as acrosome proper, anterior acrosome) and the head cap (also known as equatorial segment, posterior acrosome) (Lin et al., 2013). The acrosome cap sits on top of the nucleus in the anterior- dorsal part of the head and contains numerous digestive enzymes that facilitate the passage of the sperm through the cumulus cell layer and the zona pellucida during fertilization (Jin et al., 2011). The head cap is much thinner and covers much of the lateral surface of the head (Lalli and Clermont, 1981). The perinuclear theca is a cytoskeletal structure that covers most of the nucleus except in the area of perifossal zone near the base of the head where the tail is attached (Lin et al., 2013). The neck region contains the connecting piece, which is the structural base of the tail and forms the surface for articulation with the head. In the middle piece, there is also a helically-arranged mitochondrial sheath. The sperm tail is specialized flagellum that provides the spermatozoon with the motility necessary for to ascend to the site of fertilization and penetrate the extracellular coats around the oocyte (Yanagimachi, 1981).

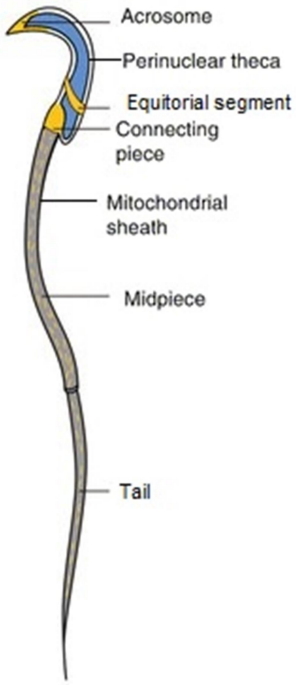


Fig. 2: Structure of rat spermatozoon

2.15.2 Sperm concentration

Sperm concentration, or density, represents the number of spermatozoa per unit volume and is represented usually in millions per mm^3 or ml (Sieme, 2009). Sperm concentration is one of the major markers of fertility. Xenobiotic toxicants may directly damage spermatozoa, sertoli cells, leydig cells via ROS production or disrupt the endocrine regulation of spermatogenesis by altering hormonal balance thereby affecting sperm concentration. (Bretveld et al., 2007). Spermatogenesis and male fertility are dependent upon the presence of testosterone. In the absence of testosterone or the androgen receptor, spermatogenesis does not proceed beyond the meiosis stage (Walker, 2011). A significant reduction in sperm count was observed in adult male rats exposed to quinalphos and Diazinon (Pant and Srivastava, 2003; Adamkovicova et al., 2016).

2.15.3 Live and Dead count

The assessment of sperm vitality is one of the basic elements of semen analysis, and is especially important in samples where many sperms are immotile, to distinguish between immotile dead sperms and immotile live sperms (Bjorndahl et al.,

2003) and Eosin and nigrosine stain is commonly employed for this purpose. Pesticides have been reported to produce significant alteration in semen quality among various species of mammals (Juhler et al., 1990). Kato et al. (2001) and Prahalathan et al. (2004) suggested that ROS mediated oxidative stress to spermatogenic compartment may be considered as one of the mechanisms of abnormal and dead spermatozoa following xenobiotic exposure. Exposure to Copper (33 mg/kg) or flubendiamide (200 mg/kg) alone and both of these in combination resulted in significant decrease in percent live spermatozoa in wistar rats (Mandil et al., 2016). Diazinon at a concentration of 40 mg/ml when given orally produced a significant reduction in percent live spermatozoa in adult male wistar rats (Adamcovicova et al., 2016).

2.15.4 Hypo osmolar swelling test

The hypo osmolar swelling test evaluates the functional integrity of the sperm's plasma membrane and also serves as a useful indicator of fertility potential of sperm (Ramu and Jayendran, 2013). ROS induced lipid peroxidation is one of the major reasons for reduced membrane integrity of the spermatozoa (Sanocka and Kurpisz, 2004). Exposure to Copper (33 mg/kg) or flubendiamide (200 mg/kg) alone and both of these in combination resulted in significant decrease in host positive spermatozoa in wistar rats (Mandil et al., 2016). A significant decrease in HOST positive spermatozoa was observed in adult male wistar rats when exposed to mancozeb @500 mg/kg bwt (Girish and Reddy, 2017).

2.15.5 Acrosomal integrity

Acrosomal intactness is an important factor for acrosomal reaction to occur which results in the loss of anterior acrosomal contents and exocytosis upon interacting with zona pellucida (Ozaki et al., 2002). It is susceptible to elevated reactive oxygen species (ROS) and oxidative stress (El-Taieb et al., 2015). Several techniques have been proposed to differentiate intact from non-intact acrosome. The labelling with fluoresceinated lectins is one of the most commonly employed techniques to differentiate intact from non-intact acrosome (Talbol and Chacon, 1980). Ravula and Yenugu (2021) observed no significant change in the mean inflorescence intensity in the acrosomes of spermatozoa subjected to FITC-PNA

stain in adult male wistar rats exposed to sub lethal dosage of pyrethroid mixture for a period of 15 months.

2.16 α-tocopherol

Vitamin E (α- Tocopherol) encompasses a group of eight lipophilic molecules that are synthesized by plants starting from homogentisic acid (Gee, 2011). It includes four tocopherols and four tocotrienols. Tocopherols and tocotrienols are subdivided into alpha (α), beta (β), gamma (γ), and delta (δ) forms based on the methyl and hydroxyl substitution in their phenolic rings (Gee, 2011). The tocopherols are saturated forms of Vitamin E, whereas the tocotrienols are unsaturated and possess an isoprenoid side chain (Sheppard et al., 1993). α-Tocopherol is considered as the most abundant form in nature, and is consequently the most widely studied. Common food oils including corn, peanut, and soybean oil contain largely α-tocopherol. In contrast, tocotrienols are relatively rare in food sources and prevail in rice bran, barley, oats, and palm oil (Jensen and Lauridsen, 2007). Vitamin E is widely accepted as one of the most potent antioxidants in nature (Peh et al., 2016). The antioxidant property is attributed to the hydroxyl group from the aromatic ring which donates hydrogen to neutralize free radicals or reactive oxygen species (ROS). The antioxidant activity of α-, β-, γ-isoforms of tocotrienols and tocopherols is similar, but the γ-isoform showed weaker activity when tested in pyrogallol sulfonphthalein and 2,7-dichlorodihydro fluorescein diacetate methods (Yoshida et al., 2007). α-tocopherol is a natural form of vitamin E, and it exhibits highest vitamin E bioactivity among the eight natural forms of vitamin E. α-tocopherol is a major lipid soluble antioxidant and is known to protect cellular membranes and lipoproteins from peroxidation (Yavuz et al., 2004). α-tocopherol inhibits free radical formation (Kalender et al., 2005) and can effectively minimize lipid peroxidation and oxidative damage (Traber and Atkinson, 2007). To date several studies have been conducted to examine the protective effects of α-tocopherol on pesticide induced oxidative stress. Masaki et al. (2002) reported a significant increase in intracellular GSH in HaCaT keratinocytes cultivated in α-tocopherol. α-tocopherol reduced Cypermethrin induced increase in total Cholesterol and triglycerides by 32 % and AST by 29 % in wistar rats (Aldana et al., 2001). Tocopherol and Selenium reducedthe concentration of MDA and increased the concentration of total proteins, GSH, SOD, CAT, and GP_X in the liver and kidney of wistar rats exposed to malathion for a period of 45 days (John et al., 2001).

α-tocopherol alone and in combination with beta carotene improved semen quality in wistar rats exposed to Fenvalerate for a period of 30 days (El-Demerdash et al., 2004). Alpha tocopherol reduced increase in ALT, AST, total cholesterol, triglyceride and VLDL in wistar rats exposed to Diazinon for 7 weeks (Kalender et al., 2005). Treatment of rats with malathion and ascorbic acid or α-tocopherol or a combination of three prevented malathion induced changes in antioxidant enzyme activity and lipid peroxidation (Durak et al., 2009). α-tocopherol and selenium were effective in partially alleviating degenerative changes induced by malathion in the liver of chicks by attenuating the process leading to lipid peroxidation (Sodhi et al., 2008). Concurrent administration of α-tocopherol was found to be very effective in ameliorating the toxicity produced by flubendiamide and copper (alone and in combination) in wistar rats particularly by reducing oxidative damage in terms of lipid peroxidation and improving anti-oxidant system in cells (Mandil et al., 2016). Effect of α-tocopherol on serum biochemical profile has also been reported in a few studies. Manning et al. (2004) observed a significant fall in plasma glucose following 3-month administration of α-tocopherol in over weight individuals. Oxidative stress is partly responsible for the development of insulin resistance and therefore antioxidants like α-tocopherol and ascorbic acid which reduce ROS levels can improve glycemic control and reduce plasma glucose level (Evans et al., 2003). Peluzio et al. (2001) observed a significant lowering of total cholesterol following 6 weeks treatment of apolipoprotein E- deficient mice fed on high fat atherogenic diet with α-tocopherol. α-tocopherol down regulates hepatocyte CYP27A1 gene responsible for the synthesis of sterol-27-hydroxylase, an enzyme crucial for the sterol biosynthesis pathway resulting in reduced cholesterol synthesis (Landrier et al., 2010). Various other therapeutic effects of α-tocopherol have also been reported in different studies. Ehterami et al. (2019) reported accelerated wound healing following application of α-tocopherol based gel in wound healing rat model. Palipoch et al. (2014) reported reduction in hepatic congestion following treatment with α-tocopherol following cisplatin induced oxidative injury in adult male wistar rats. Nephroprotective potential of α-tocopherol has been reported by Othman et al. (2011) who observed restoration of normal kidney histoarchitecture following co-treatment with α-tocopherol in adult male rats exposed to sublethal dosage of malathion. Cardioprotective effect of α-tocopherol against lipid peroxidation was reported by Gornicka et al. (2019) who observed reduced cardiac lipid peroxidation following treatment with α-tocopherol in

growing male rats subjected to physical efforts. Anti-atherosclerotic potential of α-tocopherol was reported by Terasawa et al. (2000) who observed reduction in plaque buildup in arterial walls of hyperlipidemic adult male mice following treatment with α-tocopherol. Mandil et al. (2020) reported restorative potential of α-tocopherol as he observed normal histoarchitecture of splenic tissue following concurrent treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of flubendiamide.



Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

Place of investigation

Present study was carried out in the Laboratories of the Department of Veterinary Pharmacology and Toxicology, Veterinary Pathology and Central Instrumentation Laboratory of College of Veterinary Science and Animal Husbandry, DUVASU, Mathura.

3.1Chemicals used

Following chemicals, reagents and diagnostic kits were used in the present study:

Chemicals/Diagnostic kits	**Brand/Make**
1-Chloro-2,4-dinitrobenzene (CDNB)	Sigma Aldrich
1Kb Plus DNA ladder	Thermo Fischer scientific
2-mercaptoethanol	VWR Life science
5,5' Dithiobis (2-nitrobenzoic acid) (DTNB)	Sigma Aldrich
Acetate buffer	Sigma Aldrich
Acid phosphatase assay kit	Sigma Aldrich
Agarose	VWR Life science
Albumin kit	Span diagnostic
ALT kit	Span diagnostic
AST kit	Span diagnostic
Bilirubin kit	Span diagnostic
Bovine serum albumin	Sigma Aldrich
BUN kit	Span diagnostic
Calcium chloride	Merck
Calcium kit	Span diagnostic
Cartap commercial grade (Caldan^{R} 50 SP)	Dhanuka Agritech (India)
Creatinine kit	Span diagnostic
D-fructose	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
EDTA	VWR Life science
EDTA (disodium salt)	Sigma Aldrich

Ethanol (absolute)	Merck
FITC PSA	Sigma Aldrich
GGT kit	Coral
Glucose kit	Span diagnostic
Glutathione	Sigma Aldrich
HDL kit	Span diagnostic
HEPES	Sigma Aldrich
Hydrogen peroxide	Merck
LDH kit	Arkray
LDL kit	Span diagnostic
Magnesium chloride	Merck
Methanol	Sigma Aldrich
Methylthiazolyldiphenyl-tetrazolium bromide (MTT)	Sigma Aldrich
Nicotinamide adenine dinucleotide hydrogen (NADH)	Sigma Aldrich
para-Nitrophenyl phosphate (PNPP)	Sigma Aldrich
PCR master mix	Thermo Fischer scientific
Phosphate buffered saline tablet	VWR Life science
Potassium chloride	Merck
Power Up™ SYBR™ Green Master mix	Thermo Fischer scientific
Pyrogallol	Sigma Aldrich
Rat/mouse testosterone ELISA kit	Demeditech diagnostic
RevertAid™ First Strand cDNA synthesis kit	Thermo Fischer scientific
RNA isolation kit	Thermo Fischer scientific
RNA Zap	Sigma Aldrich
Sodium bicarbonate	Merck
Sodium chloride	Merck
Sodium citrate	Sigma Aldrich
Sodium hydroxide	Rankem
Sodium hypophosphate	Merck
Sodium lactate	Sigma Aldrich
Sodium pyruvate	Sigma Aldrich
Thiobarbituric acid (TBA)	Sigma Aldrich
Total Cholesterol kit	Span diagnostic
Total protein kit	Span diagnostic

Trichloroacetic acid (TCA)	Sigma Aldrich
Triethanolamine buffer	Sigma Aldrich
Tris base	VWR Life science
Tris EDTA buffer	Sigma Aldrich
α tocopherol	Sigma Aldrich

3.2 Equipment/Instruments used

Following equipments/instruments were used in the present study:

Equipment	**Make/Company**
Autoclave	Sonar, India
Biophotometer	Eppendorf
CO_2 Incubator	Eppendorf
Compound microscope	Motic
Electronic balance	Shimadzu
ELISA reader (Multiskan™FC)	Thermo Fischer Scientific
Fluorescent microscope (EVOS™M5000)	Thermo Fischer Scientific
Gel documentation system (iBright™)	Thermo Fischer scientific
Haematology autoanalyzer (MS4Se™)	Melet Schloesing
Horizontal Electrophoresis system	BioRad
Hot air oven	Instron
Micro centrifuge	Tarsons
Microtome	Leica
pH meter	Systronics
Phase contrast microscope (Nikon TS-400)	Nikon
Refrigerated Micro centrifuge	Eppendorf
Refrigerated Centrifuge	REMI, C-30BL
RT-PCR system (Quant Studio™3)	Thermo Fischer scientific
Semi-autoanalyzer	Erba, Manheim
Shaker	Genei
Thermocycler (T-100 Thermal cycler)	BioRad

Tissue homogenizer	Heidolph
Ultra-low deep freezer,	New Brunswick Scientific
UV cabinet	Thomas Scientific
UV-VIS Spectrophotometer	ECIL
Water bath	Macflow

3.3 Experimental animals

Healthy adult male Wistar rats weighing 130-150 gm were procured from Indian Institute of Toxicology Research (CSIR-IITR), Lucknow. Before start of the experiment, animals were kept in laboratory conditions for a period of fifteen days for acclimatization and maintained under standard managemental conditions with daily 12 hours of light-dark cycle along with *ad libitum* balanced pelleted feed (Ashirwad feed industries, Chandigarh) and clean drinking water. All the animals were kept under constant observation during entire period of study. Experimental protocol involving use of adult male rats was approved by IAEC approval no. IAEC/20/3 (via letter No. 131/IAEC/20 Dated 30.12.2020) as per guidelines of CPSEA, Govt. of India.

3.4 Experimental design

Forty-two rats (42) were grouped into seven groups of six animals each. Group I, II served as control, vehicle control respectively, while animals of group (III-VII) received orally α-tocopherol (100 mg/kg), cartap (16.25 mg/kg), cartap (32.5 mg/kg), cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg) and cartap (32.5 mg/kg) + α-tocopherol (100 mg/kg) respectively. Animals were treated with cartap hydrochloride at the dose rate of 16.25 mg/kg and 32.5 mg/kg body weight which is equivalent to $1/20^{th}$and $1/10^{th}$ of the reported LD_{50} of cartap in adult male rats respectively. The reported LD_{50} (oral) of cartap in adult male rats is 325 mg/kg body weight (WHO, 2004). Cartap was administered alone and with α-tocopherol (100 mg/kg) for a period of 28 days via oral gavage. Solution of cartap was prepared by dissolving in distilled water and α-tocopherol was dissolved in corn oil. The compound used in the present study was Caldan^{R}50 SP (50% Cartap hydrochloride) by Dhanuka Agritech, Gurgaon procured from local market. Details of the experimental design were as follows:

Groups	Treatment	Mode of administration	Exposure period	Number of animals
I.	Control	Oral	28 days	6
II.	Vehicle Control (Corn oil)	Oral	28 days	6
III.	α-tocopherol (100 mg/kg)	Oral	28 days	6
IV.	Cartap hydrochloride (16.25 mg/kg)	Oral	28 days	6
V.	Cartap hydrochloride (32.5 mg/kg)	Oral	28 days	6
VI.	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	Oral	28 days	6
VII.	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	Oral	28 days	6

3.5. General parameters

During the entire study period animals were closely observed for any apparent clinical signs or symptoms of toxicity. Body weight was measured on 7^{th}, 14^{th}, 21^{st} and 28^{th} day of experiment, and after end of experimental period of 28 days animals were sacrificed by cervical dislocation and organ weights (relative and absolute) were assessed for heart, liver, kidneys, spleen, lungs and testes.

3.6Haemato- biochemical parameters

3.6.1 Collection of blood samples

Blood samples of experimental rats were collected on day 29 of the study period from retro-orbital plexus in EDTA (for haematological analysis) and heparinized vials (for plasma collection) on day 29 after overnight fasting. Glucose estimation was done on the day of collection while other biochemical parameters were determined at the end of experiment in plasma samples. Plasma was collected by centrifuging the blood collected in heparinized vials at 3000 rpm for 20 minutes.

Plasma was collected in a 2 ml micro centrifuge tube and stored at -20^0 C for further analysis.

3.6.2 Hematological parameters

Total erythrocyte count, hemoglobin, packed cell volume, total leukocyte count, differential leukocyte count and Platelet count were estimated using hematological cell counter (MS4Se^{TM}, Melet schloesing).

3.6.3 Plasma biochemical parameters

Total protein, Albumin, Globulin, Bilirubin, Calcium, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Creatinine, BUN, Glucose, Total Cholesterol, HDL and LDL and Triglycerides in plasma were estimated by commercially available kits (Span diagnostics Ltd, India) with the help of semi-auto analyser (Erba, Manheim).

3.7Oxidative stress markers in testes and liver

Levels of different oxidative stress related parameters in testes and liver were determined by employing different assay by using double beam UV-VIS spectrophotometer.

3.7.1 Preparation of tissue homogenate

200 mg of tissue sample was weighed and taken in 2 ml of chilled saline. 200 mg sample weighed separately and taken in 2 ml of 0.02 M EDTA for GSH estimation. The homogenates were prepared by using tissue homogenizer under cold condition and were centrifuged for 10 min at 3000 rpm. The supernatant was used for estimation of different oxidative stress markers.

(a) Lipid peroxidation

The extent of lipid peroxidation was evaluated in terms of malonaldehyde (MDA) production, determined by method as described by Rehman (1984).

Reagents

1) TCA (10 %): 10 g of TCA was dissolved in distilled water and final volume was made up to 100 ml.
2) 0.67 % TBA: 0.67 g of TBA was dissolved in 100 ml of distilled water and warmed up for complete dissolution.

Procedure

1 ml of homogenate supernatant was incubated at 37^0C for 2 h. To each sample 1 ml of 10 % TCA was added. After thoroughly mixing, it was centrifuged at 2000 rpm for 10 min. To 1ml of supernatant liquid an equal amount of 0.67 % TBA was added and kept in boiling water bath for 10 min, cooled and diluted with 1ml distilled water. The absorbance was read at 535 nm.

Calculation

The concentration of LPO was calculated by using the molar extinction coefficient (EC) of MDA- TBA complex at 535 nm, i.e., 1.56 x 10^8 /M/cm and result was expressed in nM MDA formed /g of wet tissue.

$$\text{LPO (nM MDA formed /g tissue)} = \frac{\text{OD} \times \text{total volume of reaction mixture}}{\text{EC} \times \text{amount of sample}} \times 10^9 \times \text{DF} \times 2$$

(b) Reduced glutathione (GSH)

GSH was determined by estimating free –SH groups, using DTNB method of Sedlak and Lindsay (1968). For determining reduced glutathione, 10 % tissue homogenate was prepared in 0.02 M EDTA.

Reagents

1) DTNB (0.01M): 99 mg of DTNB in 25 ml of methanol.
2) 1M Tris-buffer (pH 8.9): 121g of Tris base was dissolved in 950 ml of distilled water. pH was adjusted to 8.9 and final volume was made up to 1000 ml by addition of distilled water.
3) TCA (50 %): 50 g of TCA was dissolved in distilled water and final volume was made up to 100ml.

Procedure

To 1 ml of homogenate supernatant, 0.8 ml of distilled water and 0.2 ml of 50% TCA solution were added and kept at room temperature for 15 min. This solution was centrifuged at 3000 rpm for 15 min. 0.4 ml of supernatant was taken and to this 0.8 ml of Tris buffer was added, followed by 0.2 ml DTNB (0.01M) and after mixing, the absorbance was read at 412 nm within 5 min. Reagent blank had no sample and sample blank was without DTNB.

Calculation

The concentration of GSH was calculated by using the extinction coefficient (EC) 1300 M^{-1} cm^{-1} and results were expressed in mM GSH/g of wet tissue.

$$\text{GSH (Mm/g of tissue)} = \frac{\text{OD} \times \text{total volume of reaction mixture}}{\text{EC} \times \text{amount of sample taken}} \times 1000 \times \text{DF}$$

DF = Dilution factor

(c) Superoxide Dismutase

Superoxide dismutase was estimated as per the method described by Madesh and Balasubramanian (1998).

Reagents

1) 100 µM – Pyrogallol (6.3 mg of Pyrogallol was dissolved in 5 ml of distilled water; 1ml of this solution was added to 100 ml of distilled water).
2) 1.25 mM MTT (2.58 mg MTT was dissolved in 5ml distilled water)
3) Phosphate buffer saline

Procedure

Different reagents were added in sample, control and blank as shown below. The absorbance was read at 570 nm against distilled water.

Reagent	Test /sample	Blank	Control
PBS	650 µl	650 µl	650 µl
MTT	30 µl	30 µl	30 µl
Homogenate	10 µl	-	-
Pyrogallol	75 µl	75 µl	75 µl
Incubate at RT for 5 min			
DMSO	750 µl	750 µl	750 µl
Homogenate	-	-	10 µl
DW	-	10 µl	-
Take OD at 570 nm			

Calculation

Superoxide Dismutase was expressed as SOD units / mg of protein.

$$Y\ \% = \frac{\text{OD of test}}{\text{OD of blank}}$$

SOD (U /mg protein) = (mg protein in 10 µl homogenate /Y %) x 50 x DF

where,

DF = dilution factor

Y % = % inhibition of MTT by SOD

(d) Catalase

Catalase in tissue homogenate was estimated and calculated by the method of Bergmeyer (1983).

Reagents

1) Phosphate buffer (50 mM pH 7.0)

A) KH_2PO_4 - 1.37 g/ 200 ml DW (50 mM)

B) Na_2HPO_4 - 1.42 g/ 200 ml DW (50 mM)

Solution A and B were mixed in a ratio of 1:1.5 (v/v) and pH was adjusted to pH 7.0.

2) H_2O_2 (10 mM) 0.1 ml H_2O_2 of 30 % was diluted to 100 ml DW.

Procedure

In a test tube, 2 ml phosphate buffer and 10 µl homogenate were added and the contents were transferred to cuvette. By adding 1 ml of H_2O_2 directly into the cuvette, the reaction was started. OD was recorded at every 30 second for 3 min at 240 nm against blank (DW).

Calculation

The activity of catalase was expressed as mM H_2O_2 utilized /min/ mg protein and calculated using the formula:

Catalase mM H_2O_2 utilized /min/ mg protein =

$$\frac{\Delta OD/time}{0.067} \times \frac{\text{total volume of reaction}}{\text{amount of sample take}} \times 1/\text{ mg protein in 10 µl}$$

(e) Glutathione-S-transferase (GST)

Glutathione-S-transferase activity in kidney was estimated by method of Habig et al., 1974. 1 ml of phosphate buffer (0.3M; pH 6.5) and 0.1 ml of CDNB (30

mM CDNB in 95% alcohol) was taken to which 0.1 ml of tissue homogenate was added. 1.7 ml of distilled water was added to make final volume to 2.9 ml. The reaction mixture was incubated at 37ºc for 5 minutes in BOD incubator and reaction was started by adding 0.1 ml of 30 mM glutathione. The absorbance was measured at 60 sec intervals for 5 min at 340 nm. Reaction mixture without homogenate was used as blank. Calculation was done by using molar extinction coefficient (EC) of CDNB - GSH conjugate (9.6 mM cm^{-1}) and result was expressed in µM of CDNB-GSH conjugate formed per minute per mg of protein.

$$GST\ (\text{M min}^{-1}\ \text{mg}^{-1}\ \text{protein} = \frac{\Delta\text{OD x Total volume x 1}}{\text{EC x Protein in mg x time}}$$

where,

EC = 9.6 is millimolar extinction co-efficient

(f) Total Protein

Total protein was estimated by method of Lowry et al. (1951).

Reagents

1) Reagent A: 2 % Sodium Carbonate in 0.1N NaOH (2 g Na_2CO_3 in 100 ml of 0.1N NaOH)
2) Reagent B: 1 % copper sulphate (100 mg of $CuSO_4$ was dissolved in 10 ml of distilled water and stored in amber coloured bottle)
3) Reagent C: 2 % sodium potassium tartarate (200 mg of sodium potassium tartarate was dissolved in10 ml of distilled water)
4) Reagent D (Alkaline copper sulphate): It was prepared by mixing 49 ml of reagent A with 0.5 ml of each of reagent B and C just before estimation.
5) Folin-Ciocalteau reagent (1N): 2 ml Folin-Ciocalteau reagent was diluted with 2 ml of distilled water. Fresh solution was prepared.
6) Bovine Serum Albumin: 50 mg of BSA was dissolved in 50 ml of distilled water and used as standard.

Procedure

To 20 µl of sample (1:100) in a test tube 980 µl of distilled water and 2.5 ml alkaline copper sulphate solution were added. The reaction mixture was allowed to stand for 10 minutes at room temperature. Thereafter, Folin-Ciocalteau reagent (0.25 ml) was added and tubes were shaken immediately. The whole mixture was allowed to stand for 30 minutes at room temperature and absorbance was read at 750 nm. For

BSA standard, 100 µl was added to 900 µl, for blank to 1000 µl of distilled water and other solutions were added as per the sample.

Calculation

Protein (µg/ml) = OD of test/OD of standard x 100 x1/0.02

3.8 Testicular injury biomarkers

3.8.1 Sorbitol dehydrogenase (SDH)

The SDH estimation was done as per method described by Gerlach and Walter (1974).The SDH assay is based on inter conversion of fructose and sorbitol. During reduction of fructose, an equimolar amount of NADH is oxidized to NAD. Testicular tissue was homogenized in chilled 0.1 mol Tris EDTA buffer (pH 8.0) at a tissue concentration of 10mg/ml and then homogenizing mixture was centrifuged at 10,000 g for 30 min at 4^0C. The supernatant was used for both SDH and LDH enzymes. Reaction mixture contained 2.35ml of triethanolamine buffer (100mM, pH 7.6 at 25^0 C), 0.5 ml D fructose (1.1 M) and 0.05 ml NADH (12.8 mM, freshly prepared) mixed well and read the absorbance at 340nm, 25^0C. Oxidation of NADH results in decrease in absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to SDH activity in the sample, where one unit of SDH activity is defined as 1 µ mol of D-fructose converted to D-sorbitol/minute or substrate transformed /minute /mg protein at pH 7.6 at 25^0C.

3.8.2 Lactate dehydrogenase (LDH)

Lactate dehydrogenase activity in testicular tissues was measured using the commercial kits (Span diagnostics Ltd, India). For lactate dehydrogenase estimation, same tissue homogenate was used as that for sorbitol dehydrogenase as described earlier. The LDH assay is based on inter conversion of lactate and pyruvate. During the reduction of pyruvate, an equimolar amount of NADH is oxidized to NAD. Oxidation of NADH results in a decrease in absorbance at 340 nm. Rate of decrease of absorbance at 340 nm is directly proportional to LDH activity in sample, where one unit of LDH activity is defined as 0.1 µmol of substrate transformed/minute at pH 7.6 at 25°C.

3.8.3 γ-glutamyl transpeptidase (γ-GT/GGT)

The GGT activity was measured using the commercially available kit (Coral, India) and activity expressed as IU/L. Testicular tissue was homogenized in ice-cold

0.1M Tris-hydrochloride buffer having pH 7.4 at a tissue concentration of 10 mg/ml, and then the homogenized mixture was centrifuged at 10,000 xg for 30 min at 4ºC. The supernatant was used for enzyme activity estimation.

3.8.4 Acid Phosphatase

Testicular tissue was homogenized in 0.02 mol Tris hydrochloride solution of pH 7.5, at a tissue concentration of 10 mg/ml. Acid phosphatase was then estimated using commercially available kit (Sigma Aldrich) and activity was expressed as IU/L.

3.9 Plasma testosterone levels

Plasma testosterone levels were estimated using commercially available ELISA kit (Demeditec Diagnostics GmbH, Germany).

3.10 Histopathological examination

Rats of each group were humanely sacrificed by cervical dislocation after 28 days of experimentation and were subjected to necropsy. Gross pathological examination of the organs was carried out followed by their further processing for histopathological alterations. Gross lesions were studied in liver, testes, lungs, kidneys, heart, and spleen. After gross pathological examination, the tissues were fixed in 10 % neutral buffered formalin and were processed for paraffin embedding technique.

(1) Dehydration of tissue and preparation of blocks and slides

Tissues from 10 % neutral buffered formalin were taken and to remove formalin from the tissue were kept under running tap water for overnight

↓

Water was removed as much as possible with the help of tissue paper

↓

Tissue was dehydrated in ascending grades of ethanol for 30 min each

↓

50 % ethanol

↓

70 % ethanol

↓

90 % ethanol

↓

Absolute ethanol - I

↓

Absolute ethanol - II

↓

Kept in acetone for 20 minutes

↓

Kept in xylene and benzene mixture (1:1) for 20 minutes

↓

The tissue was kept in molten paraffin for 3 hrs

↓

Blocks were prepared by adding molten paraffin to the tissue and kept at room temp. for 0.5 hr for solidification, then kept in refrigerator at 4˚C for 1 h

↓

Sections (5-6µm) were cut using microtome (Leica, ICC50) and tissue sections were transferred on glass slides

↓

Slides were kept in oven for 1 h to melt the paraffin wax

2) Hematoxylin and Eosin staining (H & E staining)

Xylene I (2 min)

↓

Xylene II (2 min)

↓

Xylene + Alcohol (1:1; 2 min)

↓

Absolute ethanol (2 min)

↓

90, 80, 70 and 50 % ethanol (for 2 min each)

↓

Distilled water (2 min)

↓

Hematoxylin (3 min)

↓

Tap water (1-2 min)

↓

Acid water (few dips)

↓

Ammonia water (few dips)

↓

Eosin (1 min)

↓

80 and 90 % ethanol (few dips each)

↓

Absolute ethanol I (few dips)

↓

Absolute ethanol II (few dips)

↓

Xylene + Alcohol (1:1; few dips)

↓

Xylene I (3 min)

↓

Xylene II (3 min)

↓

Slides were dried and mounted in DPX and kept for 3 h to dry the DPX solution.

↓

After fixing, the slides were examined under microscope to determine the histological details of different organs.

3.11HSP-70 gene expression studies

Testes tissue homogenates were subjected to molecular analysis for determining the relative mRNA expressions of HSP-70 gene as per method described below:

a) Isolation of total RNA and determination of RNA concentration and its purity

All the procedure of RNA isolation were performed using materials that were treated with in 0.1 % diethyl pyro carbonate (DEPC) and further autoclaved before use. The samples frozen in liquid nitrogen and stored at -80^{0}C were thawed and subsequently used for RNA isolation. Total RNA was isolated from testes samples by using Pure Link RNA mini-Kit (Thermo Fischer Scientific, USA) by following manufacturer's instructions. The purity of the RNA was checked by Bio-photometer plus (Eppendorf, Germany) which quantifies as follows:

$$1\ OD_{260} = 40\ \mu g/ml$$

The RNA samples showing A_{260}/A_{280} ratio 1.8-2.0 and A_{260}/A_{230} ratio $\geq$1.5 were used for cDNA synthesis.

b) c-DNA synthesis

cDNA synthesis was carried out from the isolated total RNA using RevertAid® First Strand c-DNA Synthesis kit (K1622, Thermo Fischer Scientific,

USA) by following manufacturer's instructions. Briefly, 1000 ng RNA was mixed thoroughly with 1 µl of oligo dT primer (10 pmol/µl) and volume of each tube was made to 12 µl using nuclease-free water. The solution was incubated at 65 °C for 5 min in thermo-cycler immediately cooled on ice after incubation. To this, 2 µl d-NTPs (10 mM), 4 µl reaction buffer (5x), 1 µl RevertAid Reverse Transcriptase (200 U/µl) and 1µl RiboLock™ RNase inhibitor were added to make the total volume 20 µl. Afterwards, the tubes were incubated at 42 ºC for 60 min and 70 ºC for 5 min in a thermo-cycler. The overall steps of cDNA synthesis were performed in two reactions.

A) Reaction A

SN	Component(s)	Volume or concentration
1.	RNA template	1000 ng
2.	Oligo dT primer	1 µl
3.	Nuclease free water	to 12 µl
	Total volume	**12 µl**

B) Reaction B

SN	Component(s)	Volume or
1.	Reaction buffer (5X)	4.0 µl
2.	d-NTP mix (10mM)	2.0 µl
3.	Ribo Lock RNAase inhibitor (20 U/µl)	1.0 µl
4.	Reverse transcriptase enzyme	1.0 µl
	(RevertAid Reverse Transcriptase (200 U/µl)	
	Total volume	8 µl
	Grand total (A+B)	**12+8 = 20 µl**

c) Primer sequences

Primer sequence was obtained from the previous publication and respective gene sequence was checked from NCBI nucleotide database. The cDNA was subjected to amplification with the primer sets for HSP-70 and GAPDH at various annealing temperatures to optimize the PCR conditions. Reverse transcription real time polymerase reaction (RT-qPCR) analysis was carried out after checking each primer for its specificity.

Gene	Sequence of Primers	Amplicon size (bp)	Annealing Temp. (°C)	Reference
HSP-70	F: 5′-CTCGTGCGTGGGCGTGTTCC-3′ R: 5′-TTTCCCTTGTAGTTCACCTG-3′	285	61	Bahaloo et al., 2019
GAPDH	F: 5′-AAGGCTGAGAACGGGAAACT-3′ R: 5′-TACTCAGCACCAGCATCACC-3′	101	62	Turchetti et al., 2015

d) Polymerase chain reaction (PCR):

The cDNA was checked for quality by performing PCR with standard GAPDH primer. The polymerase chain reaction was standardized for these above-mentioned genes (HSP-70 and GAPDH) using cDNA samples. The reaction was carried out at different annealing temperatures and the optimum annealing temperature of gene was determined. The protocol used was as follows (Sambrook and Russel, 2012).

Composition of PCR mixture

Composition	Volume
PCR master mix (2X)	6.25 µl
Forward primer (10 pmol/µl)	1 µl (HSP-70)/ 1µl (GAPDH)
Reverse primer (10 pmol/µl)	1 µl (HSP-70)/ 1µl (GAPDH)
Template	1.0 µl
Nuclease free water	up to 12.5 µl

Thermal cycling conditions

SN	Steps	Temperature and duration
1.	Initial denaturation	95 ºC for 5 min.
2.	Denaturation	95 ºC for 1 min.
3.	Annealing	61 ºC for 1 min (for HSP70) 62 ºC for 1 min (for GAPDH)
4.	Extension	72 ºC for 1 min
5.	Step 2-4 repetition	40 cycles
6.	Final extension	72ºC for 5 min
7.	Held at	4ºC

f) Real time Polymerase chain reaction (RT-PCR)

RT-PCR reactions were run on Real-Time PCR system (QuantStudio3, Applied Bio-system, USA) and analysed by using Quant Studio™ Design and Analysis Software's v1.2.x.

RT-PCR reaction conditions

RT-PCR was performed using SYBR Green master mix (PowerUpSYBR^{TM} Green master mix [2X]; Thermo Fischer Scientific, USA). The 10 µl reaction mixture consisted of 5 µl SYBR Green master mix,0.5 µlfrom 10 pmol/µl stock solution of forward and reverse primers of HSP-70 and GAPDH (as mentioned in the Table 11), and 1µl of (1:4 dilution) of cDNA and volume was made up to 10 µl with nuclease free water (NFW). The real-time PCR reaction was started with UDG activation at 50 °C for 2 min, Taq polymerase activation at 95 ºC for 2 min followed by 40 cycles of amplification with denaturation at 95 ºC for 15 sec, annealing at (respective temperature as mentioned in Table 10) for 15 sec and extension at 72 ºC for 1 min each.

To assess the specificity of the amplified product, dissociation curve was generated at temperature of 60 °C through 95 °C. The results were expressed as threshold cycle values (C_T). This value is the cycle number when the fluorescence of the reporter dye is appreciably higher than the background fluorescence. The threshold, automatically adjusted by the instrument, was used for the generation of C_T values. To study the relative change in gene expression, the $2^{-\Delta\Delta Ct}$ method was used as described previously by Livak and Schmittgen (2001). The formula used to calculate the fold change in gene expression was "fold change =$2^{-\Delta\Delta Ct}$ " [where $\Delta\Delta C_T$ = ($C_{T,\ target\ gene}$ - $C_{T,\ GAPDH}$) treatment - ($C_{T,\ target\ gene}$ -$C_{T,\ GAPDH}$) control]. The gene-specific amplification was corrected for the difference in input of RNA by taking housekeeping gene GAPDH to account. For all the experimental groups (except Control) evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to control (i.e., fold change in control = 1). The results were analysed in comparison with the C_T (minimum threshold of amplification) value of the target gene and the reference gene (GAPDH).

RT-PCR thermal cycle conditions

S. No.	Component	Volume (per well)	Step
1	PowerUpSYBR^{TM} green Master Mix (2X)	5 µl	**Segment 1**
2.	Forward primer (10 pmol/µl)	0.5 µl (HSP-70)/ 0.5 µl (GAPDH)	1- 50ºC : 2 min 2- 95 ºC : 2min 1 cycle
3.	Reverse primer (10 pmol/µl)	0.5 µl (HSP-70)/ 0.5 µl (GAPDH)	**Segment 2** 3- 95 ºC: 15 sec
4.	cDNA Template (1:4 dilution from stock)	1.0 µl	4-AT*: 15 sec 5- 72 ºC :60 sec Repeat for 40 cycles
5.	Nuclease free water (NFW)	3 µl	**Segment 3** 95 ºC: 15 sec 60 ºC: 60 sec 95 ºC: 15 sec 1 cycle
	Final volume	10 µl	

*AT: Respective Annealing temperature (61^0C for HSP-70 and 62^0C for GAPDH)

e) Agarose gel electrophoresis

DNA amplified by PCR was subjected to agarose gel electrophoresis as described by Sambrook and Russel (2012). Four hundred fifty milligram (1.5%) agarose was dissolved in 30 ml 1X TAE buffer by heating and allowed to cool to 50 ºC. To this, 1.5 µl ethidium bromides (final concentration 0.5 µg/ml) was added and mixed thoroughly. The mixture was poured onto a gel-casting tray fitted with acrylic comb and allowed to solidify. Once the gel had formed, a few ml of TAE was added near the comb, which was later removed carefully and the gel was immersed in an electrophoresis tank containing 1X TAE buffer. One µl of 6X DNA loading dye was mixed with 5 µl of RT-PCR amplified samples and loaded into the wells. Electrophoresis was carried out at 85 volts until the tracking dye reached the end of the gel. The DNA samples were visualized as bands under UV-illumination and documented. The size of the PCR products amplified was calculated from the standard 1kb plus DNA ladder (Invitrogen, USA).

3.12 Sperm parameters studies

3.12.1 Collection of semen

Collection of epididymal spermatozoa was done within 10 min of collection of testes. Spermatozoa were collected from of the epididymis into the petri dish containing SP-TALP medium by using epidydimal slicing method as described by Kempinas and Carvalho (1988). Following sacrifice, the cauda epididymis was quickly removed from the testes and spermatozoa were released into the petri dish containing the media by cutting the cauda epididymis longitudinally with a pair of fine-pointed scissors and compressing with forceps. Collected spermatozoa were kept in SP- TALP in the respective Petri dishes at 37^0 C for further processing. Sperm concentration, live and dead count, membrane integrity and acrosomal integrity of spermatozoa were evaluated using hemocytometer method, Eosin and Nigrosine stain, hypoosmotic swelling test (100 mOsm/L hypoosmotic solution) and FITC-PSA respectively.

Composition of SP-TALP media (100 ml) (Parrish et al., 1988)

Compound	Quantity
Sodium Chloride	100.0 mM
Potassium Chloride	3.1 mM
Sodium bicarbonate	25.0 mM
Sodium dihydrogen phosphate	0.3 mM
Lactate (Sodium salt)	21.6 mM
Calcium chloride	2.0 mM
Magnesium chloride	0.4 mM
HEPES	10.0 mM
Sodium pyruvate	1.0 mM
Bovine serum albumin (BSA)	6 mg/ml

3.12.2 Spermatozoa concentration

Semen was diluted using diluting fluid consisting of 2.9 g of sodium citrate and 0.05 g of eosin Y (water soluble) in 100 ml of formalized distilled water. Concentration of spermatozoa in the semen samples was estimated using hemocytometer (Improved Neubauer's chamber) as per the method describe below:

i. Hemocytometer was well cleaned, dried and placed on a level surface. A clean dry cover slip was placed over the hemocytometer.

ii. A small drop of diluted semen formed by using pipette to charge the hemocytometer through capillary action.

iii. The hemocytometer was then left undisturbed for 10 minutes to ensure the settling of spermatozoa.

iv. Total number of spermatozoa in the given sample was counted in five secondary squares, namely, top right, top left, bottom right, bottom left and the central squares of the central primary square.

v. Each secondary square contains 16 tertiary squares, so total number of spermatozoa were counted in 80 tertiary squares. The average of the counts on both sides of hemocytometer chamber was considered for calculating the total number of spermatozoa per ml of neat semen sample.

