EVALUATION OF CARTAP-INDUCED IMMUNOTOXICITY FOLLOWING SUBACUTE EXPOSURE IN MICE

Thesis

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Master of Veterinary Science
(Veterinary Pharmacology)

2015

Dedicated to....

Maa Pitambara

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Certificate

This is to be certified that the research work embodied in this thesis entitled **"Evaluation of cartap-induced immunotoxicity following subacute exposure in mice"** *submitted by* **Dr. Laxman Prasad Sharma, Roll No. M-5330,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Pharmacology** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Laxman Prasad Sharma, Roll No. 5330,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

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We the undersigned members of Advisory Committee of **Dr. Laxman Prasad Sharma, Roll No. 5330,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Pharmacology**, *agree that the thesis entitled* **"Evaluation of cartap-induced immunotoxicity following subacute exposure in mice"** *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

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Laxman

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(Laxman Prasad Sharma)

ABBREVIATIONS

%	:	Percent (age)
μg	:	Microgram
μl	:	Microliter
μM	:	Micromole
AChE	:	Acetylcholine esterase
AEBSF	:	4-(2- Aminoethyl)benzenesulfonylfluoride
ALP	:	Alkaline phosphatase
ALT	:	Alanine aminotransferase
AST	:	Aspartate aminotransferase
ATP	:	Adenosine Tri Phosphate
b.wt.	:	Body weight
BSA	:	Bovine Serum Albumin
CAT	:	Catalase
CDNB	:	1-chloro-2, 4-dinitrobenzene
CK	:	Creatine kinase
CNS	:	central nervous system
con-A	:	concanavalin-A
DAB	:	Diaminobenzidine
DCFH-DA	:	2'7'-dichlorofluorescin diacetate
DEPC	:	Diethyl pyrocarbonate
DF	:	Dilution Factor
$DiOC_6$	:	3,3-dihexyl-oxacarbocyanine iodide
DLC	:	Differential Leucocyte Count
DMEM	:	Dulbeco's modified eagle medium
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribo Nucleic Acid
DTH	:	Delayed type hypersensitivity
DTNB	:	5,5'-dithiobis-2-nitrobenzoic acid
EC	:	Molar extinction coefficient
EDTA	:	Ethylene Diamine Tetraacetic Acid
ELISA	:	Enzyme-linked immunosorbent assay
et al.	:	and others/ et. alibi
FBS	:	Fetal bovine serum
FCA	:	Freund's complete adjuvant
Fig.	:	Figure
FITC-annexin-V-PI	:	Fluorescein isothiocyanate annexin-V-propidium iodide

gm	: Gram (s)
GPx	: Glutathione Peroxidase
GR	: Glutathione Reductase
GSH	: Reduced Glutathione
GSSG	: Oxidized Glutathione
GST	: Glutathione S-Transferase
H and E	: Haematoxilin and Eosin
h	: Hour(s)
H_2O	: Water
H_2O_2	: Hydrogen Peroxide
HBSS	: Hanks Balance Salt Solution
IAEC	: Institutional Animal Ethics Committee
i.e.	: That is
i.p	: *intra peritoneal*
i.v.	: *intra venous*
IM	: Imidacloprid
K_2HPO_4	: Dipotassium hydrogen phosphate
KCl	: Potassium chloride
kg	: Kilogram
KH_2PO_4	: Potassium dihydrogen phosphate
LD_{50}	: Lethal Dose-50
LDH	: Lactate dehydrogenase
LMP	: Low melting point
LPO	: Lipid Peroxidation
LPS	: Lipopolysaccharides
M	: Molar
MDA	: Malondialdehyde
mg	: Milligram(s)
$MgCl_2$	: Magnesium chloride
min	: Minute(s)
ml	: Milliliter
mM	: Milimole
MTP	: Mitochondrial transmembrane potential
MTT	: Thiozolyl blue tetrazolium bromide
mRNA	: Messenger ribonucleic acid
n	: Number of observations
NAC	: N-Acetyl Cysteine
NADP	: Nicotinamide adenine dinucleotide phosphate
NADPH	: Nicotinamide adenine dinucleotide phosphate (reduced)
NaOH	: Sodium hydroxide

nM	:	Nanomoles
NO	:	Nitric oxide
NOAEL	:	No observed adverse effect level
NOS	:	Nitrous oxide synthatase
^{O}C	:	Degree Celsius
OD	:	Optical density
OECD	:	Organization for Economic Co-operation and Development
$OH^{\cdot}$	:	Hydroxyl Radical
$ONOO^{-}$	:	Peroxynitrite Anion
p.	:	Page
p.o.	:	Per os
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase chain reaction
PI	:	Propidium Iodide
pp.	:	Pages
ppm	:	Parts per million
ROS	:	Reactive Oxygen Species
rpm	:	Revolutions per minute
RPMI-1640	:	Rosewell Park Memorial Institute-1640
s	:	Second
S.E.	:	Standard Error
SDH	:	Sorbitol dehydrogenase
SDS-PAGE	:	Sodium Dodecyl Sulphate polyacrylamide gel electrophorosis
SH	:	Sulfhydryl
SOD	:	Superoxide Dismutase
TBA	:	Thiobarbituric acid
TBE	:	Tri-Borate-EDTA buffer
TCA	:	Trichloroacetic Acid
TLC	:	Total Leucocyte Count
TNF	:	Tumour Necrosis Factor
UV	:	Ultraviolet
WHO	:	World Health Organization
γ-GT	:	Gamma-glutamyltranspeptidase
ΔOD	:	Change in optical density
$\Delta\Psi$M	:	Mitochondrial Transmembrane Potential

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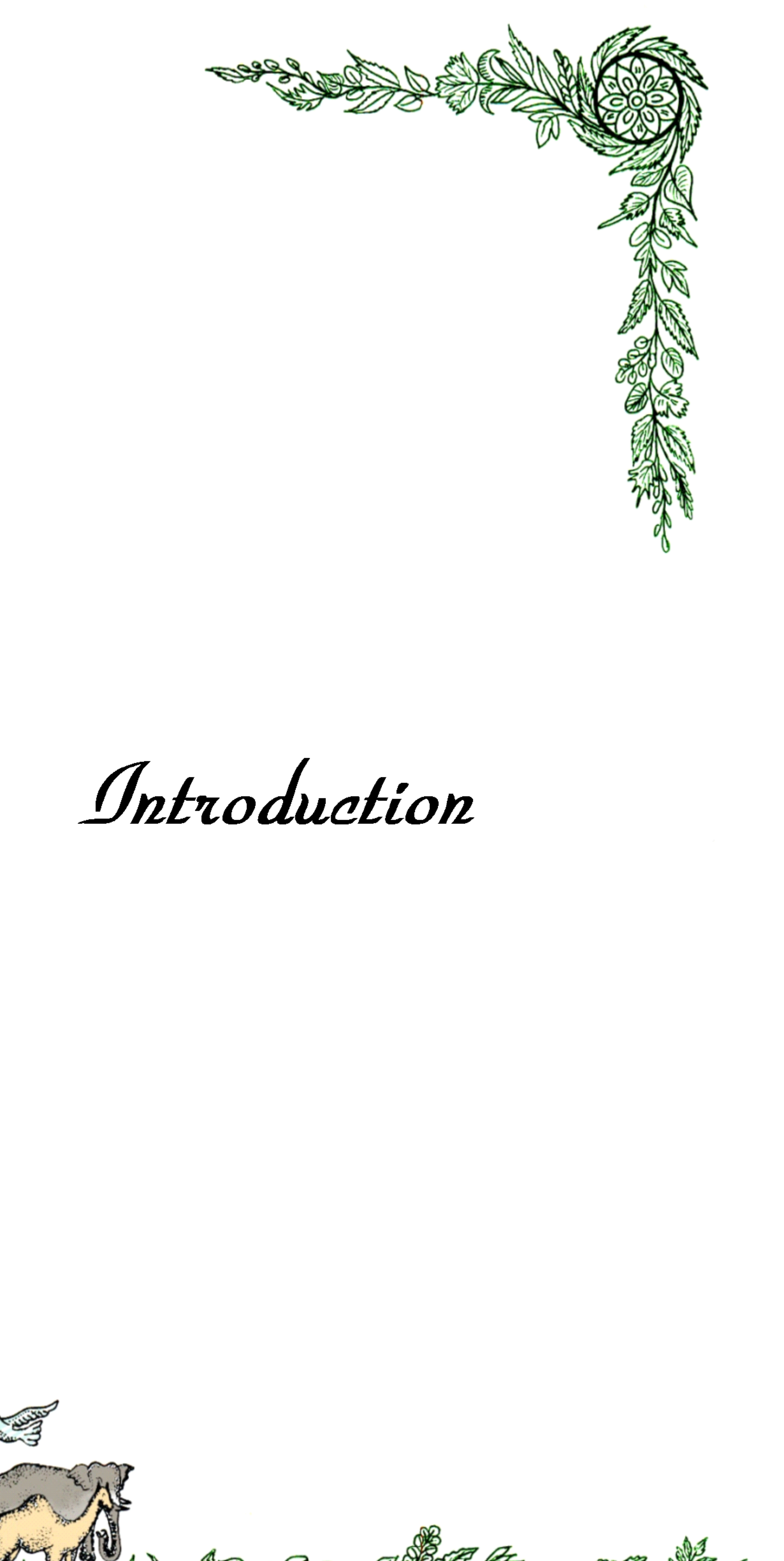