Calculation was done as per the following method:

Area of 1 tertiary square = $1/20 \times 1/20$ mm^2

Volume of 1 tertiary square = $1/4000$ mm^3

Number of spermatozoa in 80 tertiary squares = n

Volume of 80 tertiary squares = $1/4000 \times 80 mm^3$ (where 80/4000 mm^3 volume has 'n' number of spermatozoa)

Therefore, the Volume correction factor = $n/80 \times 4000 = n \times 50$

Dilution factor (d) = 20

Total number of spermatozoa in1 mm^3 of neat semen has = $n \times d \times 50$

Spermatozoa concentration (per ml) = $n \times 20 \times 50 \times 1000$

$= n \times 10^6$

3.12.3 Live and dead spermatozoa count

To determine the percentage of live or dead spermatozoa following exposure to lead acetate, Eosin-Nigrosin staining technique was used as per the method of Hancock (1952).

Composition of Eosin and Nigrosin stain (Hancock, 1952)

Eosin – Y (Water Soluble)	0.6% w/v
Nigrosin (Water Soluble)	5% w/v
Sodium citrate dihydrate	3% w/v

pH of the stain was adjusted to 7.0 by adding a few drops of 0.1 M NaH_2PO_4 or 0.1 M Na_2HPO_4 and filter through paper.

For this, 10µl of semen sample was mixed with 10µl of stain on a glass slide kept at 37^0C on the dry bath plate. Smears were made on clean, grease-free slide and air dried. Minimum 200 spermatozoa were counted in each slide and were identified as live or dead under the oil-immersion (100X) objective. Stained spermatozoa appearing eosinophilic or pinkish in colour against the dark background were dead and those which remained unstained against the dark background were counted as live. Percentage of the live spermatozoa was calculated using the following equation:

$$\% \text{ Live spermatozoa} = \frac{\text{Total No. of live spermatozoa}}{\text{Total number of spermatozoa observed}} \times 100$$

3.12.4 Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling (HOS) test was performed as per the method described by Matson (2009). Hypo-osmotic solution of 100 mOsm/l was prepared as follows:

Chemicals	Quantity
Sodium citrate	0.49g
Fructose	0.90g
Triple distilled water	100 ml

100 µl of hypo-osmotic solution, having an osmotic strength of 100 mOsm/l was mixed with 20 µl of semen (10×10^6/ml) and incubated at 37^0C for one hour in a dry bath. Following incubation, a drop of the well mixed solution was taken on a clean, grease-free slide and covered with a cover slip. Sperm tail coiling was considered as the effect of hypo-osmotic solution and sperm tails were classified as non-coiled, coiled, and strongly coiled. Total 200 spermatozoa were counted in different fields under 40X objective of the phase contrast microscope. Percentage of swollen spermatozoa was calculated by using the equation given below:

$$\text{HOST Positive spermatozoa (\%)} = \frac{\text{No. of spermatozoa with coiled tailed}}{\text{Total number of spermatozoa observed}} \times 100$$

3.12.5 Acrosomal integrity

Acrosome plays vital role in fertilization of ovum and hence percentage of intact Acrosomes is a good indicator of the fertilization potential of spermatozoa in semen samples. Acrosomal integrity of sperm cells was evaluated by employing the FITC-PSA (Sigma) staining method of Mendoza et al. (1992). Briefly, diluted semen samples (10×10^6/ml) were suspended in PBS (Ca^{2+} and Mg^{2+} free) and centrifuged at 223×g for 3 minutes. Sperm's pellet was washed three times in PBS (pH =7.4). After three washings, the sperm pellet was resuspended in PBS. 10 µl of the sample was taken and a thin and uniform smear was prepared on clean grease-free glass slide. The slide was kept for air drying. Air dried slides were incubated with FITC-PSA (100 µg/ml in PBS, pH= 7.4) for 30 minute at room temperature in dark. Slides were removed and air dried. After air drying, slides were observed using EVOS^{TM} M5000 imaging system (Thermo Fischer Scientific, USA). Two hundred spermatozoa were differentiated according to the fluorescence patterns in their acrosomes (bright fluorescence acrosome-intact, no fluorescence or only fluorescence of the equatorial segment means loss of acrosome).

3.13. Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis of data was performed using SPSS- 20 by one-way ANOVA followed by tukey's-b multiple comparison *post-hoc* tests. A value of $p<0.05$ was considered as statistically significant.



Results

CHAPTER-4

RESULTS

The present work was designed to study the toxicity of cartap (16.25 mg/kg and 32.5 mg/kg) following sub-acute (28 days) exposure and ameliorative potential of α-tocopherol (100 mg/kg) and its effects on general body parameters, haemato-biochemical parameters, oxidative stress biomarkers, testicular injury biomarkers, plasma testosterone levels, sperm parameters, histopathology and quantitative gene expression of HSP-70 gene in testes in adult male wistar rats.

4.1 Effect of oral administration of α- tocopherol (100 mg/kg) on general toxicity parameters in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg).

4.1.1 Clinical signs: Following 28 days daily oral administration of cartap (16.25 mg/kg and 32.5 mg/kg) and α-tocopherol (100 mg/kg) alone and in combination with α- tocopherol (100 mg/kg), no clinical signs of toxicity were observed in animals of any of the treatment groups and animals exhibited normal behaviour and activities throughout the period of study.

4.1.2 Body weight gain: Percent body weight gain of different treatment groups following 28 days daily oral administration of cartap (16.25 mg/kg and 32.5 mg/kg) alone and in combination with α- tocopherol (100 mg/kg) are presented in Table 1 and illustrated in Fig 3. Percent body weight gain in various groups did not differ significantly ($p>0.05$) from each other except in group V. Percent body weight gain was found to be (13.89±1.31%) in group V which was significantly ($p<0.05$) lower compared to that in control group (31.43±3.31%). Co-treated groups VI and VII showed no significant ($p>0.05$) change in percent body weight gain compared to cartap alone treatment groups IV and V respectively as evident from the data presented in Table 1 and illustrated in Fig 3.

4.1.3 Absolute (g) and relative organ weights (g/100g body weight):

4.1.3.1 Absolute organ weight: The absolute organ weights (g) data of lungs, liver, spleen, right kidney, left kidney, heart, left testes and right testes of rats of different treatment groups are summarized in Table 2 and illustrated in Fig 4. After 28 days

exposure to cartap and α- tocopherol no significant ($p>0.05$) change in the absolute organ weights of lungs, liver, spleen, right kidney, left kidney, heart, left testes and right testes was observed in any of the treatment groups compared to control group as evident from the data present in Table 2 and illustrated in Fig 4.

4.1.3.2 Relative organ weight: The relative organ weights (g/100g body wt.) data of lungs, liver, spleen, right kidney, left kidney, heart, left testes and right testes of rats of different treatment groups are summarized in Table 3 and illustrated in Fig 5. After 28 days exposure to cartap and α- tocopherol no significant ($p>0.05$) change in the relative organ weights of lungs, liver, spleen, right kidney, left kidney, heart, left testes and right testes was observed in any of the treatment groups compared to control group as evident from the data present in Table 3 and illustrated in figure 5.

Table 1: Effect of oral administration of α- tocopherol (100 mg/kg) on weekly body weight and percent weight gain in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	Weekly body weight (g)					Percent body weight gain (%)
		0-day weight (g)	1st	2nd	3rd	4th	
I	Control	170.33±5.31[a]	180.5±5.81[a]	194±6.67[a]	202.16±7.59[a]	212.33±8.06[a]	31.43±3.31[b]
II	Vehicle Control (Corn oil)	167±8.10[a]	183.5±5.33[a]	192.83±5.71[a]	208±6.15[a]	217.83±5.49[a]	34.07±4.65[b]
III	α-tocopherol (100 mg/kg)	165±5.42[a]	173±1.63[a]	183.16±1.81[a]	198.16±6.01[a]	210.16±1.70[a]	32.113±2.17[b]
IV	Cartap hydrochloride (16.25 mg/kg)	165.33±3.53[a]	175±3.49[a]	188±4.23[a]	194.33±4.71[a]	202.5±6.37[a]	28.16±1.87[b]
V	Cartap hydrochloride (32.5 mg/kg)	172.11±4.63[a]	185±5.13[a]	195.33±6.00[a]	198.83±6.02[a]	202.83+5.89[a]	13.89±1.31[a]
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	170.09±3.38[a]	189±4.98[a]	205.17±8.62[a]	211.83±9.37[a]	219.67±10.38[a]	29.11±1.67[b]
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	174.22±0.83[a]	186±0.73[a]	202.33±6.54[a]	206±6.96[a]	210.83±7.04[a]	16.02±2.40[a]
P-value		0.796	0.141	0.180	0.552	0.525	0.00

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

Table 2: Effect of oral administration of α- tocopherol (100 mg/kg) on absolute organ weight in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	Absolute organ weight (g)							
		Lungs	Liver	Left kidney	Right kidney	Spleen	Heart	Left testes	Right testes
I	Control	1.37±0.04[a]	6.88±0.39[a]	0.68±0.05[a]	0.70±0.04[a]	0.54±0.05[a]	0.89±0.05[a]	1.40±0.15[a]	1.36±0.16[a]
II	Vehicle Control (Corn oil)	1.19±0.05[a]	6.89±0.79[a]	0.76±0.05[a]	0.71±0.04[a]	0.48±0.06[a]	0.85±0.05[a]	1.45±0.08[a]	1.50±0.09[a]
III	α-tocopherol (100 mg/kg)	1.14±0.03[a]	6.58±0.30[a]	0.67±0.03[a]	0.67±0.04[a]	0.42±0.04[a]	0.72±0.03[a]	1.35±0.05[a]	1.32±0.06[a]
IV	Cartap hydrochloride (16.25 mg/kg)	1.11±0.02[a]	6.10±0.22[a]	0.70±0.03[a]	0.72±0.03[a]	0.49±0.01[a]	0.76±0.03[a]	1.42±0.04[a]	1.34±0.13[a]
V	Cartap hydrochloride (32.5 mg/kg)	1.19±0.03[a]	6.96±0.37[a]	0.76±0.03[a]	0.76±0.03[a]	0.45±0.01[a]	0.90±0.06[a]	1.37±0.12[a]	1.50±0.03[a]
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	1.34±0.09[a]	6.24±0.59[a]	0.72±0.05[a]	0.73±0.05[a]	0.47±0.03[a]	0.89±0.06[a]	1.58±0.05[a]	1.58±0.05[a]
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	1.23±0.06[a]	6.22±0.46[a]	0.76±0.02[a]	0.78±0.01[a]	0.42±0.07[a]	0.78±0.03[a]	1.39±0.12 [a]	1.40±0.12[a]
P-value		0.105	0.757	0.589	0.615	0.080	0.440	0.770	0.525

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

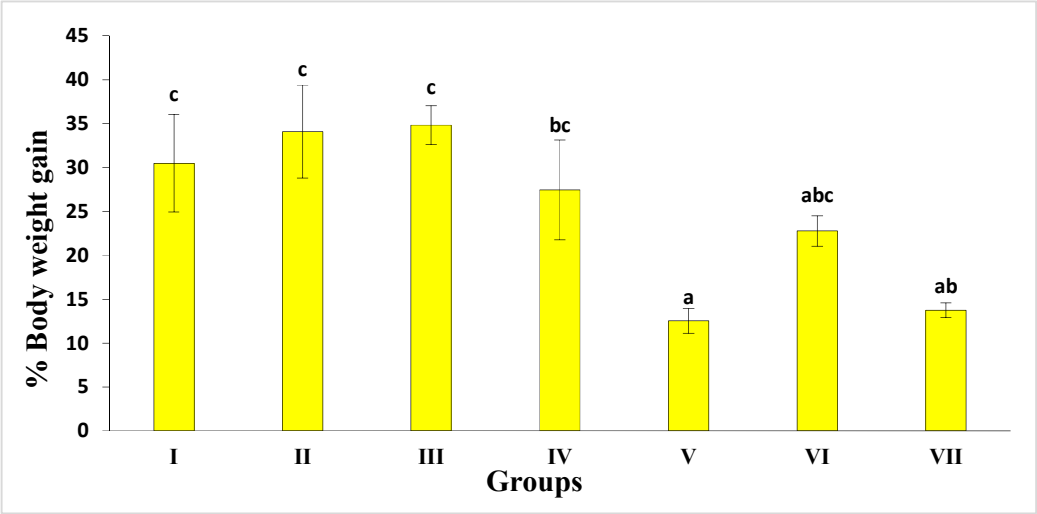


Fig. 3: Effect of oral administration of α- tocopherol (100 mg/kg) on percent body weight gain in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).
Different superscripts on bars indicate significant (P<0.05) differences.

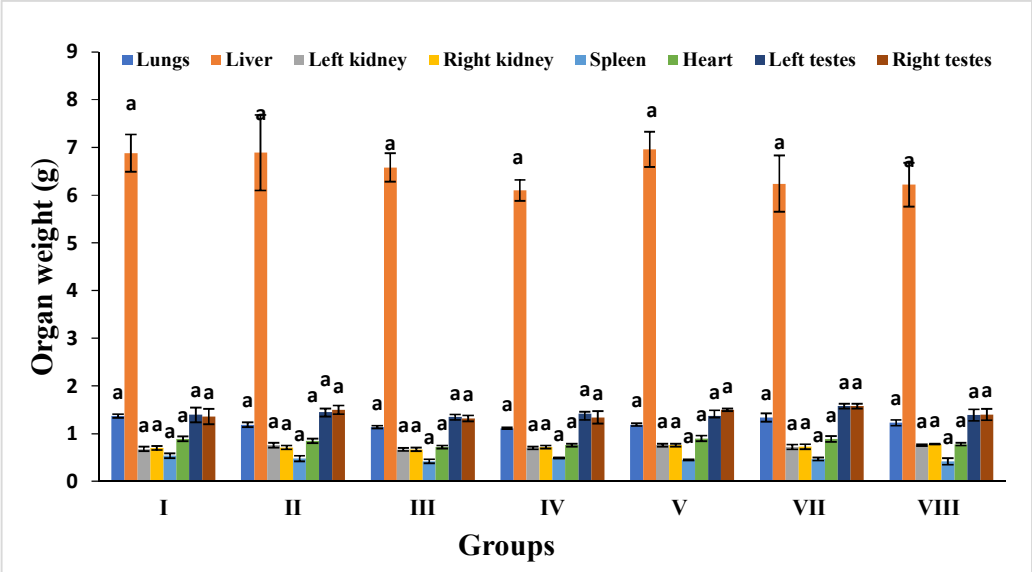


Fig. 4: Effect of oral administration of α- tocopherol (100 mg/kg) on absolute organ weight in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).
Different superscripts on bars indicate significant (P<0.05) differences.

Table 3: Effect of oral administration of α- tocopherol (100 mg/kg) on relative organ weight in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	Relative organ weight (g/100g)							
		Lungs	Liver	Left kidney	Right kidney	Spleen	Heart	Left testes	Right testes
I	Control	0.65±0.02[a]	3.23±0.09[a]	0.32±0.01[a]	0.33±0.01[a]	0.25±0.02[a]	0.41±0.02[a]	0.66±0.07[a]	0.64±0.07[a]
II	Vehicle Control (Corn oil)	0.68±0.03[a]	3.52±0.16[a]	0.30±0.01[a]	0.28±0.01[a]	0.24±0.02[a]	0.43±0.02[a]	0.64±0.03[a]	0.65±0.03[a]
III	α-tocopherol (100 mg/kg)	0.54±0.16[a]	3.12±0.30[a]	0.32±0.08[a]	0.32±0.03[a]	0.34±0.01[a]	0.31±0.02[a]	0.63±0.01[a]	0.64±0.02[a]
IV	Cartap hydrochloride (16.25 mg/kg)	0.55±0.01[a]	3.01±0.08[a]	0.348±0.01[a]	0.35±0.01[a]	0.24±0.01[a]	0.37±0.01[a]	0.70±0.01[a]	0.65±0.05[a]
V	Cartap hydrochloride (32.5 mg/kg)	0.58±0.02[a]	3.44±0.01[a]	0.37±0.02[a]	0.36±0.01[a]	0.22±0.01[a]	0.41±0.02[a]	0.68±0.06[a]	0.74±0.03[a]
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	0.62±0.05[a]	2.85±0.05[a]	0.33±0.02[a]	0.33±0.02[a]	0.21±0.01[a]	0.41±0.01[a]	0.72±0.04[a]	0.73±0.04[a]
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	0.59±0.03[a]	2.95±0.02[a]	0.36±0.01[a]	0.34±0.01[a]	0.19±0.08[a]	0.37±0.01[a]	0.66±0.06[a]	0.66±0.05[a]
P-value		0.061	0.080	0.117	0.111	0.088	0.368	0.911	0.623

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

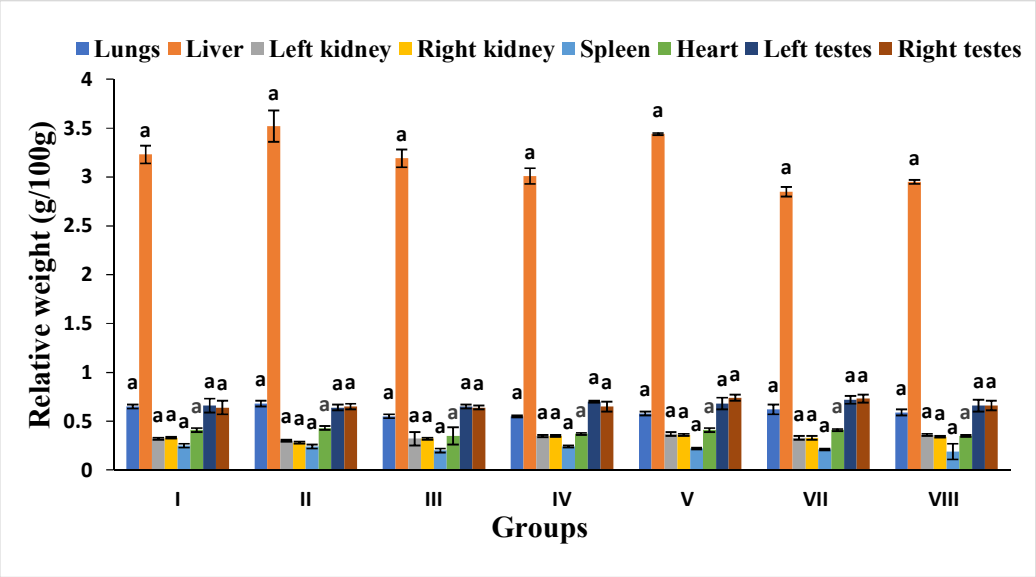


Fig. 5: Effect of oral administration of α- tocopherol (100 mg/kg) on relative organ weight in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant ($P<0.05$) differences.

4.2 Haematological parameters:

4.2.1 Erythrocytic parameters and platelet count: Data on effect of α- tocopherol (100 mg/kg) on different erythrocytic parameters and platelet count in rats of different treatment groups exposed to cartap (16.25 mg/kg and 32.5 mg/kg) once daily by oral route for 28 days is presented in Table 4 and illustrated in Fig 6-9. After 28 days exposure to cartap alone and co treatment α- tocopherol with cartap, non-significant ($p>0.05$) change was observed in RBC count, haemoglobin concentration and platelet count in any of the treatment groups compared to control group.

4.2.2 Leucocytic parameters: Following 28 days exposure to α- tocopherol (100 mg/kg) and cartap (16.25 mg/kg and 32.5 mg/kg) both alone and in combination, the data obtained on leucocytic parameters has been summarised in Table 5 and illustrated in Fig 10-15. Similar to erythrocytic parameters and platelet count, after 28 days exposure to cartap and α- tocopherol no significant ($p>0.05$) change was observed in WBC count, lymphocytes %, neutrophils %, eosinophils %, basophils % and % monocytes in any of the treatment groups compared to control group.

4.3 Blood biochemical parameters: Blood biochemical parameters in rats of different treatment groups exposed to cartap (16.25 mg/kg and 32.5 mg/kg) and α- tocopherol (100 mg/kg) both alone and in combination by oral route for 28 days is presented in Table 6-7 and illustrated in Fig 16-29. Compared to the control group (82.53 ±5.97 mg/dl) there was a significant ($p<0.05$) increase in blood glucose level in cartap group V (111.75±5.41 mg/dl). A significant ($p<0.05$) decrease in glucose level was found in α- tocopherol alone treatment group compared to control group (82.53 ±5.97 mg/dl). Following co-treatment of rats with α-tocopherol and cartap (group VI and VII), the blood glucose levels were reduced moderately in group VI and VII but non-significantly ($p>0.05$) compared to cartap alone treatment groups IV and V respectively. Compared to control group (82.53 ±5.97 mg/dl) non-significant ($p>0.05$) change was observed in co-treatment groups VI (82.57±4.72 mg/dl) and VII (95.53±4.68 mg/dl).

No significant ($p>0.05$) change in total protein, albumin and globulin levels was observed in any of the treatment groups compared to control group.

Compared to the control group (85.76 ± 6.23 mg/dl) there was no significant ($p>0.05$) difference in cholesterol level in both the cartap groups (IV and V). A significant ($p<0.05$) decrease in cholesterol level was observed in co-treated groups VI and VII compared to the cartap alone treated groups (IV and V). Furthermore, cholesterol level in co-treatment groups VI and VII was significantly ($p<0.05$) lower compared to the control group also.

No significant ($p>0.05$) change in plasma HDL and LDL levels was observed in any of the treatment groups compared to control group.

At both the doses (16.25 mg/kg and 32.5 mg/kg) cartap produced non-significant ($p>0.05$) increase in triglyceride level compared to control group. Co-treatment with α-tocopherol and cartap produced a significant ($p<0.05$) decrease in triglyceride level in the rats of group VI (91.24 ± 6.03 mg/dl) and VII (93.67 ± 2.09 mg/dl) compared to cartap alone treatment groups IV (122.25 ± 5.34 mg/dl) and V (125.80 ± 3.56 mg/dl) as well as control group (116.7 ± 4.73 mg/dl).

No significant ($p>0.05$) change was observed in plasma concentration of total bilirubin in any of the treatment groups (II to VII) compared to the control group (I).

Group IV and V showed a non-significant ($p>0.05$) change in plasma AST level compared to the control group. Compared to cartap alone treatment groups IV and V, non-significant ($p>0.05$) change was found in co-treatment groups (VI and VII). Compared to control group, non-significant ($p>0.05$) change in plasma AST level was observed in cartap and α-tocopherol co-treated groups VI and VII respectively. Compared to control group (97.61 ± 4.1 U/L), non-significant ($p>0.05$) change in AST level was observed in cartap and α-tocopherol co-treated groups VI (87.94 ± 1.59 U/L) and VII (102.36±4.93 U/L).

Cartap at lower dosage group IV (16.25 mg/kg; 33.99±1.45 U/L) did not show any significant ($p>0.05$) change in plasma ALT level but showed a significant ($p<0.05$) increase in ALT level at higher dosage in group V (32.5 mg/kg; 42.37±2.25 U/L) compared to the control group. Following co-treatment with α-tocopherol and cartap, non-significant ($p>0.05$) change was observed in co-treatment groups VI and VII compared to cartap alone treatment group IV and V respectively.

No significant change ($p>0.05$) was observed in plasma BUN level at low dose of cartap (group IV;16.25 mg/kg) when compared to control group. At higher dosage (32.5 mg/kg) cartap produced a significant ($p<0.05$) increase in plasma BUN level (23.42 ± 1.09 mg/dl) when compared to control group (15.89 ± 1.41 mg/dl). Co-treatment groups VI and VII did not show any significant ($p>0.05$) change in plasma BUN level compared to cartap alone treatment groups IV and V respectively. When compared to control group (15.89 ± 1.41 mg/dl), plasma BUN levels were significantly increased ($p<0.05$) in co-treated group VI (20.75 ±0.82 mg/dl) and VII (22.45±0.68 mg/dl).

No significant ($p>0.05$) change was observed in plasma creatinine levels in any of the treatment groups compared to control group.

Compared to control group (9.35±0.25 mg/dl) there was no significant ($p>0.05$) change in plasma calcium level in cartap alone treated groups (IV and V). A moderate but not significant ($p>0.05$) decrease in plasma calcium levels was observed co-treated group VI (7.73±0.29 mg/dl) compared to lower dosage cartap group IV (9.03±0.23 mg/dl). However, compared to higher dosage cartap group V (9.9 ± 0.14 mg/dl) plasma calcium level was significantly ($p<0.05$) decreased in group VII (7.77 ± 0.11 mg/dl). Group VI (7.73±0.29 mg/dl) and VII (7.77 ± 0.11 mg/dl) showed significant ($p<0.05$) decrease in plasma calcium level compared to control group (9.35±0.25 mg/dl).

Table 4: Effect of oral administration of α- tocopherol (100 mg/kg) on certain erythrocytic indices and platelet count in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	RBC (10^6/mm^3)	Hb (g/dl)	PCV (%)	Platelets (10^3/mm^3)
I	Control	6.69±0.27 [a]	13.11±0.40[a]	35.33±0.88[a]	577.33±55.44[a]
II	Vehicle Control (Corn oil)	7.18±0.59 [a]	13.44±0.47[a]	36.96±3.33[a]	533.16±56.17[a]
III	α-tocopherol (100 mg/kg)	6.40±0.35 [a]	13.21±1.27[a]	34.15±2.68[a]	526.33±44.71[a]
IV	Cartap hydrochloride (16.25 mg/kg)	6.83±0.30 [a]	14.40±0.42[a]	36.93±0.77[a]	520.55±26.87[a]
V	Cartap hydrochloride (32.5 mg/kg)	6.77±0.82 [a]	15.58±0.63[a]	37.76±0.73[a]	537.42±38.38[a]
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	6.82±0.15 [a]	16.11±0.53[a]	34.88±1.44[a]	535.00±44.70[a]
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	6.74±0.15 [a]	16.15±0.90[a]	37.21±0.27[a]	542.72±49.7[a]
P-value		0.947	0.466	0.313	0.996

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-Hoc* test

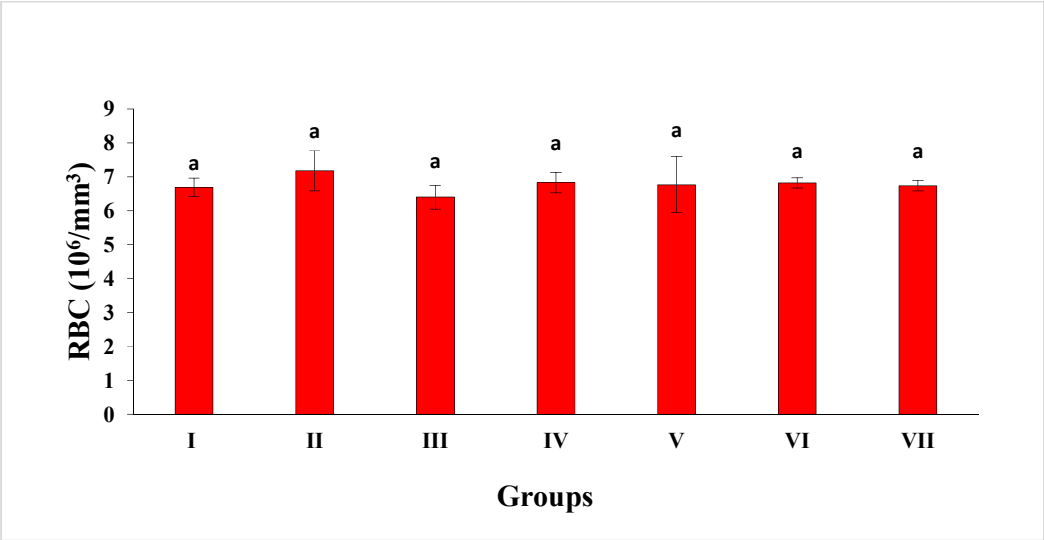


Fig. 6: Effect of oral administration of α- tocopherol (100 mg/kg) on RBC count in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

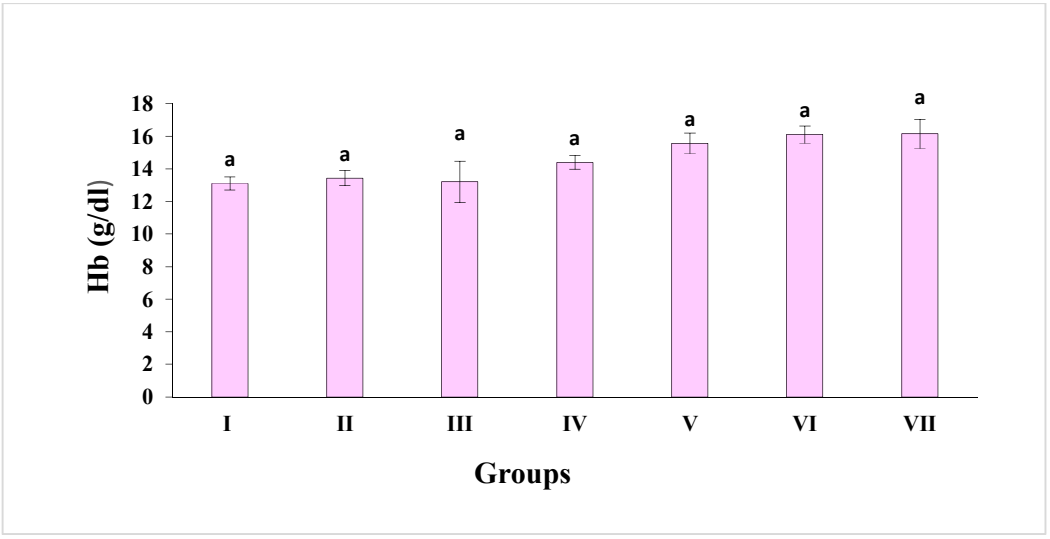


Fig. 7: Effect of oral administration of α- tocopherol (100 mg/kg) on Haemoglobin (Hb) level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

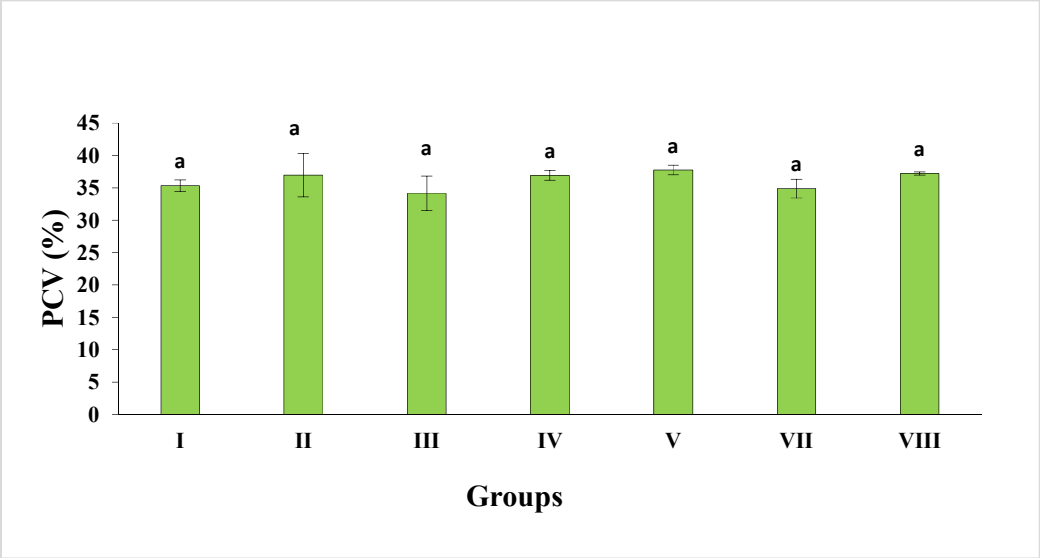


Fig. 8: Effect of oral administration of α- tocopherol (100 mg/kg) on Packed Cell Volume (PCV) in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

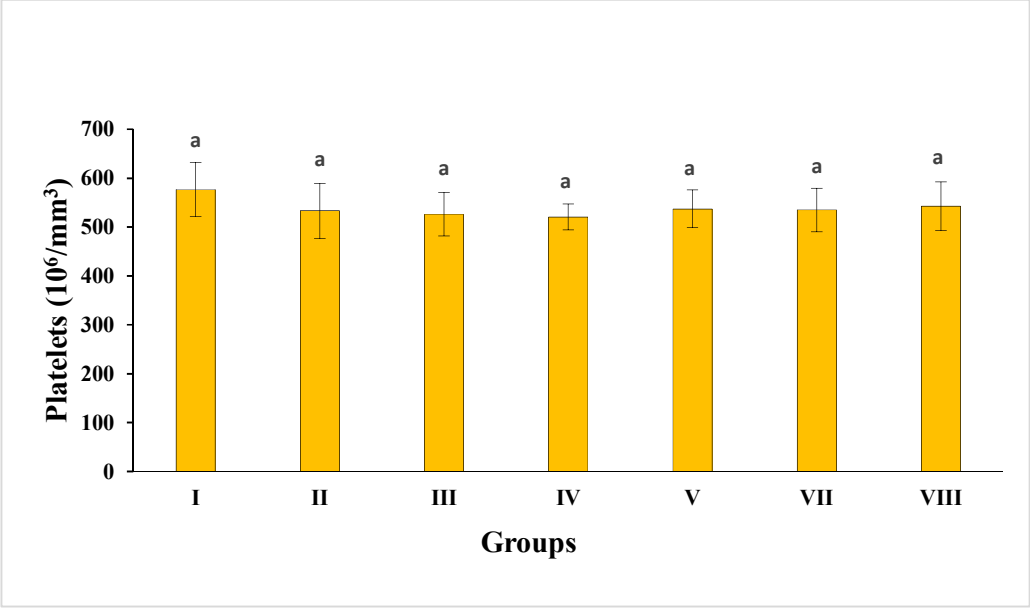


Fig. 9: Effect of oral administration of α- tocopherol (100 mg/kg) on Platelet count in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

Table 5: Effect of oral administration of α- tocopherol (100 mg/kg) on certain leucocytic indices count in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	WBC (10^3/mm^3)	Neutrophils (%)	Basophils (%)	Lymphocytes (%)	Eosinophils (%)	Monocytes (%)
I	Control	8.69±0.95[a]	25.86±2.56[a]	0.23±0.04[a]	69.18±1.62[a]	0.80±0.11[a]	1.63±0.11[a]
II	Vehicle Control (Corn oil)	7.99±0.32[a]	26.73±1.68[a]	0.25±0.04[a]	69.58±1.82[a]	0.86±0.11[a]	1.83±0.10[a]
III	α-tocopherol (100 mg/kg)	7.8±0.56[a]	25.01±2.88[a]	0.28±0.04[a]	68.32±1.61[a]	0.80±0.12[a]	1.72±0.10[a]
IV	Cartap hydrochloride (16.25 mg/kg)	7.93±0.77[a]	29.46±1.78[a]	0.30±0.05[a]	65.16±1.33[a]	0.83±0.11[a]	1.85±0.13[a]
V	Cartap hydrochloride (32.5 mg/kg)	8.77±0.43[a]	29.70±2.31[a]	0.25±0.05[a]	69.26±0.26[a]	1.00±0.09[a]	1.88±0.1[a]
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	7.92±0.52[a]	27.76±1.48[a]	0.30±0.05[a]	66.93±0.65[a]	0.93±0.067[a]	1.71±0.13[a]
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	7.89±0.23[a]	25.13±2.78[a]	0.23±0.07[a]	70.23±2.09[a]	0.95±0.06[a]	2.21±0.19[a]
P-value		0.661	0.412	0.939	0.385	0.605	0.772

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

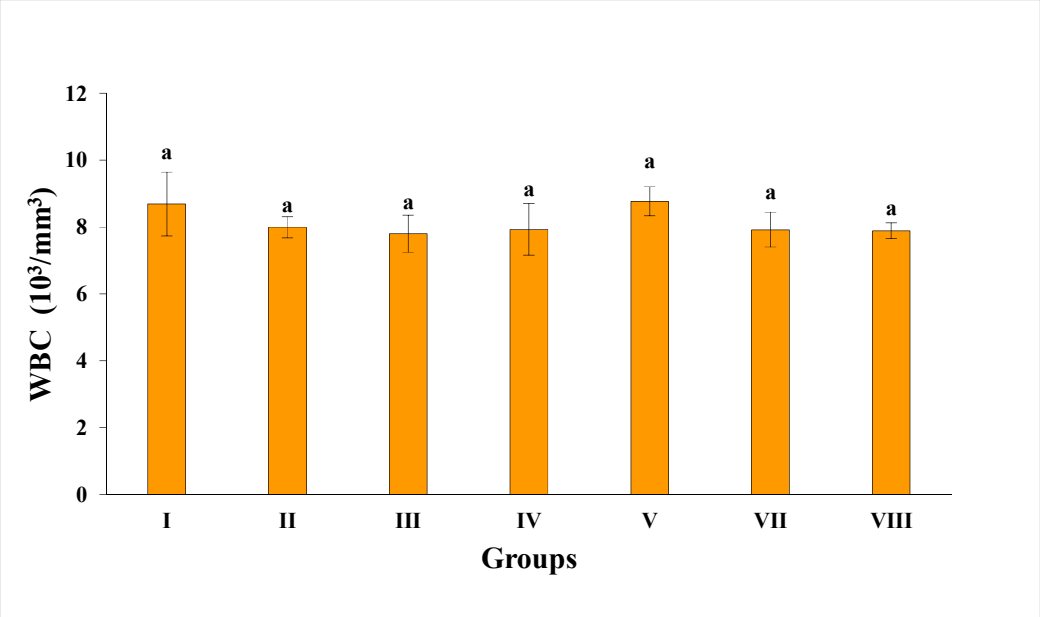


Fig. 10: Effect of oral administration of α- tocopherol (100 mg/kg) on WBC count in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

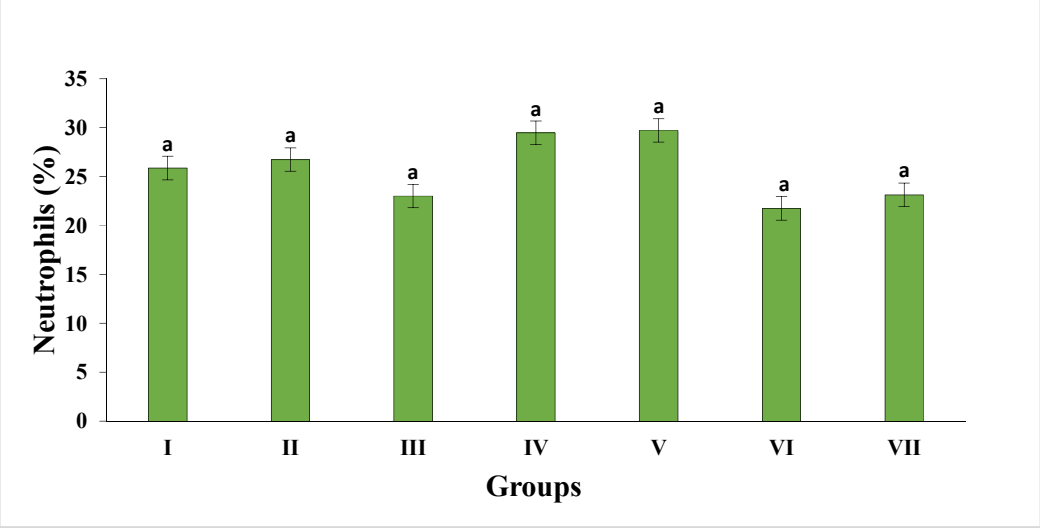


Fig. 11: Effect of oral administration of α- tocopherol (100 mg/kg) on % Neutrophils in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

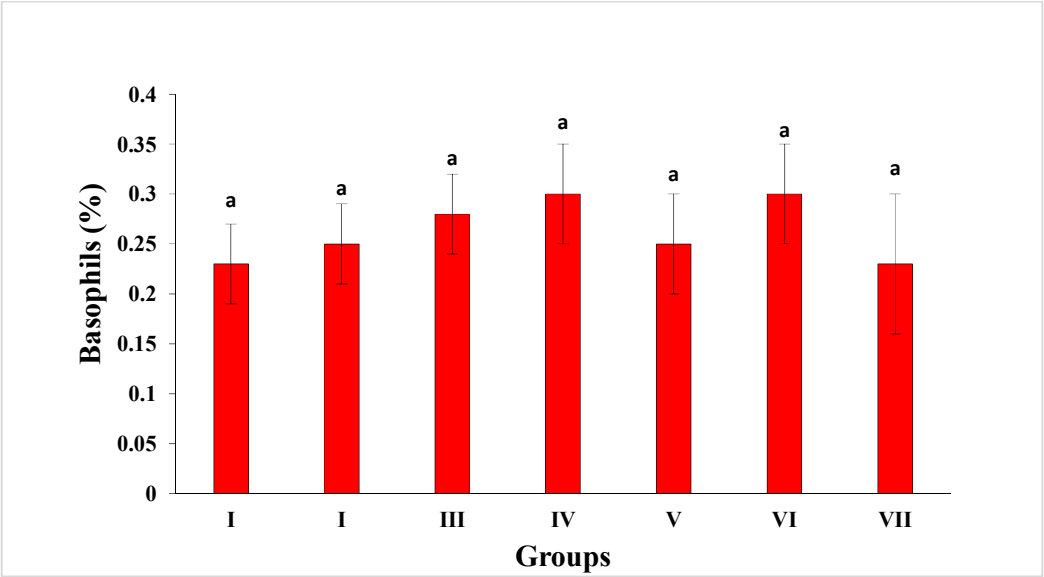


Fig. 12: Effect of oral administration of α- tocopherol (100 mg/kg) on % Basophils in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

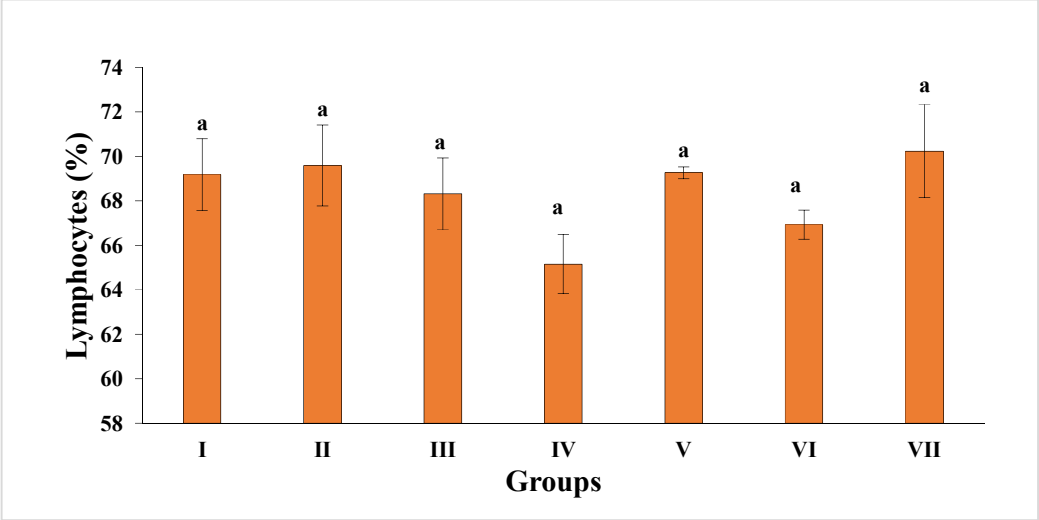


Fig. 13: Effect of oral administration of α- tocopherol (100 mg/kg) on % Lymphocytes in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

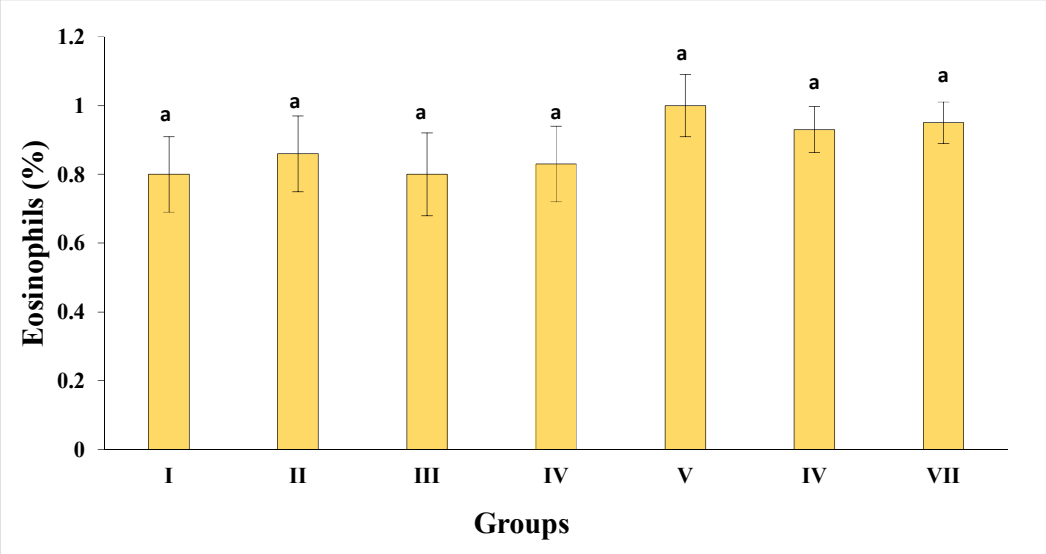


Fig. 14: Effect of oral administration of α- tocopherol (100 mg/kg) on % Eosinophils in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

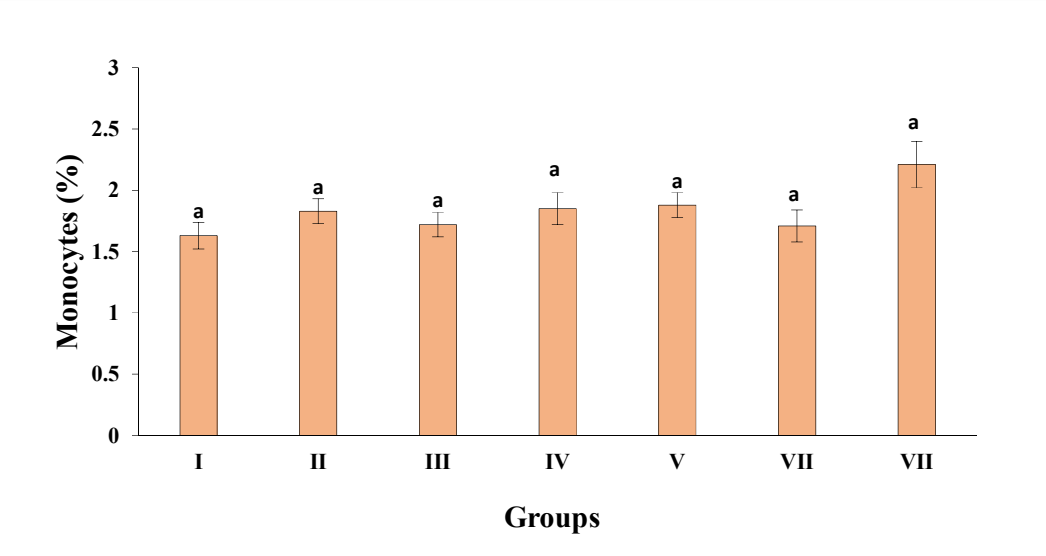


Fig. 15: Effect of oral administration of α- tocopherol (100 mg/kg) on % Monocytes in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

Table 6: Effect of oral administration of α- tocopherol (100 mg/kg) on certain blood biochemical parameters in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	Glucose (mg/dl)	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	Triglycerides (mg/dl)
I	Control	82.53 ±5.97^{bc}	7.60 ± 0.33^{a}	4.13 ± 0.19^{a}	3.34 ± 0.51^{a}	85.76 ± 6.23^{b}	32.73 ± 1.50^{a}	31.08 ± 4.25^{a}	116.7 ± 4.73^{b}
II	Vehicle Control (Corn oil)	71.5 ± 6.86^{ab}	6.22 ± 0.71^{a}	4.18 ± 0.41^{a}	2.05 ± 0.61^{a}	77.19 ± 5.74^{ab}	28.59 ± 2.29^{a}	24.40 ± 4.90^{a}	107.67 ± 4.95^{ab}
III	α-tocopherol (100 mg/kg)	61.44±3.50^{a}	6.17 ± 0.54^{a}	3.53 ± 0.19^{a}	3.81 ± 0.93^{a}	80.37 ± 7.13^{ab}	32.62 ± 1.45^{a}	25.19 ± 5.14^{a}	112.75 ± 5.11^{b}
IV	Cartap hydrochloride (16.25 mg/kg)	89.24±3.70^{bc}	6.33 ± 0.40^{a}	3.6 ± 0.15^{a}	2.64 ± 0.35^{a}	93.05 ± 4.10^{b}	34.92 ± 2.17^{a}	32.95 ± 2.74^{a}	122.25 ± 5.34^{b}
V	Cartap hydrochloride (32.5 mg/kg)	111.75±5.41^{d}	6.08 ± 0.31^{a}	3.77 ± 0.14^{a}	2.36 ± 0.29^{a}	95.06 ± 5.18^{b}	32.28 ± 2.82^{a}	33.56 ± 2.61^{a}	125.80 ± 3.56^{b}
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	82.57±4.72^{bc}	6.61 ± 0.30^{a}	3.96 ± 0.18^{a}	3.24 ± 0.52^{a}	60.35 ± 3.35^{a}	30.77 ± 0.73^{a}	26.61 ± 2.22^{a}	91.24 ± 6.03^{a}
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	95.53±4.68^{cd}	6.89 ± 0.31^{a}	3.99 ± 0.16^{a}	2.9 ± 0.28^{a}	64.06 ± 4.08^{a}	30.58 ± 1.59^{a}	25.83 ± 2.53^{a}	93.67 ± 2.09^{a}
P-value		0.00	0.208	0.241	0.296	0.00	0.370	0.371	0.00

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

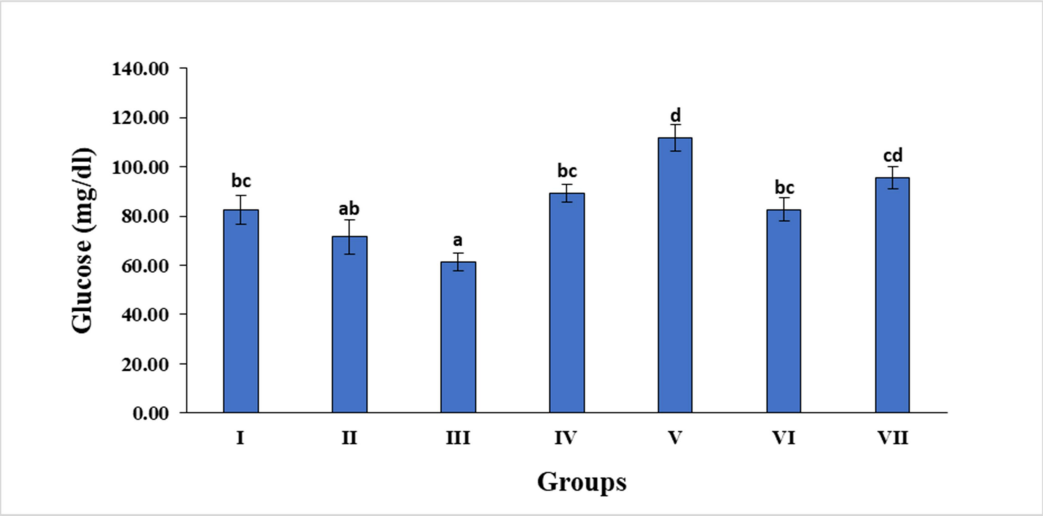


Fig. 16: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Glucose level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

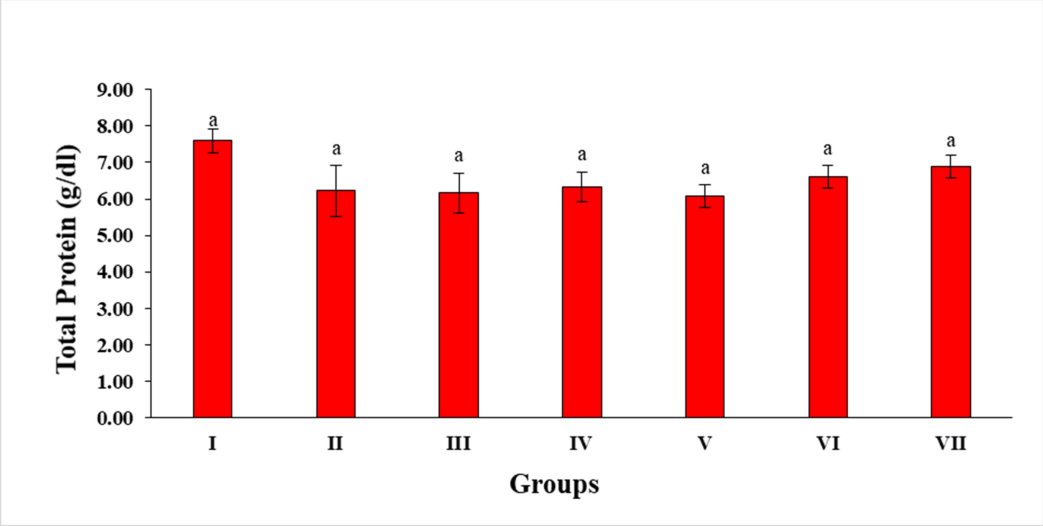


Fig. 17: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Total Protein level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

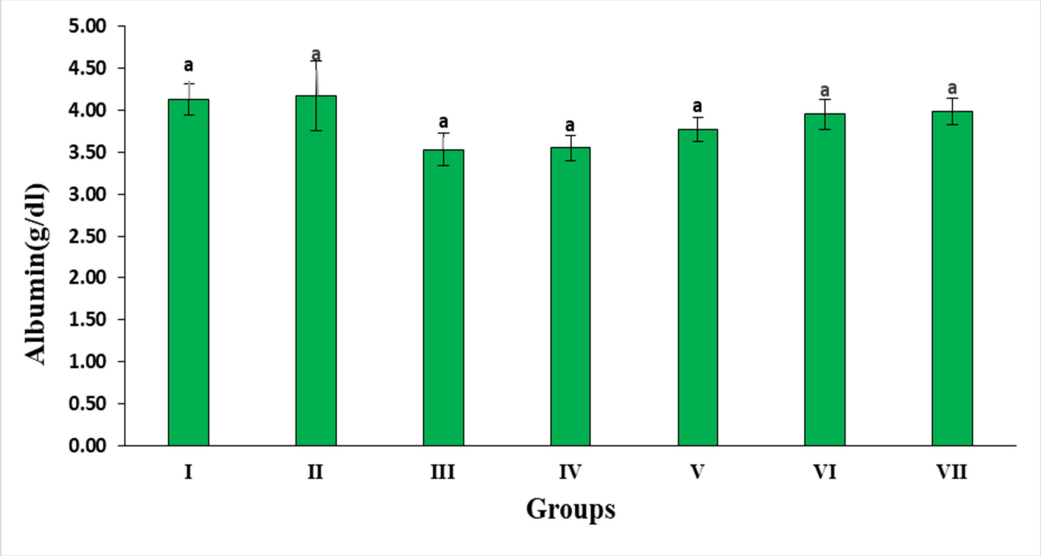


Fig. 18: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Albumin level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

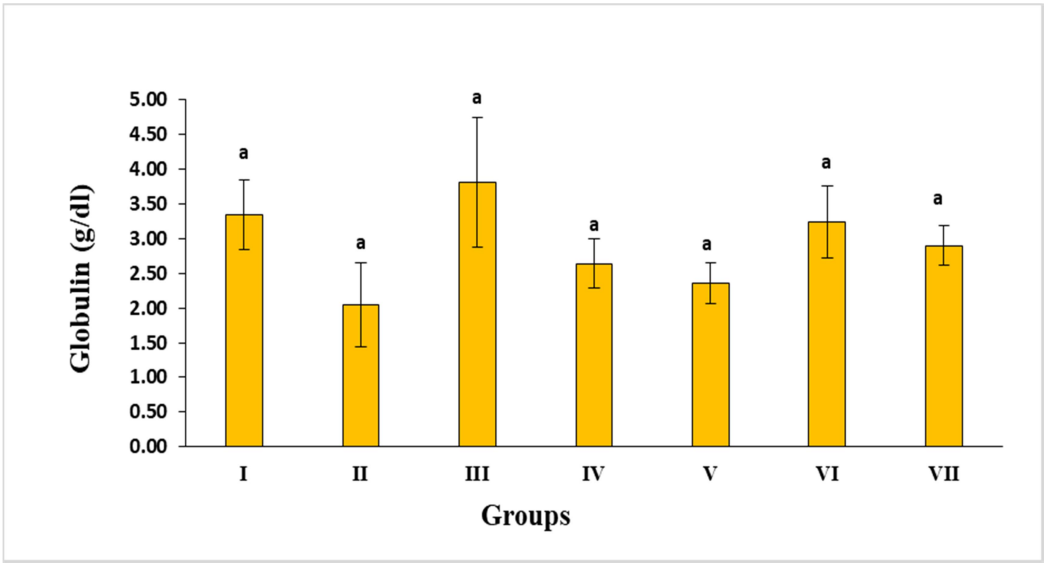


Fig. 19: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Globulin level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

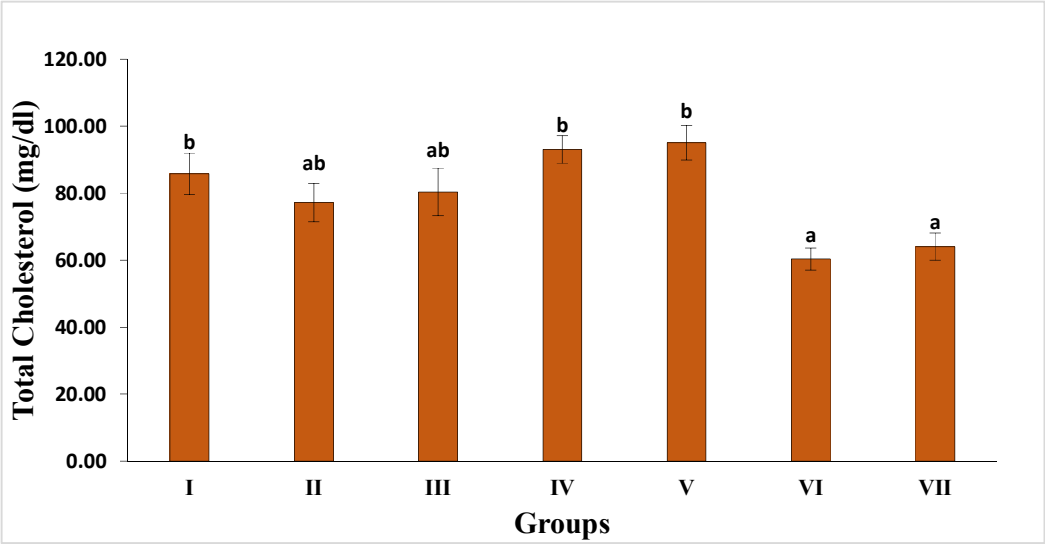


Fig. 20: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Total Cholesterol level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

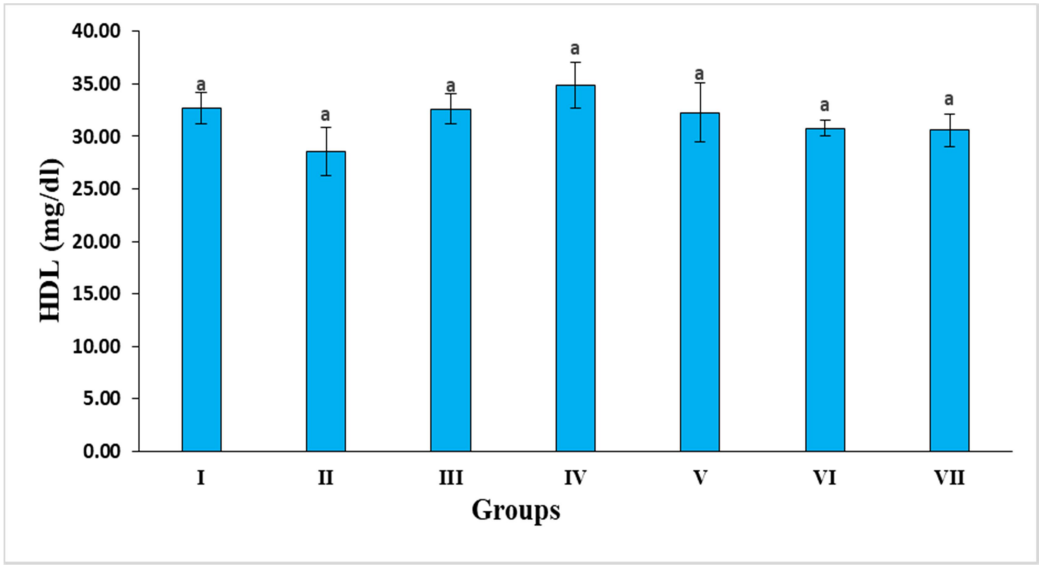


Fig. 21: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma HDL level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

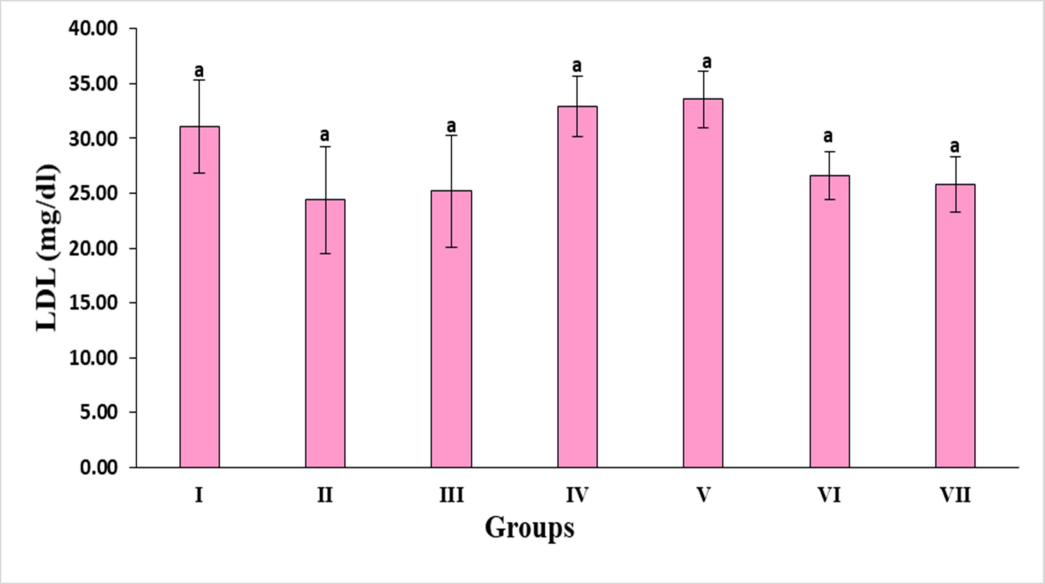


Fig. 22: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma LDL level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

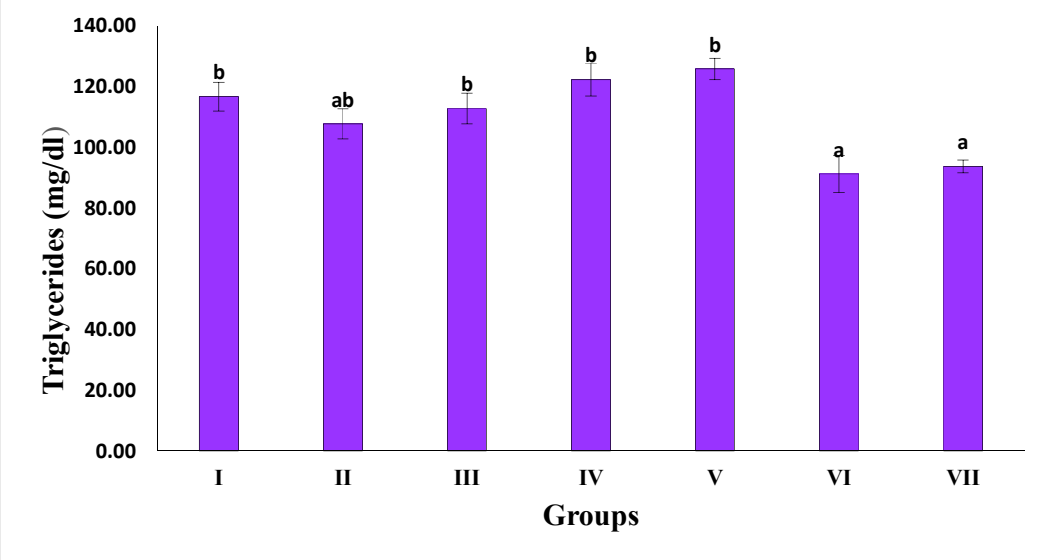


Fig. 23: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Triglycerides level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

Table 7: Effect of oral administration of α- tocopherol (100 mg/kg) on certain biochemical parameters in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	Total Bilirubin (mg/dl)	AST (U/L)	ALT (U/L)	BUN (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)
I	Control	0.17 ± 0.02^{a}	97.61 ± 4.1^{abc}	32.42 ± 1.42^{ab}	15.89 ± 1.41^{ab}	0.95 ± 0.07^{a}	9.35 ± 0.25^{bc}
II	Vehicle Control (Corn oil)	0.2 ± 0.04^{a}	101.82 ± 3.85^{abc}	28.43 ± 2.83^{a}	12.65 ± 0.83^{a}	0.96 ± 0.03^{a}	8.75 ± 0.44^{abc}
III	α-tocopherol (100 mg/kg)	0.21 ± 0.04^{a}	85.355 ± 5.09^{a}	26.82 ± 1.12^{a}	18.66 ± 0.95^{bc}	0.97 ± 0.04^{a}	8.13 ± 0.61^{ab}
IV	Cartap hydrochloride (16.25 mg/kg)	0.22 ± 0.04^{a}	100.28 ± 3.94^{abc}	33.99 ± 1.45^{ab}	19.54 ± 0.93^{bcd}	0.97 ± 0.01^{a}	9.03 ± 0.23^{abc}
V	Cartap hydrochloride (32.5 mg/kg)	0.22 ± 0.02^{a}	113.79 ± 2.69^{c}	42.37 ± 2.25^{c}	23.42 ± 1.09^{d}	0.98 ± 0.04^{a}	9.9 ± 0.14^{c}
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	0.17 ± 0.02^{a}	87.94 ± 1.59^{ab}	32.12 ± 1.22^{ab}	20.75 ± 0.82^{cd}	0.92 ± 0.03^{a}	7.73 ± 0.29^{a}
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	0.24 ± 0.02^{a}	102.36 ± 4.93^{bc}	38.96 ± 1.22^{bc}	22.45 ± 0.68^{cd}	0.89 ± 0.03^{a}	7.77 ± 0.11^{a}
P-value		0.549	0.00	0.00	0.00	0.341	0.00

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

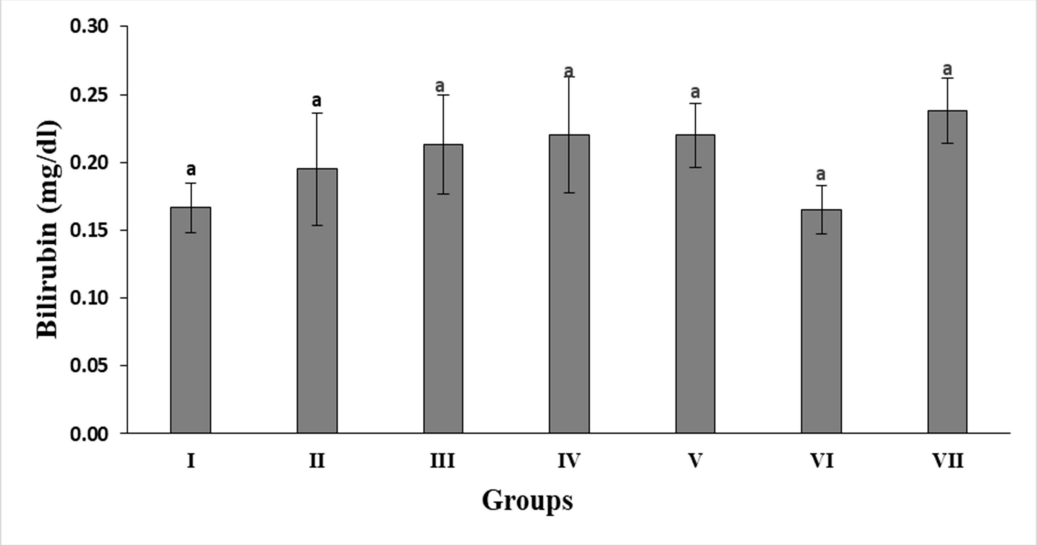


Fig. 24: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Total bilirubin level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts in bars show significant (P<0.05) differences.

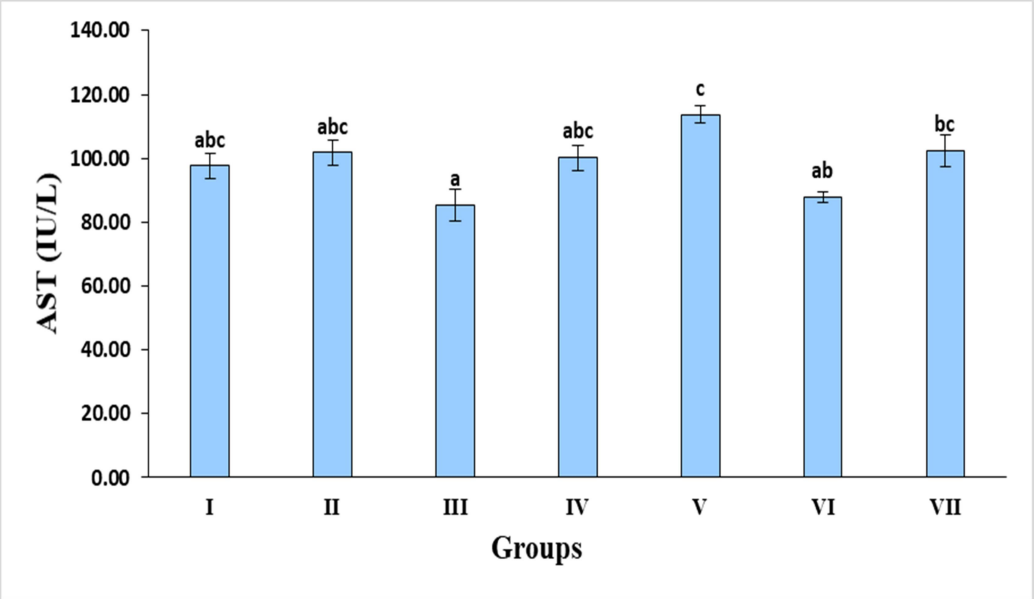


Fig. 25: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma AST level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts in bars show significant (P<0.05) differences.

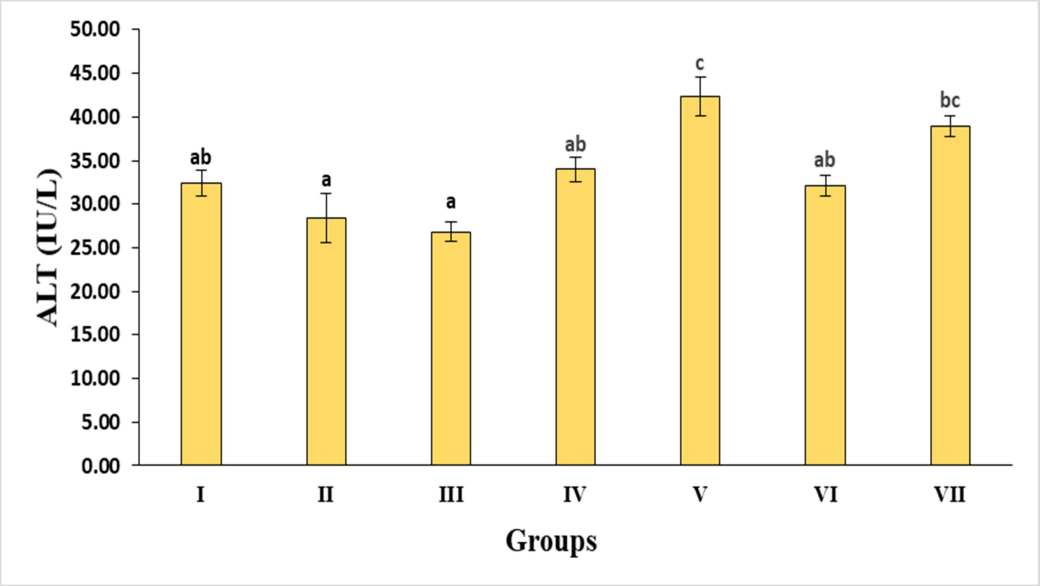


Fig. 26: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma ALT level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

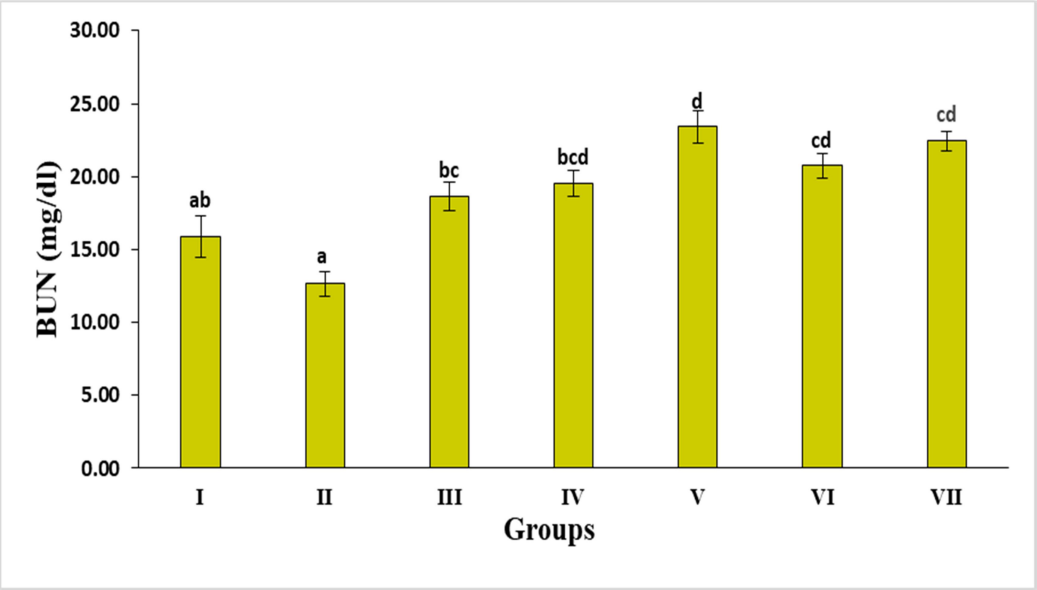


Fig. 27: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma BUN level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

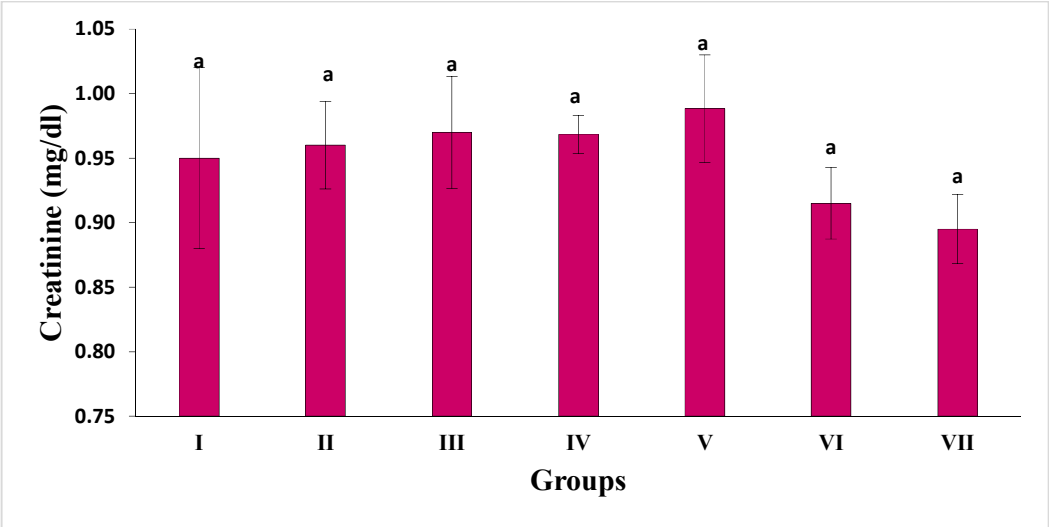


Fig. 28: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Creatinine level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

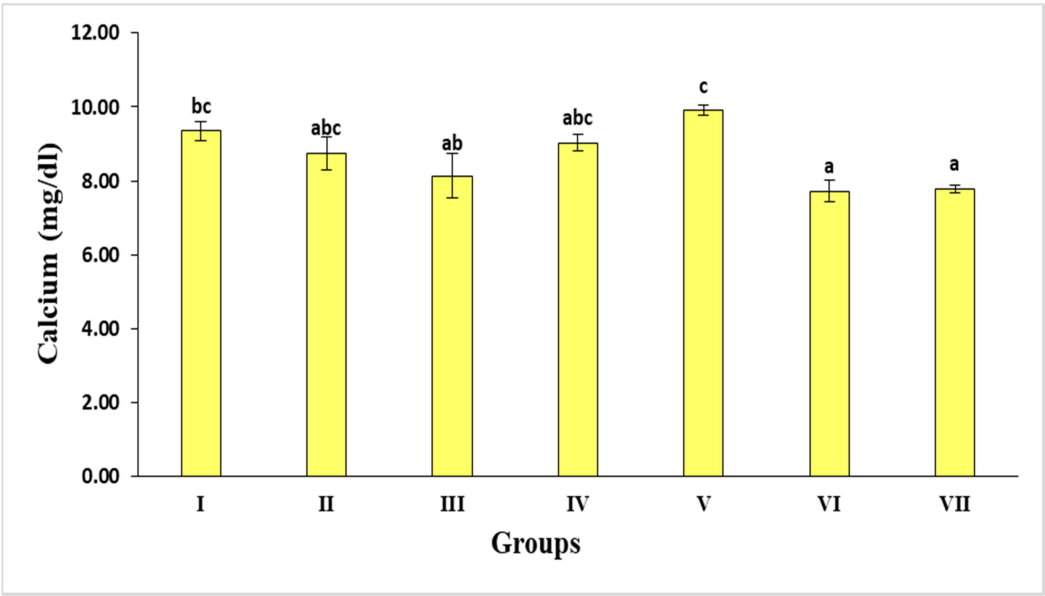


Fig. 29: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Calcium level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

4.4 Effect of alpha tocopherol on cartap induced oxidative stress biomarkers in liver and testes of rats:

4.4.1 Oxidative stress biomarkers in Liver: Data on oxidative stress biomarkers in Liver in rats of different treatment groups following 28 days exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and alpha tocopherol (100 mg/kg) alone and in combination is presented in Table 8 and illustrated in Fig 30-35. There was a significant ($p<0.05$) decrease in hepatic protein concentration in cartap alone treatment groups IV (6.08±0.09) and V (6.04±0.09) compared to control group (6.61±0.09). Co-treatment groups VI (6.5±0.05) and VII (6.53±0.08) showed a significant ($p<0.05$) increase in hepatic protein concentration compared to cartap alone group IV (6.08±0.09) and V (6.04±0.09) respectively. Compared to control group (6.61±0.09) no significant ($p>0.05$) change was observed in cartap and α-tocopherol co-treated groups VI (6.5±0.05) and VII (6.53±0.08).

Hepatic MDA levels were significantly ($p>0.05$) increased in cartap alone treatment groups IV (22.86±2.79) and V (35.71±5.56) compared to control group (6.31±1.10). Moderate but non-significant ($p>0.05$) decrease in MDA levels was observed in cartap co-treated group VI (14.29±1.015) compared to cartap alone treatment groups IV (22.86±2.79). However, when compared to cartap alone group V (35.71±5.56) the MDA level was significantly ($p<0.05$) decreased in cartap co-treated group VII (24.63±3.66). Although, compared to control group (6.31±1.10) there was no significant ($p>0.05$) change in hepatic MDA level was observed in group VI (14.29±1.015), but MDA level was significantly increased in cartap co-treated group VII (24.63±3.66). It shows less effect of α-tocopherol at higher dose of cartap.

No significant ($p>0.05$) change in SOD activity was observed in liver of cartap group IV (7.38±0.55) compared to control group (6.62±0.32). A significant ($p<0.05$) decrease in hepatic SOD activity was observed in cartap group V (4.95±0.21) compared to control group (6.62±0.32). Cartap and α-tocopherol co-treated groups VI and VII showed no significant ($p>0.05$) change in SOD activity compared to cartap alone groups IV and V. No significant ($p>0.05$) change in SOD activity was observed in cartap co-treatment groups VI (6.22±0.25) and VII (5.44±0.11) compared to control group (6.62±0.32).

Hepatic catalase activity was found to be non-significantly ($p>0.05$) reduced in cartap alone groups IV (1.07±0.07) and V (0.98±0.22) compared to control group (1.76±0.19). No significant ($p>0.05$) change in catalase activity was also observed in cartap co-treatment groups (VI and VII) compared to cartap alone treatment groups (IV and V). Further, no significant ($p>0.05$) change was observed in cartap and α-tocopherol co-treated groups (VI and VII) compared to control group.

Cartap alone treatment group IV (0.1±0.007) showed no significant ($p>0.05$) change in hepatic GSH levels compared to control group (0.12±0.006). Although at higher dosage of cartap in group V (0.07±0.007) GSH levels were significantly ($p<0.05$) decreased compared to control group (0.12±0.006). Further, no significant ($p>0.05$) change GSH levels was observed in cartap and α-tocopherol co-treatment groups VI and VII compared to cartap alone treatment groups IV and V. Non-significant ($p>0.05$) difference was observed in hepatic GSH levels in co-treatment group VI (0.09±0.004) compared to control group (0.12±0.006), but in group VII (0.08±0.008) a significant ($p<0.05$) decrease in hepatic GSH level was found compared to control group (0.12±0.006).

Non-significant ($p>0.05$) change in hepatic GST activity was observed in cartap alone groups IV (0.4±0.07) and V (0.22±0.04) compared to control group (0.45±0.03). No significant ($p>0.05$) change in GST levels were observed in cartap and α-tocopherol co-treated groups VI and VII compared to cartap alone treatment groups IV and V. Compared to control group, no significant ($p>0.05$) change was observed in hepatic GST levels in cartap and α-tocopherol co-treatment groups VI (0.46±0.12) and VII (0.25±0.03).

4.4.2 Oxidative stress biomarkers in testes: Effect of alpha tocopherol (100 mg/kg) on oxidative stress biomarkers in testes in rats of different treatment groups following 28 days exposure to cartap (16.25 mg/kg and 32.5 mg/kg) is presented in Table 9 and illustrated in Fig 36-41. There was a significant ($p<0.05$) decrease in testicular protein concentration in cartap alone treatment groups IV (5.63±0.07) and V (5.59±0.04) compared to control group (6.13±0.13). Co-treatment group VI (5.98±0.06) showed no significant ($p>0.05$) change in testicular protein compared to cartap alone group IV (5.63±0.07). However, compared to cartap alone group V (5.59±0.04) testicular protein level was significantly increased in co-treatment group VII (6.09±0.08). However, no significant ($p>0.05$) change was observed in cartap and α-tocopherol co-

treated groups VI (5.98±0.06) and VII (6.09±0.08) compared to control group (6.13±0.13).