Introduction

1 INTRODUCTION

Pesticides are used globally in agrarian and non-agrarian economy countries to fulfill the increasing demand of food of growing population on earth and control of various pests including unwanted weeds that are harmful to the growing plants along with that they also control many insect vectors of many disease of animals including man and plants.

Pesticides are substances meant for attracting, destroying, or mitigating any pest (US Environmental, 2007). According to FAO (2002) pesticide as: any substance or mixture of substances intended for preventing, destroying, or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals, causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances that may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant, or agent for thinning fruit or preventing the premature fall of fruit. Also used as substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport.

They are a class of biocide. In general, a pesticide is a chemical or biological agent (such as virus, bacterium, antimicrobial or disinfectant) that deters, incapacitates, kills, or discourages pests. Target pests include insects, plant pathogens, weeds, mollusks, birds, mammals, fish, nematodes (roundworms) and microbes that destroy property, cause nuisance, spread disease or are disease vectors. Globally, consumption of pesticide is several million

tons per year. Pesticide occupy a special position among the chemical to which man can be exposed, in that they are readily diffuse into the environment for the purpose of killing or damaging some form of life. Although pesticides have enormous number of benefits, along with these compounds have many drawbacks, such as potential toxicity to humans and other desired species either direct or indirect exposure. According to the Stockholm Convention on Persistent Organic Pollutants, 9 of the 12 most dangerous and persistent organic chemicals are pesticides (Gilden *et al.*, 2010). Although pesticide is a broader term, includes all of the following: herbicide, insecticide, insect growth regulator, nematicide, termiticide, molluscicide, piscicide, avicide, rodenticide, predacide, bactericide, insect repellent, animal repellent, antimicrobial, fungicide, disinfectant (antimicrobial), and sanitizer (Carolyn *et al.*, 2013).

Several classes of pesticide like organochlorine, organophosphate, carbamate, thiocarbamate, pyrithroids, neoniconamides, rotenones, fumigants, rodenticides, etc. now used in agricultural field as well as for house hold purpose also that which undesirable species including human can be exposed either by inhalation of fumes or ingestion of the pesticides that are hazardous to them. India ranked as 2nd largest manufacturer of basic pesticide in Asia and 12th globally (Gupta, 2004) with annual turnover of 90,000 tons. Chemically, pesticide/ insecticide belongs to the organochlorine, organophosphorus, carbamate, thiocarbamate, pyrithrin or pyrithroids, neonicotinamide, zinc-phosphides etc.

Thiocarbamates are semisulphur analogue of carbamate and exist as a salt or ester of carbamic acid, its ester form present in two isoform one is S- thiocarbamate or O-thiocarbamate. Thiocarbamates herbicide mainly belongs to the S- thiocarbamate isoform. All thiocarbamates pesticides are liquid or solid at room temperature, with high vapour pressure, low melting point and soluble in most of the organic solvents (IARC 1976; Worthing and Walker, 1983). All are volatile in nature, parent compound and their sulphone and sulfoxide derivative are stable at acidic medium (pH 2) and less stable at alkaline medium (pH > 10) (Casida *et al.*, 1974).

Thiocarbamates pesticide are used in the form of insecticide, herbicide and fungicide in agriculture and as a biocide for industrial and house hold application; some compounds used

for controlling insect vector in public health programme too. It is produced in massive amount every year because of wide range of application worldwide. Like other pesticide, thiocarbamate also reach the soil by various routes like direct application as well as by drift from foliage treatment. All thiocarbamates loss from soil by a various mechanism but mostly by volatilization which is depend on the moisture content of soil and another less important mechanism is evaporation, depend on soil organic matter and clay content (Fang, 1975; Anderson and Domsch, 1980). Thiocarbamates disappear within several weeks from soil (Gray, 1971), soil microbe play significant role in degradation of thiocarbamates from soil (Kaufman, 1967). Microbe hydrolyzes the ester linkage of compound and form mercaptans and secondary amine which convert into 2, 3- dichloroallyl alcohol by transthiolation then to acid by oxidation reaction (Fang *et al*., 1964; Kaufman, 1967).

Thiocarbamates are sensitive to light, photo degradation depend on state of compound, hence minimum effect on solid state due to poor penetration of UV light through compound (Demarco and Hayes, 1979). Absorption of UV light break the carbonyl bond (C-S bond) and produce free radical, this radical combine with the proton 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). Thiocarbamatesare readily absorbed by the plants and metabolized by thiol sulphur oxidation to corresponding sulfoxide (Hubbell and Casida, 1977; Carringer *et al*., 1978; Chen and Casida, 1978), this reactive sulfoxide react with the glutathione or cystiene to give carbamylated derivative (Hovarth and Pulay, 1980). Herbicidal action of the thiocarbamates is due to reactive sulfoxide intermediate (Casida *et al*., 1974).

Thiocarbamate pesticides enter into the animal and human body either by ingestion (mistakenly) or by inhalation of aerosol during the spraying of pesticide in agriculture field.They are rapidly absorbed through the skin and mucous membrane of digestive tract or lungs. Fang *et al*. (1964) reported that when rat given Pebulate @ 0.16–1.95 mg/kg b.wt. approximately 80% eliminated within 3 days and small amount were detected in organ and tissue like lung, liver and kidney. Eptc @ 0.6–103 mg/animal almost complete elimination of compound within 15 h and 35 h in low dose and higher doses, respectively (Ong and Fang, 1970). Approximately

97% of orally administered Molinate @ 72 mg/kg b.wt. eliminated within 78 h without differences in male and female animals and tissue residue level decrease 13.8% to 3.7% within period of week (Debaun *et al.*, 1978a).

Thiocarbamate metabolized into the body by main two mechanism one is sulfoxidation followed by conjugation with glutathione then acetylated and eliminated as S-carbamoyl mercapturic acid (Hubbell and Casida,1977; Chen and Casida, 1978), another mechanism is oxidation by liver microsome NADH involve attack on α- carbon of ethyl and propyl group, β carbon of the propyl group. Metabolites are hydroxylated at α-carbon to nitrogen and sulphur decomposes at physiological pH, carbonyl sulfoxide further metabolized to carbon dioxide (Dalvi *et al.*, 1974; Chen and Casida, 1978).