Testicular MDA levels were significantly ($p>0.05$) increased in cartap alone treatment groups IV (27.03±2.37) and V (32.55±6.02) compared to control group (8.69±2.28). No significant ($p>0.05$) change in MDA levels was observed in cartap co-treated groups VI and VII compared to cartap alone treatment groups IV and V respectively. Compared to control group (8.69±2.28) no significant ($p>0.05$) change in testicular MDA level was observed in cartap and α-tocopherol co-treated group VI (20.68±3.89), However, MDA level was significantly ($p<0.05$) increased in cartap co-treated group VII (32.67±8.38) compared to control group (8.69±2.28).

No significant ($p>0.05$) change in SOD activity was observed in cartap group IV (5.08±0.19) compared to control group (5.76±0.41). A significant ($p<0.05$) decrease in testicular SOD activity was observed in cartap (32.5 mg/kg) group V (4.29±0.14) compared to control group (5.76±0.41). Cartap and α-tocopherol co-treated groups VI and VII showed no significant ($p>0.05$) change in SOD activity compared to cartap alone groups IV and V. No significant ($p>0.05$) change in SOD activity was observed in cartap co treatment groups VI and VII compared to control group.

Testicular catalase activity was found to be significantly ($p<0.05$) reduced in cartap group V (0.84±0.28) compared to control group (2.06±0.15). No significant ($p>0.05$) change in catalase activity was observed in cartap co-treatment groups (VI and VII) compared to cartap alone treatment groups (IV and V). No significant ($p>0.05$) change was observed in cartap and α-tocopherol co-treated group VI (2.02±0.16) compared to control group (2.06±0.15), however, it was significantly ($p<0.05$) reduced in co-treated group VII (0.85±0.20).

Cartap alone treatment groups IV (0.095±0.005) and V (0.1±0.01) did not show significant ($p>0.05$) change in testicular GSH level compared to control group (0.12±0.008). No significant ($p>0.05$) change in GSH levels was observed in cartap and α-tocopherol co-treatment groups (VI and VII) compared to cartap alone treatment groups (IV and V). Similarly, no significant difference was observed in testicular GSH levels in co-treatment groups VI (0.11±0.003) and VII (0.09±0.005) compared to control group (0.12±0.008).

There was no significant ($p>0.05$) change in testicular GST activity was in cartap group IV (0.55±0.06) compared to control group (0.69±0.07). However, higher dosage cartap group V (0.35±0.07) showed a significant ($p<0.05$) decrease in GST activity compared to control group (0.69±0.07). No significant ($p>0.05$) change in GST activity was observed in cartap and α-tocopherol co-treated group VI and VII compared to cartap alone treatment groups IV and V. Compared to control group (0.69±0.07) non-significant ($p>0.05$) difference was observed in testicular GST activity in co-treatment group VI (0.63±0.04) compared to control group. The GST activity was found to be significantly ($p<0.05$) decreased in co-treated group VII (0.34±0.06) compared to control group (0.69±0.07).

Table 8: Effect of oral administration of α- tocopherol (100 mg/kg) on certain hepatic oxidative stress biomarkers in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg body and 32.5 mg/kg body weight).

Groups	Treatment	Protein in hepatic tissue (mg/ml)	LPO (nM MDA/g tissue)	SOD (U/mg of protein)	Catalase (mM H_2O_2 utilized/min/mg of protein)	GSH (Mm GSH/g tissue)	GST (µM of CDNB-GSH conjugate min^{-1} mg^{-1} protein)
I	Control	6.61 ± 0.09^{bc}	6.31 ± 1.10^{a}	6.62 ± 0.32^{bc}	1.76 ± 0.19^{abc}	0.12 ± 0.006^{b}	0.45 ± 0.03^{ab}
II	Vehicle Control (Corn oil)	6.54 ± 0.15^{bc}	8.07 ± 1.46^{a}	5.39 ± 0.22^{ab}	1.83 ± 0.28^{bc}	0.09 ± 0.005^{ab}	0.35 ± 0.02^{ab}
III	α-tocopherol (100 mg/kg)	6.73 ± 0.11^{c}	5.71 ± 1.36^{a}	7.38 ± 0.55^{c}	2.33 ± 0.24^{c}	0.11 ± 0.001^{b}	0.55 ± 0.06^{b}
IV	Cartap hydrochloride (16.25 mg/kg)	6.08 ± 0.09^{a}	22.86 ± 2.79^{b}	6.14 ± 0.37^{abc}	1.07 ± 0.07^{ab}	0.1 ± 0.007^{ab}	0.4 ± 0.07^{ab}
V	Cartap hydrochloride (32.5 mg/kg)	6.04 ± 0.09^{a}	35.71 ± 5.56^{c}	4.95 ± 0.21^{a}	0.98 ± 0.22^{a}	0.07 ± 0.007^{a}	0.22 ± 0.04^{a}
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	6.5 ± 0.05^{bc}	14.29 ± 1.015^{ab}	6.22 ± 0.25^{abc}	1.3 ± 0.14^{ab}	0.09 ± 0.004^{ab}	0.46 ± 0.12^{ab}
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	6.53 ± 0.08^{bc}	24.63 ± 3.66^{b}	5.44 ± 0.11^{ab}	0.92 ± 0.14^{a}	0.08 ± 0.008^{a}	0.25 ± 0.03^{a}
P-value		0.001	0.00	0.00	0.00	0.00	0.008

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

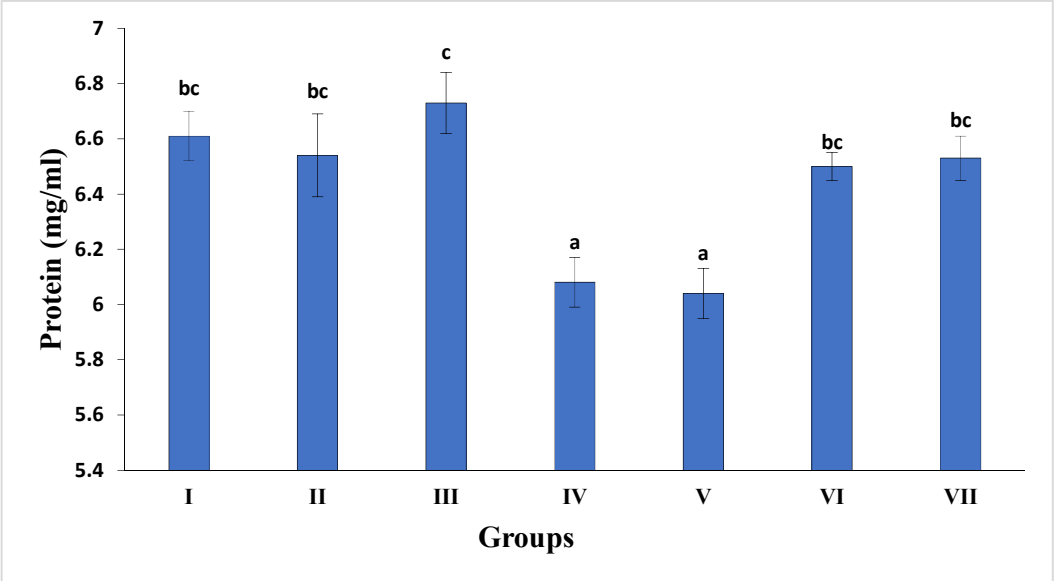


Fig. 30: Effect of oral administration of α- tocopherol (100 mg/kg) on hepatic Total Protein in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).
Different superscripts on bars indicate significant (P<0.05) differences.

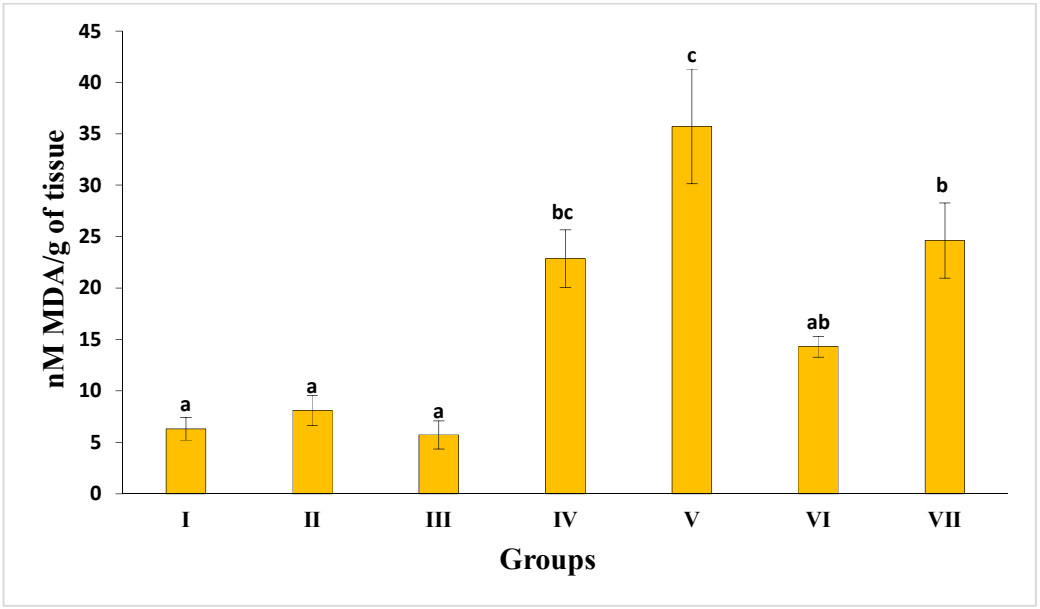


Fig. 31: Effect of oral administration of α- tocopherol (100 mg/kg) on hepatic MDA level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).
Different superscripts on bars indicate significant (P<0.05) differences.

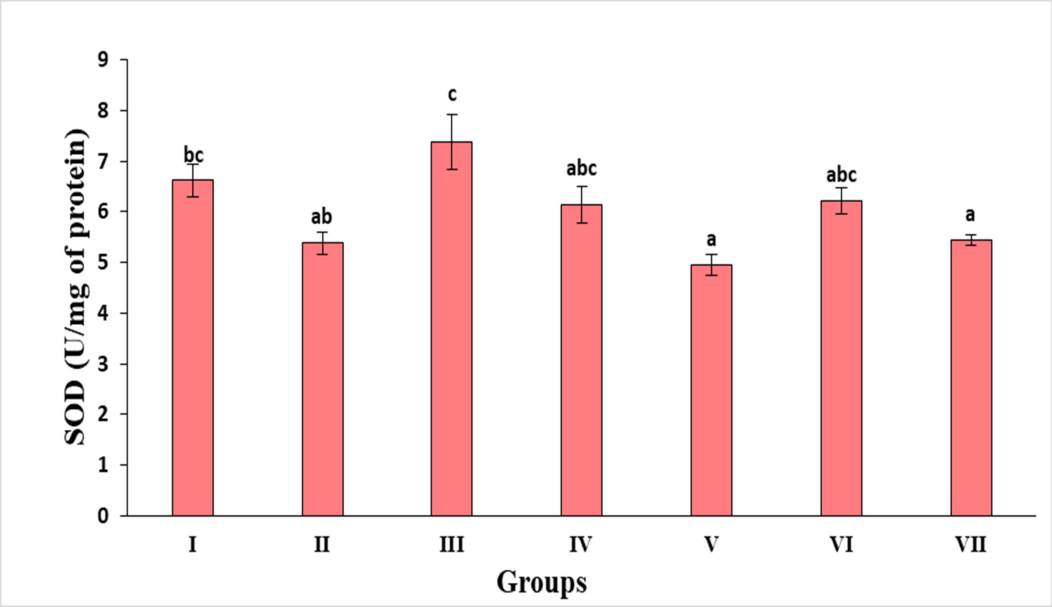


Fig. 32: Effect of oral administration of α- tocopherol (100 mg/kg) on hepatic SOD level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).
Different superscripts on bars indicate significant (P<0.05) differences.

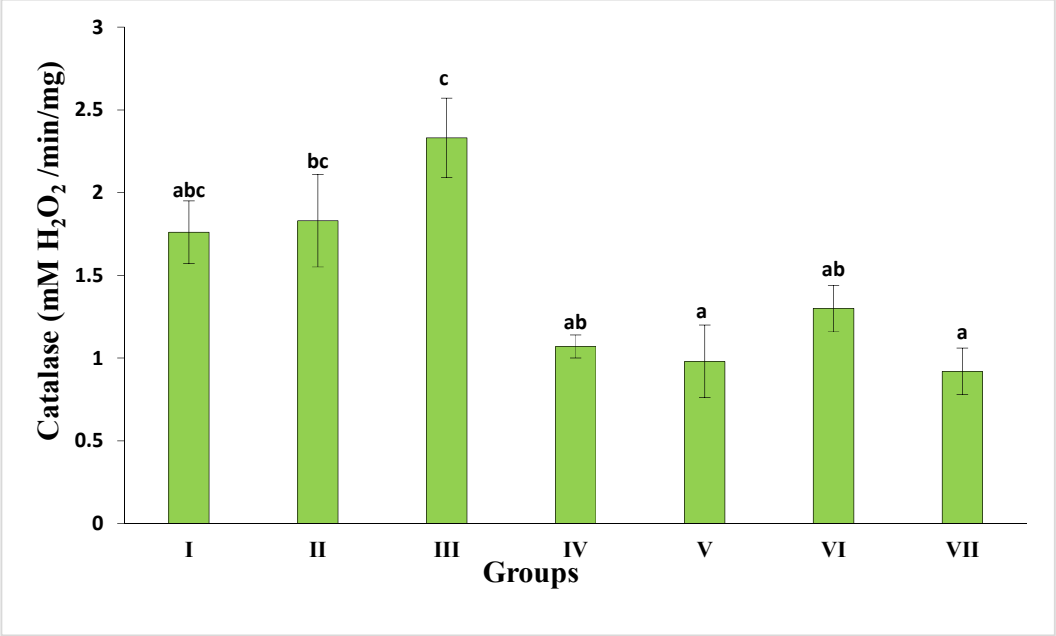


Fig. 33: Effect of oral administration of α- tocopherol (100 mg/kg) on hepatic Catalase level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).
Different superscripts on bars indicate significant (P<0.05) differences.

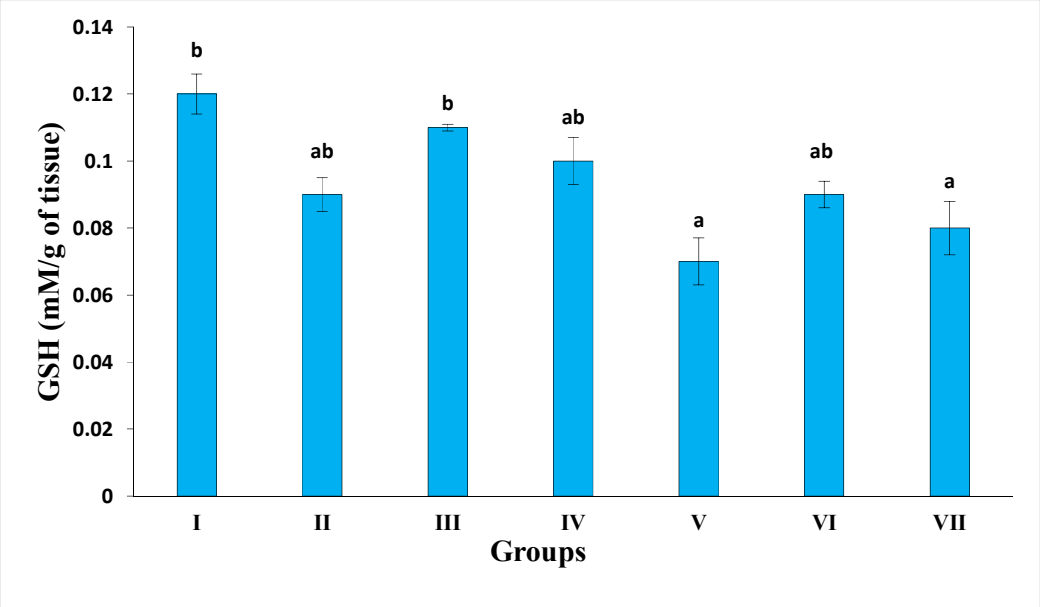


Fig. 34: Effect of oral administration of α- tocopherol (100 mg/kg) on hepatic GSH level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

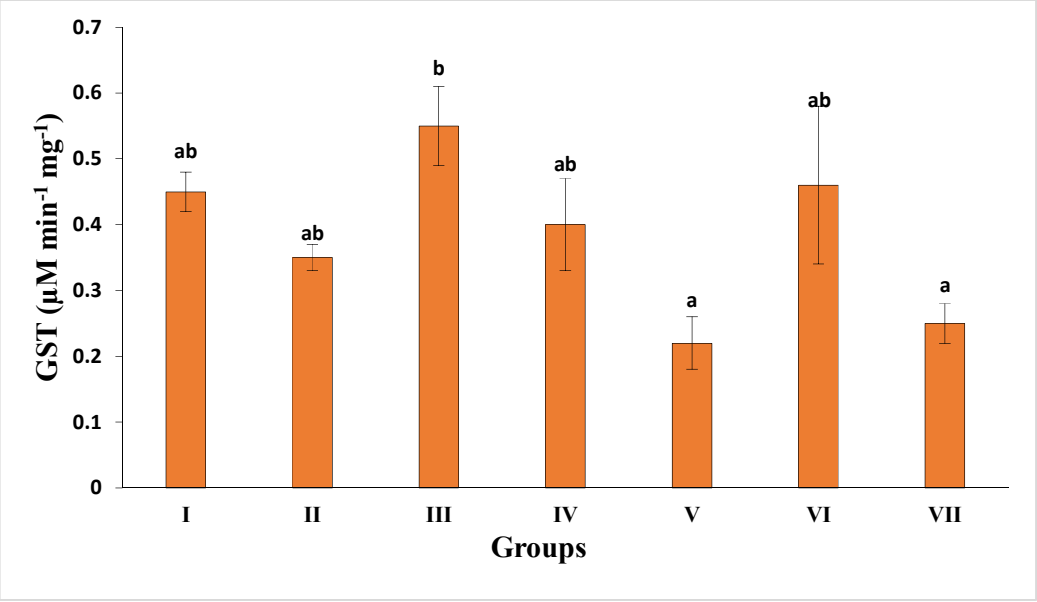


Fig. 35: Effect of oral administration of α- tocopherol (100 mg/kg) on hepatic GST level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

Table 9: Effect of oral administration of α- tocopherol (100 mg/kg) on certain testicular oxidative stress biomarkers in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Groups	Treatment	Testicular Protein (mg/ml)	LPO (nM MDA/g tissue)	SOD (U/mg of protein)	Catalase (mM H_2O_2 utilized/min/mg of protein)	GSH (Mm GSH/g tissue)	GST (µM of CDNB- GSH conjugate min^{-1} mg^{-1} protein)
I	Control	6.10 ± 0.13^{b}	8.69 ± 2.28^{a}	5.76 ± 0.41^{b}	2.06 ± 0.15^{bc}	0.12 ± 0.008^{bc}	0.69 ± 0.07^{b}
II	Vehicle Control (Corn oil)	5.75 ± 0.21^{ab}	9.46 ± 1.89^{a}	5.34 ± 0.19^{ab}	1.51 ± 0.29^{b}	0.10 ± 0.008^{ab}	0.56 ± 0.08^{ab}
III	α-tocopherol (100 mg/kg)	5.96 ± 0.05^{ab}	8.84 ± 1.30^{a}	5.38 ± 0.40^{ab}	2.72 ± 0.28^{c}	0.14 ± 0.01^{c}	0.51 ± 0.12^{ab}
IV	Cartap hydrochloride (16.25mg/kg)	5.63 ± 0.07^{a}	27.03 ± 2.37^{b}	5.08 ± 0.19^{ab}	1.62 ± 0.21^{ab}	0.095 ± 0.005^{ab}	0.55 ± 0.06^{ab}
V	Cartap hydrochloride (32.5 mg/kg)	5.59 ± 0.04^{a}	32.55 ± 6.02^{b}	4.29 ± 0.14^{a}	0.84 ± 0.28^{a}	0.1 ± 0.01^{ab}	0.35 ± 0.07^{a}
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	5.98 ± 0.06^{ab}	20.68 ± 3.89^{ab}	5.25 ± 0.26^{ab}	2.02 ± 0.16^{bc}	0.11 ± 0.003^{abc}	0.63 ± 0.04^{ab}
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	6.09 ± 0.08^{b}	32.67 ± 8.38^{b}	4.69 ± 0.09^{ab}	0.85 ± 0.20^{a}	0.09 ± 0.005^{ab}	0.34 ± 0.06^{a}
P-value		0.00	0.00	0.00	0.00	0.00	0.00

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

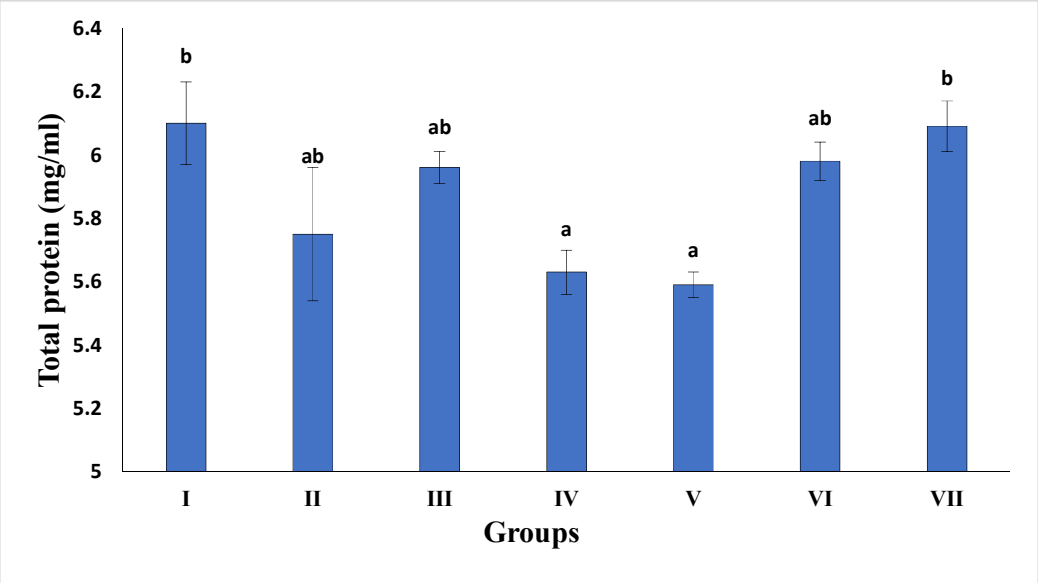


Fig. 36: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular Total Protein in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

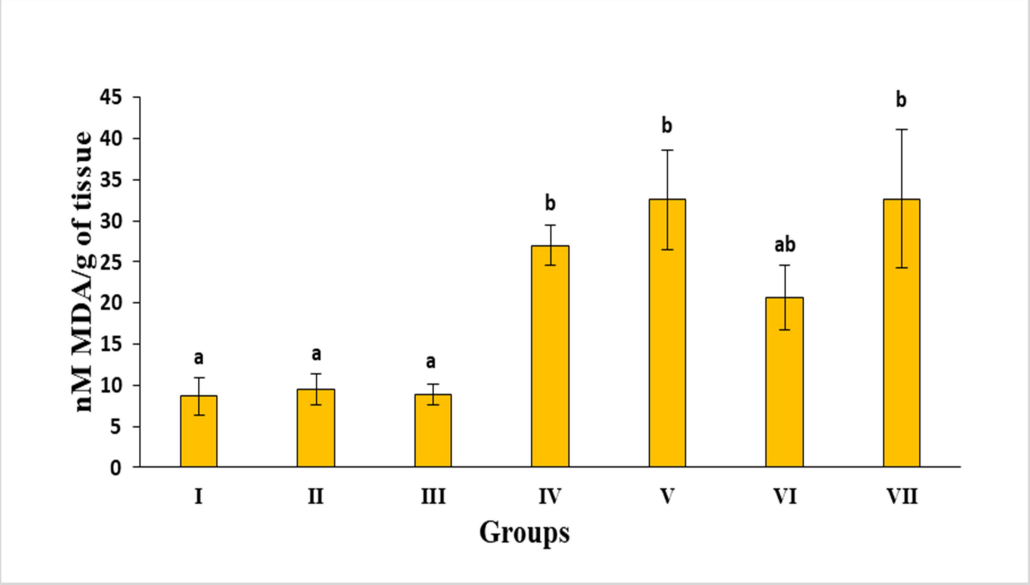


Fig. 37: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular MDA level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

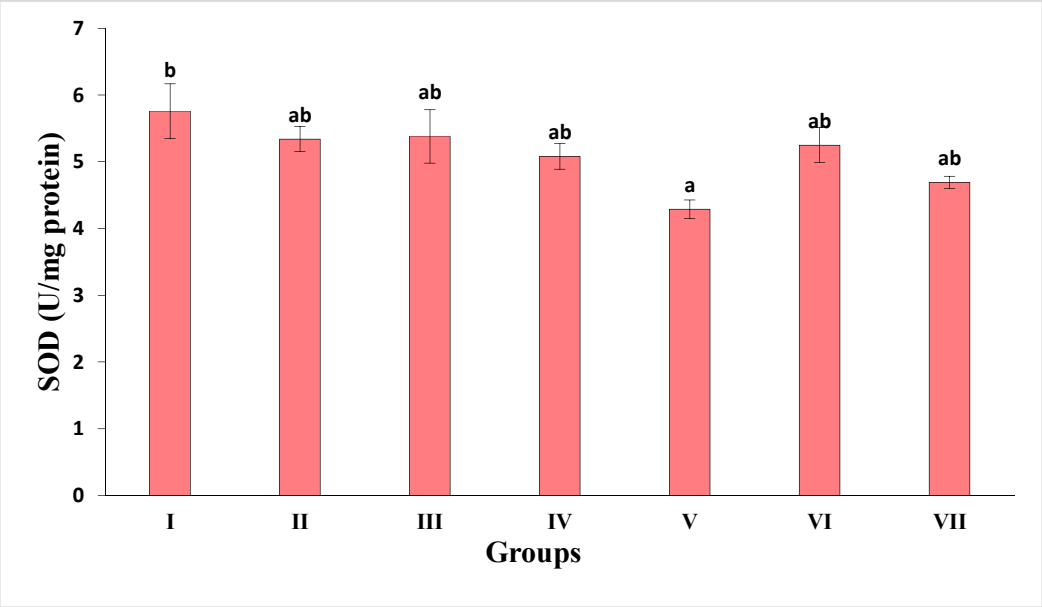


Fig. 38: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular SOD level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

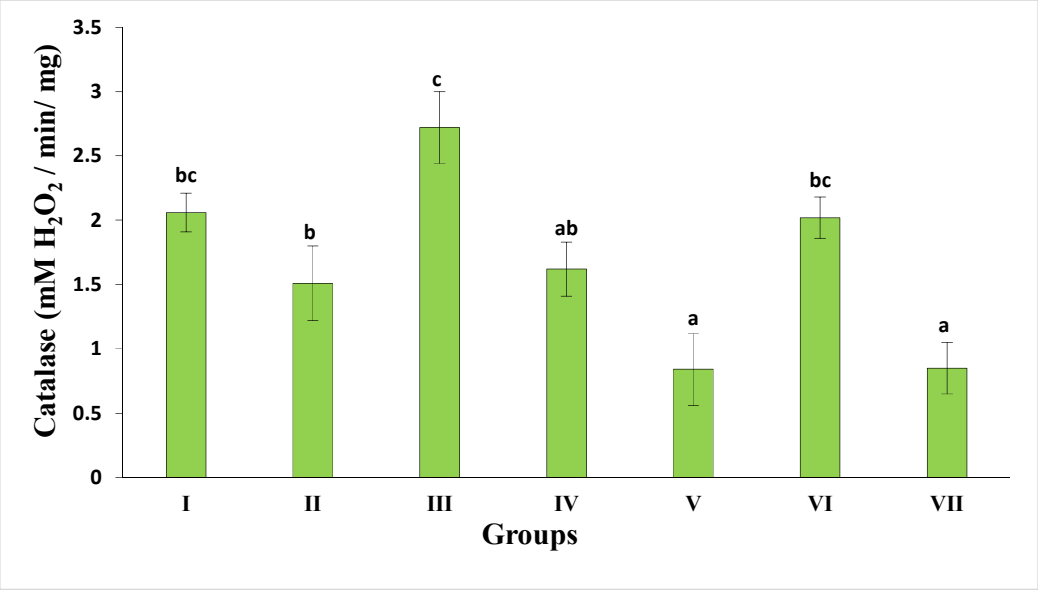


Fig. 39: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular Catalase level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

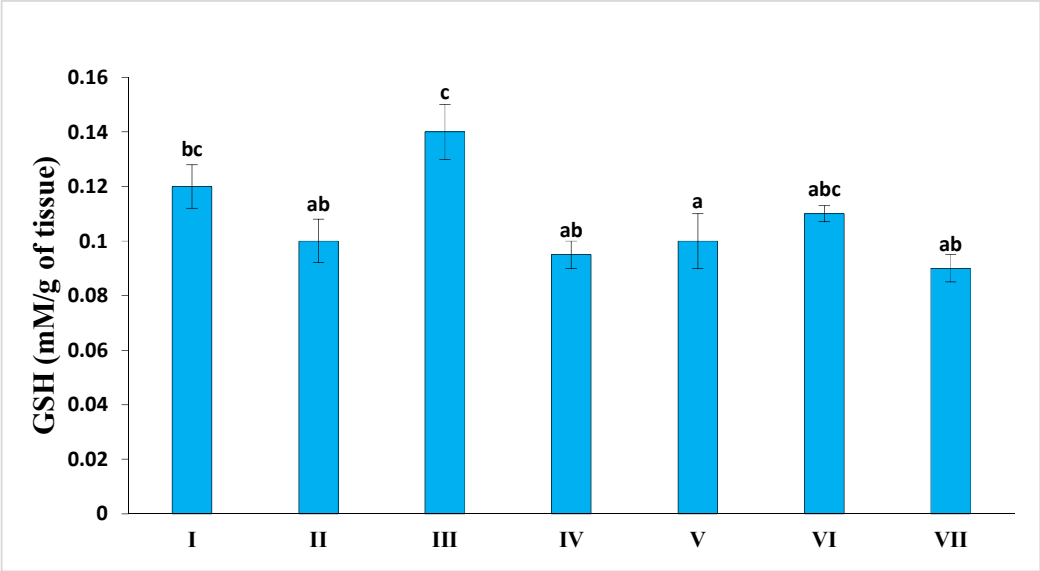


Fig. 40: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular GSH level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

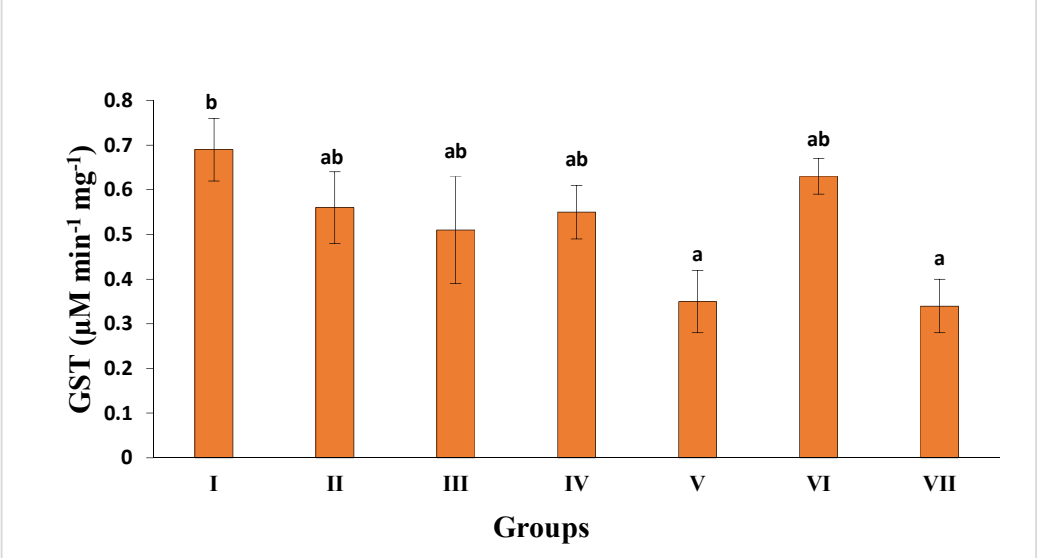


Fig. 41: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular GST level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

4.5 Effect of α-tocopherol on cartap induced insult to certain testicular injury biomarkers and testosterone level: Effect of cartap (16.25 mg/kg and 32.5 mg/kg) and α-tocopherol (100 mg/kg) alone and in combination on certain testicular injury biomarkers and plasma testosterone level in male rats of different treatment groups is presented in Table 10 and illustrated in Fig 42-46.

Compared to control group (0.3±0.06 IU/L) non-significant ($p>0.05$) change was observed in testicular acid phosphatase (ACP) activity in cartap group IV (0.77±0.2 IU/L). However, cartap group V (1.49±0.11 IU/L) showed a significant ($p<0.05$) increase in testicular ACP activity compared to control group (0.3±0.06 IU/L). Co-treatment with α-tocopherol and cartap in group VI and VII resulted in non-significant ($p>0.05$) change in testicular ACP activity compared to cartap alone treatment groups (IV and V) respectively. Co-treated group VI (0.63±0.12 IU/L) showed a non-significant ($p>0.05$) change in ACP activity while a significant ($p<0.05$) increase was observed in co-treated group VII (1.34±0.12 IU/L) compared to control group (0.3±0.06 IU/L).

SDH activity was found to be 0.09±0.021 in control group. No significant ($p>0.05$) alteration in testicular sorbitol dehydrogenase (SDH) activity was observed in different treatment groups (II-VII) compared to control group.

Testicular lactate dehydrogenase (LDH) level was increased moderately but non-significantly ($p>0.05$) at lower dosage of cartap in group IV (2709±300.45 IU/L). At higher dosage of cartap in group VII, LDH level was found to be 3404.16±424.29 IU/L, which was significantly ($p<0.05$) higher compared to control group (1927.66±270.66 IU/L). Co-treatment with α-tocopherol and cartap (group VI and VII) resulted in a moderate but not significant ($p>0.05$) decrease in testicular LDH activity compared to cartap alone treatment groups IV and V respectively. Both co-treated groups VI (1892.83±120.83 IU/L) and VII (2715.33±147.18 IU/L) showed no significant ($p>0.05$) change in testicular LDH levels compared to control group (1927.66±270.66 IU/L).

Cartap alone group IV (5.52±0.69 IU/L) showed non-significant ($p>0.05$) change in testicular gamma glutamyl transpeptidase (GGT) activity compared to control group (4.03±0.77 IU/L). However, a significant ($p<0.05$) increase in testicular GGT activity was observed in cartap alone group V (7.3±0.49 IU/L) compared to control group (4.03±0.77 IU/L). Co-treatment with α-tocopherol and cartap in group

VI and VII resulted in a non-significant ($p>0.05$) decrease in testicular GGT activity compared to cartap alone treatment groups (IV and V) respectively. Co-treatment groups VI (4.6±0.62 IU/L) and VII (6.24±0.61 IU/L) showed no significant ($p>0.05$) change in testicular GGT levels compared to control group (4.03±0.77 IU/L).

Plasma testosterone levels were significantly ($p<0.05$) decreased in cartap alone groups IV (0.69±0.05 ng/ml) and V (0.55±0.03 ng/ml) compared to control group (0.98±0.06 ng/ml). Non-significant ($p>0.05$) change was observed in cartap co-treated groups VI and VII compared to cartap alone treatment groups IV and V. Testosterone levels were found to be 0.73±0.05 ng/ml and 0.64±0.06 ng/ml in co-treatment group VI and VII respectively. These observations indicate that Testosterone levels were significantly ($p<0.05$) decreased in co-treatment groups VI and VII compared to control group (0.98±0.06 ng/ml).

Table 10: Effect of oral administration of α- tocopherol (100 mg/kg) on certain testicular injury biomarkers and testosterone levels in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg body and 32.5 mg/kg body weight).

Groups	Treatment	Testicular injury biomarkers and testosterone levels				
		ACP (IU/L)	SDH (U/ml enzyme /mg protein/ml)	LDH (IU/L)	GGT (IU/L)	Testosterone (ng/ml)
I	Control	0.30±0.06^{a}	0.09±0.02^{a}	1927.66±270.66^{a}	4.03±0.77^{a}	0.98±0.06^{d}
II	Vehicle Control (Corn oil)	0.49±0.04^{a}	0.06±0.01^{a}	1544.13±254.38^{a}	4.01±0.78^{a}	0.89±0.04^{bcd}
III	α-tocopherol (100 mg/kg)	0.44±0.09^{a}	0.05±0.01^{a}	1705.16±257.63^{a}	3.98±0.1^{a}	0.92±0.03^{cd}
IV	Cartap hydrochloride (16.25 mg/kg)	0.77±0.2^{a}	0.1±0.01^{a}	2709±300.45^{ab}	5.52±0.69^{ab}	0.69±0.05^{ab}
V	Cartap hydrochloride (32.5 mg/kg)	1.49±0.17^{b}	0.11±0.01^{a}	3404.16±424.29^{b}	7.3±0.49^{b}	0.55±0.03^{a}
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	0.63±0.12^{a}	0.06±0.01^{a}	1892.83±120.83^{a}	4.6±0.62^{a}	0.73±0.05^{abc}
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	1.34±0.12^{b}	0.07±0.005^{a}	2715.33±147.18^{ab}	6.24±0.61^{ab}	0.64±0.06^{a}
P-value		0.00	0.055	0.00	0.002	0.00

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

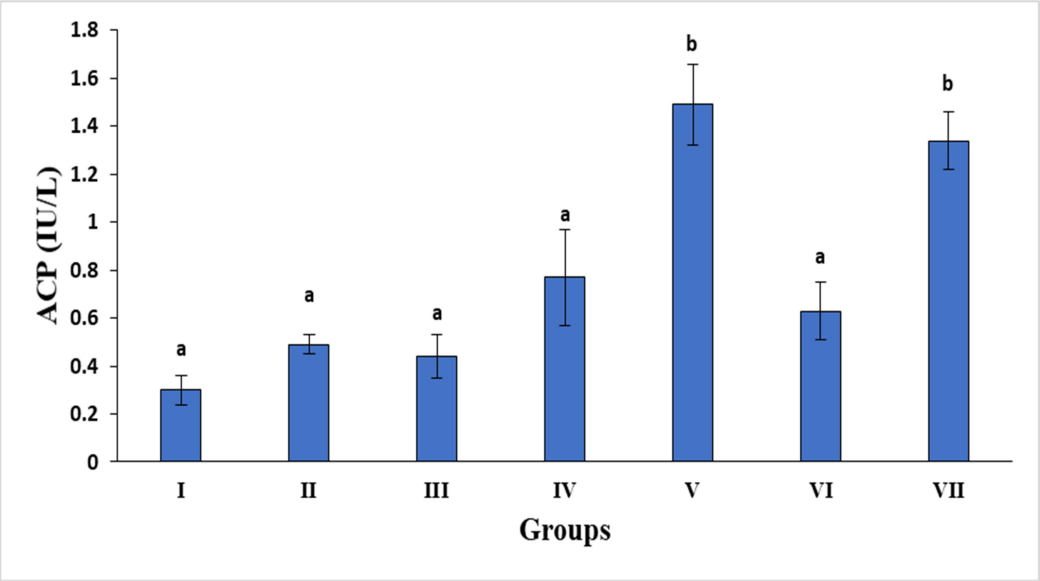


Fig. 42: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular ACP level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

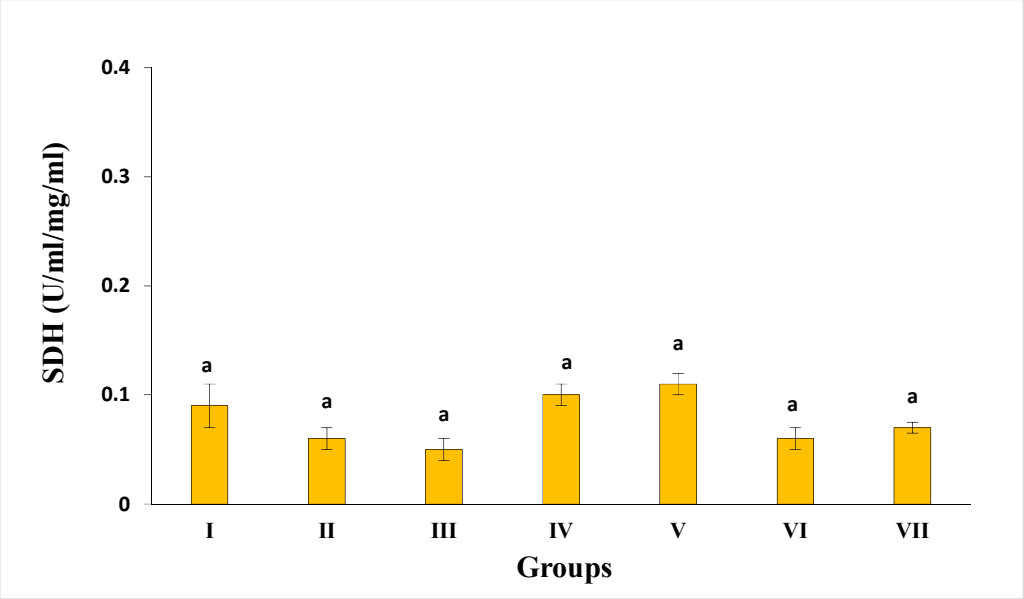


Fig. 43: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular SDH level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

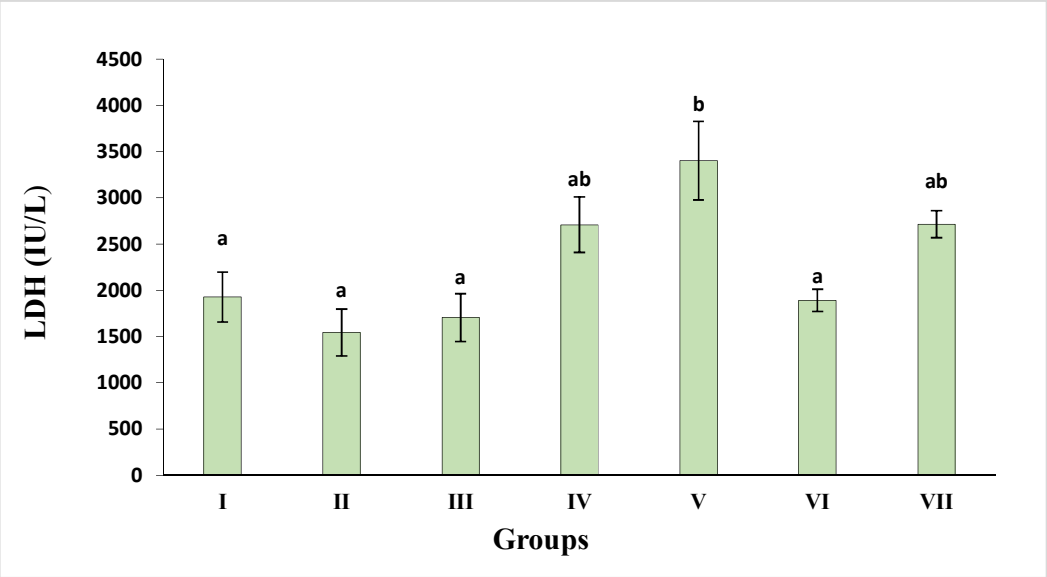


Fig. 44: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular LDH level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

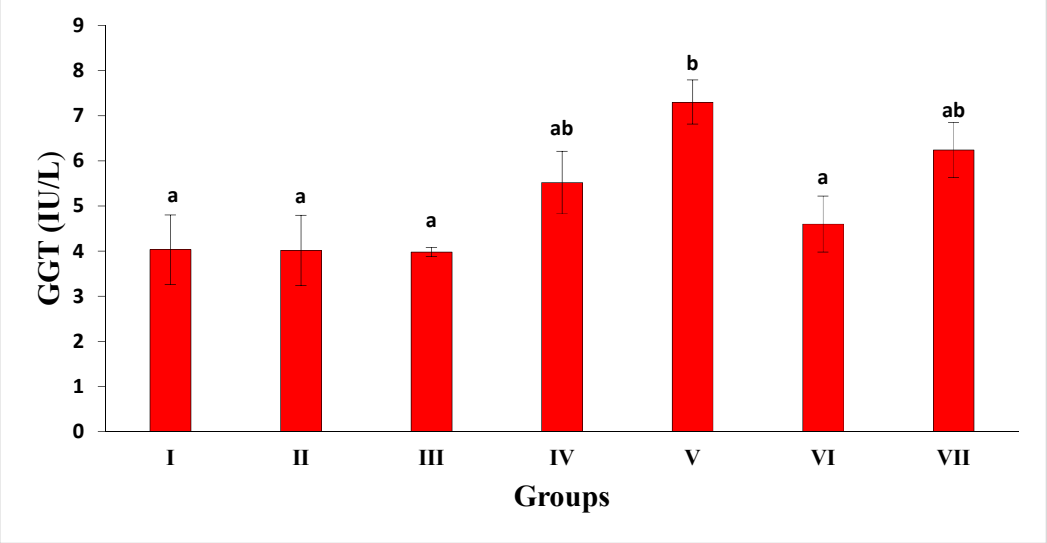


Fig. 45: Effect of oral administration of α- tocopherol (100 mg/kg) on testicular GGT level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

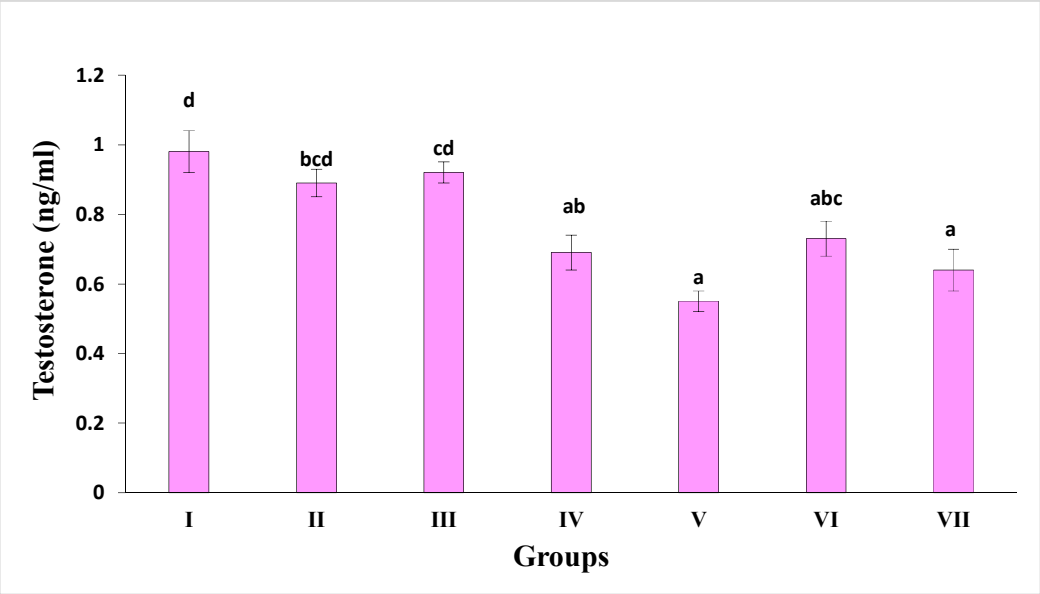


Fig. 46: Effect of oral administration of α- tocopherol (100 mg/kg) on plasma Testosterone level in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

4.6 Effect of α-tocopherol on cartap induced gross and histopathological changes in lungs, liver, kidneys, spleen, heart, testes and trachea.