Bis (thiocarbamate) derivative of 2-(dimethyl amino) propane -1, 3-dithiol is known as cartap. Cartap is a first synthetic organonitrogen insecticide having structural similarity with the natural insecticide nereistoxin derived from the marine annelid worm *Lumbriconereis heteropoda* and isolated in 1934 (Okaichi and Hashimoto, 1962; Sakai, 1969). It was first synthesized and used in Japan in1964 (Chiba *et al.*, 1967; Ray, 1991; Tomlin, 2000) and categorized as a relatively safe compound and now used worldwide (IPCS, 2000-2001). IPCS (2002) categorized cartap as a moderately toxic substance (Toxicity class-II). Cartap normally used as a hydrochloride has broad spectrum of activity against insect and first choice for control of chewing and sucking pests e.g. weevil and caterpillar in paddy and diamond black moth in cabbage field, resulting paralysis of insect.

It's used in India began in or after 1988 after the agreement with Japan from where technical product was imported. In India two formulations are available, one is 50% soluble powder and another is 4% granular form (Abbasi and Krishnan, 1993). Toxicity of cartap is rarely occur, it is very toxic to aquatic organism and produce adverse effect for a period of time in aquatic environment.

Cartap cause generation of reactive oxygen species in time and dose dependent manner by Ca^{2+}dependent mechanism, resulting oxidative damage to cells and induced oxidative stress (Gordeeva *et al.*, 2003; Suzuki *et al.*, 2003). Supinski *et al.*(1999), Trump and Berezesky,

(1995) suggested that increased concentration of Ca^{2+}causes activation of phospholipase A_2, phospholipase C, protease and several endonuclease, where as other activate kinase, intracellular messengers e.g. $IP_{3,}$ cADRP, and nitric oxide. Increased concentration of intracellular Ca^{2+} by cartap exhibit adverse effect on cytoskeleton, including loss of F-fibers, loss of actin plasmalema attachments and destabilization of microtubule resulting formation of cytoplasmic bleb in C_2 C_{12} myocyte cell line (Bennet and Weeds, 1993; Trump and Berezesky, 1995).

Cartap cause increase the activity of creatine kinase (CK) and lactate dehydrogenase (LDH) and their isoenzyme in serum and muscle indicating muscular damage (Gupta *et al*., 2002), leak of CK and LDH indicate muscle damage due to depletion of adenosine triphosphate and phosphocreatine (Gupta *et al*., 1994; Angulo and Lomonate, 2003; Gupta and Goad, 2000).

The extensive use of pesticides in public health program as well as for agriculture purpose has caused severe environmental pollution and potential health hazards. The persistence and extreme stability of various pesticides in the natural environment are the ultimate source of contamination of feed and water at the dietary level. Toxicity of pesticide results or caused not due to intake of a single or few low doses of a given pesticide, but due to continuous oral intake of very small quantities over a reasonable period of time; resulting toxicity .

Problems and/or outbreaks are reported to occur among animals and human from insecticide toxicity, which usually occurs either from direct exposure to insecticides or indirectly from contaminated feeds or water by such chemicals. Prolonged exposure to insecticides causes chronic neurological syndrome, malignant tumors, immunosuppressive action, teratogenic effect, abortion and decreased male fertility in experimental animals (Nafstad *et al.,* 1983; Meeker *et al.,* 2006; Yousef, 2010).

The immune system plays a vital role in maintaining health of animal and human being and several evidence indicate that immune system can be the target for immunotoxic effects of a certain variety of chemicals including the environmental pollutants, such as polychlorinated biphenyls, chlorinated dibenzo-p-dioxins, pesticides and heavy metals (Krzystyniak *et al.,* 2005) by some unknown mechanism of immunotoxicity. There are numerous factors that

influence toxicity; for instance, external factors such as housing of the animals, stress, exercise, feeding schedule and intrinsic factors such as hormonal status or genetic makeup of animal make each case unique. Also feedback or compensatory mechanisms associated with immunoregulation could further complicate these predictions. In many instances, it is these compensatory mechanisms that trigger immune mediated diseases and cellular dysfunction.

The susceptibility of the immune system to toxic damage depends on many factors. Host resistance to infectious agents and spontaneous neoplasm's depends on immunocompetant cells, which are subject to continuous proliferation and differentiation and because of that they become excessively susceptible to variety of chemical and physical agents. It has been hypothesized that altered immune function may be an indicator of increased incidence for the development of immunologically based diseases such as cancer, hypersensitivity and autoimmunity (Luster and Rosenthal, 1993; Esser and Jux, 2009). The immune system have highly organized co-operation and regulation between various type cells, which is ensured on one hand by soluble mediators like immunoglobulins, immune hormones and cytokines and on other hand by intercellular interactions at the level of membrane receptors and anti receptors. All agents that affect the fine balance between mechanisms mentioned can cause agent specific or species specific immunity damage which in majority of cases results in immunosuppression (Kacmar *et al.,* 1999).

The exposure of animals to residual concentration of pesticides can lead to immunosuppression either directly or with participation of stress mechanisms and that of the neuroendocrine system. Immunosuppression leads to change in longevity of life span, increased susceptibility to infectious diseases and decreased immune response to vaccination. Thus there is an urgent need to obtain more information regarding the manner in which various pesticides alter the immune system. Modulation of immune response through stimulation or suppression may help in maintaining a disease free state.

Earlier, most of the studies on toxicity of pesticides have been focused on enzyme alterations, gross pathological effects, mutagenic and carcinogenic potential of these agents. According to Pesticide Manual (1994) very few ingredients present in pesticides that are used

in our country are subjected to immunotoxicological studies. Rapid development of agrochemical industries and extensive use of such agents in agriculture field as well as for public welfare program makes it necessary to investigate not only the acute and chronic toxicity but also immunotoxic effects of these compounds.

In recent years the effects of pesticides on immune response have received great attention. Luster *et al.* (1990) have designated that immune system as sensitive indicator of toxicity assay especially for environmental contaminants and pollutants which may have residual effects in the ecosystem. Taking into account the importance of immunotoxicity (Moore *et al.*, 1982) suggested that all newly introduced compounds should be tested for immunotoxicity before their introduction into the market.

It is now clear that important changes in host immunity may occur after pesticide ingestion. Many pesticide chemicals like Pyrethroids (Prater, 2003), Organophosphates (Galloway and Handy, 2003), Organochlorines (Kumar *et al.,* 2002) are known to cause immunosuppression. Moreover, immunomodulation by agrochemicals at low level of dietary intake in the form of toxic residue in food might decrease the resistance to infectious agents and cause breakdown to protection by vaccination (Rodgers, 1996).

Oxidative stress has been identified as one of the major factor for immunomodulation in any species of animal. Oxygen is essential to sustain life; conversely, breakdown products of oxygen such as reactive oxygen species (ROS) can adversely affect the cellular function and survival (De Lamirande and Gagnon, 1995). When ROS generation is increased beyond the capacity to cellular antioxidants, it results into oxidative stress.

There are several studies on immunotoxicity and oxidative stress of most groups of pesticides viz. organochlorine, organophosphates, carbamates, and synthetic pyrithroids; however, reports are lacking on detailed study on immunotoxicity of thiocarbamates groups of pesticide to which cartap belongs.

There are very few studies related to acute, chronic, as well as carcinogenic potential and reproductive toxicity of cartap hydrochloride are reported but, immunotoxicity of cartap hydrochloride is yet to be studied in details.

This investigation will help in understanding the specific immunological alterations caused by cartap hydrochloride and to explore probable mechanism involve; therefore the proposed study will be undertaken with the following objectives:

Objectives:

- **To investigate the immunotoxic potential of subacute exposure of cartap in mice.**
- **To investigate the role of oxidative stress and apoptosis in cartap- induced immunotoxicity in mice.**

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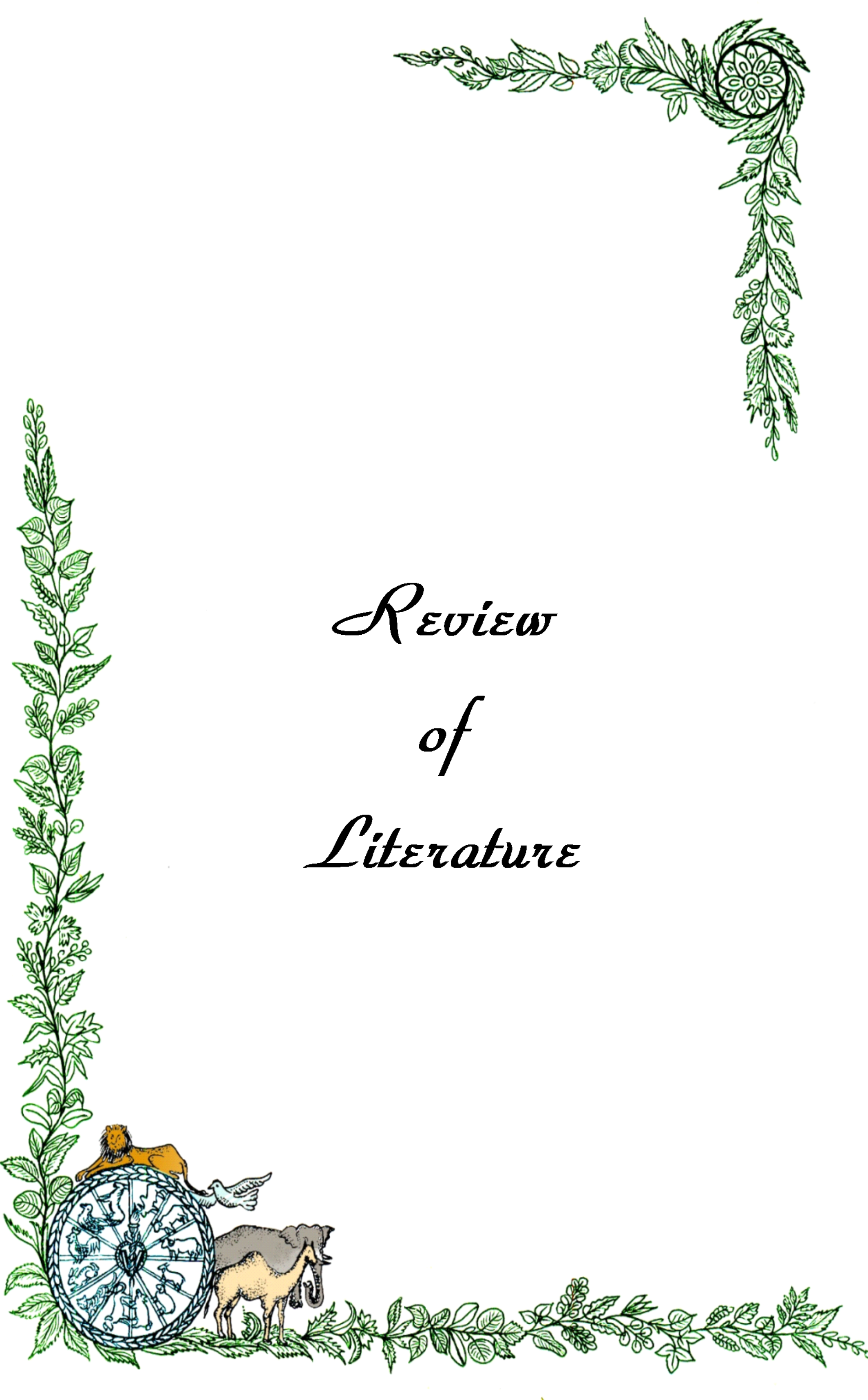

Review of Literature

2 REVIEW OF LITERATURE

The high industrialization and production through rapid development in an agriculture practices, animal husbandry and human civilization have brought by using a number of synthetic chemical in our environment. Pesticide may be any chemical, physical and biological agent that will help in controlling or killing of undesirable pest which may be plants or insects or animals or microorganisms. Pesticide is a generic name for a variety of chemical agents that are classifying on the basis of their use and organism killed. The term "pesticide" includes insecticides, herbicides, rodenticides as well as disinfectants, fumigants and wood preservatives. These compounds play vital role in controlling agricultural, industrial, home/garden and public health pests throughout the world. Because pesticide have potential to reduce the incidences of vector borne diseases with in lower cost, provide better quality food and service to society, that is why public has been tolerating their uses (Aspelin, 1998). However, these economic and health benefit are not achieved without any risk and possible adverse health effects to humans, domestic as well as wild animal and the surrounding natural environment where these compound are used. It has been estimated that 85% to 90% of the pesticide used in agriculture field never reach to target organism instead of this it is dispersed into the atmospheric air, water, and soil (Repetto and Baliga, 1996). FAO/WHO joint expert committee on pesticide in food (1965) has recommended the establishment of data on maximum safe level of pesticides for different animals species mainly on the basis of development of clinical overt toxicity. But their extensive and continuous use even at recommended level caused problem to health hazard to human being as well as for domestic and wild fauna.

Man and animals are continuously exposed to various pesticides either directly or indirectly via their residue in feed, water, dairy products, meat, eggs, vegetables, soil and air (Dahiya and Chauhan, 1982; Agnihotri *et al*., 1987; Ram *et al*., 1987). Pesticides compounds not only cause clinical toxicity but also interfere in various physiological homeostatic mechanisms. The high emerging incidences of infectious diseases even after proper vaccination of population of human being and animals has attract the attention toward the environmental pollutant and their effect on immune system of animal body.

Both cellular and humoral immune system plays a crucial role in maintaining the body integrity and providing the defense against any foreign challenges, any alteration in this homeostatic mechanism will cause deleterious effects. Decreased immunocompetance may encounter by a pathogens while increased immune function may be beneficial or detrimental to the host as it may enhanced disease resistance to the development of autoimmunity or allergic reactions. In general low level of pesticide exposure some time enhances the immune function but sudden exposure to high level or low level exposure for a longer period of time cause immunosuppression by direct or indirect mechanism like action on lymphoid cells, lymphoid cells distribution and immunoglobulin metabolism, T cell and B cell co-operation and macromolecule biosynthesis; hence altering humoral and cellular immune responses.

2.1. Cartap hydrochloride

2.1.1. Chemical (IUPAC) name:

[*S, S2* -(2-dimethylaminotrimethylene) bis (thiocarbamate) hydrochloride].Cartap hydrochloride an organonitrogenous thiocarbamates insecticide , has been recognized as a nereistoxin analogue and categorized as a relatively safe compound; since it was first synthesized and used in Japan in 1964 (Chiba *et al*., 1967; Ray, 1991; Tomlin, 2000). It is a derivative of nereistoxin which is a naturally occurring insecticidal substance isolated from the marine segmented worms *Lumbrinereis heteropoda* and *L.brevicirra*. Nereistoxin was isolated by Nitta in 1934, the structure was elucidated by Okaichi and Hashimoto (1962) and synthesized in 1965 by Hagiwara *et al*. Cartap hydrochloride is one of the most potent of the nereistoxin derivatives (Chemical abstracts Service, CAS No.15263-52-2) chemical name 1, 3-di (carbamoylthio)-2-dimethylaminopropane; Technical grade of cartap hydrochloride contains 98.2% of pure compound /ingredient. In India it is available as 25% and 50% water soluble

powder, 2% dust, 4% and 10% granule and 2% fine granules under various brand names such as Caldran^{R}, Padan^{R}, Patap^{R}, Sanvex^{R}, Thiobel^{R}, Vegetox, NTD-2, TA-7, and TI-1258.It is primarily used to control rice stem borer and leaf folder pests in rice paddy field and diamond back moth and aphids in cabbage and cauliflower crops.