Gross changes were observed following necropsy in lungs, liver, kidneys, spleen, heart and testes as illustrated in Fig 47-52. Grossly, no changes were observed in any of the organs except in lungs which showed mild congestion and swelling in cartap alone treatment groups (IV and V) as illustrated in Fig 47. The swelling was more severe in cartap group V (32.5 mg/kg) compared to cartap group IV (16.25 mg/kg). Following co-treatment with α-tocopherol there was a visible reduction in swelling in lungs in both co-treated groups (VI and VII) however lungs remained slightly congested.

Histopathological changes in lungs, liver, kidneys, spleen, heart, testes and trachea of different treatment groups following 28 days exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α-tocopherol (100 mg/kg) alone and in combination are illustrated in Figure 53-66.

Lungs showing normal histoarchitecture of alveoli and alveolar walls were observed in control (I), vehicle control (II) and α-tocopherol group (III). Mild congestion and thickening of alveolar walls was observed in cartap group IV (16.25 mg/kg) while moderate congestion and thickening of alveolar as well as bronchiolar wall was observed in cartap group V (32.5 mg/kg). Only mild congestion was observed in co-treated group VI, however mild congestion along with alveolar wall thickening was observed in co-treated group VII.

Liver of control (I), vehicle control (II) and α-tocopherol group (III) showed normal histoarchitecture of hepatocytes and sinusoids. Mild congestion in the central vein was observed in cartap alone group IV (16.25 mg/kg). However, central vein of group V (32.5 mg/kg) showed moderate congestion. Normal histoarchitecture was observed in cartap and α-tocopherol co-treatment groups (VI and VII) similar to control group.

Normal histoarchitecture of glomeruli and tubules was observed in kidneys of control (I) vehicle control (II) and α-tocopherol group (III). Constriction of glomeruli along with degeneration of tubules was observed in kidneys of cartap alone group IV (16.25 mg/kg). Along with constriction of glomeruli and degeneration of the

tubules moderate congestion was also observed in kidneys of cartap alone group V (32.5 mg/kg). Only mild congestion of tubular capillaries along with normal histoarchitecture was observed in cartap and α-tocopherol co-treatment groups (VI and VII).

Control (I), Vehicle control (II) and α-tocopherol (III) group showed normal histoarchitecture of white pulp and red pulp in spleen. Normal histoarchitecture was also observed in cartap alone group IV (16.25 mg/kg). However, Cartap alone group V (32.5 mg/kg) showed mild congestion in the red pulp of spleen. Normal histoarchitecture was observed in cartap and α-tocopherol co-treatment groups (VI and VII).

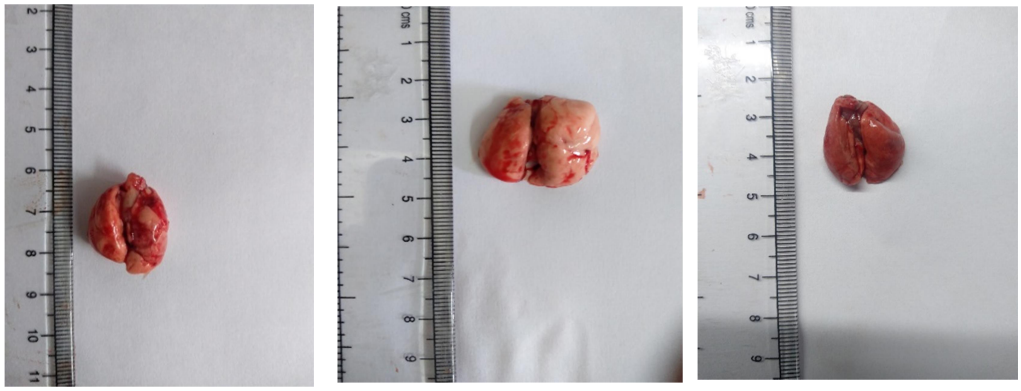
Cardiac tissue showing normal arrangement of myocardial fibres was observed in control (I), vehicle control (II) and α-tocopherol group (III). Group IV (Cartap, 16.25 mg/kg) also showed normal arrangement of myocardial fibres in cardiac tissue. However, mild degeneration of myocardial fibres along with haemorrhage was observed in cardiac tissue of group V (Cartap, 32.5 mg/kg). Mild disruption of myocardial fibres was observed in cardiac tissue of cartap alone group VII (32.5 mg/kg) + α-tocopherol) while normal histoarchitecture was observed in co-treated group VI (16.25 mg/kg + α-tocopherol).

Seminiferous tubules in testicular tissue of control (I), vehicle control (II) and α-tocopherol (III) group were normally arranged and showed normal histoarchitecture. Cartap exposure in group IV (16.25 mg/kg) produced mild degeneration of seminiferous tubules compared to group V (32.5 mg/kg) where it produced moderate degeneration. Testicular tissue in both the cartap alone groups (IV and V) had detached germ cells within seminiferous tubules. Tissue sections of co-treatment groups (VI and VII) showed mild congestion in tubular capillaries.

Normal histoarchitecture of cartilage and ciliated epithelium in tracheal tissue was observed in control (I), vehicle control (II) and α-tocopherol (III) group. Cartap at the dose rate of 16.25 mg/kg in group IV showed no visible effect as the tissue histoarchitecture remained unchanged. However, cartap at the dosage of 32.5 mg/kg in group V showed mild degeneration of cartilage and ciliated epithelium. Normal histoarchitecture of cartilage and ciliated epithelium was apparent in co-treated group VI but the tracheal tissue of co-treated group VII showed mild degeneration of cartilage.



(I) Control (II) Vehicle Control (III) α- tocopherol (100 mg/kg)

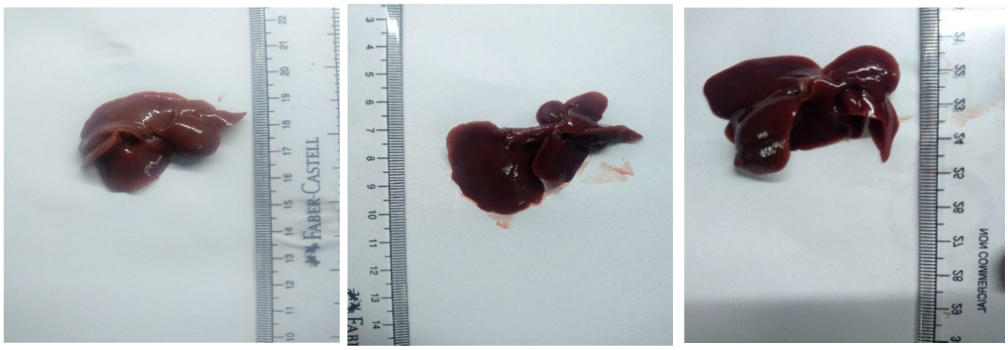


(IV) Cartap (16.25 mg/kg) (V) Cartap (32.5 mg/kg) (VI) Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg)

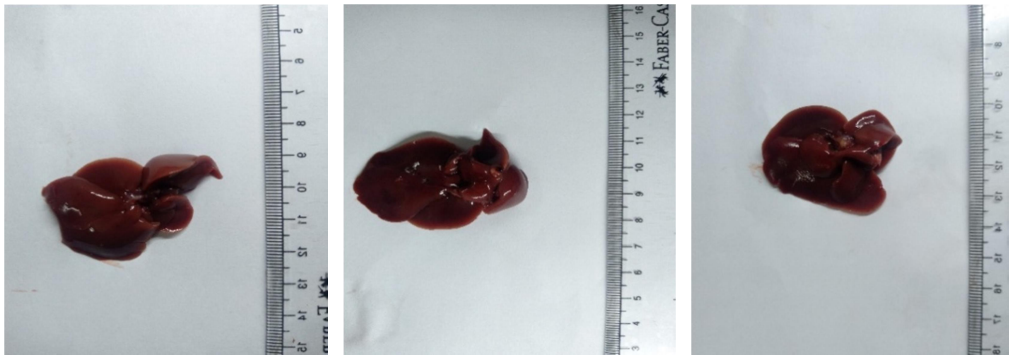


(VI) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg)

Fig. 47: Photographs showing gross changes in lungs of various treatment groups following 28-day exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α- tocopherol (100 mg/kg) alone and in combination.



(I) Control **(II) Vehicle Control** **(III) α- tocopherol (100 mg/kg)**



(IV) Cartap (16.25 mg/kg) **(V) Cartap (32.5 mg/kg)** **(VI) Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg)**



(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg)

Fig. 48: Photographs showing gross changes in liver of various treatment groups following 28-day exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α-tocopherol (100 mg/kg) alone and in combination.



(I) Control



(II) Vehicle Control



(III) α- tocopherol (100 mg/kg)



(IV) Cartap (16.25 mg/kg



(V) Cartap (32.5 mg/kg)



(VI) Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg)



(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg)

Fig. 49: Photographs showing gross changes in kidneys of various treatment groups following 28-day exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α- tocopherol (100 mg/kg) alone and in combination.



(I) Control



(II) Vehicle Control



(III) α- tocopherol (100 mg/kg)



(IV) Cartap (16.25 mg/kg)



(V) Cartap (32.5 mg/kg)



(VI) Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg)



(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg)

Fig. 50: Photographs showing gross changes in spleen of various treatment groups following 28-day exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α- tocopherol (100 mg/kg) alone and in combination.



(I) Control



(II) Vehicle Control



(III) α- tocopherol (100 mg/kg)



(IV) Cartap (16.25 mg/kg



(V) Cartap (32.5 mg/kg)

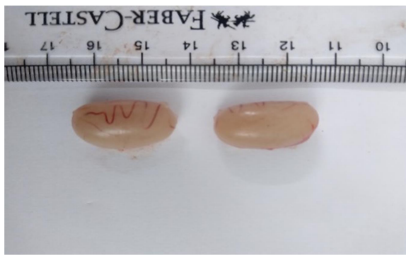


(VI) Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg)

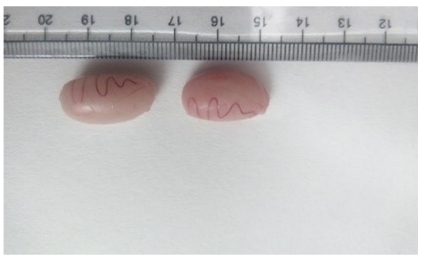


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg)

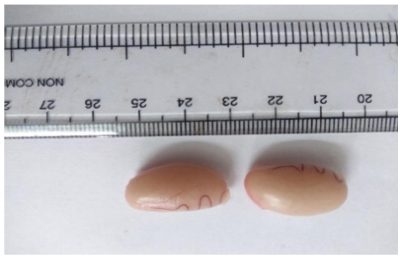
Fig. 51: Photographs showing gross changes in heart of various treatment groups following 28-day exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α-tocopherol (100 mg/kg) alone and in combination.



(I) Control



(II) Vehicle Control



(III) α- tocopherol (100 mg/kg)



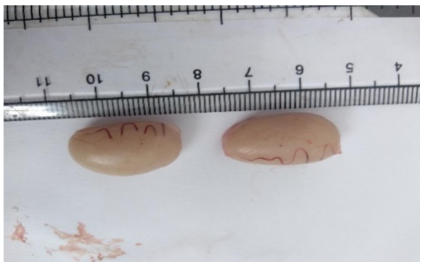
(IV) Cartap (16.25 mg/kg)



(V) Cartap (32.5 mg/kg)

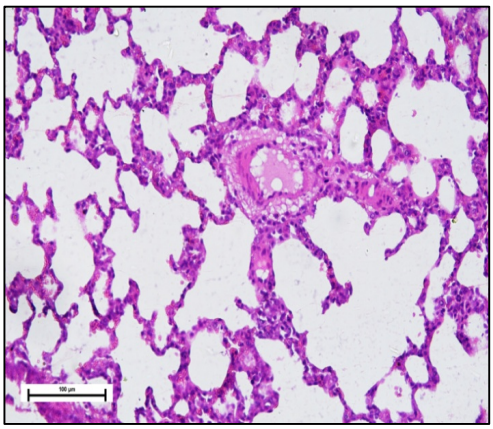


(VI) Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg)

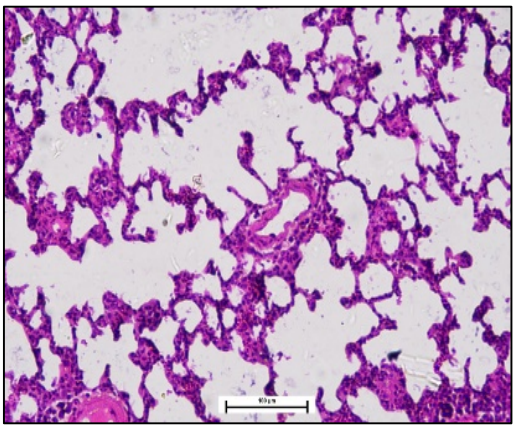


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg)

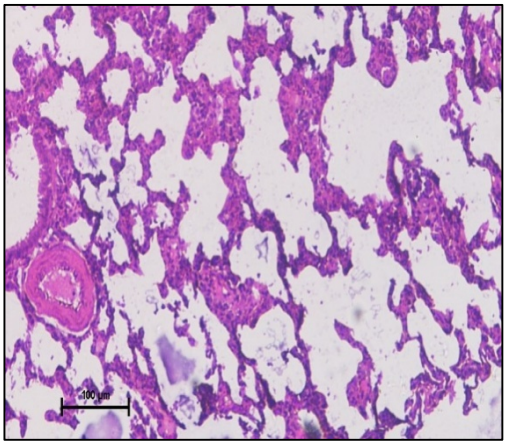
Fig. 52: Photographs showing gross changes in testes of various treatment groups following 28-day exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α-tocopherol (100 mg/kg) alone and in combination.



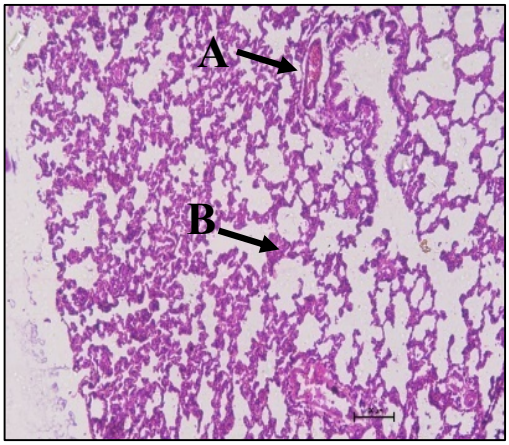
(I) Control: Lung tissue section showing normal histoarchitecture of alveoli and alveolar walls.



(II) Vehicle Control: Lung tissue section showing normal histoarchitecture of alveoli and alveolar walls.

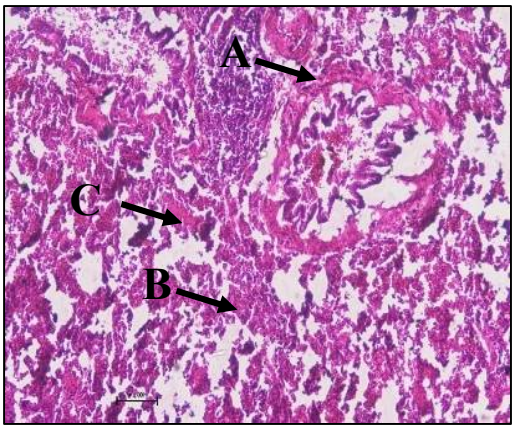


(III) α-tocopherol (100 mg/kg): Lung tissue section showing normal histoarchitecture of alveoli and alveolar walls.

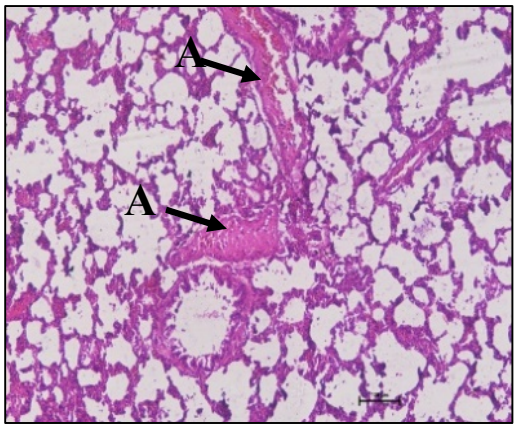


(IV) Cartap (16.25 mg/kg): Lung tissue section showing: A. Mild congestion of alveolar capillaries: B. Thickening of alveolar wall.

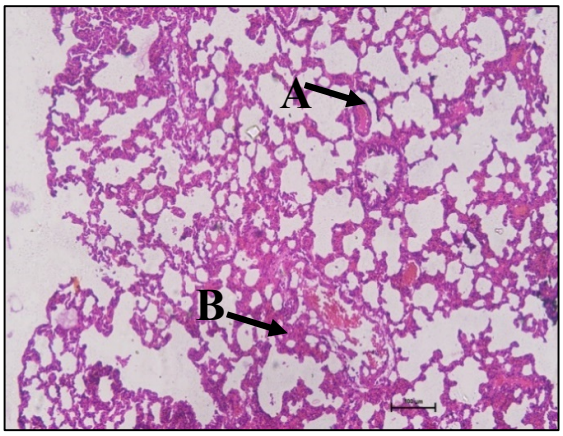
Fig. 53: Representative microphotographs of lung tissue sections of rats from group I, II, III and IV (200X, H&E).



(V) Cartap (32.5 mg/kg): Lung tissue section showing:
A. Thickening of bronchiolar wall.
B.Thickening of alveolar wall.
C. Moderate congestion.

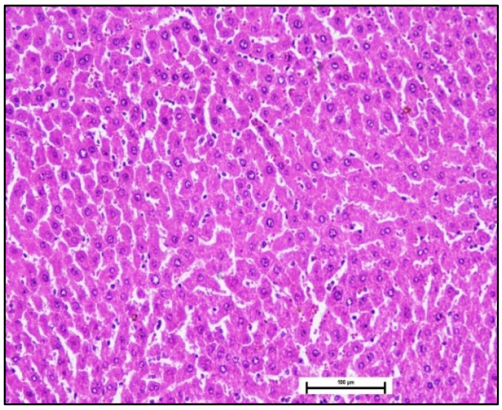


(VI) Cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg): Lung tissue section showing:
A. Mild congestion of alveolar capillaries.

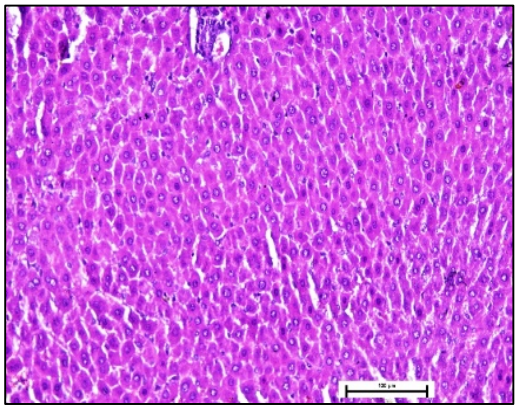


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg): Lungs tissue section showing:
A. Mild congestion
B Thickening of alveolar walls

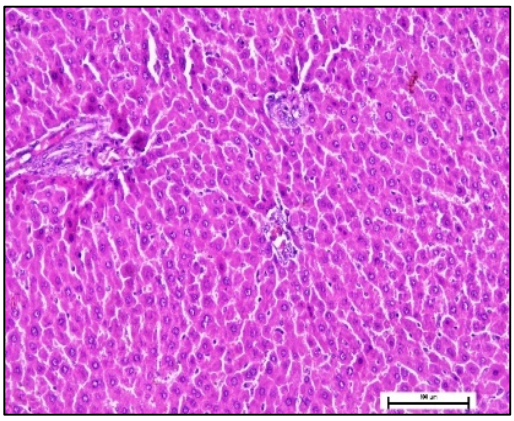
Fig. 54: Representative microphotographs of lung tissue sections of rats from group V, VI and VII (200X, H&E).



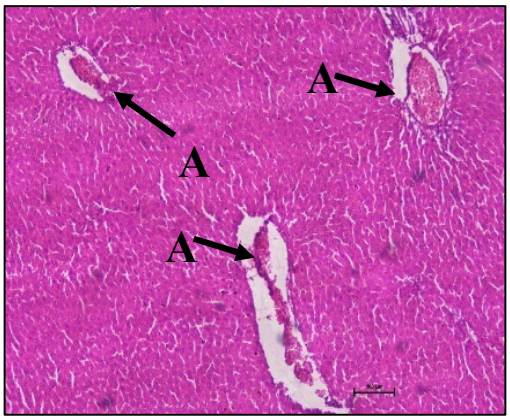
(I) Control: Liver tissue section showing normal histoarchitecture of hepatocytes and sinusoids.



(II) Vehicle Control: Liver tissue section showing normal histoarchitecture of hepatocytes and sinusoids.

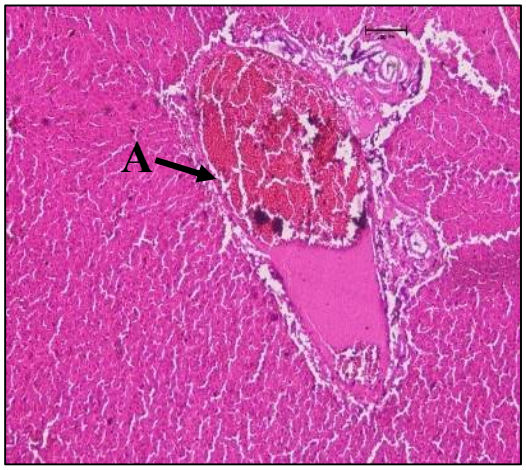


(III) α- tocopherol (100 mg/kg): Liver tissue section showing normal histoarchitecture of hepatocytes and



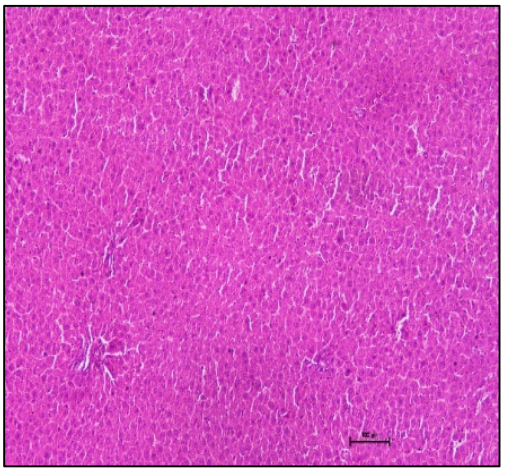
(IV) Cartap (16.25 mg/kg): Liver tissue section showing:
A. Mild Congestion in central vein of liver.

Fig. 55: Representative microphotographs of liver tissue sections of rats from group I, II, III and IV (200X, H&E).

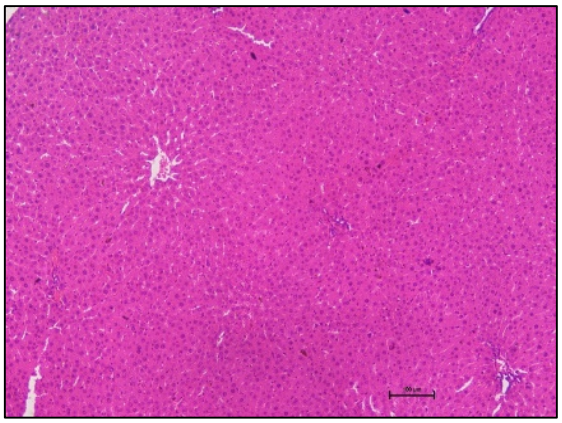


(V) Cartap (32.5 mg/kg): Liver tissue section showing:

A. Moderate congestion in central vein of liver.

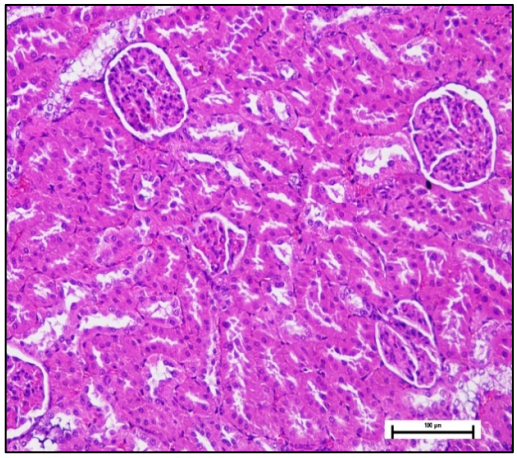


(VI) Cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg): Liver tissue section showing normal histoarchitecture of hepatocytes and sinusoids.

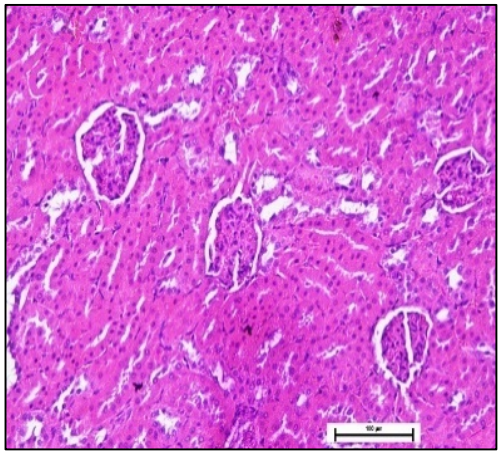


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg): Liver tissue section showing normal histoarchitecture of hepatocytes and sinusoids.

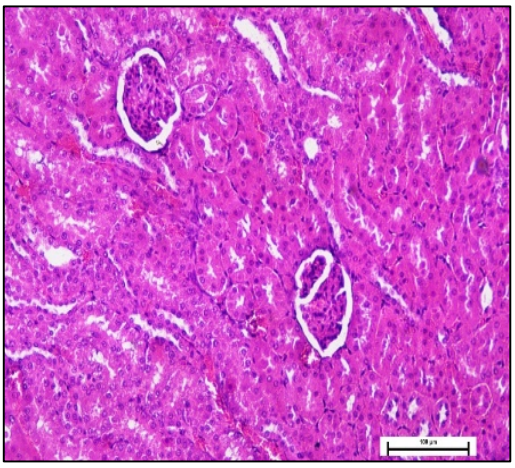
Fig. 56: Representative microphotographs of liver tissue sections of rats from group V, VI and VII (200X, H&E).



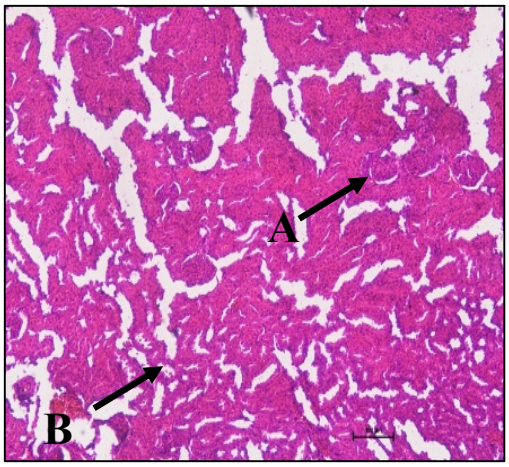
(I) Control**:** Kidney tissue section showing normal histoarchitecture of glomeruli and tubules.



(II) Vehicle Control: Kidney tissue section showing normal histoarchitecture of glomeruli and tubules.

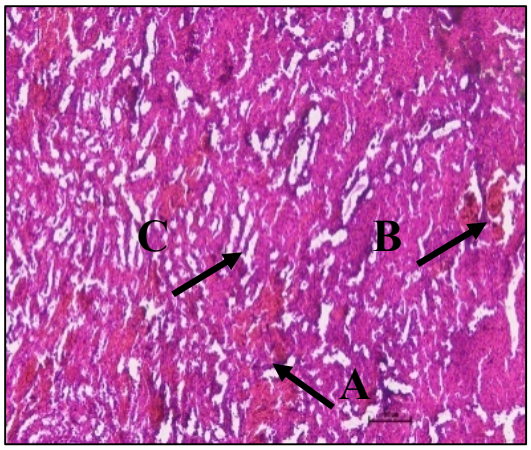


(III) α- tocopherol (100 mg/kg): Kidney tissue sectionshowing normal histoarchitecture of glomeruli and tubules.

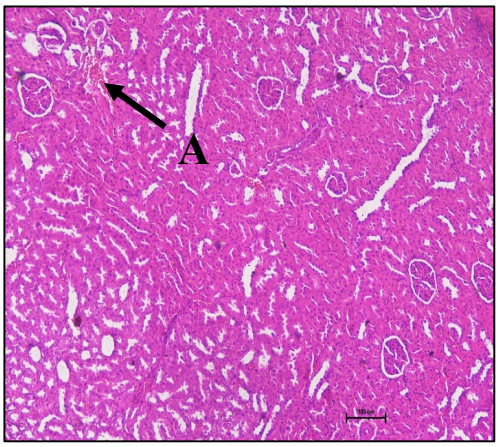


(IV) Cartap (16.25 mg/kg): Kidney tissue section showing
A. Constriction of glomeruli
B. Degeneration of tubules

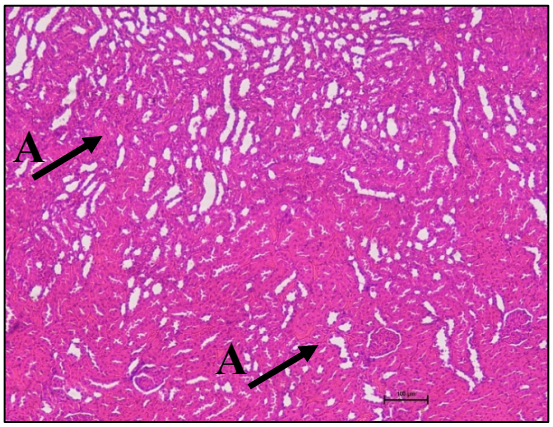
Fig. 57: Representative microphotographs of kidney tissue sections of rats from group I, II , III and IV (200X, H&E).



(V) Cartap (32.5 mg/kg): Kidney tissue section showing:
A. Constriction of glomeruli
B. Moderate congestion
C. Degeneration of tubules

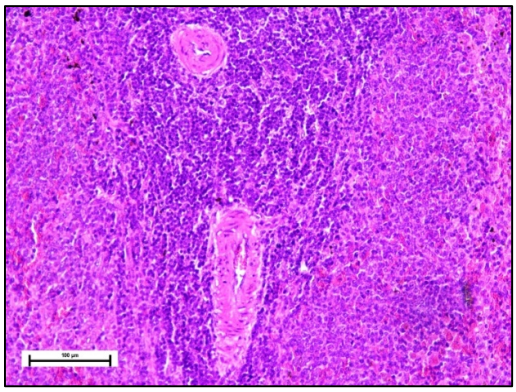


(VI) Cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg): Kidney tissue section showing:
A. Mild congestion of tubular capillaries.

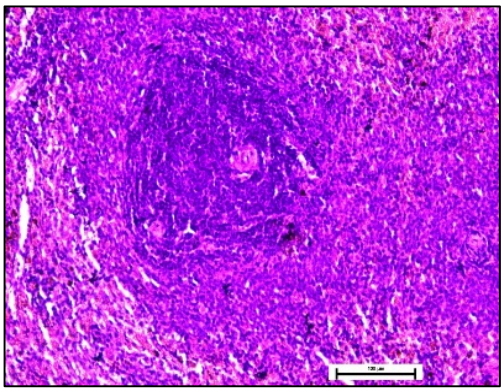


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg): Kidney tissue section showing
A. Mild congestion of tubular capillaries.

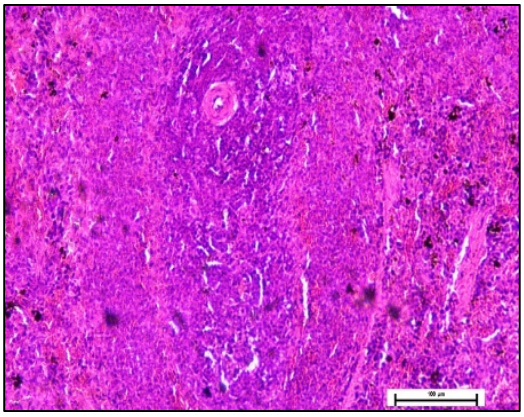
Fig. 58: Representative microphotographs of kidney tissue sections of rats from group V, VI and VII (200X, H&E).



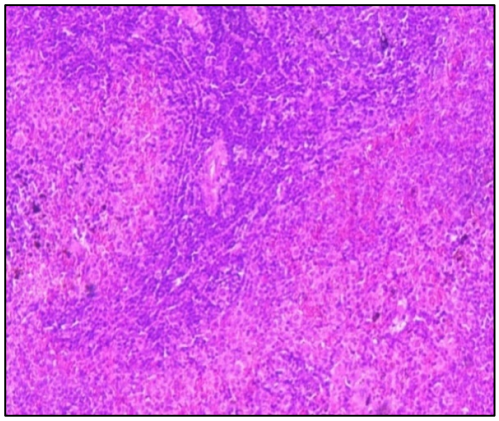
(I) Control**:** Spleen tissue section showing normal histoarchitecture of white pulp and red pulp.



(II) Vehicle Control: Spleen tissue section showing normal histoarchitecture of white pulp and red pulp.

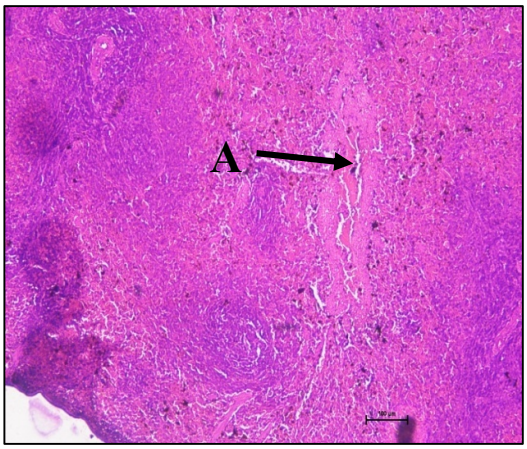


(III) α- tocopherol (100 mg/kg): Spleen tissue section showing normal histoarchitecture of white pulp and red pulp.

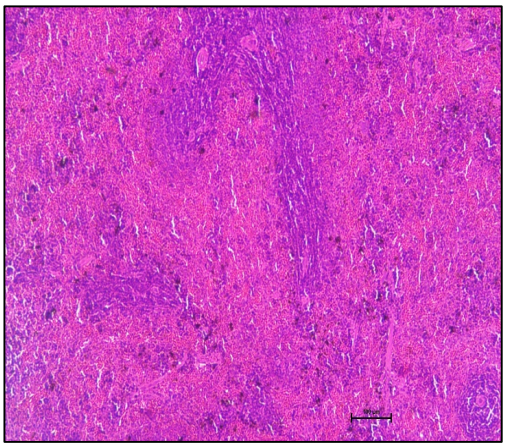


(IV) Cartap (16.25 mg/kg): Spleen tissue section showing normal histoarchitecture of white pulp and red pulp.

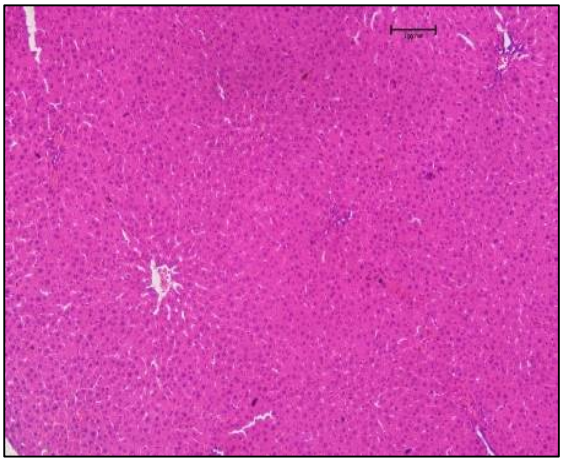
Fig. 59: Representative microphotographs of spleen tissue sections of rats from group I, II, III and IV (200X, H&E).



(V) Cartap (32.5 mg/kg): Spleen tissue section showing:
A. Mild congestion in red pulp.