2.1.2. Structural formula [$C_7H_{16}ClN_3O_2S_2$]

Fig. 1: Structure of cartap hydrochloride

2.1.3 Physiochemical properties

Table 1: Physiochemical properties of cartap hydrochloride

Sr no.	Parameter	Properties
1	Physical appearance	A crystalline solid with slight odour
2	Colour	White to light brown.
3	CAS number	15263-52-2
4	Molecular formula	[$C_7H_{16}ClN_3O_2S_2$]
5	Molecular weight	273.81
6	Melting point	179 – 181°C (decomp)/354.2- 357.8 ° F(decomp)
7	Solubility	Soluble in water 25 gm / liter at 25°C
8	Chemical family	2-dimethylaminopropane-1,3-dithiol
9	Symbol	Xn, N
10	Vapour pressure (mmHg)	Negligible
11	Flammability	Not flammable
12	pH (1% solution)	3.0 - 4.5
13	Storage stability	Period of 2 years (if kept in closed container)
14	Hygroscopicity	Slightly hygroscopic
15	Volatility	Negligible
16	Solubility in other solvent (e.g. alcohol, acetone)	Very slightly soluble in methanol and ethanol.Insoluble in acetone, diethyl ether, ethyl acetate, chloroform, benzene and n-hexane.
17	Moisture content	1.04 %
18	Density :	0.691 g/ml

(SOURCE: Universal Crop Protection (Pvt) Ltd., 2005; Villa Crop Protection (Pvt.) Ltd., 2005; JMPR, 1976; British Crop Protection Council, Survey, UK, 2000; Nagarjuna Agrichem Ltd., 2013; Tomlin, 2000**)**

2.1.4. Toxicokinetics

Cartap hydrochloride is rapidly absorbed, metabolized and excreted in urine both in rats and mice. There are no apparent differences in absorption and excretion of 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 of animal as might be expected that predominantly water-soluble nature of compound. It is stable in acidic media, but hydrolyzed in alkaline and neutral medium/solution. In rat, orally administered cartap hydrochloride primarily metabolized by hydrolysis of the carbonyl carbon and oxidation of the sulphur atom to the sulfoxide (SO), sulfone (SO_2) and ultimately the sulfate (SO_3H) (Kamesaki *et al*., 1976a). In mammals, nereistoxin is methylated and oxidized at the sulfur atom as well as undergoing a series of oxidative demethylation reactions at the dimethylamino moiety. A small quantity of products was observed to correspond to sulfates.

2.1.5. Mechanism of action

Cartap is a synthetic analogue of thiocarbamates group of insecticide has structural similarity with nereistoxin. Nagata *et al*. (1997) by using single patch clamp technique on pheocytochroma cells of rat showed that cartap hydrochloride act on the nicotinic acetylcholine receptor (nAchR) in open state and cause burst of sub-conductance of action potential. Ray (1991) showed that it is structurally similar to the nereistoxin and may act on neuromuscular junction and produce acute toxic symptoms or death of animal due to respiratory failure. Cartap cause marked contraction with twitch depression of isolated mouse phrenic nerve diaphragm (Liao *et al.,* 2000). (O' malley *et al*., 1990) suggests that effect of cartap hydrochloride could be myogenic in nature because α-bungarotoxin and tetradotoxin selectively inhibit the nAchR and Na^+ channels were unable to abolish the cartap induced contraction. Fleckenstein (1983) suggested that cartap induced muscular contractions are primarily due to influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular pool by inhibiting the Ca^{2+}-ATPase pump (Hosey and Lezduuski, 1988; Lai *et al*., 1988) resulting increase in the intracellular Ca^{2+} level by unloading of Ca^{2+} from sarcoplasmic reticulum vesicles.

2.2. Toxicological data

2.2.1 Reproductive toxicity

In two generation reproductive study, by Oslan and Busey (1972) in a group of 10 male and 20 female rats, given cartap in feed @ 0, 100, 1000 ppm for 9 weeks prior to mating, at 1000 ppm F_1A generation showed; reduction in body weight, feed consumption, impairment of fertility. Weaning survival index was lower in F_1b generation than F_1A generation. Only slight incomplete ossification was seen in F_2b group of progeny at 1000 ppm.

2.2.2. Teratogenicity

Mizutani *et al*. (1971) conducted experiment on group of mice and rat having 20, 12, 14 and 21, 12,17 pregnant female animals in each group respectively; administered cartap by stomach tube @ 0, 50, 100 mg/kg b. wt. and from day 8 to day 13 of mice and from day 9 to day 15 of rat gestation period respectively. Mice and rat were sacrificed at 19th and 21st days of pregnancy and fetus were delivered by caesarian section, only slight skeletal abnormality was seen in skeletal (fusion of vertebrae) at 50 mg/kg (1.8%) and 100 mg/kg (1.7%) in mice and 14.4% of bilateral twin thoracic vertebral centrae at 100 mg/kg b. wt. in rats.

2.2.3. Eye irritation

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

2.2.4. Dermal toxicity

2.2.4.1. Rabbit

Davies *et al*. (1974) applied the cartap on intact as well as abraded skin of group of rabbit (5 animals in each group) at dose level 0, 15, 60 and 240 mg/kg b.wt./day and observed the death of rabbit at highest dose level within a week. Treatment continued on 15 and 60 mg/ kg b.wt. for 3 consecutive weeks and there was only mild dermal irritation in 2nd week and reduced body weight gain, food consumption and incoordination of hind limb at 60 mg/ kg b.wt. especially in 3rd week of treatment.

2.2.4.2. Mouse

Aramaki (1972) showed no toxicity signs, when cartap was applied @ 0.2 ml of 5-10% aqueous solution on the intact skin of the back of mouse, during the observation period of 7 days.

2.2.5 Short term studies

2.2.5.1. Mouse

Tsubura *et al*. (1976) fed cartap @ 0, 15, 45 and 135 mg/kg b.wt./day for a period of 3 months to a group of animals (8 – 9 animals per group) of each sex and observed only slight reduction in body weight gain and food efficiency at highest dose level.

2.2.5.2. Rat

Rivett *et al*. (1972) fed the cartap @ 0, 10, 20 and 40 mg/kg b.wt./day to the two groups of animal of different sex (each having 15 animals) and observed the reduction in body weight gain and food consumption in male animal at highest dose level.

2.2.6. Long term study

2.2.6.1. Mouse

Hunter *et al*. (1974) performed the 80 weeks long term study on the two groups of animals of different sex (40 animals in each group); fed the cartap @ 0, 10, 20 and 40 mg/kg b.wt.in diet and shown that only slight reduction in body weight gain during first 52 weeks at 20 mg/kg b.wt. and after 80 weeks additionally reduced activity of erythrocyte cholinesterase and plasma cholinesterase in male and female animal respectively at highest dose level. Heart and thyroid weight increased at 20 mg/kg and 40 mg/kg in female and male animals, respectively.

2.2.6.2. Rat

Hunter *et al*. (1975) fed the cartap to the group of rats of either sex (each having 45 animals) @ 0, 10, 20 and 40 mg/kg b.wt. for a period of 104 weeks and showed no toxic symptoms except reduction in body weight gain at highest and sub highest dose level to male and female animal respectively.

2.2.7 Acute toxicity symptoms

2.2.7.1. Animal

Rabbit treated with dose of cartap above the 7.5 mg/kg b.wt. showed severe signs of toxicity, such as dullness, miosis, ventral recumbancy, tremor, ataxia, convulsion and dyspnoea, become cyanotic and die within 20 minutes (Koyama, 1988).

2.2.7.2. Humans

Signs of acute exposure of cartap include salivation, nausea, vomiting, abdominal pain, and tremor of the arms and legs. In severe case, convulsions, respiratory failure and followed by death (Koyama, 1988).

2.2.8 Food residue

Concentration of cartap in the rice plant was recorded as 1-2 mg/kg immediately after application of compound in dust and fine granular form. Leaf blade contained higher amount of residue than leaf sheath (Koyama *et al*., 1975).

2.3 Pesticide induced immunotoxicity

The immunotoxicity of a wide range of pesticides has been established in laboratory animals. However, impacts of pesticide on the immune system are important and can have far reaching results, especially for exposures that occur whilst immune function is still developing – from conception until around age 18 when the thymus gland gains maturity (Corsini *et al.,* 2008). Exposure to pesticides may result in decreased immunocompetance which can result in more severe and prolonged infections, and the development of cancer; or immunostimulation which can lead to immune mediated diseases, hypersensitivity reactions, inflammatory responses, asthma, allergies and autoimmune diseases (Corsini *et al.,* 2008).

Pesticides and various environmental contaminants are the compounds of interest that have the ability to induce various immune responses. For instance, various studies have shown that chlordane, methylmercury, and carbofuran etc. all induce immunotoxic effects on murine splenocytes *in vivo* (Thompson *et al.,* 1998; Jeon *et al.,* 2001). Toxicity of pesticide may occur through oxidative damage to immune organs or by modulation of signal transduction pathways, including chronic effects of altered metabolism of immune organs.

Cytotoxicity refers to the cell-killing potential of a chemical compound. In immune cells, cytotoxicity usually occurs via two specific mechanisms of cell death, either apoptosis or necrosis (Wylie *et al.,* 1980).

The immune response is affected by various ways, including antibody generation, interleukin-2 production, T-cell proliferation and increase in auto antibodies. One of the modes of action is mediated by the Fas ligand/Fas pathways in which a membrane ligand crosslink's with the target cell surface receptors to induce apoptosis (Kovacic, 2003).

2.4 Apoptosis and necrosis

Apoptosis and necrosis are two modes of cell death that have own unique morphological, biochemical and physiological characteristics. Necrosis is "accidental" cell death. It is a pathological process, which occurs when cells are exposed to a violent physical or chemical insult, such as in hypoxia, ischemia, chemical exposure, temperature fluctuations, disruption of membrane structure and exposure to toxins. Necrosis begins when the cell is unable to maintain its own homeostasis mechanism, leading to an influx of water and extracellular ions into the cells. Morphological characteristics of necrosis include loss of membrane integrity and swelling of the cytoplasm, mitochondria and endoplasmic reticulum, which leads to cell rupture. Upon rupture, the contents of the cytosol, including lysosomal enzymes are released into the extracellular environment. The major biochemical processes of necrosis are the loss of ion homeostasis, digestion of DNA and post-lytic random DNA fragmentation (late event). The physiological significance is that extensive tissue injury can result due to lysosomal enzyme action, inflammatory responses are likely to be triggered and phagocytic activity of macrophages is increased leading to higher incidences of respiratory burst and ROS generation (Wylie *et al.,* 1980). Incontrast, apoptosis is a mode of cell death that occurs even under normal physiological conditions. It is considered "normal" cell death or a form of "programmed cell death." It is a process in which unwanted or useless cells are eliminated during development and other normal biological processes.

Chemically-Induced Apoptosis

Certain chemicals have been known to induce immunotoxicity by triggering cell death via necrosis or apoptosis (Corcoran *et al.,* 1994). Any significant loss of a cell population or

particular cell type can be devastating to the proper functioning of an organ. There are numerous reasons to evaluate the level of apoptosis or necrosis in exposed cells. First, the two types of cell death differ markedly in context of how they develop and by fundamentally different processes. Second, the occurrence and circumstances surround either event, permits inferences to be drawn regarding the intracellular mechanisms underlying them. Third, each event has different physiological implications by identifying predictable results of exposure (i.e. ROS generation, gene activation) of compounds could be classified based upon toxic potential (Wylie *et al.,* 1980). The significance of identifying either event is a fundamental issue when addressing chemical exposures because under normal circumstances apoptosis is a natural event that occurs in living tissue naturally, whereas necrosis is often a result of other mechanisms and is not initiated by intrinsic cell factors. Although both events can occur in the same exposure system, induction of apoptosis could impair the steady-state kinetics of healthy tissues in an organism could be greatly impaired and result in unpredictable, deregulatory cellular responses (Wylie *et al.,* 1980).

It is suggested that immunotoxins stimulate apoptosis by exploiting the physiological mechanisms of the cell. Some of these suggested mechanisms include: the alteration of receptor signal transduction proteins; the alteration of second messengers, such as calcium, cAMP, nitric oxide and other ROS; the alteration in gene expression; and the induction of DNA damage. In particular, ethanols, octylphenol, polychlorinated biphenyls and p-chloronitrobenzene, cypermethrin have been implicated in inducing apoptosis in murine splenocytes (Slukvin and Jerrells, 1995; Menon *et al.,* 1996; Li *et al.,* 1999; Sankar *et al.,* 2012).

2.5 Oxidative stress

Free radicals are molecules which contain one or more unpaired electrons in their outer orbit (Younes, 1999; Halliwell, 1996). A free radical species may be extremely reactive and can initiate chain reactions by extracting an electron from a neighboring molecule to complete its own orbital (Marks *et al.,* 1996; Younes, 1999). There are many forms of free radical species, such as oxygen-centered, carbon-centered or nitrogen-centered (Kalyanaraman, 1982;

Kehrer, 1993; Halliwell, 1996). Many of these free radical species are formed when chemicals are metabolized to one or more reactive intermediates (Comporti and Pompella, 1994). Free radicals, called reactive oxygen species (ROS) can form (Yu, 1994). These include species such as superoxide anion (O_2-•), hydrogen peroxide (H_2O_2).

Oxidative stress is associated with the generation of free radical species beyond the capacity of antioxidant mechanism of cell or malfunctioning of antioxidant defense mechanism (Hei *et al*., 1998). Reactive oxygen species (ROS) induce oxidative stress by peroxidation of lipid of cell membrane, cross linking of DNA and protein which are the electron rich sites within the cells (Romero *et al*., 1998). Reactive oxygen species (ROS) play significant role in induction of apoptosis by mitochondrial pathway under both physiological as well as pathological conditions. ROS cause disruption of mitochondrial membrane (Gupta *et al*., 2003), resulting increased release of cytochrome-C in cytosol, leading to activation of caspase 3 (Sen *et al*., 2004). Activation of caspase 3 cause to DNA breakage, nuclear chromatin condensation, cytoplasmic shrinkage, dilation of endoplasmic reticulum (Sgonc *et al*., 1994) and finally cause apoptosis of the cell (Hengartner, 2000). ROS induction/generation by cartap hydrochloride is depending on time and dose of the toxic compound (Liao *et al*., 2006).

2.6 Cytotoxicity

Mosmann (1983) estimated the cytotoxic potential of cartap *in-vitro* on C_2 C_{12} monolayer myoblast cell line of mus musculus by MTT colometric method, treated with final concentration of cartap @ 0, 10, 30, 100, 300 and 1000µM or an equal volume of DMEM-C at 37°C in 5% CO_2 for 24 h and revealed that no effect at 10µM and at 30 µM and myoblast cell showed many long radiating finger like pseudopodia (bleb formation or rounding of cell and cell lysis). Hence cartap not only cytotoxic in nature but its cytotoxic effect depends on dose and time.

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Materials and Methods

3 MATERIALS AND METHODS

The present work was designed for the evaluation of immunotoxicity induced by cartap a thiocarbamates group herbicide belongs to Type-II class of pesticide in swiss albino mice. Since oxidative stress has been implicated as its important mechanism of action, therefore it was also studied. The present work was conducted at Toxicology laboratory, Center for Animal Disease Research and Diagnosis (CADRAD) and Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

3.1 EXPERIMENTAL ANIMALS

Male swiss albino mice (4-6 weeks old) were procured from Laboratory Animal Resource section of the Indian Veterinary Research Institute (IVRI), Izatnagar. The body weights of mice were in the range of 15-16 gm at the beginning of the experiments. Mice were housed in polystyrene cages (six mice per cage) with free access to food and water, a 12/12 hr light–darkness cycle and an ambient temperature of 20–25°C. They were housed one week for acclimatization before starting the experiments. Standard feed and water was provided *ad libitum*. All procedures employed in this study were approved by the Institutional Animal Ethics Committee (IAEC).

3.2 CHEMICALS

Cartap technical grade (> 98.2% purity) was offered generously by M/s, cheminova Industries Ltd. Mumbai, India.