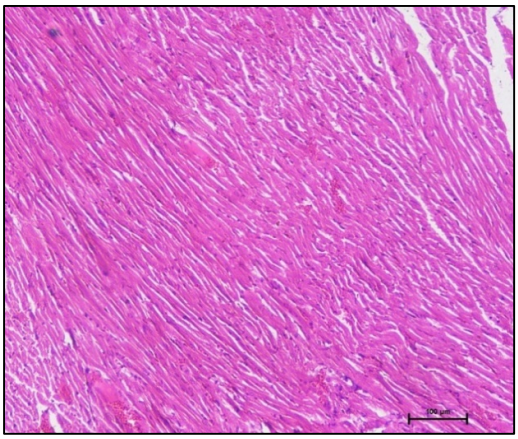


(VI) Cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg): Spleen tissue section showing normal histoarchitecture of white pulp and red pulp.

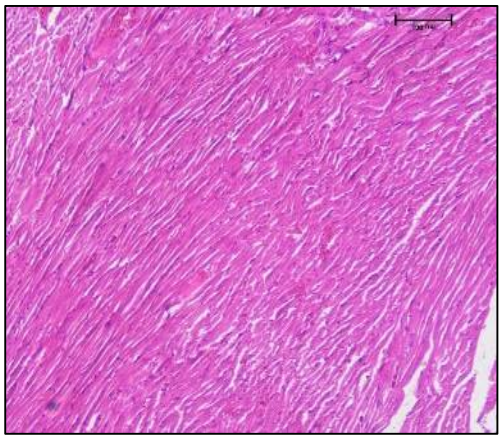


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg): Spleen tissue section showing normal histoarchitecture of white pulp and red pulp.

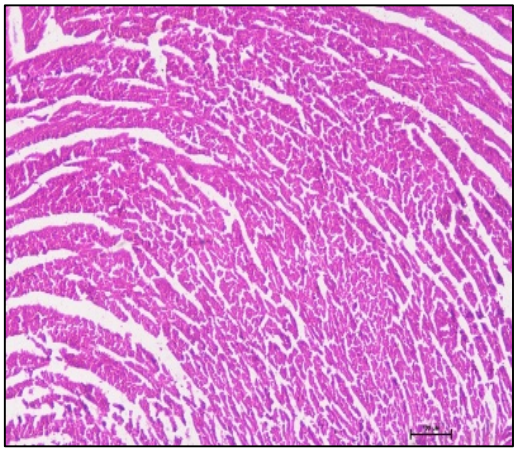
Fig. 60: Representative microphotographs of Spleen tissue sections of rats from group V, VI and VII (200X, H&E).



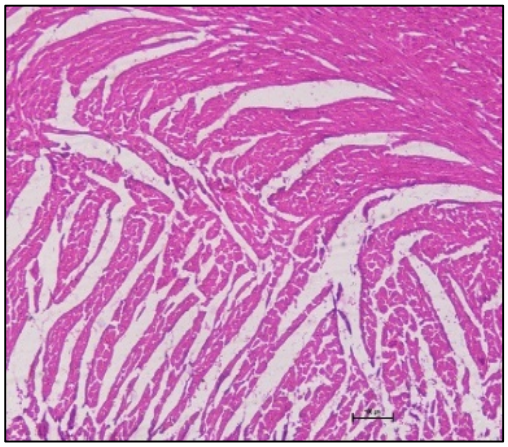
(I) Control**:** Heart tissue section showing normal arrangements of myocardial fibres.



(II) Vehicle Control: Heart tissue section showing normal arrangements of myocardial fibres.

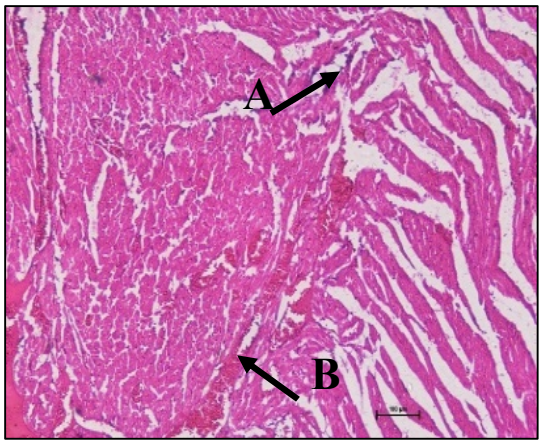


(III) α- tocopherol (100 mg/kg): Heart tissue section showing normal arrangements of myocardial fibres.

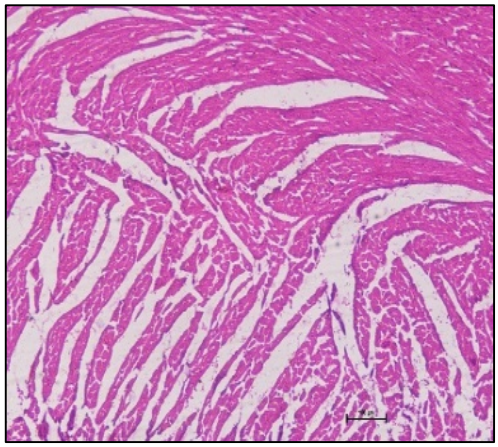


(IV) Cartap (16.25 mg/kg): Heart tissue section showing normal arrangements of myocardial fibres.

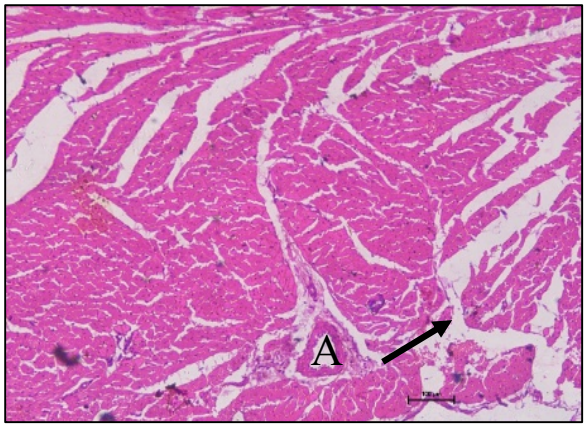
Fig. 61: Representative microphotographs of Heart tissue sections of rats from group I, II, III and IV (100X, H&E).



(V) Cartap (32.5 mg/kg): Heart tissue section showing:
A. Mild degeneration of myocardial fibres
B. Hemorrhage

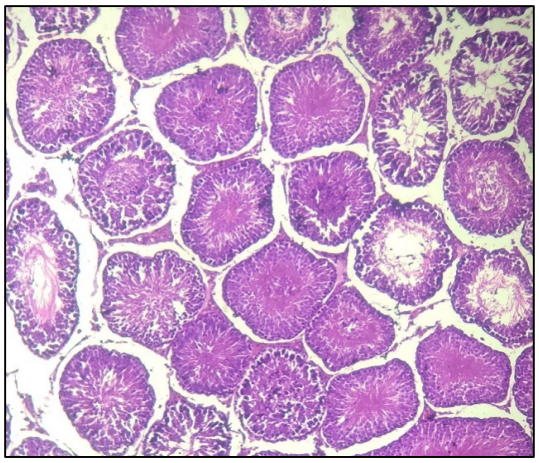


(VI) Cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg): Heart tissue section showing normal arrangements of myocardial fibres.

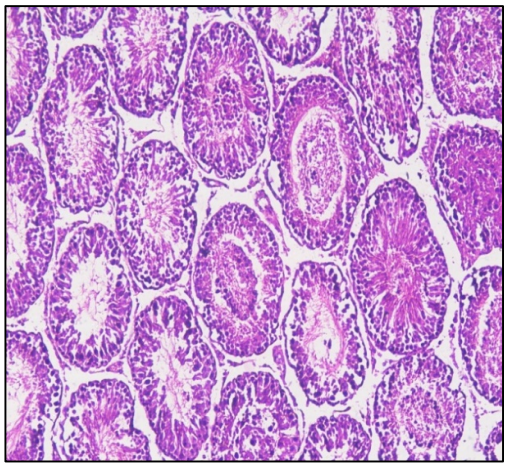


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg): Heart tissue section showing:
A. Mild disruption of myocardial fibres.

Fig. 62: Representative microphotographs of Heart tissue sections of rats from group V, VI and VII (100X, H&E).



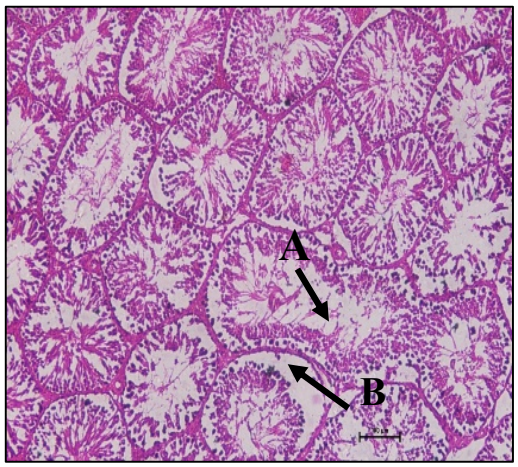
(I) Control**:** Testis tissue section showing normal histoarchitecture of seminiferous tubules.



(II) Vehicle Control: Testis tissue section showing normal histoarchitecture of seminiferous tubules.

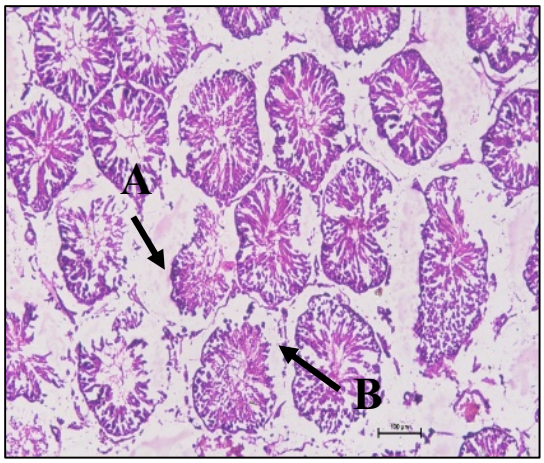


(IV) α- tocopherol (100 mg/kg): Testis tissue section showing normal histoarchitecture of seminiferous tubules.

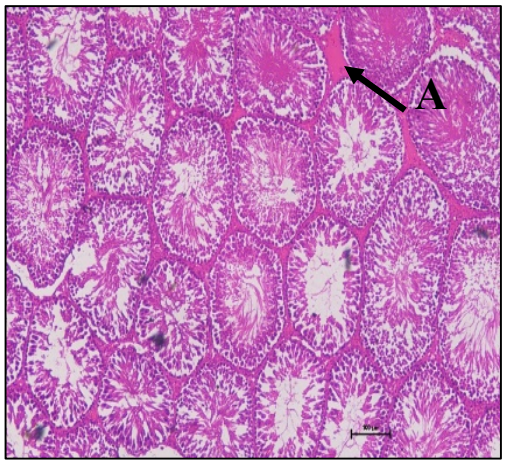


(V) Cartap (16.25 mg/kg): Testis tissue section showing:
A. Mild degeneration of seminiferous tubules
B. Detachment of germ cells.

Fig. 63: Representative microphotographs of testes tissue sections of rats from group I, II, III and IV (100X, H&E).



(V) Cartap (32.5 mg/kg): Testis tissue section showing:
A. Moderate degeneration of seminiferous tubules
B. Detachment of germ cells.

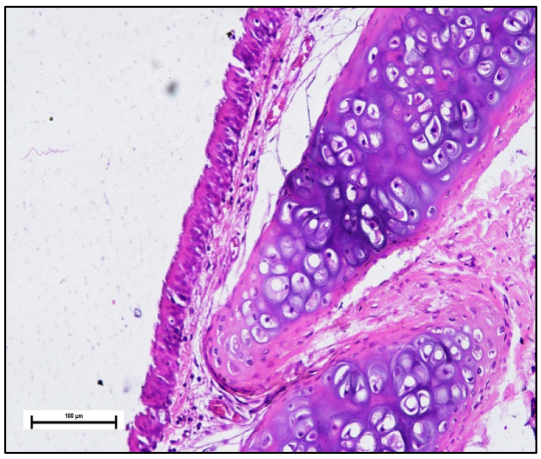


(VII) Cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg): Testis tissue section showing:
A. Mild congestion of seminiferous tubular capillaries.

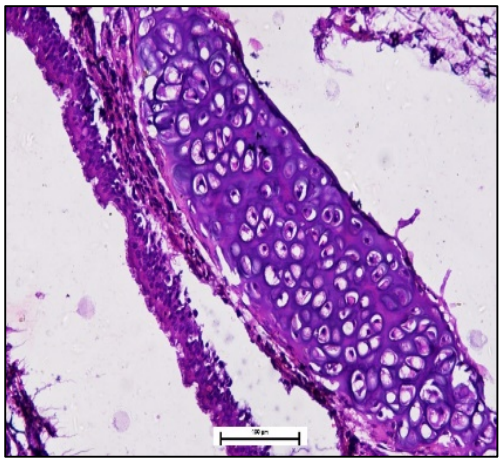


(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg): Testis tissue section showing:
A. Mild congestion of seminiferous tubular capillaries.

Fig. 64: Representative microphotographs of testes tissue sections of rats from group V, VI and VII (100X, H&E).



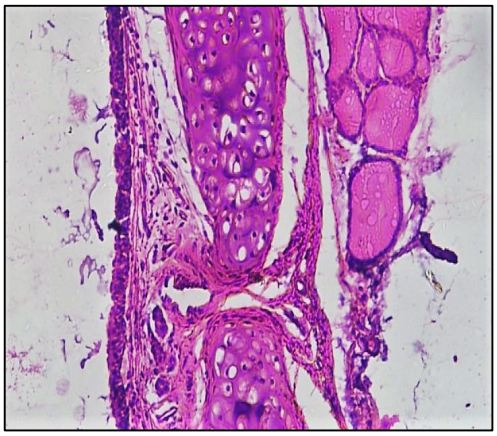
(I) Control: Tracheal tissue section showing normal histoarchitecture of cartilage and ciliated epithelium.



(II) Vehicle Control: Tracheal tissue section showing normal histoarchitecture of cartilage and ciliatedepithelium.

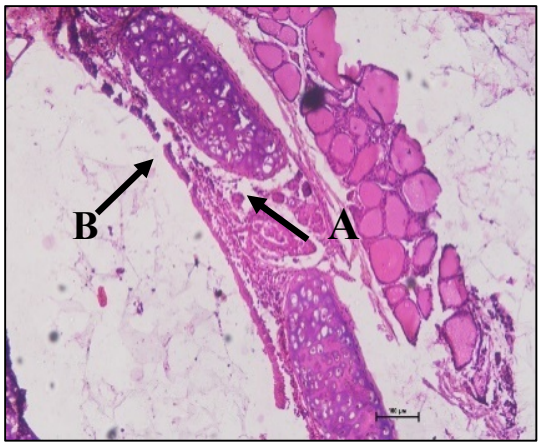


(III) α- tocopherol (100 mg/kg): Tracheal tissue section showing normal histoarchitecture of cartilage and ciliated

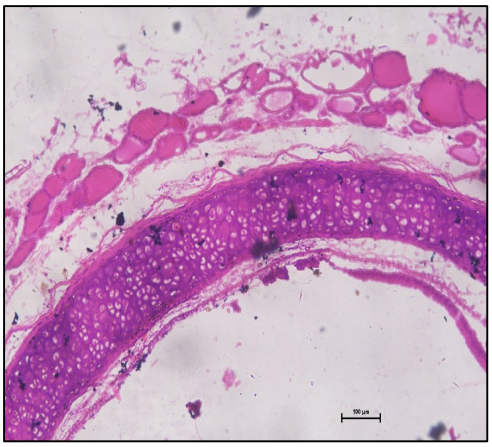


(IV) Cartap (16.25 mg/kg): Tracheal tissue section showing normal histoarchitecture of cartilage and ciliated epithelium.

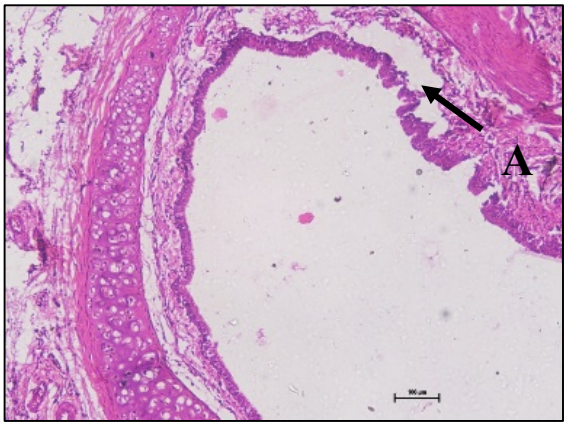
Fig. 65: Representative microphotographs of tracheal tissue sections of rats from group I, II, III and IV (200X, H&E).



(V) Cartap (32.5 mg/kg): Tracheal tissue section showing:
A. Mild degeneration in cartilage
B. Mild degeneration of ciliated epithelium.



(VII) Cartap (16.25 mg/kg) + α-tocopherol (100 mg/kg): Tracheal tissue section showing normal histoarchitecture of cartilage and ciliated epithelium.



(VII) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg): Tracheal tissue section showing:
A. Mild degeneration of cartilage.

Fig. 66: Representative microphotographs of tracheal tissue sections of rats from group V, VI and VII (200X, H&E).

4.7 Effectof α- tocopherol on mRNA expression of HSP-70 in testicular tissue of rats in different treatment groups following 28 days oral exposure to cartap.

(A) RT-PCR amplification of the genes: Figure 69 shows the representative image of agarose gel electrophoresis of RT-PCR products of HSP-70 (285 bp) and GAPDH (101 bp) genes in the testicular tissue of rats from different experimental groups.

(B) Effect on mRNA expression of HSP-70: To further assess the degree of testicular injury following exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and the effect of α-tocopherol (100 mg/kg) alone and in combination, the mRNA expression profile of HSP-70, one of the bioindicators of chemical toxicity in cells was evaluated. Real time PCR amplification plot and dissociation curves of HSP-70 gene are shown in Fig.68 (A). Representative real time PCR amplification plot and melt curve for GAPDH are shown in Fig 68 (B). As illustrated in Fig. 67 and summarized in Table 11, the fold change in mRNA expression of testicular HSP-70 was significantly ($p<0.05$) increased to 8.17±1.55 and 27.70±4.79 in cartap alone treatment groups IV and V respectively compared to control group (1.05±0.24). No significant ($p>0.05$) change in the fold change in mRNA expression of testicular HSP-70 was observed in α-tocopherol and cartap co-treatment groups VI and VII compared to cartap alone treatment groups IV and V respectively. Compared to control group, the fold change in mRNA expression of testicular HSP-70 was significantly ($p<0.05$) increased in both co -treatment groups VI (6.46±0.58) and VII (26.7±2.07).

Table 11: Effect of oral administration of α- tocopherol (100 mg/kg) on mRNA expression of HSP-70 in testicular tissue of rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg body and 32.5 mg/kg body weight).

Groups	Treatment	HSP 70 (Fold Change)
I	Control	1.05±0.24 [a]
II	Vehicle Control (Corn oil)	1.18±0.14 [a]
III	α-tocopherol (100 mg/kg)	0.97±0.17 [a]
IV	Cartap hydrochloride (16.25 mg/kg)	8.17±1.55 [b]
V	Cartap hydrochloride (32.5 mg/kg)	27.70±4.79 [c]
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	6.46±0.58 [ab]
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	26.7±2.07 [c]
P-value		0.00

Values (Mean ±SEM; n=3) bearing different superscripts in the same column differed significantly (P<0.05) using one way ANOVA following Tukey's-b multiple comparison *post-Hoc* test.

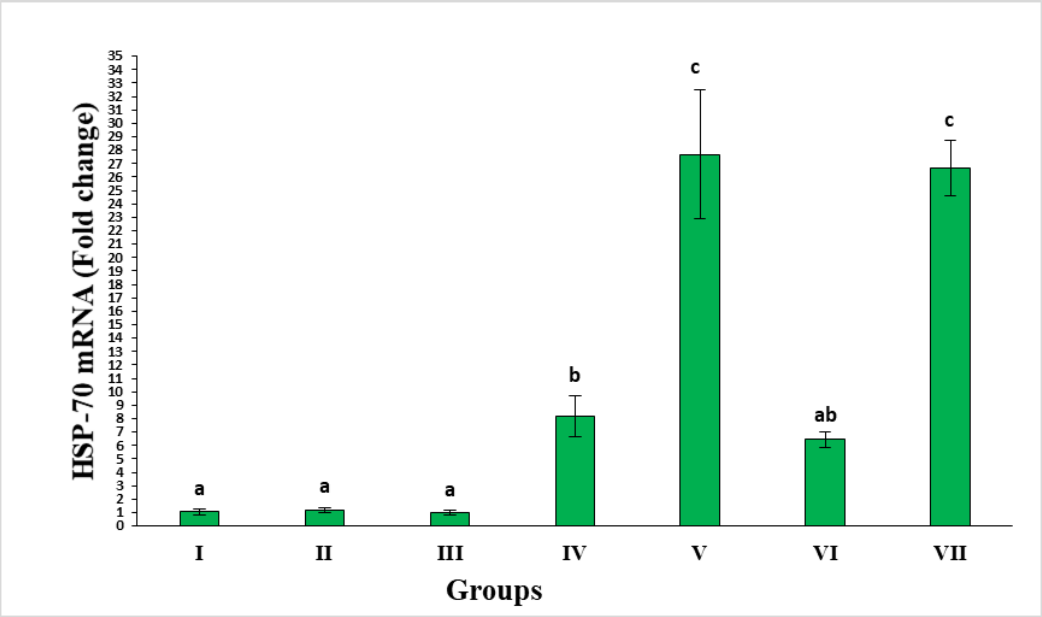
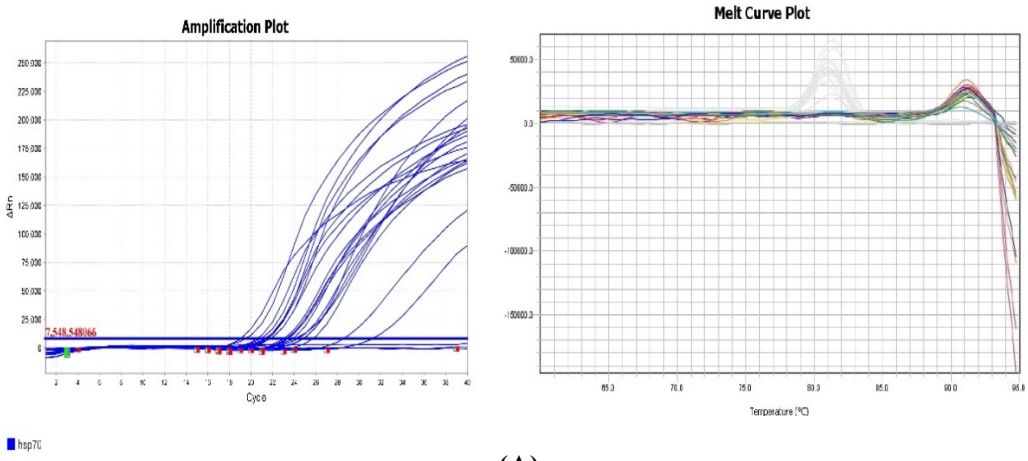
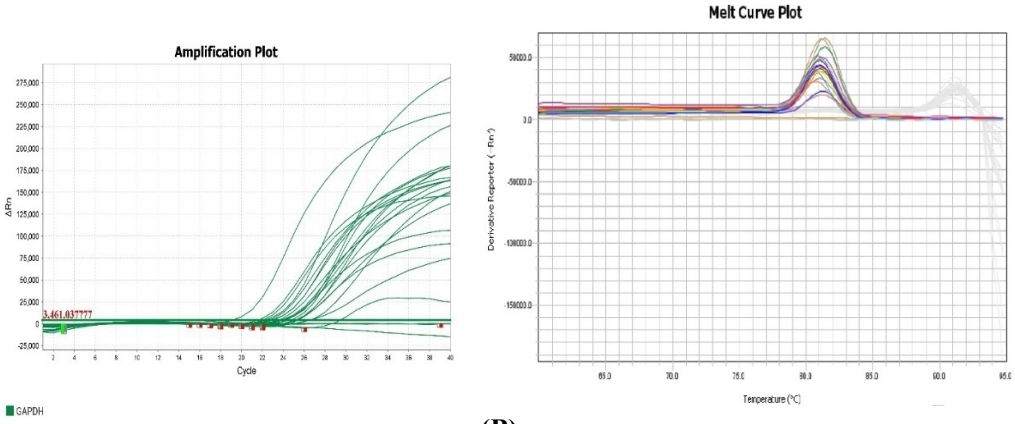


Fig. 67: Effect of oral administration of α- tocopherol (100 mg/kg) on mRNA expression of HSP-70 in testicular tissue of rats in different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg).
Different superscripts on bars indicate significant (P<0.05) differences.



(A)



(B)

Fig. 68: RT-qPCR analysis of HSP-70 and GAPDH gene in rat testes. (A) Amplification plot and Melt curve of HSP-70 and (B) amplification plot and Melt curve of GAPDH gene.

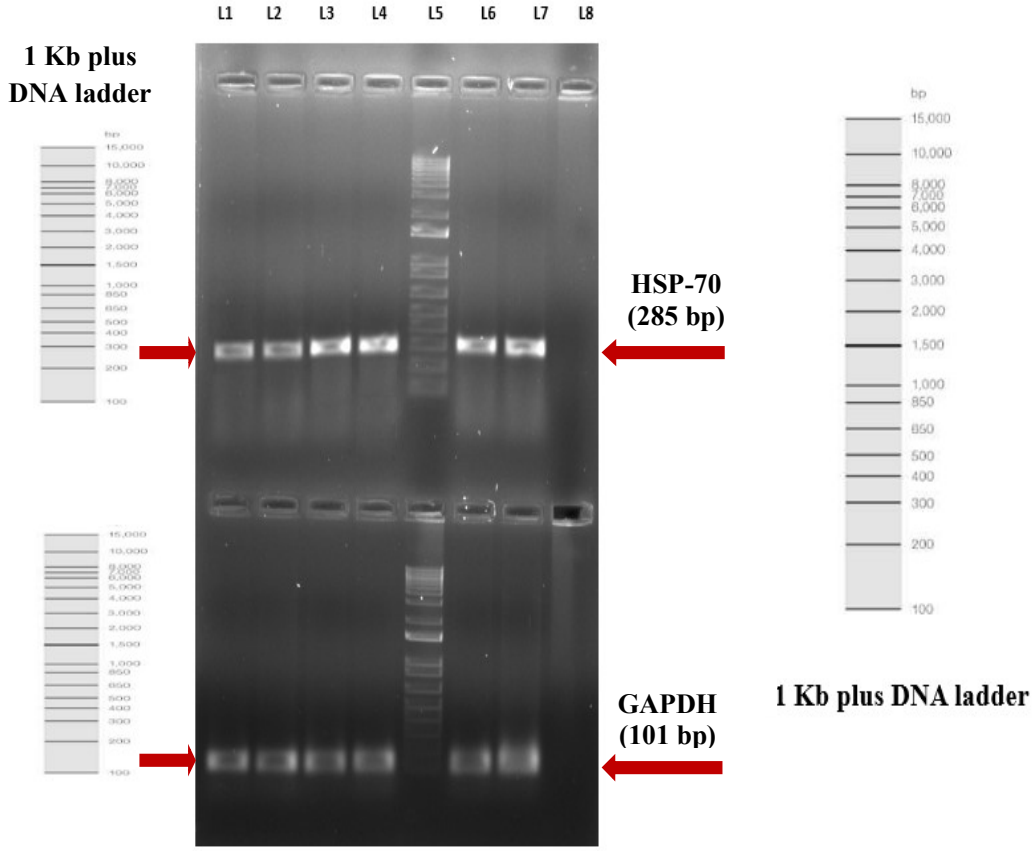


Fig. 69: Representative agarose gel image showing the single amplified RT-PCR amplicon for HSP-70 (285 bp) and GAPDH (101 bp). L1= Control; L2= α-tocopherol (100mg/kg); L3= Cartap (16.25 mg/kg); L4= Cartap (32.5 mg/kg); L5= 1 Kb plus DNA ladder; L6= Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg); L7= Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg); L8= NTC (Non-template control).

4.8 Effect of α-tocopherol on alterations in certain physical attributes of semen induced by cartap

Following 28 days exposure to cartap (16.25 mg/kg and 32.5 mg/kg) andα-tocopherol (100 mg/kg) alone and in combination the effect on certain physical attributes of semen is presented in Table 12 and illustrated in Fig 70-73. Data presented in Table 12 showed that sperm concentration in cartap alone group IV (34.5±1.87) did not change significantly ($p>0.05$) change compared to control group (49.5±5.57). However, a significant ($p<0.05$) decrease in sperm concentration was observed in cartap alone group V (25±2.67) compared to control group (49.5±5.57). Co-treated cartap groups (VI and VII) showed non-significant ($p>0.05$) change in sperm concentration compared to cartap alone treatment groups (IV and V). Non-significant ($p>0.05$) change in sperm concentration was observed in cartap co-treated group VI (35±3.28) compared to control group (49.5±5.57). However, a significant ($p<0.05$) decrease in sperm concentration was observed in co-treated group VII (28.67±2.85) compared to control group (49.5±5.57).

Percentage of live sperm cells data indicate significant ($p>0.05$) decrease in live spermatozoa in cartap alone treatment groups IV (33.35±4.30) andV (29.92±5.91) compared to control group (55.92±5.89). Co-treated cartap groups VI and VII showed non-significant ($p>0.05$) change in percentage of live sperms compared to cartap alone treatment groups IV and V. Compared to control group (55.92±5.89), non-significant ($p>0.05$) change was observed in percentage of live spermatozoa in co-treated group VI (38.08±2.41), but it was significantly ($p<0.05$) decreased in co-treated group VII (36.44±3.56).

Data presented in Table 12 and illustrated in Fig 72 showed that a significant ($p<0.05$) decrease in per cent hypo osmotic swelling positive (HOS+ve) sperms was observed in rats of cartap alone treatment groups IV (16.25±1.83) and V (14.08±1.75) compared to control group (28.48±1.79). A non-significant ($p>0.05$) increase in percentage HOS+ve sperms was observed in co-treated group VI (20.87±1.26) compared to cartap alone group IV (16.25±1.83). However, a significant ($p<0.05$) increase in per cent HOS+ve sperms was observed in co-treated group VII (20.19±1.70) compared to cartap alone group V (14.08±1.75). Further, per cent HOS+ve spermatozoa were significantly ($p<0.05$) decreased in co-treated groups VI (20.87±1.26) and VII (20.19±1.70) compared to control group (28.48±1.79).

Data present in Table 12 and shown in Fig 73 summarize the effect of cartap on acrosomal integrity of rat spermatozoa following exposure to cartap and α-tocopherol. Perusal of data revealed that compared to control (77.75±3.63 %) there was no significant change in % intact acrosome in different treatment groups (II-VII). This result indicates that there was no effect of cartap alone and in combination with α-tocopherol on the acrosomal integrity of rat spermatozoa.

Table 12: Effect of oral administration of α- tocopherol (100 mg/kg) on certain physical attributes of semen following 28-day exposure to cartap (16.25 mg/kg and 32.5 mg/kg).

Groups	Treatment	Concentration (millions/ml)	% Live	% HOS +ve	% Spermatozoa showing intact acrosome
I	Control	49.5 ± 5.57^{b}	55.92 ± 5.89^{b}	28.48 ± 1.79^{c}	77.75 ± 3.63^{a}
II	Vehicle Control (Corn oil)	49.67 ± 8.42^{b}	53.63 ± 6.42^{b}	27.15 ± 1.26^{c}	76.00 ± 2.48^{a}
III	α-tocopherol (100 mg/kg)	48.67 ± 4.80^{b}	54.48 ± 3.67^{b}	28.68 ± 0.26^{c}	77.00 ± 2.27^{a}
IV	Cartap hydrochloride (16.25 mg/kg)	34.5 ± 1.87^{ab}	33.35 ± 4.30^{a}	16.25 ± 1.83^{ab}	73.25 ± 2.86^{a}
V	Cartap hydrochloride (32.5 mg/kg)	25 ± 2.67^{a}	29.92 ± 5.91^{a}	14.08 ± 1.75^{a}	70.75 ± 1.43^{a}
VI	Cartap hydrochloride (16.25 mg/kg) + α-tocopherol (100 mg/kg)	35 ± 3.28^{ab}	38.08 ± 2.41^{ab}	20.87 ± 1.26^{b}	72.00 ± 1.22^{a}
VII	Cartap hydrochloride (32.5 mg/kg) + α-tocopherol (100 mg/kg)	28.67 ± 2.85^{a}	36.44 ± 3.56^{a}	20.19 ± 1.70^{b}	70.25 ± 1.65^{a}
P-value		0.001	0.00	0.00	0.177

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly ($P < 0.05$) using one way ANOVA following Tukey's-b multiple comparison *post-hoc* test.

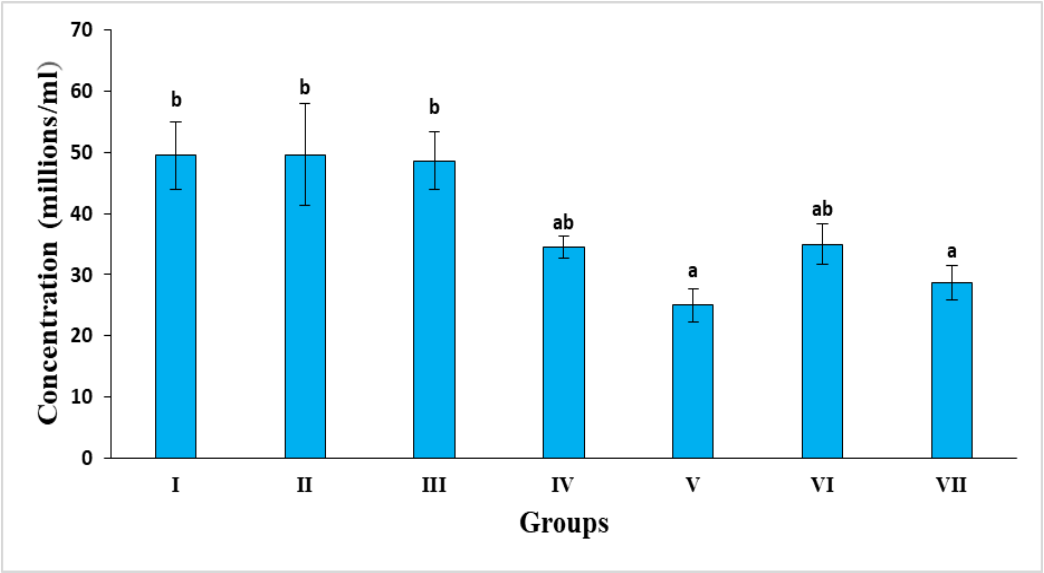


Fig. 70: Effect of oral administration of α- tocopherol (100 mg/kg) on sperm concentration in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

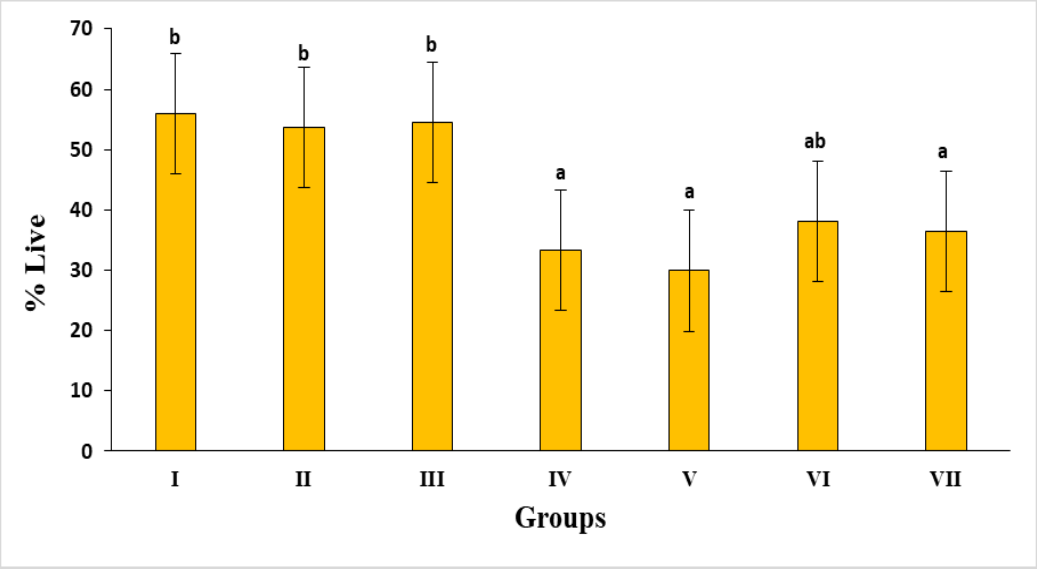


Fig. 71: Effect of oral administration of α- tocopherol (100 mg/kg) on % live spermatozoa in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

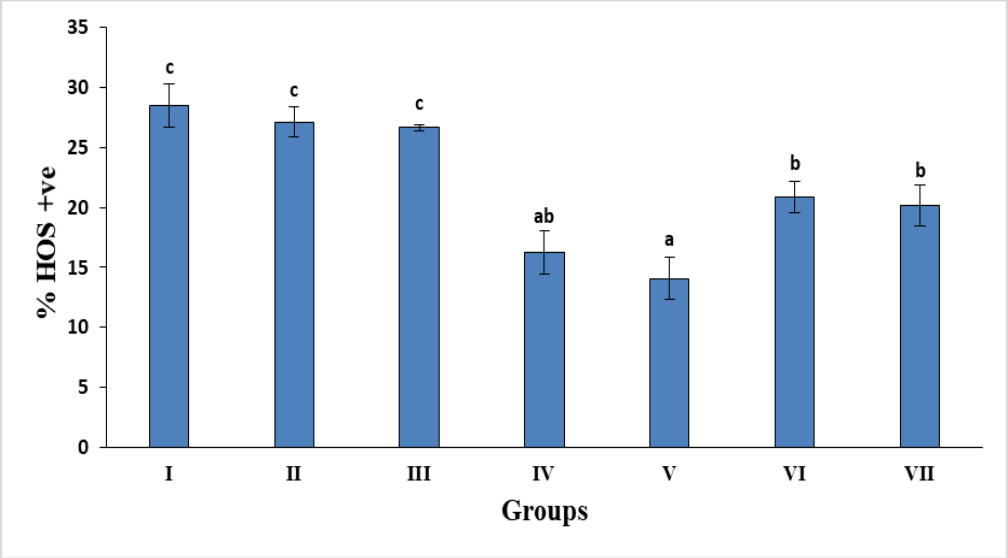


Fig. 72: Effect of oral administration of α- tocopherol (100 mg/kg) on % HOS +ve spermatozoa in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

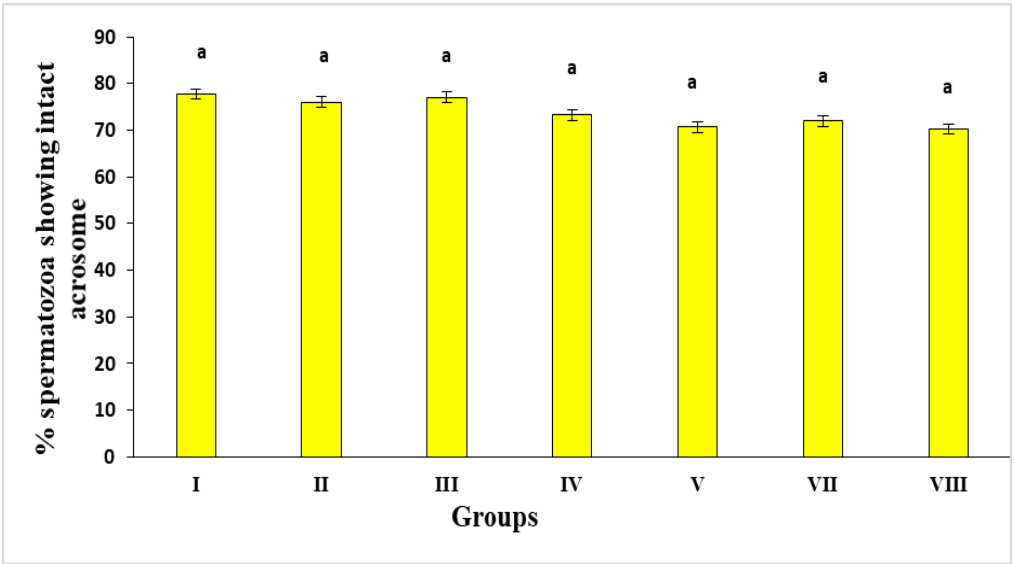


Fig. 73: Effect of oral administration of α- tocopherol (100 mg/kg) on % spermatozoa showing intact acrosome in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg body weight).

Different superscripts on bars indicate significant (P<0.05) differences.

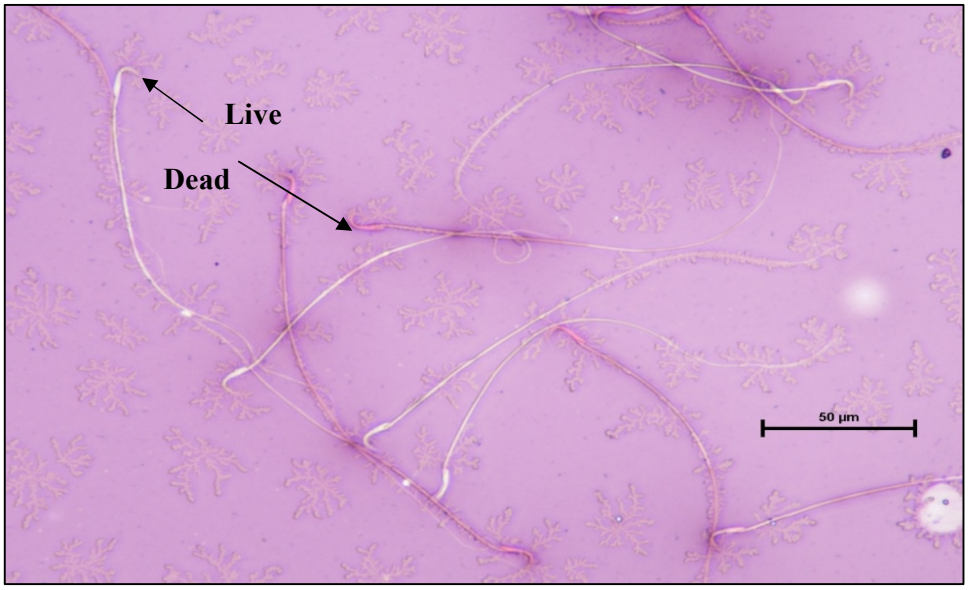


Fig. 74: Representative picture of live and dead spermatozoa (Eosin and Nigrosine stain). Live spermatozoa appear colourless while dead are pink.