β-actin monoclonal antibody produced in mouse, Freund's complete adjuvant, concanavalin-A (con-A), lipopolysaccharide from E-coli 055-B5 (LPS), MTT (Thiozolyl blue tetrazolium bromide) dye, trizol, tris (hydroxymethyl)-aminomethane, propidium iodide, antibiotic and antimycotic solution, protease inhibitor cocktail, L-glutathione reduced, 1-chloro-2, 4-dinitrobenzene (CDNB), DTNB (5, 5'-dithiobis (2-nitrobenzoic acid), $DiOC_6$ (3,3-dihexyl-oxacarbocyanine iodide) were purchased from Sigma Chemicals Co. (USA). Dulbecco's modified eagle medium (DMEM) containing (high glucose with L-glutamine, pyridoxine hydrochloride and sodium bicarbonate without sodium pyruvate) and fetal bovine serum was procured from Gibco (UK). Fas and Fas-L polyclonal primary antibodies were purchased from Santacruz Biotechnology, USA. Goat anti-rabbit IgG-HRP conjugate and protein estimation kit by Bradford method were procured from Genei, Bangalore, India. Fluorescein isothiocyanate annexin-V-propidium iodide (FITC-annexin-V-PI) apoptosis detection kit with propidium iodide and cell staining buffer were obtained from Biolegend Inc., (San Diego, CA, USA). RNase solution, glycine, Tris HCl, diaminobenzidine (DAB) were purchased from Bangalore, Genei™, India. 100 bp plus DNA ladder were obtained from Thermo Scientific, USA. DNA tissue isolation kit (NucleoSpin® Tissue) was procured from MACHERY-NAGEL GmbH & Co. Germany. RNase Zap and DEPC treated water was procured from Life Technologies, USA. Nitrocellulose membrane (0.45 µ), blotting pads, commassie brilliant blue-R-250, acrylamide-bis-acrylamide were purchased from Bio-Rad Laboratories Inc., USA. DCFH-DA (2',7'-dichlorofluorescin diacetate) was purchased from Calbiochem, EMD Biosciences, Germany. Thiobarbituric acid (TBA), trichloro acetic acid (TCA), dimethyl sulphoxide (DMSO), methanol, Sodium dodecyl sulphate (SDS), EDTA was purchased from Merck (Germany). Sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium citrate (all extrapure AR), bovine serum albumin (BSA) and isopropanol were procured from SRL Laboratories, India. Dulbecco's phosphate buffer saline (DPBS without calcium and magnesium) and haematoxylin and eosin stains were obtained from (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Plastic ware such as centrifuge tubes (15 ml and 50 ml), microcentrifuge tubes (1.5 & 2 ml), microtips (2-200 µl and 200-1000 µl) and gloves were procured from Tarsons Products Ltd, Kolkata, India.

3.3 DOSES AND EXPOSURE SCHEDULES

The basis for selection of three test doses of cartap was its oral LD_{50}. Oral LD_{50} of technical grade cartap in mice has been reported to be 150- 225 mg/kg b.wt. (Ray, 1991; Tomlin, 1997). We experimentally determined its oral LD_{50} by preliminary dose range finding study and it was found to be 156 mg/kg b.wt. in swiss albino mice. Accordingly, three test doses of cartap for the 28 days subacute toxicity study were, high dose: 15 mg/kg (1/10th of LD_{50}); medium dose: 7.5 mg/kg (1/20th of LD_{50}) and low dose: 5 mg/kg (1/40th of LD_{50}).

Mice were divided in four groups (six mice per group) and were treated with different doses of cartap. Mice were administered cartap by oral gavage for consecutive 28 days. Distilled water was used as vehicle for cartap. The different mice groups and their respective doses were mentioned in the table 1. Each dose was adjusted as per body weight (100μl per 10 gm body weight) of each mouse. Cartap doses were prepared on every alternate day and thoroughly mixed before administering orally. All dosing was performed between 14:30 and 15:00 hr as far as possible, and body weights were recorded daily at the time of dosing.

For each of the following set of experiments, different subsets of mice were used i.e. four separate 28 days toxicity studies were performed. The important experimental subsets are mentioned below:

Set-1: Body weight, absolute organ weight, Relative organ weight, Hematological parameters, Serum biochemical analysis, histopathology of various organ such as liver, kidney, brain, heart, spleen and lung, estimation of oxidative stress in liver, kidney and brain .

Set-2: Delayed type hypersensitivity (DTH) response, Western blot analysis for the expression of Fas and Fas-L proteins, T and B lymphocyte proliferation assay, IFN-γ by ELISA.

Set-3: Serum antibody titre (HA), Quantization of IL-4, Flow cytometric analysis of splenic lymphocytes for the detection of apoptosis and necrosis and DNA fragmentation assay.

Table: 2. Dosage and experimental schedule for *In vivo* sub-acute immunotoxicity study

Group	Treatment	Dose/Concentration (In per kg b.wt.)	Route	Period of exposure
I	Control (distilled water)	10 ml/kg	Oral	28 days
II	Cartap 5	5 mg/kg	Oral	28 days
III	Cartap 7.5	7.5 mg/kg	Oral	28 days
IV	Cartap 15	15 mg/kg	Oral	28 days

3.4 General toxicity parameters

3.4.1 Clinical signs/symptoms

Mice of all groups were closely observed twice a day throughout the period of experiment for clinical signs and mortality.

3.4.2 Body weight and organ weights

The body weight of mice was recorded at weekly interval. Vital organs, *viz.,* spleen, thymus, liver, kidneys, brain, lung and heart were excised at the time of necropsy, lightly blotted with tissue paper and weighed. Calculated organ weight was represented as absolute organ weight and relative organ weight. Relative organ weight was calculated as mentioned below.

$$\text{Relative organ weight} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100$$

3.4.3 Hematological parameters

At the end of exposure period, blood samples were collected (0.5 ml of peripheral blood from retro-orbital plexus) in vacationer containing anticoagulant, EDTA. Various haematological end-points such as hemoglobin concentration (Hb), hematocrit/packed cell volume (PCV), total erythrocytes count (TEC), total leucocytes count (TLC) and differential leucocyte counts (DLC) was carried out. Differential leukocyte count (lymphocytes, neutrophils, monocytes and eosinophil) was determined by observing 100 WBCs in blood smears stained with Giemsa stain. All haematological parameters were done manually by standard protocol.

3.4.4 Serum biochemical evaluation

For serum, 0.5-1 ml of blood was collected from mice at the time of sacrifice in the sterile clot activator (vacationer) tubes and allowed to clot. These samples were then centrifuged at 5000 rpm for 10 min to separate serum. Serum samples were preserved at -20°C till the estimation of biochemical parameters. Serum samples were analyzed to evaluate various end-points biochemical parameters such as albumin, total protein, globulin, albumin: globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and serum creatinine , Blood urea nitrogen (BUN), lactate dehydrogenase (LDH) by commercial kits (Span Diagnostic Ltd., Surat, India) by employing ultraviolet spectrophotometer [Eppendorf- Biospectrometer (kinetic)].

3.5.5 Histopathology

The tissues of liver, kidney, brain, heart, spleen, and lung were collected in 10% formalin from mice of each group at the end of treatment period. Paraffin embedded sections of these tissues were cut at 5-6 µm thickness and stained with haematoxylin and eosin following the procedure of Lillie (1965).

3.5 Oxidative stress related biochemical parameters

Different oxidative stress related and internal antioxidant defense mechanism related biochemical parameters were evaluated in liver, kidney, and brain. A single beam UV-VIS spectrophotometer [Eppendorf- Biospectrometer (kinetic)] was used for the estimation of various oxidative stresses related biochemical indices.

3.5.1 Preparation of liver, kidney, brain and spleen homogenates

Samples of liver, kidney and brain were weighed and 10% tissue homogenate was prepared in ice-cold phosphate buffer (pH 7.4) with the help of a powerful homogenizer (Ultra Turrax T18 basic, IKA, Germany). A 200 mg of sample was taken in 2 ml of ice-cold phosphate buffer saline. For estimation of reduced glutathione (GSH) separate 200 mg of sample was taken in 2 ml of 0.02 M EDTA. The homogenate (10%) prepared with homogenizer (IKA, Germany) under ice-cold condition was centrifuged for 10 min at 3000 rpm and the supernatant was stored at -20°C till assay.

3.5.2 Lipid peroxidation

The extent of lipid peroxidation (LPO) was evaluated in terms of malondialdehyde (MDA) production, determined by the thiobarbituric acid (TBA) method (Shafiq-U-Rehman, 1984).