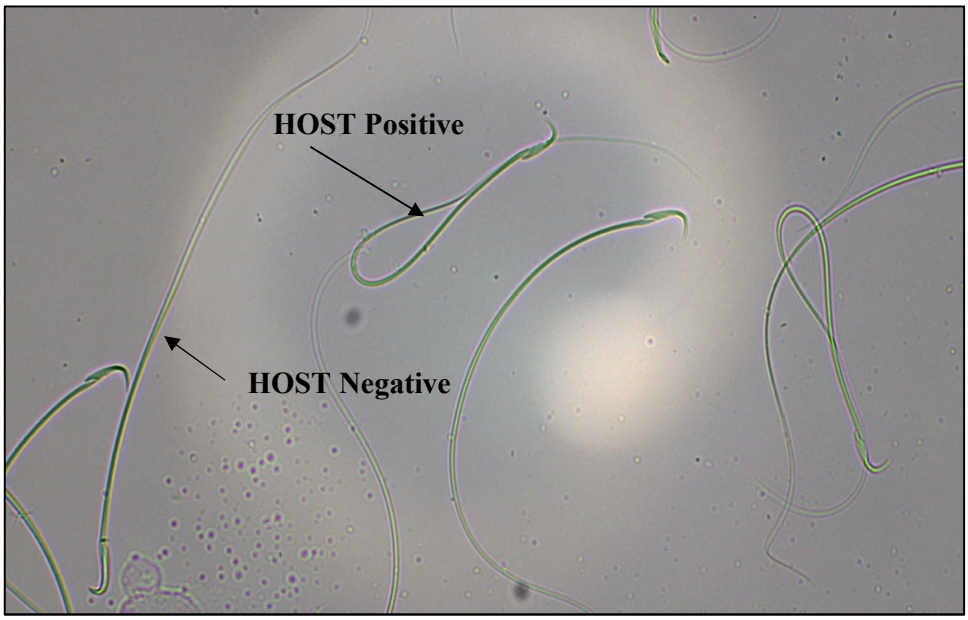
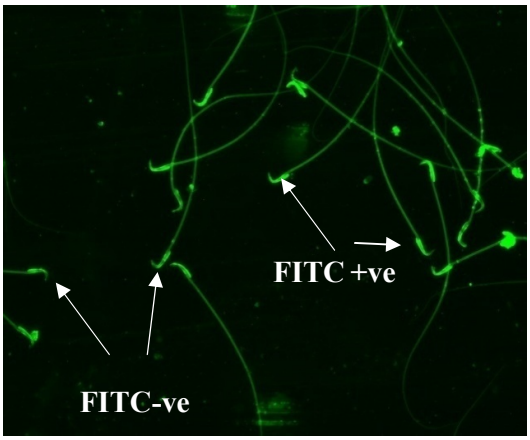
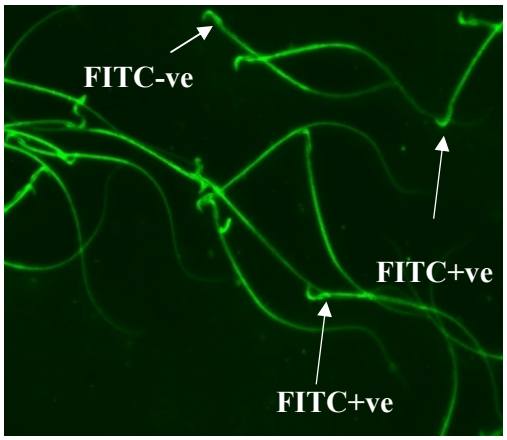


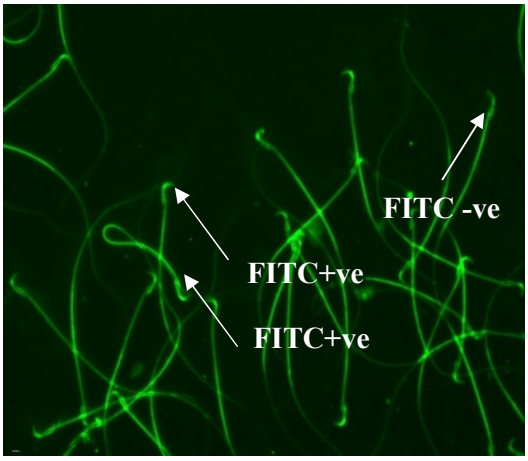
Fig. 75: Rat spermatozoa showing HOS-positive (curled tail) and HOS-negative response (no curled tail).



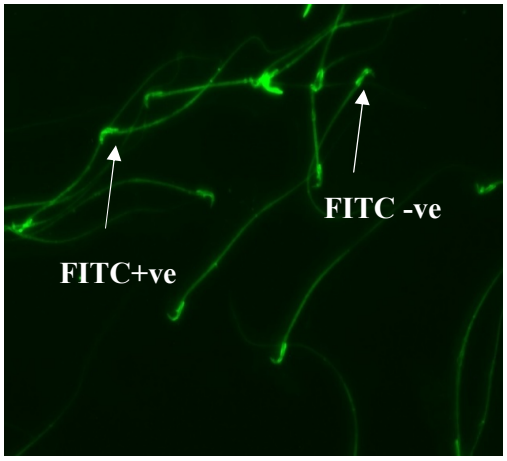
(I) Control



(II) Vehicle Control

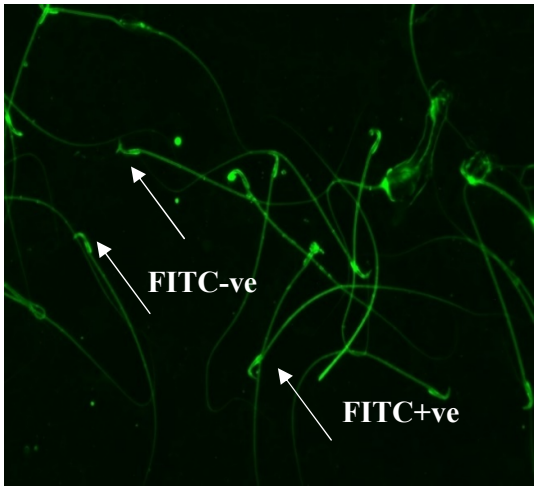


(III) α- tocopherol (100 mg/kg)

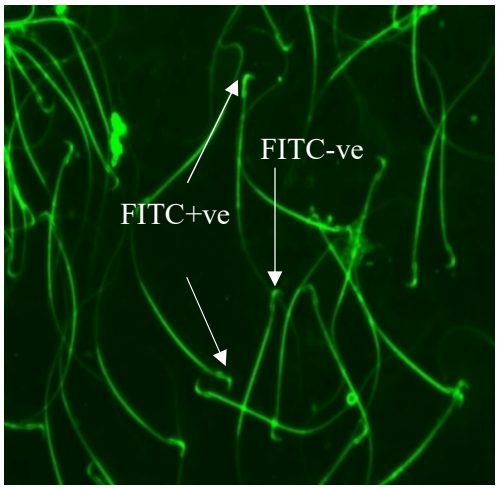


(IV) Cartap (16.25 mg/kg)

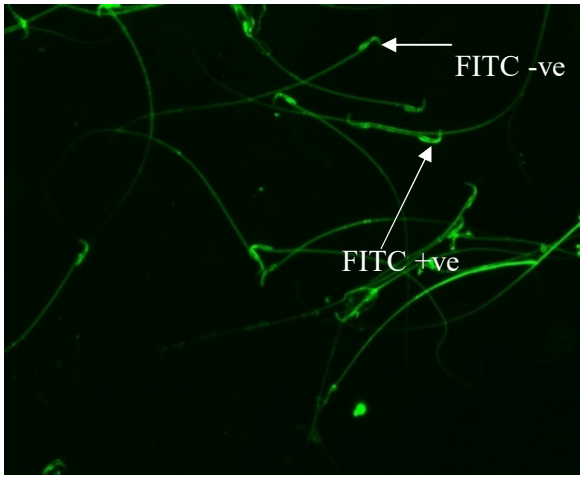
Fig. 76: Representative pictures of FITC-PSAstained rat spermatozoa in different treatment groups. FITC +ve spermatozoa show fluorescence in acrosomal region while FITC -ve do not show or show partial fluorescence.



(V) Cartap (32.5 mg/kg)



(VI) Cartap (16.25 mg/kg) + α- tocopherol (100 mg/kg)



(VI) Cartap (32.5 mg/kg) + α- tocopherol (100 mg/kg)

Fig. 77: Representative pictures of FITC-PSA stained of rat spermatozoa in different treatment groups. FITC +ve spermatozoa show fluorescence in acrosomal region while FITC -ve do not show or show partial fluorescence.



Discussion

CHAPTER-5

DISCUSSION

The present work was undertaken to understand the toxicity of cartap (16.25 mg/kg and 32.5 mg/kg) following sub-acute (28 days) exposure and ameliorative potential of α- tocopherol (100 mg/kg) and its effects on general body parameters, hematology, Biochemical parameters, oxidative stress biomarkers (Liver and testes), testicular injury biomarkers (ACP, SDH, GGT, and LDH), testosterone levels in plasma, sperm parameters (Live and Dead count, Membrane integrity and Acrosomal intactness), histopathology and gene expression of HSP-70 gene in testes in adult male wistar rats.

5.1 Effect of oral administration of α- tocopherol (100 mg/kg) on general toxicity parameters in rats of different treatment groups following 28 days oral exposure to cartap (16.25 mg/kg and 32.5 mg/kg)

5.1.1 Clinical signs

Exposure of rats to cartap (16.25 mg/kg and 32.5 mg/kg) for a period of 28 days did not produce any apparent change in behavior of animals or produce any clinical signs of toxicity. Rats of all the treatment groups exhibited normal activities and behavior throughout the period of study. Our observation is in agreement to the findings of Sharma et al., (2021) who in a similar study in male mice following subacute (28 days) exposure to sublethal dosage of cartap observed no overt signs of toxicity. These findings are indicative of lack of clinical signs and symptoms of toxicity following cartap exposure in rats.

5.1.2 Body weight gain

Oral exposure to cartap did not alter percent body weight gain except in cartap alone group V (32.5 mg/kg) which was significantly lower compared to control group. Our observation is in agreement to the findings of Mizutani et al. (1971) who observed retardation in weight gain following sub lethal exposure to cartap in pregnant female rats. In a similar study by Olsen and Busey (1972), pregnant female rats exposed to sublethal dosage of cartap for 9 weeks showed slower weight gain compared to normal healthy female rats. Similar findings were also observed by

Berczy and Cobb (1973) in adult male as well as female rats exposed to sublethal concentration of cartap for 3 weeks, who observed retarded body weight gain in exposed rats. Hunter et al. (1975) reported similar fall in body weight gain in adult male rats following sublethal exposure to cartap. Hassan et al. (2019) and Akinmoladun et al. (2020) observed a significant fall in body weight gain in adult male wistar rats exposed to sublethal dosage of imidacloprid and rotenone respectively. Hassan et al. (2019) suggested that pesticide induced decrease in weight gain might be attributed to the impairment in food assimilation due to tissue damage by pesticide resulting in decreased absorption of nutrients from the gut. These findings indicate negative effect of cartap on body weight gain. Following co-treatment with α-tocopherol and cartap, no significant change in body weight was observed at either of the dose rates in group VI and VII which is in agreement to the findings of Mandil et al. (2016) who in a similar study with pesticide flubendiamide observed no significant improvement in weight gain following concurrent exposure to α-tocopherol. This may indicate the inability of α-tocopherol to ameliorate xenobiotic induced decline in body weight gain.

5.1.3 Relative and absolute organ weight

Oral exposure of rats to cartap did not produce any significant change in relative as well as absolute organ weight of lungs, liver, right kidney, left kidney, spleen, heart, right testes and left testes in cartap alone (IV and V) and co-treated groups (VI and VII) any of the treatment groups compared to control group. Contrary to our observation Sharma et al. (2021) observed significant reduction in liver and kidney weight in male mice exposed to sublethal dosage of cartap for a period of 28 days. Earlier studies showed that pesticides, such as lambda cyhalothrin, imidacloprid, atrazine, primiphos-methyl and simazine reduced weights of spleen and thymus significantly in exposed rats and mice (Morgan and Osman, 2007; Badgujar et al., 2013; Zhang et al., 2011; Kim et al., 2007; Ren et al., 2013). In a similar study by Mandil et al. (2016), no significant change in organ weights was observed in rats exposed to sub lethal concentration of flubendiamide. These observations indicate the species-specific effects of cartap on organ weight in mice as well as a general affinity of pesticides for spleen and thymus in rats. This affinity seems to be missing in cartap toxicity in rats producing little to no variation in different organ weights in exposed rats.

5.2 Hematological parameters

Exposure of rats to cartap (16.25 mg/kg and 32.5 mg/kg) did not produce any significant change in any of hematological parameters in any of the treatment groups compared to control group. Our observation is in agreement to the findings of Rivett et al. (1972) who observed hematological values in normal range following sublethal exposure to cartap in adult male rats. However, in similar studies on exposure of rats to pesticides a dose dependent reduction in lymphocytes, RBC and Hb concentration was observed following exposure to Endosulfan, chlorpyrifos-ethyl, chlorpyrifos-methyl and methomyl (Mossa and Abbassy, 2012). Sankar et al. (2012) also observed lymphocytopenia and neutrophilia in cypermethrin treated rats. These findings indicate that pesticides in general produce cytotoxic effect in various cells of blood resulting in reduction of their respective count and somewhat trigger an immune response resulting in neutrophilia. Cartap on the other hand seems to be lacking in haemocytotoxic as well as immunostimulatory properties in rats as shown by other pesticides in similar studies.

5.3 Blood biochemical parameters

A significant increase in blood glucose level was observed at higher dose rate of cartap (32.5 mg/kg) in group V compared to control group. This is in agreement with the findings of Berczy and Cobb (1973) who observed similar elevation in plasma glucose levels following sublethal exposure to cartap in adult male rats. α-tocopherol alone treatment group had significantly lower plasma glucose level compared to control group, Manning et al., (2004) also observed a significant fall in plasma glucose following 3-month administration of α-tocopherol in over weight individuals. It was suggested by Evans et al. (2003) that oxidative stress is partly responsible for the development of insulin resistance and therefore antioxidants like α-tocopherol and ascorbic acid, which reduce ROS levels, can improve glycemic control and reduce plasma glucose level. Administration of α-tocopherol along with cartap in co- treatment groups (VI and VII) produced moderate but non-significant reduction in plasma glucose levels compared to cartap alone groups IV and V respectively. However, in blood glucose levels there was no significant change in co-treatment groups (IV and V) when compared to control group. Our findings are in agreement to the findings of El-Demerdash et al., (2004) who in a similar study in rats with pesticide Fenvalerate observed elevation in plasma glucose level and its level

was reduced significantly following concurrent exposure to α-tocopherol, and suggested that the increase in blood glucose by pesticide treatment may indicate disrupted carbohydrate metabolism due to enhanced breakdown of liver glycogen possibly mediated by increase in adrenocorticotrophic and glucagon hormones or reduced insulin activity. From the above findings we can conclude that cartap produces hyperglycemia in exposed rats which can be attributed to pesticide induced hormonal imbalance particularly of glucagon and insulin. In the present study, α-tocopherol, which has an apparent hypoglycemic effect, was able to somewhat reduce glucose levels indicating towards restoration of hormonal balance to a certain degree by α-tocopherol leading to moderate reduction in blood glucose levels in co-treatment groups (VI and VII).

No significant change was observed in the plasma protein fraction (Total protein, albumin and globulin) following treatment with cartap in any of the treatment groups compared to control group. Our findings are in agreement to the findings of Tizhe et al., (2013) who observed no significant alteration in plasma protein fraction following sublethal exposure to herbicide glyphosate in adult male wistar rats. Lower serum albumin indicates malnutrition, liver disease or inflammatory disease while higher albumin may be caused by acute infections, burns and stress (Caraceni et al., 2013). Serum albumin levels tend to be normal in various hepatic conditions like acute viral hepatitis, drug related hepatotoxicity and obstructive jaundice (Thapa and Walia, 2007). Lower serum globulin indicates infection, inflammatory disease or immune disorder while higher levels may be indicative of neoplasia such as multiple myeloma, Hodgkin's disease or malignant lymphoma (Busher, 1990). Our findings indicate that cartap has no effect on serum protein fraction but that isn't a reliable marker of lack or presence of systemic toxicity.

Following 28 days exposure to cartap (16.25 mg/kg and 32.5 mg/kg) there was a moderate but non-significant increase in plasma total cholesterol level in cartap alone groups (IV and V) compared to control group. Our observation is in agreement with the findings of Eissa and Zidan (2010) who observed mild but non-significant rise in total cholesterol following sublethal exposure to vertimec for 30 days in adult male wistar rats. Following co-treatment with α-tocopherol and cartap a significant fall in total cholesterol was observed in co treated groups (VI and VII) which is in agreement to the findings of Peluzio et al. (2001), who observed a significant

lowering of total cholesterol following 6 weeks treatment of apolipoprotein E-deficient mice fed on high fat atherogenic diet with α-tocopherol. Landrier et al. (2010) observed that α-tocopherol downregulates hepatocyte CYP27A1 gene responsible for the synthesis of sterol-27-hydroxylase, an enzyme crucial for the sterol biosynthesis pathway resulting in reduced cholesterol synthesis. It has been suggested by Pothu et al. (2019) that pesticide induced hypercholesterolemia might be due to endocrine imbalance particularly that of thyroid hormones resulting in slower BMR and increased serum cholesterol. These findings suggest that cartap might be involved in altering thyroid hormonal balance resulting in increased plasma cholesterol level which was lowered by α-tocopherol.

In the present study, there was no significant change in plasma HDL and LDL levels in any of the treatment groups compared to control group. Contrary to our observation Rai et al. (2009) observed a significant decrease in serum HDL and an increase in serum LDL following 7-day sublethal exposure to cartap and carbofuran both alone and in combination in adult male wistar rats albeit at a higher dose rate of cartap (195 mg/kg) than the ones used in our study. It was later observed by Kheirallah et al. (2021) that serum HDL and LDL levels were around normal range and no significant change was observed following sub lethal exposure to cypermethrin in adult male wistar rats. It has been suggested by Lehtonen (1985) that HDL and LDL levels in plasma are primarily dietary dependent and xenobiotic induced alteration in plasma levels can be due to inhibition or stimulation of lipid degradation enzymes like adipose lipase. From our findings and the above-mentioned observations, we can conclude that cartap has little or no effect on lipid degradation enzymes at the dose rate used in our study.

A moderate but non-significant increase in plasma triglycerides level was observed in cartap alone treatment groups (IV and V) compared to control group. Rai et al. (2009) also observed a significant increase in serum triglyceride levels following 7 days sublethal exposure to cartap and carbofuran both alone and in combination in adult male wistar rats and hypothesized that serum triglyceride levels might be elevated due to xenobiotic induced stimulation of catecholamines which stimulates lipolysis and increase fatty acid production. The higher increase in this study can be due to higher dose rate of cartap (195 mg/kg) compared to the dose used in our study (16.25 mg/kg and 32.5 mg/kg). It can therefore be concluded that cartap might be

involved in stimulation of catecholamines induced lipolysis. A significant fall in plasma triglyceride level was observed in co-treated groups (α-tocopherol + cartap) compared to cartap alone treatment groups as well as control group. Contrary to our finding, Costa et al. (2005) observed no significant change in plasma triglyceride levels following treatment of obese diabetic rats with α-tocopherol. Similarly, Saraf et al. (2011) following 45-day sublethal exposure to malathion and concurrent administration of α-tocopherol in adult male wistar rats observed no significant change in serum triglyceride levels. It can be therefore concluded that α-tocopherol has no effect on serum triglyceride level and this significant decrease in serum triglyceride level in our study might be indicating that α-tocopherol is somehow interfering with the lipolytic activity of cartap.

Compared to control group no significant change in plasma creatinine level was observed following sublethal exposure to cartap in any of the treatment groups. Contrary to our findings a significant increase in plasma creatinine levels was observed by Sharma et al. (2021) following sublethal exposure to cartap for 28 days in adult male mice. Similarly, Bhilegaonkar et al (1995), following benfuracarb toxicity in wistar rats observed significant increase in plasma creatinine levels. Creatinine is a waste product from the normal breakdown of muscles in the body and is removed from blood by kidneys (Shahbaz, 2021). As suggested by Peralta et al. (2011) an increased plasma creatinine is a marker of impaired kidney function particularly chronic kidney disease but does not necessarily suggest any acute change since plasma creatinine accumulation occurs gradually in response to renal failure and is mostly a chronic change.

Plasma BUN level was significantly increased in cartap group (V; 32.5 mg/kg) compared to control group. Our observation is in agreement to findings of Sharma et al. (2021) who reported a significant increase in plasma BUN following subacute exposure to cartap in adult male mice. Saxena and Saxena (2010) also observed a significant increase in plasma BUN following sublethal exposure to cypermethrin for 28 days in adult male wistar rats. Similarly, Elevated BUN is correlated to increased protein catabolism, decreased water intake and impaired kidney function (Hosten, 1990). Our observation therefore is an indicator of nephrotoxic nature of the compound. Co-treatment of α-tocopherol with cartap in co-treatment groups (VI and VII) did not produce any significant change in plasma BUN levels compared to cartap

alone treatment groups. Although BUN levels were significantly increased in co treated groups (VI, VII) compared to control group. Our findings are in agreement to the findings of El-Demerdash et al. (2004) who observed no significant change in plasma BUN levels following concurrent exposure to α-tocopherol in adult male wistar rats exposed to sublethal dosage of Fenvalerate. These findings indicate that α-tocopherol was ineffective in improving cartap induced increase in plasma BUN levels.

No significant change in plasma bilirubin levels was observed in any of the treatment groups compared to control group. Contrary to our observation Yousef et al. (2006) observed significant increase in plasma bilirubin levels following 30-day exposure to deltamethrin in adult male wistar rats. Similarly, Zidan (2015) also observed significant increase in plasma bilirubin following 90 days exposure to malathion in adult male wistar rats. Elevation of total bilirubin may be due to impaired intra-hepatic excretion/regurgitation of unconjugated or conjugated bilirubin from hepatocytes to bile ducts (Friedman et al., 2003). Hyperbilirubinemia is usually secondary to hepatocellular injury or cholestasis (intrahepatic and extra-hepatic) (Beckingham and Ryder, 2001). However, in our study we did not find any change in plasma bilirubin levels suggesting that cartap did not produce any hepatocellular damage or the damage wasn't enough to produce hyperbilirubinemia.

In our study, plasma ALT levels were significantly increased at higher dose rate of cartap (32.5 mg/kg) compared to control group, which is in agreement to the findings of Sharma et al. (2021) who observed significant increase in plasma ALT following subacute exposure to cartap in adult male mice. Similarly, Mossa and Abassy, (2012) observed significant increase in plasma ALT following sublethal exposure to mixture of chlorpyrifos-ethyl, chlorpyriphos-methyl and methomyl in adult male wistar rats. ALT is primarily localized in hepatocytes and appearance of the enzyme in plasma is an indicator to hepatocellular damage (Huang et al., 2006). Serum ALT elevation may not be accompanied by elevation in bilirubin, a condition called anicteric hepatitis since ALT levels tend to rise even in mild hepatic injury which may not translate to increased bilirubin. Anicteric hepatitis is usually a finding of xenobiotic induced hepatocellular injury (Malakouti et al., 2017). It can therefore be concluded that cartap produces hepatoxicity resulting in cellular damage and release of ALT in plasma. However, the injury could be of mild nature since bilirubin

level was not increased significantly. Following co-treatment with α-tocopherol and cartap a mild but not significant decrease was observed in plasma ALT in co-treated groups (VI and VII) compared to cartap alone groups (IV and V). This observation is somewhat in agreement to the findings of lida et al. (2009) who observed significant decrease in plasma ALT levels following treatment of carbon tetrachloride exposed rats with α-tocopherol. It can be concluded that α-tocopherol showed some reparative potential in our study, however the extent of restoration wasn't enough.

Similar to ALT we observed an increase in plasma AST but only moderately and non-significantly at higher dose rate of cartap (32.5 mg/kg) compared to control group. Abdou et al. (2012) also observed a significant increase in plasma AST following sublethal exposure to cypermethrin for 30 days in adult male wistar rats. AST is localized in various organs and tissues primarily in liver kidney and skeletal muscles (Huang et al., 2006). Hence an elevation in plasma AST levels could be an indicator of liver, kidney or skeletal muscle damage or a combination of three. In co-treated groups (VI and VII) a mild but not significant decrease in plasma AST was observed compared to cartap alone groups (1V and V). This observation is in agreement to the findings of lida et al. (2009) who observed significant decrease in plasma AST levels following treatment of carbon tetrachloride exposed rats with α-tocopherol. Similar to ALT the overall decrease in AST wasn't significant.

Following 28 days exposure to cartap, a non-significant decrease and increase in plasma calcium levels was observed in cartap alone treatment group IV and V respectively compared to control group. Mishra et al. (2005) also observed a significant decrease in plasma calcium level following 28 days exposure to sublethal concentrations of cartap in teleost fish. Following co-administration of α-tocopherol along with cartap there was a significant decrease in plasma calcium level in co-treated group VII compared to cartap alone treatment group V. Norazlina et al. (2004) in a study in adult male wistar rats kept on vitamin e deficient diet for 3 months reported a sudden increase in parathyroid activity. Therefore, fall in calcium levels in our study might be due to the combined effect of restoration of tissue architecture by α-tocopherol as well as a possible fall in parathyroid activity resulting in reduced calcium resorption in co-treatment groups (VI and VII).

5.4: Effect of alpha tocopherol on cartap induced oxidative stress biomarkers in testes and liver of rats: Oxidative stress refers to a serious imbalance between reactive oxygen species (ROS) production and antioxidant defence. Sies (1991) defined it as "a disturbance in the pro-oxidant and antioxidant balance in favour of the former, leading to potential damage.

5.4.1 Oxidative stress biomarkers in testes

Following 28 days exposure to cartap testicular total protein concentration was significantly reduced in cartap alone group IV and V in both groups compared to control group. Our observation is in agreement to the findings of Ndonwi et al. (2019) who observed a significant decrease in hepatic total protein in adult female (nulliparous) wistar rats exposed to sublethal dosage of imidacloprid, chlorpyriphos and lambda cyhalothrin alone in all treatment groups. Similarly, Mandil et al. (2016) also observed moderate but not significant decrease in testicular protein in adult male rats exposed to sublethal concentrations of flubendiamide. Cartap produces decrease in testicular protein concentration which might be because of protein degradation following tissue damage as suggested by Klaunig et al. (2018) who hypothesized xenobiotic induced protein degradation as one of the reasons for xenobiotic induced fatty liver and other hepatic disorders. Co-treated group VI showed a moderate but not significant increase in testicular total protein compared to cartap alone group IV (16.25 mg/kg). Compared to cartap alone group V (32.5 mg/kg) the testicular total protein concentration was significantly increased in co-treated group VI. This observation is in agreement to the findings of Saraf et al. (2011) who observed a significant increase in total protein in kidney and liver following treatment with α-tocopherol in adult male wistar rats exposed to sublethal concentrations of malathion. From these findings we can conclude that α-tocopherol was effective in restoring pesticide induced reduction in testicular total protein concentration which might be because of restoration of tissue histoarchitecture by α-tocopherol.

Lipid peroxidation occurs as a result of attack of xenobiotic induced reactive oxygen species on the lipid component of plasma membrane. This results in the formation of malondialdehyde, thereby indicating oxidative stress (Ayala et al., 2014). In the present study, testicular MDA levels were significantly increased in cartap alone treatment groups (IV and V) compared to control group. Our observation is in agreement to the findings of Sharma et al. (2021) who observed a significant increase

in MDA levels in brain and liver following 28-day subacute exposure to cartap in adult male mice. Zervos et al. (2011) observed a significant increase in MDA level in brain tissue following sublethal exposure to Endosulfan in adult male rats. Our findings indicate that cartap produces oxidative stress by lipid peroxidation in testes. No significant change in MDA levels were observed in cartap co-treated groups (VI and VII) compared to cartap alone groups (IV and V). Contrary to our observation, Saraf et al. (2011) observed a significant decrease in MDA levels in kidney and liver following treatment with α-tocopherol in adult male rats exposed to sub lethal concentrations of malathion. The antioxidant property of α-tocopherol is attributed to the hydroxyl group from the aromatic ring which donates hydrogen to neutralize free radicals or reactive oxygen species (ROS) (Yoshida et al., 2007). This indicates that α-tocopherol is able to reduce pesticide induced increase in MDA levels but it was not observed in our study. It might be indicative of interference of cartap in free radical neutralization reaction involving α-tocopherol or reduced concentration of α-tocopherol in testis.

Superoxide dismutases (SOD) are antioxidant enzymes involved in the neutralization of superoxide free radical by converting it into oxygen and hydrogen peroxide (Hayyan et al., 2016). The toxicity of several xenobiotics including pesticides has been suggested to be mediated by formation of free radicals derived from reduction of molecular oxygen, forming superoxide anion and hydroxyl radical. Decreased SOD activity suggests accumulation of superoxide anions which are also responsible for increased lipid peroxidation (Bus and Gibson, 1982). Testicular superoxide dismutase activity was significantly reduced at higher dose rate cartap group (V; 32.5 mg/kg) compared to control group. Our observation is in agreement to the findings of Otitoju et al. (2008) who in similar study observed a significant decrease in SOD activity in brain of rats exposed to sublethal concentration of permethrin. Similarly, Mansour et al. (2017) also observed a significant decrease in plasma SOD activity following subacute exposure to Methomyl in male adult wistar rats. From our observation and similar findings, we can conclude that cartap produces a significant decrease SOD enzyme activity in testicular tissue. Following treatment with α-tocopherol and cartap no significant change was observed in co-treated groups (VI and VII) compared to cartap alone groups (IV and V). Contrary to our findings Eroglu et al. (2013) observed a significant increase in erythrocytic SOD activity

following treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of dichlorvos. Our observation is in agreement to the findings of Mandil et al. (2016) who did not observe any significant change in testicular SOD activity following treatment with α-tocopherol in adult male wistar rats exposed to sub lethal dosage of flubendiamide. These contrasting findings might be due to varying concentration α-tocopherol in different anatomical sites as suggested by Hymøller et al. (2018) who reported different concentrations of alpha tocopherol in plasma, liver, heart, lungs, brain and abdominal fat of male adult minks kept on α-tocopherol rich diet. Thus, we can conclude that cartap induced decrease in testicular SOD activity was not restored by α-tocopherol.

Among the reactive species, hydrogen peroxide is freely diffusible and is relatively long-lived (Grigoras, 2017). It acts as a weak oxidizing as well as reducing agent; however, it is not very reactive, but it is the progenitor of many other reactive oxygen species (ROS) (Nandi et al., 2019). Catalase breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water in a two-step reaction (Glorieux and Calderon, 2017). Following 28 days subacute exposure to cartap there was a significant decrease in the activity of testicular catalase in group V (32.5 mg/kg) compared to control group. Our observation is in agreement to the findings Sharma et al. (2021) who observed a significant decrease in catalase activity in brain liver and kidney of adult male mice exposed to sublethal dosage of cartap. Alva et al. (2012) reported a significant decrease in catalase activity in liver and heart following subacute exposure to endosulfan in adult male wistar rats. In a similar study Marouani et al. 2017 observed a significant decrease in testicular catalase activity following subacute exposure to DDT in adult male wistar rats. From our observation and similar findings in other studies we can conclude that cartap produces reduction in testicular catalase activity. Co-treated group VI and VII showed no significant change was observed in catalase activity compared to cartap alone treatment groups (IV and V) except for a mild but non-significant increase in enzymatic activity when α-tocopherol was co-treated with cartap group IV at lower dose (16.25 mg/kg). Our observation is in agreement to the findings of Amin and Hashem (2012) also observed a significant increase in catalase activity following treatment with α-tocopherol in the erythrocytes of catfish exposed to sublethal concentration of deltamethrin. Fahim et al. (2013) also observed who observed significant improvement in reduced catalase

activity in the heart of adult male wistar rats exposed to sublethal dosage of paraquat. Shah, (2016) reported a significant increase in plasma catalase activity in adult male Japanese quails fed on alpha tocopherol rich diet for 1 month and suggested that tocopherol induced neutralization of free radicals and ROS lead to decreased load over antioxidant enzyme system and increased catalase activity. These findings indicate the ability of α-tocopherol to restore pesticide induced reduction in enzyme activity to a certain extent. However, at higher dose of cartap (VII), α-tocopherol was ineffective as indicated by our finding.

Glutathione in reduced state protects cells by neutralizing reactive oxygen species. The ratio of reduced glutathione to oxidized glutathione within cells is a measure of cellular oxidative stress (Pastore et al., 2001). Following exposure to cartap there was no significant change in testicular reduced glutathione (GSH) levels in cartap alone groups (IV and V) compared to control group. Contrary to our finding Gupta et al (2019) and Sharma et al. (2021) observed significant reduction in GSH levels in rat and mice exposed to sublethal concentration of cartap. Mossa et al. (2015) observed a significant decrease in GSH levels in kidney and liver of adult male albino rats exposed to sub lethal dosage of fipronil. These findings indicate that pesticides in general produce oxidative stress by reducing the levels of glutathione. However, cartap does not have much effect on glutathione levels in testes. This might be indicative of lower concentration of cartap in testes which may not be enough to produce any change in glutathione levels. Similarly, co-treated groups (VI and VII) did not show any significant change in GSH levels compared to cartap alone groups (IV and V). Masaki et al. (2002) reported a significant increase in intracellular GSH in HaCaT keratinocytes cultivated in α-tocopherol. Thus, it can be concluded that cartap might be interfering with α-tocopherol leading to reduced effect on GSH levels in testes.

Glutathione-S-transferases (GST) are detoxifying enzymes that catalyze the conjugation of variety of electrophilic substrates including free radicals to thiol group of GSH, producing less toxic forms (Finkel, 2003). Xenobiotics may induce inhibition of the enzyme leading to accumulation of peroxide, which promotes lipid peroxidation, modulation of DNA and altered gene expression resulting in cell death (Stoh and Bagchi, 1995; Calviello et al., 2006). Decrease in GST activity reflects inadequate detoxification or accumulations of ROS, resulting in oxidative damage and

indicates a compromised cellular defence against xenobiotics (Hayes and Pulford, 1995). Testicular glutathione-s-transferase (GST) was found to be significantly reduced in group V (32.5 mg/kg) following exposure to cartap at higher dose (32.5 mg/kg) compared to control group. Our observation is in agreement to the findings of Sharma et al. (2021) who observed a significant decrease in GST in liver, kidney and brain of adult mice exposed to sub lethal dosage of cartap. Jaiswal (2016) also observed a significant decrease in brain GST level in adult male wistar rats exposed to sublethal concentrations of carbofuran. No significant change was observed in testicular GST levels in co-treated group (VI and VII) compared to cartap alone groups (IV and V) which is in agreement to the findings of Mandil et al. (2016) who observed no significant change in testicular GST activity following treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of flubendiamide. Therefore, we can conclude that α-tocopherol is ineffective in reducing cartap induced fall in testicular GST activity which might be due to similar lack of effect of α-tocopherol to produce any significant change in testicular GSH as observed earlier.

5.4.2 Oxidative stress biomarkers in Liver

In the present study, hepatic total protein concentration was significantly reduced following 28 days exposure to cartap in cartap alone treatment groups (IV and V). Our observation is in agreement to the findings of Ndonwi et al. (2019) who observed a significant decrease in hepatic total protein in adult female wistar rats exposed to sublethal dosage of imidacloprid, chlorpyriphos and lambda cyhalothrin alone in all treatment groups. Similarly, Abdoul-Soud et al. (2011) reported a significant decrease in hepatic protein concentration in adult male wistar rats exposed to sublethal dosage of malathion. These findings suggest that cartap produces decrease in hepatic protein concentration which might be because of protein degradation following tissue damage. Co-treatment with α-tocopherol lead to a significant increase in hepatic total protein in co-treatment groups (VI and VII) compared to cartap alone treatment groups (IV and V). This observation is in agreement to the findings of Saraf et al. (2011) who observed a significant increase in total protein in kidney and liver following treatment with α-tocopherol in adult male wistar rats exposed to sublethal concentrations of malathion. From these findings we can conclude that α-tocopherol was effective in restoring pesticide induced reduction

in hepatic total protein concentration which might be because of restoration of tissue histoarchitecture by α-tocopherol.

Hepatic MDA levels were significantly increased in cartap alone treatment groups (IV and V) compared to control group. Our observation is in agreement to the findings of Gupta et al. (2019) who observed a significant increase in hepatic MDA levels in adult male wistar rats exposed to sublethal dosage of cartap. Similar findings were reported by Sharma et al. (2021) who observed significant increase in MDA levels in liver and kidney following sublethal exposure to cartap in adult male mice. This indicates that cartap exposure produces oxidative damage in the lipid component of hepatocytes leading to lipid peroxidation. Following co- treatment with α-tocopherol (100 mg/kg) and cartap in group VI (16.25 mg/kg) there was moderate but non-significant decrease in hepatic MDA levels compared to cartap alone group IV (16.25 mg/kg). However, a significant decrease in hepatic MDA levels was observed in co-treated group VII compared to cartap alone group (V). Our observation is in agreement to the findings of Saraf et al. (2011) who observed a significant decrease in MDA levels in liver and kidney following treatment with α-tocopherol in adult male rats exposed to sub lethal concentrations of malathion. This indicates that α-tocopherol is able to significantly reduce pesticide induced increase in MDA levels in liver but not in testes. This might be because of higher concentration of α-tocopherol reaching hepatic tissue since liver is the major metabolizing site in the body thus producing maximal effect as indicated by Banudevi et al. (2006) who reported greater reduction in hepatic MDA levels compared to lungs and kidneys in following concurrent exposure to α-tocopherol in male albino rats exposed to a mixture of Polychlorinated biphenyls (PCB).

Hepatic superoxide dismutase (SOD) activity was found to be significantly reduced at higher dose rate of cartap (32.5 mg/kg) in group V compared to control group. Our observation is in agreement to the findings of Sharma et al. (2021) who observed a significant decrease in hepatic SOD activity in male adult mice exposed to sublethal dosage of cartap. Jaiswal et al. (2017) also observed a significant decrease in hepatic SOD activity in adult male wistar rats exposed to sublethal dosage of carbofuran pesticide. This indicates the ability of cartap to reduce the activity SOD in liver similar to that in testis. Following treatment with α-tocopherol (100 mg/kg) and cartap (16.25 mg/kg) no significant change was observed in hepatic SOD levels in co-

treated group VI (16.25 mg/kg + α-tocopherol) compared to cartap alone group IV (16.25 mg/kg) Compared to cartap alone group V (32.5 mg/kg) a moderate but non-significant increase in hepatic SOD activity was observed in co-treated group VII (32. 5 mg/kg) which is in agreement to the findings of Eroglu et al. (2013) who observed a significant increase in erythrocytic SOD activity following treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of dichlorvos. Similarly, Othman et al. (2011) observed a significant increase in SOD activity in liver and kidney of rats following concurrent exposure to α-tocopherol in adult male wistar rats exposed to sublethal dosage of malathion. These findings suggest that α-tocopherol is effective in increasing the hepatic SOD activity in both erythrocytes as well as in liver in pesticides (Cartap, Dichlorvos and Malathion) toxicity. Our findings indicate that α-tocopherol is effective in increasing hepatic (Non-significant) SOD activity reduced by cartap.

In the present study, following subacute exposure to cartap, there was a non-significant decrease in the activity of hepatic catalase in cartap group V (32.5 mg/kg) compared to control group. Gupta et al. (2019) observed a significant decrease in hepatic catalase activity following sublethal dosage of cartap in adult male rats. Alva et al. (2012) also observed a significant decrease in catalase activity in liver and heart following subacute exposure to endosulfan in adult male wistar rats. Our results showed that cartap at higher dose (32.5 mg/kg) produces decrease in catalase in both liver and testes. Following treatment with α-tocopherol and cartap no significant change in catalase activity was observed in co-treated groups (VI and VII) compared to cartap alone groups (IV and V). Our observation is in agreement to the findings of Mandil et al. (2016) who observed no significant change in testicular catalase activity following treatment with α-tocopherol in adult male rats exposed to sublethal dosage of flubendiamide. This indicates that α-tocopherol was ineffective in restoring catalase activity in liver similar to that in testes indicating that cartap might be interfering with the anti-oxidant activity of α-tocopherol.

Following exposure to cartap at higher dose (32.5 mg/kg) in group V, there was a significant decrease in hepatic reduced glutathione (GSH) level was found compared to control group. Our observation is in agreement to the findings of Gupta et al. (2019) who observed a significant decrease in hepatic GSH levels following sub lethal exposure to cartap in adult male wistar rats. Mossa et al. (2015) also observed a

significant decrease in GSH levels in kidney and liver of adult male albino rats exposed to sub lethal dosage of fipronil. Following co-treatment with α-tocopherol and cartap no significant change was observed in hepatic GSH levels in co treated groups (VI and VII) compared to cartap alone groups (IV and V). Contrary to our findings Othman et al. (2011) who reported a significant increase in hepatic GSH levels following treatment with α-tocopherol in adult male rats exposed to sublethal dosage of Malathion. Our results indicate that α-tocopherol was ineffective in restoring hepatic GSH levels following sublethal exposure to cartap which might be due to cartap producing any hindrance in the antioxidant activity of α-tocopherol.

Hepatic Glutathione-s-transferase (GST) activity was found to be moderately but non-significantly reduced following exposure to cartap at higher dose (32.5 mg/kg) in group V. Our observation is in agreement to the findings of Sharma et al. (2021) who observed a significant decrease in GST activity in liver, kidney and brain of adult male mice exposed to sub lethal dosage of cartap. Jaiswal (2016) observed a significant decrease in brain GST activity in adult male wistar rats exposed to sublethal concentrations of carbofuran. In another study by Ozcelebi et al. (2021) it was found that there was a significant decrease in brain GST activity following sublethal exposure to fenarimol in adult male wistar rats. No significant change was observed in hepatic GST activity following co-treatment with α-tocopherol and cartap in co-treated groups (VI and VII) compared to cartap alone groups (IV and V). Contrary to our findings El- Demerdash et al. (2004) observed a significant increase in GST activity in brain following treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of fenvalerate. Therefore, we can conclude that α-tocopherol is ineffective in improving cartap induced fall in hepatic GST activity which might be due to cartap producing any hindrance in the antioxidant activity of α-tocopherol.

5.5 Effect of α-tocopherol on cartap induced insult to certain testicular injury biomarkers and testosterone levels

Acid phosphatase (ACP) is a lysosomal enzyme and plays an important role in catabolism, pathological necrosis, autolysis, differentiation, phagocytosis, cell death and tissue damage (Weissmann, 1967; Sugar, 1997). It is primarily present in bone, prostate, platelets, erythrocytes, testes and spleen (Garnero et al. 2008). Increase in ACP activity in testes could be due to release of this enzyme from lysosomes of

degenerating cells, rapid catabolism of injured germ cells, enhanced cell membrane permeability with disturbance in transphosphorylation (Hodgen and Sherins, 1973). Following exposure to cartap there was a significant increase in testicular ACP activity in cartap alone group V (32.5 mg/kg) compared to control group which is in agreement to the findings of Eissa and Zidan, (2010) who observed a significant elevation in plasma acid phosphatase in adult male rats exposed to sublethal dosage of Vertimec insecticide. Similarly, Ngoula et al., (2014) also observed a significant increase in testicular acid phosphatase in adult male wistar rats exposed to Dimethoate. Increase in ACP activity in testes could be due to release of this enzyme from lysosomes of degenerating cells following exposure to cartap. Following treatment with α-tocopherol a moderate but non-significant decrease in ACP activity was observed in co-treated VI and VII groups compared to cartap alone groups IV and V respectively. Our observations are in agreement to the findings of Yousef et al. (2006) who observed a significant decrease in plasma ACP activity following co-treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of deltamethrin. This decrease in ACP activity might be due to reduction in degenerating cells indicating a reparative potential of α-tocopherol.

Sorbitol Dehydrogenase (SDH) is a cytosolic enzyme in polyol pathway and catalyzes the conversion of sorbitol to fructose with NAD^+ as the coenzyme (Kabbani et al., 2004). In testes the activity of SDH is associated with the maturation of germinal epithelial layer of seminiferous tubule (Mills and Means, 1972). Rounded immature germinal epithelial cells undergo successive mitotic and meiotic divisions and a metamorphic change to produce spermatozoa (Cheng and Mruk, 2010). The activity of SDH is increased markedly during the maturation of germinal epithelium and is an indicator of testicular maturation (Jana et al., 2006). Arrested development of spermatogonia cells of germinal epithelium is correlated to fall in SDH activity (Shen and Lee, 1984). In the present study, no significant alteration in SDH activity was observed in testes of rats in any of the treatment groups compared to control group. Contrary to our finding, Lonare et al. (2014) reported significant decrease in testicular SDH in adult male wistar rats exposed to sublethal dosage of imidacloprid. However, Mandil et al. (2016) reported no significant change in SDH levels in testes of adult male wistar rats exposed to sublethal dosage of flubendiamide which is in

agreement to our finding. Thus, we can conclude from these findings that cartap has no effect on testicular SDH/maturation of germinal epithelium for spermatogenesis.

Lactate dehydrogenase (LDH) is an intracellular enzyme which is capable of reversible formation of pyruvate and lactate in all eukaryotic and prokaryotic cells. This enzyme is ubiquitously present in all tissues and serves as an important checkpoint of gluconeogenesis and DNA metabolism (Farhana et al., 2021). The increase in testicular LDH activity suggests deterioration of germinal epithelium (Teng et al., 2016). A significant increase in LDH activity was observed in testes of cartap group V (32.5 mg/kg) compared to control group. Our observation is in agreement with the findings of Mansour and Mossa, (2010) who observed a significant increase in plasma LDH in adult lactating female rats on oral administration of chlorpyrifos. In a similar study, Jaiswal et al. (2018) also observed a significant increase in LDH activity in the brain of adult male wistar rats following sublethal exposure to Carbofuran. These findings suggest that cartap might be damaging the germinal epithelium of testes. Following co-treatment with α-tocopherol a moderate but non-significant decrease in LDH activity was observed in co-treated groups VI and VII compared to cartap alone groups IV and V respectively which is in agreement to the findings of Yousef et al. (2006) and Mandil et al. (2016) who reported a significant decrease in LDH activity following co-treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of deltamethrin and flubendiamide in plasma and testes respectively. Therefore, our results indicate some degree of restoration of germinal epithelium by α-tocopherol.

Gamma-glutamyl transpeptidase (GGT) is a transferase type of enzyme that catalyzes the transfer of gamma-glutamyl functional groups to an acceptor that may be an amino acid, or a short chain peptide (Tate and Meister, 1985; Whitfield, 2001). It plays a major role in amino acid transport for the synthesis of specific proteins known to be secreted by Sertoli cells (Tate and Meister 1981). Increase in its activity is due to impaired function of Sertoli cells (Hodgen and Sherins, 1976). A significant increase in the activity of testicular γ-GT was observed in cartap group V (32.5mg/kg) compared to control group which is in agreement with the findings of Omayma et al., 2019 who observed a significant increase in plasma γ-GT activity in adult male wistar rats exposed to Lambda cyhalothrin. Similarly, Mandil et al. (2016) also reported a significant increase in γ-GT levels in testes of adult male wistar rats

exposed to sublethal dosage of flubendiamide. Increase in γ-GT activity in the present study, could be due to damage of sertoli cells following exposure to cartap. Following co-treatment with α-tocopherol a moderate but non-significant decrease in γ-GT activity was observed in co-treated groups VI and VII compared to cartap alone groups IV and V respectively. Our observation is in agreement to the findings of Mandil et al. (2016) who observed a significant decrease in testicular γ-GT activity following co-treatment with α-tocopherol in rats exposed to sublethal dosage of flubendiamide. In another study Elayapillai et al. (2017) also reported reduced sertoli cell damage in adult male wistar rats exposed to polychlorinated biphenyl (PCB's) following concurrent exposure to α-tocopherol. Therefore, we can conclude that reduction in GGT activity might be due to some degree of restoration of Sertoli cell by α-tocopherol.

In males, testosterone plays a key role in the development of male reproductive tissues such as testes and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair (Mooradian et al., 1987). Alterations of reproductive hormone levels may lead to a decline in semen quality (i.e., concentration, motility, and morphology) and sperm DNA damage/fragmentation (Panuwet et al., 2017). In the present study, a significant decrease in plasma testosterone level in both cartap alone treatment groups (IV and V) was observed compared to control group. Our observation is in agreement to the findings of Mandil et al. (2016) who observed a significant decrease in plasma testosterone levels following sub lethal exposure to flubendiamide in adult male wistar rats. Similarly, Arican et al. (2020) also observed a significant decrease in plasma testosterone levels in male Sprague Dawley rats exposed to sublethal dosage of Acetaprimid. Pesticides are responsible for decreasing testosterone concentration by inhibiting release of FSH or LH (Slimani et al., 2011). Pesticides induce apoptosis of Leydig cells and thus decreasing overall production of testosterone (Kaur et al., 2014). Following co-treatment with α-tocopherol non-significant change was observed in plasma testosterone levels in co-treated groups (IV and V) compared to cartap alone groups (IV and V) which is in agreement to the findings of Mandil et al. (2016) and Kong et al. (2017) who observed non-significant change in plasma testosterone levels following treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of flubendiamide and acetaprimid respectively. It was

suggested by Kong et al. (2017) that xenobiotic induced fall in testosterone levels might be indicative of ROS mediated Leydig cell damage. Thus, we can conclude that fall in plasma testosterone levels might be due to Leydig cell damage or cartap inhibits release of FSH or LH. Following exposure to cartap in the present study, α-tocopherol failed to restore testosterone levels which might be due to the failure of α-tocopherol to repair Leydig cell damage or restore FSH and LH hormonal levels.

5.6 Effect of α-tocopherol on cartap induced gross and histopathological changes in lungs, liver, kidneys, spleen, heart, testes and trachea

Gross examination following necropsy revealed no changes in any of the organs except in lungs which showed mild congestion and swelling in both cartap alone treatment groups (IV and V). Our observation is in agreement to the findings of El din et al. (2014) who reported profused congestion in lungs of adult male wistar rats exposed to sublethal dosage of lambda cyhalothrin. Pulmonary congestion i.e. distention of blood vessels in the lungs and filling of the alveoli with blood occurs as a result of an infection, high blood pressure, or cardiac insufficiencies (Pappas and Filippatos, 2011). Following co-treatment with α-tocopherol a visible reduction in swelling and congestion was observed in co-treated groups (VI and VII). Thus, we can conclude from gross findings that cartap has affinity to lungs and α-tocopherol was effective in reducing the severity of pathological change at least grossly which suggests the restorative potential of α-tocopherol.

Histopathological changes in lungs, liver, kidneys, spleen, heart, testes and trachea of different treatment groups following 28 days exposure to cartap (16.25 mg/kg and 32.5 mg/kg) and α-tocopherol (100 mg/kg) alone and in combination were observed.

Mild and moderate congestion as well as thickening of alveolar walls was observed in cartap alone treatment group IV and V respectively. Further, Group V (32.5 mg/kg) also showed thickening of bronchiolar wall which was absent in group IV (16.25 mg/kg). Our observation is in agreement to the findings of Sharma et al. (2021) who reported thickening of alveolar septa/wall along with neutrophilic infiltration following sublethal exposure to cartap in male adult mice. In another study, Ali et al. (2018) reported thickness in walls of alveoli and bronchi in adult male albino mice exposed to sublethal dosage of 2,4-D. Thickening of alveolar septa

following xenobiotic exposure is a result of direct acute pulmonary injury (Obadina et al., 2013). α-tocopherol co-treatment with cartap revealed visible reduction in alveolar wall thickness with mild congestion in group VI. However, visible thickness of alveolar walls and mild congestion was still persistent in co-treated group VII. These findings indicate the ability of cartap to produce injury in alveolar as well as bronchiolar septa and the ability of α-tocopherol to restore tissue histoarchitecture at lower dosage of cartap in group VI. Ehterami et al. (2019) reported accelerated wound healing following application of α-tocopherol based gel in wound healing rat model. These findings are indicative of healing potential of α-tocopherol against cartap induced acute pulmonary injury.

Following exposure to cartap we observed mild and moderate congestion in the central vein of liver in cartap alone group IV and group V respectively. Our observation is in agreement to the findings of Gupta et al. (2019) who reported central vein congestion following sublethal dosage of cartap in adult male wistar rats. Co-treatment with α-tocopherol and cartap revealed normal histoarchitecture in hepatic tissue of group VI and VII. Hilscher (2016) reported that hepatic congestion is a result of either right sided heart failure or obstruction of the central vein. Hence, we can conclude that cartap induced central vein congestion could be a result of occlusion of the central vein, right sided heart failure or a combination of both following exposure to cartap. α-tocopherol was effective in reducing congestion in both the co-treated groups (VI and VII). It could be indicative of hepatoprotective effect of α-tocopherol as reported by Palipoch et al. (2014), who observed reduction in hepatic congestion following treatment with α-tocopherol in cisplatin induced injury in adult male wistar rats.

Constriction of glomeruli along with degeneration of tubules was evident based on histopathological examination of kidney tissue in cartap alone group IV (16.25 mg/kg). In addition to these findings at higher dose cartap alone group V (32.5 mg/kg) also revealed mild congestion in the kidney tissue. Our observation is in agreement to the findings of Sharma et al. (2021) who observed degenerative changes around the glomerular area and in proximal renal tubule following sublethal exposure to cartap in adult male mice. These changes might be because of cartap induced kidney injury. Acute kidney injury (AKI) is one of the most common causes of renal tubular degeneration. Other less common causes of renal tubular degeneration include

hypotension and nephrotoxic drugs like Phenytoin, Quinine, Ranitidine etc (Webb and Dobb, 2004). Glomerular constriction is a result of reduced renal blood flow and is suggestive of cardiac insufficiency or hypotension (Shankland et al., 2014). Co-treatment with α-tocopherol and cartap revealed only mild congestion in the kidney tissue of group VI and VII. Renal congestion is a result of reduced cardiac output following cardiac failure (Afsar et al., 2017). These findings indicate that α-tocopherol was able to restore renal histoarchitecture to a certain degree since no degeneration was observed in both the co-treated groups (VI and VII). This might be due to the nephroprotective effect of α-tocopherol as reported by Othman et al. (2011) who reported restoration of normal kidney histoarchitecture following co-treatment with α-tocopherol in adult male rats exposed to sublethal dosage of malathion. Normal Glomeruli were restored however presence of renal congestion suggests that cardiac insufficiency was still present.

Normal histoarchitecture was observed in the spleen at lower dosage of cartap (16.25 mg/kg) in group IV but at higher dose of cartap, group V (32.5 mg/kg) revealed mild congestion in red pulp of spleen. Our observation is in agreement to the findings of El Bendary et al. (2013) who observed a splenic congestion in adult male mice exposed to sublethal dosage of Profenofos and Chlorpyrifos. Following co-treatment with α-tocopherol normal histoarchitecture of spleen was observed in co-treated groups (VI and VII). It was observed by Rotbain et al. (2017) that splenic congestion could be a result of venous thrombosis, portal hypertension or congestive heart failure. This indicates that splenic congestion observed in the present study, might be a result of vascular injury leading to thrombus formation or cardiac abnormality following exposure to cartap. α-tocopherol effectively restored the normal histoarchitecture at either of the dose rates of cartap. This might be indicative of restorative potential of α-tocopherol as reported by Mandil et al. (2020) who observed normal histoarchitecture, of splenic tissue following concurrent treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of flubendiamide.

Cartap at lower dose in group IV (16.25 mg/kg) showed normal arrangement of myocardial fibres in cardiac tissue. However, at high dose in group V (32.5 mg/kg) mild degeneration of myocardial fibres along with haemorrhage was observed. Our observation is in agreement to the findings of Bas et al. (2011), who observed

degeneration of myocardial cells following sublethal exposure to chlorpyrifos in adult male wistar rats. Following co-treatment with α-tocopherol and cartap normal histoarchitecture was observed at both of the dose rates of cartap in group VI and VII. However, mild disruption of myocardial fibers was observed in co-treated group VII. It was observed by Shimokawa and Yasuda (2008) that myocardial degeneration is primarily a result of coronary artery disease resulting in reduced myocardial blood flow. Coronary artery disease develops when the major blood vessels (coronary arteries) that supply the heart become damaged or diseased. Cholesterol-containing deposits (plaques) in the coronary arteries and inflammation are usually to blame for coronary artery disease (Malakar et al., 2019). Hemorrhage is an acute loss of blood from a damaged blood vessel and is a result of local acute trauma (Gutierrez et al. 2004). Therefore, hemorrhage in the cardiac tissue might be because of local blood vessel trauma following exposure to cartap. Thus, we can conclude that cartap induced myocardial degeneration and hemorrhage could be a result of cartap induced vascular injury and increased plasma total cholesterol levels observed earlier in our study. α-tocopherol was able to restore the tissue histoarchitecture which might be due to serum cholesterol lowering by α-tocopherol observed earlier and protective effect of α-tocopherol against atherosclerosis (buildup of plaque) as reported by Terasawa et al. (2000). Similarly, cardioprotective effect of α-tocopherol against lipid peroxidation was reported by Gornicka et al. (2019) who reported reduced cardiac lipid peroxidation following treatment with α-tocopherol in growing male rats.

Testicular tissue showing mild degeneration of seminiferous tubules and detachment of germ cells was observed at lower dosage of cartap in group IV (16.25 mg/kg). An increased severity of degeneration of seminiferous tubules along with detachment of germ cells was observed at higher dosage of cartap in group V (32.5 mg/kg). Sebastian and Raghavan (2015) also observed degenerative changes in seminiferous tubules following sublethal exposure to Endosulfan in male adult mice. Similar observation was also made by Mandil et al. (2016) who observed degenerative changes in testes following sublethal exposure to flubendiamide in adult male wistar rats. Reyes et al. (2012) observed that xenobiotic induced degenerative changes in testes could be a result of generation of ROS resulting in oxidative damage. Rodriguez and Garcia, 1998 observed a direct relation between fall in plasma FSH and testosterone level and detachment of germ cells from semeniferous tubules

following short term treatment of adult male rats with oestradiol. Co-treatment with α-tocopherol and cartap revealed only mild congestion and no degenerative changes were observed in both the co-treated groups (VI and VII). Our observation is in agreement to the findings of Mandil et al. (2016) who observed restoration of tissue histoarchitecture with only mild changes in testes following treatment with α-tocopherol in adult male wistar rats exposed to sublethal dosage of flubendiamide. As observed earlier in the present study, α-tocopherol was able to moderately reduce testicular ACP, LDH and GGT levels in both co-treated groups which might be a result of testicular tissue repair as evident from the histopathological findings.

Normal histoarchitecture of cartilage and ciliated epithelium was found in trachea of cartap alone group IV (16.25 mg/kg). However, tracheal tissue revealed mild degeneration of cartilage and ciliated epithelium in cartap alone group V (32.5 mg/kg). Our observation is in agreement to the findings of Ali et al. (2018) who observed necrosis and degeneration of tracheal and bronchiolar epithelium following sublethal exposure to 2-4 D in adult male mice. In another study, Fialho et al. (2012) also reported degeneration of articular cartilage following sublethal exposure to thiram in adult male wistar rats. Cartap induced epithelial degeneration might be due to generation of ROS as reported by Shah et al. (2020) who observed epithelial cell degeneration in human ovarian epithelial cells exposed to Dieldrin, beta-hexachlorocyclohexane and dichlorodiphenyldichloroethylene (DDE) and suggested ROS induced DNA damage as a major cause for cellular degeneration. Degeneration of cartilage occurs as a result of direct injury or inflammatory reaction (Krishnan and Grodzinsky, 2018). Thus, injury to the cartilage following cartap exposure could be the reason for degeneration of cartilage. Following co-treatment with α-tocopherol normal histoarchitecture was observed in cartap co-treated group VI but degeneration of cartilage was observed in cartap co-treated group VII. Thus, based on our observations we can conclude that α-tocopherol was able to restore epithelial cells which might be due to its ability reduce ROS levels but was unable to protect cartilage.

5.7 Effect of α- tocopherol on mRNA expression of HSP-70 in testicular tissue of rats in different treatment groups following 28 days oral exposure to cartap

70-KDa Heat shock proteins (HSP-70s) are central components of the cellular network of molecular chaperones and folding catalysts (Mayer and Bukau, 2004).

Intracellularly localized HSP-70s are important part of the cell's machinery for protein folding, performing chaperoning functions, and helping to protect cells from adverse effects of physiological stresses (Tavaria et al., 1996; Morano, 2007). HSP-70 expression is the first-tier bio-indicator of chemical induced toxicity since this gene was found to be the first inducible gene in the organism after chemical stress (Gupta et al., 2005). Stress induced HSPs act by aiding the refolding of denatured proteins, thereby preventing adverse metabolic effects arising from accumulation of misfolded proteins and proteotoxic cell death (Vos et al., 2008). Pesticides when ingested by non-target organisms, generate reactive oxygen species and oxidative stress (OS) which catalyzes unfolding and aggregation of native proteins. In response, HSP-70s are synthesized within cells and block protein degradation (Rajak and Roy, 2018). Following 28-days exposure to cartap in adult male wistar rats the expression of HSP-70 gene was accessed in testes using RT-PCR technique. The results revealed a significant increase in fold change of mRNA expression for testicular HSP-70 at both cartap alone group (IV and V) compared to control group. Our observation is in agreement to the findings of Zhang et al. (2010) who observed a significant upregulation of HSP-70 gene in lungs of adult male wistar rats exposed to sublethal Paraquat intoxication. Similarly, Ismail et al. (2013) observed a significant up regulation in testicular mRNA expression of HSP-70 gene following sub lethal exposure to deltamethrin in adult male wistar rats. From our finding we can conclude that cartap induced oxidative stress and injury as observed earlier in our study were contributing factors in up regulation of HSP-70 gene expression in testes. Following co-treatment with α-tocopherol and cartap, non-significant change in fold change was observed in both the co-treated groups (VI and VII), which might be because α-tocopherol was ineffective in significantly reducing oxidative stress and testicular injury biomarkers level as observed earlier in the present study.

5.8 Effect of α-tocopherol on alterations in certain physical attributes of semen induced by cartap

Xenobiotic toxicants may directly damage spermatozoa, sertoli cells, leydig cells via ROS production or disrupt the endocrine regulation of spermatogenesis by altering hormonal balance thereby affecting sperm concentration (Bretveld et al., 2007). Spermatogenesis and male fertility are dependent upon the presence of testosterone and in the absence of testosterone or the androgen receptor,

spermatogenesis does not proceed beyond the meiosis stage (Walker, 2011). The activities of SDH, LDH and acid phosphatase are reported to be associated with different stages of germ cells (Pant, 1995). The germinal epithelium contains germ cells i.e., spermatogonia in different stages of development and sertoli cells (Grier et al., 2016). Spermatogonia undergo spermatogenesis to form mature spermatozoa in the seminiferous tubules of the testis (Kubota and Brinster, 2018). The activity of LDH is specific for germinal epithelium activity (Aydin et al., 1997). The increase in LDH activity suggests deterioration of germinal epithelium (Teng et al., 2016). A significant decrease in sperm concentration was observed in cartap alone group V (32.5 mg/kg) compared to control group. Our observation is in agreement to the findings of Pant and Srivastava (2003) who observed a significant reduction in sperm concentration in adult male wistar rats exposed to sublethal dosage of quinalphos. In the same study the authors also reported a significant increase in the activities of testicular LDH and GGT which is also in agreement to our finding. Sertoli cells provide physical support, nutrition and release growth factors to the developing spermatogonia (Shi et al., 2018). GGT plays a major role in amino acid transport for the synthesis of specific proteins known to be secreted by Sertoli cells (Tate and Meister 1981). Increase in its activity is due to impaired function of Sertoli cells (Hodgen and Sherins, 1976). An increase in the activity of LDH and GGT in the present study, suggests damage to germ cells as well as Sertoli cells which correlates to fall in sperm concentration in the present study. Further, an increase in testicular MDA levels and fall in ant-oxidant enzyme levels of SOD, catalase and GSH suggests ROS induced oxidative damage to testes. In another study Adamcovicova et al. (2016) observed a significant reduction in sperm concentration in adult male wistar rats exposed to sublethal dosage of Diazinon. These findings indicate that cartap affects sperm concentration at sublethal dosage which might be a result of ROS induced oxidative damage in testes or reduced testosterone level as observed earlier in the study. Increased activities of testicular ACP, GGT and LDH observed earlier in our study could also be in response to fall in sperm concentration. Non-significant change in sperm concentration in co-treated groups (VI and VII) compared to cartap alone groups (IV and V) which is in agreement to the findings of El-Demerdash et al. (2004) also observed no significant change in sperm concentration following treatment with α-tocopherol in adult male wistar rats exposed to fenvalerate. Thus, we can conclude that cartap produces a significant decrease in sperm concentration at

sublethal dosage and co-treatment with α-tocopherol was not effective in restoring sperm concentration. This might be due to failure of α-tocopherol to significantly reduce oxidative stress in testes or due to failure to restore plasma testosterone levels or restore testicular enzyme levels observed earlier in the present study.

The assessment of sperm vitality is one of the basic elements of semen analysis, and is especially important in samples where many sperms are immotile, to distinguish between immotile dead sperms and immotile live sperms (Bjorndahl et al., 2003). Oxidative stress status plays a critical role in induction of sperm abnormalities and higher susceptibility of sperm DNA to denaturation and fragmentation (Sikka, 1996). Kato et *al.* (2001) and Prahalathan et al. (2004) suggested that ROS mediated oxidative stress to spermatogenic compartment may be considered as one of the mechanisms of abnormal and dead spermatozoa following xenobiotic exposure. Following 28-day treatment with cartap (16.25 mg/kg and 32.5 mg/kg) percentage live spermatozoa were significantly decreased in group IV and V compared to control group. Our observation is in agreement to the findings of Mandil et al. (2016) who observed a significant decrease in percent live spermatozoa in adult male wistar rats exposed to sublethal dosage of flubendiamide. Similarly, Adamcovicova et al. (2016) also observed a significant decrease in percent live spermatozoa following sublethal dosage of diazinon in adult male wistar rats Our findings indicate that cartap exposure resulted in significant decrease in live percent of spermatozoa which might be due to cartap induced testicular oxidative stress as observed earlier. Co-treatment with α-tocopherol resulted in a moderate but non-significant increase in live percentage in co-treated groups (VI and VII) compared to cartap alone groups (IV and V). This is in agreement to the findings of El-Demerdash et al. (2004) who observed a non-significant increase in live percent following sublethal administration to Fenvalerate. Thus, we can conclude that cartap exposure resulted in reduced percent live spermatozoa and administration of α-tocopherol did not significantly improve live spermatozoa, the reason behind this may be inability of α-tocopherol significantly reduce oxidative stress. However, moderate improvement indicates some degree of protective effect of α-tocopherol against oxidative damage to spermatozoa.

The hypo osmotic swelling (HOS) test evaluates the functional integrity of the sperm's plasma membrane and also serves as a useful indicator of fertility potential of sperm (Ramu and Jayendran, 2013). The test is based on the swelling ability when

functional spermatozoa are submitted to hypo-osmotic solution resulting in swelling and coiling of the tail (Zubair et al., 2015). ROS induced lipid peroxidation is one of the major reasons for reduced membrane integrity of the spermatozoa (Sanocka and Kurpisz, 2004). Significant decrease in percent HOS+ve sperms was observed in cartap alone treatment groups (IV and V) compared to control group. Girish and Reddy (2017) also observed significant reduction in HOS+ve sperms following sublethal exposure to mancozeb in adult male wistar rats. These findings suggest the ability of cartap to produce membrane damage in spermatozoa leading to no swelling of membrane. Co-treatment with α-tocopherol resulted in moderate but non-significant increase in HOS+ve sperms in cartap co-treated group VI compared to cartap alone group IV. A significant increase in HOS positive spermatozoa in co-treated group VII was observed compared to cartap alone group V. Contrary to our observation, Mandil et al. (2016) observed non-significant change in percent HOS positive spermatozoa following concurrent exposure to α-tocopherol in adult male wistar rats exposed to sublethal dosage of flubendiamide. These findings indicate that α-tocopherol was effective in ameliorating cartap induced damage to membrane integrity, thus indicating a protective effect of α-tocopherol against ROS induced sperm membrane damage.

An intact acrosome allows a sperm to cross the egg's protective coat and join its membrane with the egg's plasma membrane via a complex sequence of events called acrosome reaction (Beiruti and Paiardi, 2011). The acrosome reaction is an exocytotic event induced by a Ca^{2+} influx and plays an essential role during fertilization, by making spermatozoa able of penetrating the zona pellucida of egg (Crozet, 1994). Acrosome is susceptible to elevated reactive oxygen species (ROS) and oxidative stress (El-Taieb et al., 2015). No significant change in percent spermatozoa showing intact acrosome was observed in any of the treatment groups compared to control group. Ravula and Yenugu (2021) observed no significant change in the mean inflorescence intensity in the acrosomes of spermatozoa subjected to FITC-PNA stain in adult male wistar rats exposed to sublethal dosage of pyrethroid mixture for a period of 15 months. Present study showed that cartap did not produce significant damage to acrosome following 28 days oral exposure.



Summary and Conclusions

CHAPTER-6

SUMMARY AND CONCLUSIONS

The present work was designed to study the toxic effect of cartap (16.25 mg/kg and 32.5 mg/kg) following sub-acute (28 days) exposure and ameliorative potential of α- tocopherol (100 mg/kg) on general body parameters, hematology, biochemical parameters, oxidative stress biomarkers (liver and testes), testicular injury biomarkers, plasma testosterone level, seminal attributes (concentration, % live, % HOS+ve and acrosomal integrity) and histopathology in adult male Wistar rats. Quantitative real time polymerase chain reaction (qRT-PCR) was performed to evaluate the expression of HSP-70 gene in testicular tissue.

Forty-two (42) male Wistar rats were divided into seven groups of six animals each. No clinical signs of toxicity were observed throughout the period of exposure (28 days) in both cartap alone (IV and V) and co-treated groups (VI and VII). No loss of hair or treatment-related mortality was observed during the entire experiment period. Rats of all the treatment groups (I, II, III, IV, V, VI, VII) exhibited normal activities and behavior throughout the period of study.

We observed a significant decrease in per cent body weight gain at higher dose of cartap in group V (32.5 mg/kg) but not at lower dose in group IV (16.25 mg/kg) compared to control group. Our observation showed that α-tocopherol was ineffective in significantly improving per cent body weight gain in co-treatment groups (VI and VII) compared to cartap alone group (IV and V).

The findings of present study revealed non-significant change in organ weights (relative and absolute) of lungs, liver, right kidney, left kidney, spleen, heart, right testes and left testes in different treatment groups (II to VII) compared to control group. `

Our findings revealed that values for different haematological parameters (RBC, Hb, PCV, Platelets, WBC, % Neutrophils, % Lymphocytes, % Eosinophils, % Basophils and % Monocytes) did not differ significantly in different treatment groups (II to VII) compared to control group.

We observed significant elevation in plasma Glucose, ALT and BUN levels following cartap exposure in group V (32.5 mg/kg) compared to control group. Our

findings also revealed moderate (non-significant) increase in plasma Total cholesterol, Triglycerides and AST levels in both cartap alone group IV and V. Our observations revealed non-significant change in plasma protein fraction (Total protein, Albumin and Globulin), Creatinine, Total Bilirubin, HDL and LDL levels in cartap alone groups (IV and V) compared to control group. We observed non-significant decrease and increase in plasma calcium levels in cartap alone group IV and V respectively compared to control group. In our observation, α-tocopherol was able to decrease the elevated level of plasma glucose, AST, and ALT in co-treated groups (VI and VII), however the decrease was non-significant compared to cartap alone group IV and V, respectively. Furthermore, we observed plasma Total Cholesterol, Triglycerides, and Calcium levels were significantly reduced in co-treatment groups (VI and VII) compared to cartap alone groups (IV and V).

In the present study, testicular and hepatic protein levels were reduced significantly in cartap alone groups (IV and V) compared to the control group. MDA levels were found to be increased significantly in cartap alone group IV and V compared to the control group in testes and liver, indicating lipid peroxidation (LPO). In our observation, activities of antioxidant enzymes Catalase (CAT), Superoxide dismutase (SOD), and Glutathione-S-Transferase (GST) were reduced significantly in testes and liver of cartap alone group V (32.5 mg/kg) compared to the control group. We found that testicular reduced Glutathione (GSH) levels did not differ significantly between different treatment groups compared to the control group, but hepatic GSH level was reduced significantly in cartap group V (32.5 mg/kg). Perusal of data revealed that α-tocopherol was able to restore the levels of Total protein in the liver and testes of co-treated groups VI and VII around control group values but was unable to produce any significant change in SOD, GSH, and GST levels. However, we observed a moderate increase in testicular catalase activity in group VI compared to cartap alone group IV. In our observation, hepatic antioxidant enzyme activities followed a similar pattern following α-tocopherol co-treatment except for a moderate and significant increase in hepatic MDA levels in group VI and VII compared to cartap alone group IV and V, respectively.

In the present study, significant elevation was found in testicular Acid phosphatase (ACP), Lactate dehydrogenase (LDH) and Gamma-glutamyl transpeptidase (GGT) activity in cartap alone group V (32.5 mg/kg). However, we did

not observe any significant change in testicular SDH level in different treatment groups (II to VII) compared to control group. As per our observation, α-tocopherol was able to reduce (moderately but non-significantly) elevated enzyme activities in both co-treated groups (VI and VII) compared to cartap alone groups (IV and V).

In the present study, plasma testosterone levels were observed to be significantly reduced in both cartap alone treatment group IV and V compared to control group. We observed that α- tocopherol failed to produce any significant change in co-treated group VI and VII compared to cartap alone treatment group IV and V.

Gross examination following necropsy in the present study, revealed no pathological changes in liver, spleen, heart, kidneys and testes. However, we observed that lungs of cartap alone treatment groups (IV and V) showed swelling and congestion and α- tocopherol was able to reduce visible swelling but not the congestion in co-treated groups (VI and VII).

Histopathological examination revealed degenerative changes in testes, heart, kidney and trachea, congestion in lungs, liver, kidneys and spleen following cartap exposure. Testes also revealed germ cell separation in seminiferous tubules in both cartap alone groups IV and V. Kidney tissue showed constriction of glomeruli at both doses of cartap in group IV and V. We observed hemorrhage in heart of rats in cartap alone group V and thickening of alveolar septa in both group (IV and V). Thickening of bronchial septa was also found in lungs of cartap alone group V.

Perusal of data revealed a significant upregulation in testicular HSP-70 gene expression in cartap alone group IV and V compared to control group. Non-significant change in HSP-70 gene expression was observed following co-treatment with α-tocopherol in group VI and VII. HSP-70 is bioindicator of chemical induced cellular toxicity. Thus, our finding indicates the ability of cartap to induce cellular toxicity at both the doses and the failure of α- tocopherol to ameliorate it.

We observed that sperm concentration was reduced significantly in cartap alone group V (32.5 mg/kg) compared to control group. Per cent live spermatozoa and Per cent HOS+ve spermatozoa were reduced significantly in both cartap alone treated groups (IV and V) compared to control group. In the present study, we did not observe any significant change in per cent spermatozoa showing intact acrosome in

different treatment groups (II to VII) compared to control group. α-tocopherol co-treatment had no significant effect on sperm concentration, however we found moderate improvement in % live spermatozoa in co-treated group VI and VII compared to cartap alone group IV and V. % HOS+ spermatozoa were found to be significantly increased in co-treated group VII compared to cartap alone group V.

Conclusions

Based on the results of present study we can conclude that:

1. Cartap exposure produced a significant decrease in per cent body weight gain at the dose of 32.5 mg/kg.
2. Absolute and relative organ weights of lungs, liver, kidneys, spleen, heart and testes showed no significant change following cartap exposure at both the doses.
3. Haematological parameters were not significantly affected following cartap exposure.
4. Hyperglycemia and alteration in plasma lipid profile was evident based on plasma biochemical findings following cartap exposure. Plasma ALT, AST and BUN levels were also increased following cartap exposure.
5. Calcium levels in plasma showed irregular pattern in both cartap doses and requires further investigation.
6. Cartap exposure produced oxidative stress in liver and testes as suggested by significant decrease and increase in testicular total protein and MDA levels at both the doses. Also, SOD, CAT and GST levels were decreased significantly in both the organs at cartap dose of 32.5 mg/kg.
7. Testicular injury following cartap exposure was evident based on elevated levels of testicular injury biomarkers (ACP, LDH and GGT).
8. Fall in plasma testosterone levels was observed following cartap exposure.
9. Respiratory system involvement following cartap exposure was apparent based on the observed gross and histopathological findings in lungs and trachea.
10. Histopathological findings showed multi organ toxicity.
11. Histopathological findings and upregulation of HSP-70 gene in testes provided further evidence for reproductive toxicity following cartap exposure.

12. Cartap at higher dose (32.5 mg/kg) decreased seminal attributes (concentration, per cent live, per cent HOS+ve).
13. α-tocopherol (100 mg/kg) co-treatment showed improvements in levels of Glucose, Total cholesterol, Triglycerides, AST, ALT and Calcium but showed poor antioxidant activity against cartap induced oxidative stress in liver and testes. Testicular injury biomarker levels were reduced moderately but non-significantly. Seminal attributes showed moderate increase in per cent live and % HOS+ve spermatozoa. However, testosterone levels and HSP-70 gene expression did not show any appreciable change. Apparent reduction in the severity of histo-pathological changes in all the studied organs suggest restorative potential of α-tocopherol.

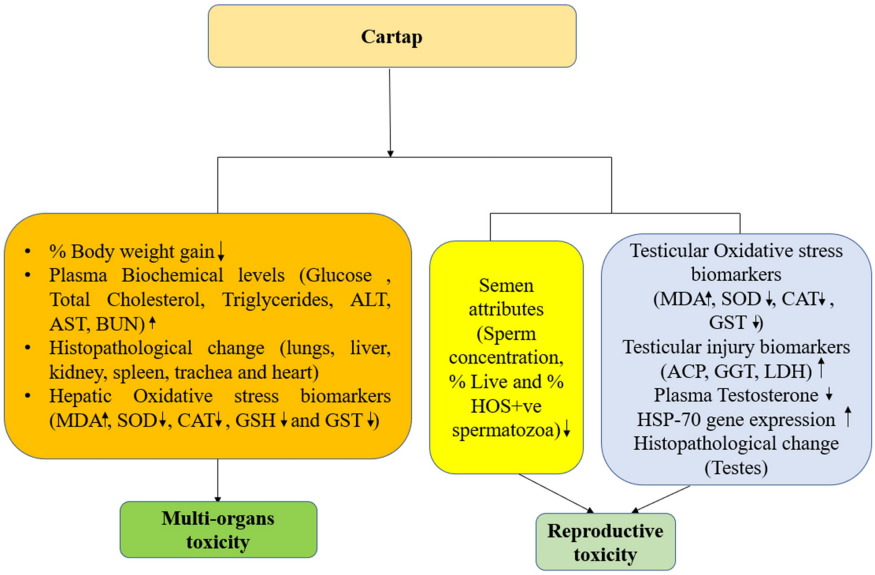
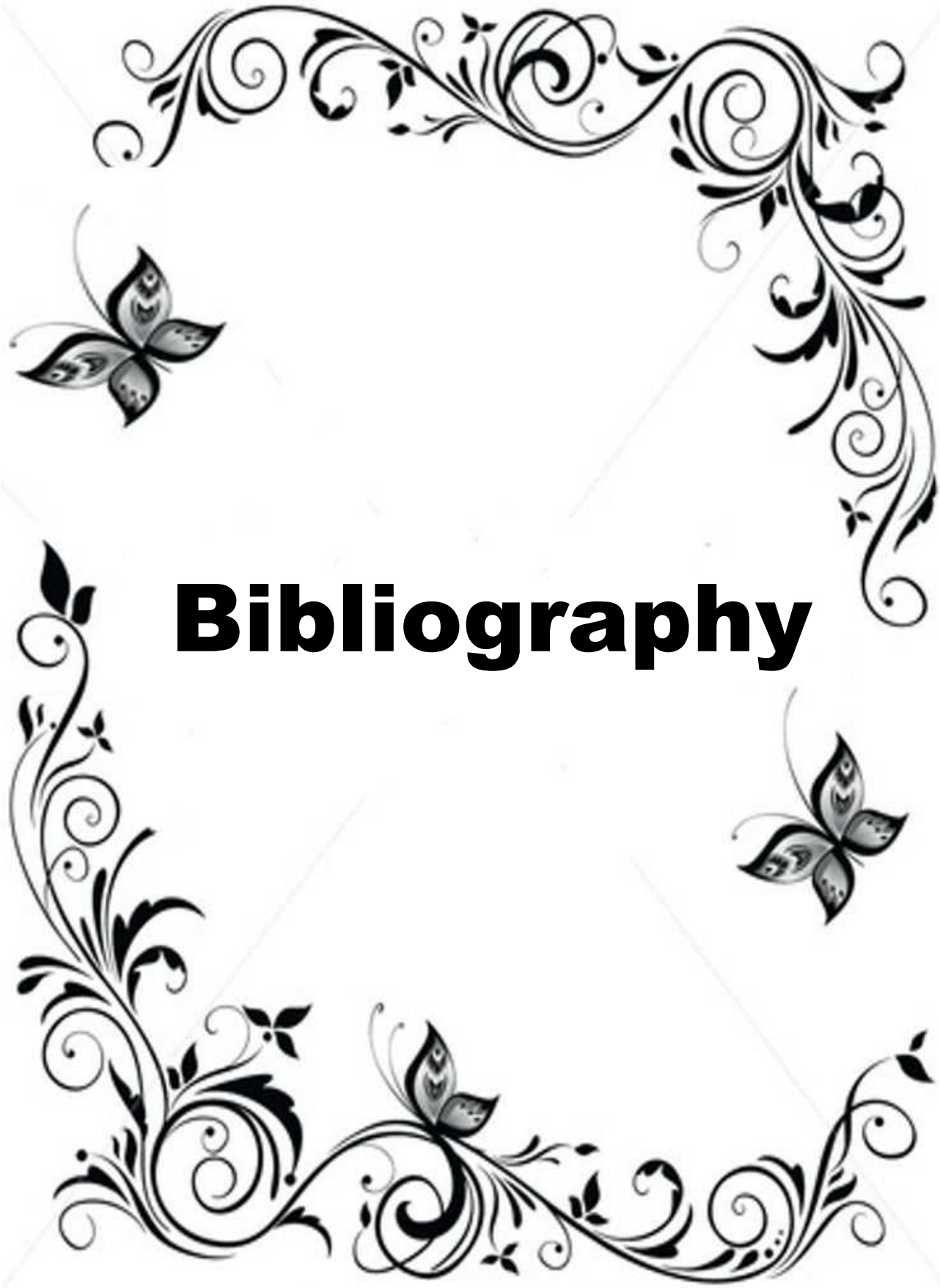


Fig. 78: Mechanistic pathways showing toxicity of cartap in male rats in the present study.



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I **Dr. Sugam Thakur**, Enrolment No **V-2109/19** undertake that I give copy right to the DUVASU, Mathura of my thesis entitled **"Studies on cartap toxicity in male rats and its amelioration by α-tocopherol."**

I also undertake that patent, if any, arising out of research work conducted during the programme shall be filed by me only with due permission of the competent authority of U.P. Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura (UP).

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