Reagents

1) Trichloroacetic Acid (TCA); 10 % solution: TCA (Sigma) (10 g) was dissolved in distilled water and the volume was made up to 100 ml with distilled water.

2) Thiobarbituric acid (TBA); 0.67 % solution: 0.67 g of TBA (Sigma) was added to 100 ml distilled water and warmed up (in water bath) for dissolving TBA.

Procedure

1 ml of tissue homogenate (supernatant) was incubated at 37 ± 0.5°C for 2 h. To each sample, 1 ml of 10% w/v TCA was added. The mixture was thoroughly mixed and centrifuged at 2000 rpm for 10 min. To 1 ml of supernatant liquid, an equal volume of 0.67% w/v TBA was added and kept in boiling water bath for 10 min, cooled and diluted with 1 ml distilled water. The absorbance was read at 535 nm.

Calculation

Calculation was done using the molar extinction coefficient (EC) of MDA-TBA complex at 535 nm, i.e., 1.56×10^8/M/cm. The amount of LPO is expressed as nanomoles of MDA formed per gm of wet tissue.

$$\text{LPO (nmol MDA formed/g tissue)} = \frac{\text{OD}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times 10^9 \times \text{DF}$$

Wherein, DF: Dilution factor = 10; EC: extinction coefficient = 1.56×10^8/M/cm; total volume of reaction mixture = 3 ml; amount of sample taken = 1 ml.

3.5.3 Reduced glutathione (GSH)

GSH was determined by estimating free-SH groups, using DTNB (dithiobis-nitro-benzoic-acid) method of Sedlak and Lindsay (1968). For the estimation of GSH, 10% homogenate was made in 0.02 M EDTA.

Reagents

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

Procedure

To 1 ml supernatant of homogenate, 0.8 ml of water and 0.2 ml of 50% TCA solution was added and incubated at room temperature for 15 min. This mixture was centrifuged at 3000 rpm for 15 min and 0.4 ml of the supernatant was taken. To it 0.8 ml of 1 M tris buffer was added, followed by 0.2 ml DTNB (0.01 M) and the absorbance was read at 412 nm within 5 min. Reagent blank contained no sample and sample blank was without DNTB.

Calculation

The concentration of GSH was calculated using the extinction coefficient 13000/M/cm and the results were expressed as μmol GSH per g tissue.

$$\text{Reduction GSH} = \frac{\text{OD}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times \text{DF} \times 100$$

3.5.4 Catalase

Catalase (CAT) activity was assayed by a spectrophotometric method as described by Bergmeyer (1983).

Reagents:

1) Phosphate buffer (50 mM pH 7.0)

   (a) 50 mM KH_2PO_4 - 1.37 g/ 200 ml

   (b) 50 mM Na_2HPO_4 - 1.42 g/ 200 ml

   Solutions (a) and (b) were mixed in a ratio of 1:1.5 (v/v) and the pH was adjusted to 7.

2) H_2O_2 (10 mM): 0.1 ml of 30% H_2O_2 was diluted to 100 ml in water. The solution was Checked at 230 nm and the concentration was adjusted using the molar extinction coefficient.

Procedure

In a test tube 1.99 ml phosphate buffer and 10 µl homogenate were added and the contents were transferred to the cuvette. The reaction was started after adding 1 ml of H_2O_2 directly into the cuvette. The optical density was recorded at every 30 sec for 3 min at 240 nm against distilled water (blank).

Calculation

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

$$\text{Catalase} = \frac{\Delta OD}{0.067} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times \frac{1}{\text{mg of protein}}$$

Wherein, ΔOD: change in absorbance per minute, i.e. either from 30 sec to 90 sec or 60 sec to 120 sec or 90 sec to 150 sec or 120 sec to 180 sec; total volume of reaction mixture = 3 ml; amount of sample taken = 1 ml.

3.5.5 Superoxide dismutase

Superoxide dismutase (SOD) was estimated as per the method described by Madesh and Balasubramanian (1998). It involves generation of superoxide by pyrogallol auto-oxidation and the inhibition of superoxide dependent reduction of the tetrazolium dye MTT [3-(4-5 dimethyl thiazol 2-xl) 2,5-diphenyl tetrazolium bromide] to its formazan, measured at 570 nm. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO), which solublize formazan crystals. The colour evolved is stable for several hours and results were expressed as SOD Units/mg of protein (one unit of SOD is the amount (g) of protein required to inhibit the MTT reduction by 50 %).

Reagents:

1) Pyragallol (100 µM): 6.3 mg of pyragallol (Sigma) was dissolved in 5 ml of distilled water. 1 ml from this solution was diluted to 100 ml with distilled water.

2) MTT (1.25 mM): 2.58 mg MTT (Sigma) was dissolved in 5 ml of distilled water.

3) Phosphate buffer saline (PBS): PBS was prepared by dissolving NaCl (8 g), KCl (0.2 g), KH_2PO_4 (0.2 g) and Na_2HPO_4 (0.94 g) in 800 ml distilled water. The pH was adjusted to 7.4 and the volume was made up to 1 liter with distilled water.

Procedure

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

Table 3: Reaction set up for determination of SOD

	Sample	Control	Blank
PBS	0.65 ml	0.65 ml	0.65 ml
MTT	30 µl	30 µl	30 µl
Homogenate	10 µl	-	-
Pyrogallol	75 µl	75 µl	75 µl
Sample , control and blank were incubated for 5 min at room temperature			
DMSO	0.75 µl	0.75 µl	0.75 µl
Homogenate	-	10 µl	-

Calculation:

Superoxide dismutase was expressed as Units/ mg of protein

$$Y\,\% = \frac{\text{OD test}}{\text{OD control}} \times 100$$

$$\text{SOD (Units / mg of protein)} = \frac{\text{Mg of protein in 1 ml homogenate}}{Y\%} \times 50 \times DF$$

3.5.6 Glutathione S-transferase (GST) activity

GST activity was determined in the liver, kidney and brain tissue homogenates by the method of Habig *et al*. (1974). The reaction mixture contained 1 ml phosphate buffer (0.3 M; pH 6.5), 0.1 ml of 1-chloro-2, 4-dinitrobenzene (CDNB; 30 mM), 0.1 ml supernatant of homogenate and 1.7 ml distilled water. The mixture was incubated at 37°C for 5 min under darkness. The reaction was initiated by adding 0.1 ml of GSH (30 mM) to the mixture. The change in absorbance was recorded at 340 nm at an interval of 30 sec for 3 min. The enzyme activity has been expressed as µmol CDNB-GSH conjugate formed/min/mg protein, using a molar extinction coefficient of 9600 $M^{-1}cm^{-1}$.

$$GST = \frac{\Delta OD}{E} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times 1000 \times \frac{1}{\text{mg of protein}}$$

Wherein, ΔOD: change in absorbance per minute, i.e. either from 30 sec to 90 sec or 60 sec to 120 sec or 90 sec to 150 sec or 120 sec to 180 sec; total volume of reaction mixture = 3 ml; amount of sample taken = 1 ml; mg of protein in 0.1 ml supernatant.

3.5.7 Total thiols (T-SH):

Total thiols were determined by measuring colour complex formed by the reaction of sulfhydryl groups with dithiobis- nitro- benzoic acid (DTNB), as per the procedure of Sedlak and Lindsay, (1968). Briefly, tissue homogenate of 0.1 ml was mixed with 0.3 ml of 0.2M Tris buffer (containing 20mM EDTA, pH 8.2) and 20 µl of 10mM DTNB was added. The mixture was brought to 2.0 ml with 1.58 ml of absolute alcohol. Colour was developed for 15 min followed by centrifugation of the contents at 3000 *g* for 15 min. The absorbance of the clear supernatants was read at 412 nm with distilled water as blank. Total quantity of thiols was estimated by using molar extinction coefficient of colour complex as 13000 and results were expressed as µmol T-SH per ml of tissue homogenate.

$$T\text{-}SH = \frac{OD}{E} \times \frac{\text{Total volume of reaction mixture}}{\text{Sample volume taken}} \times 10^6/10^3$$

3.5.8. Protein:

Protein was estimated by the method of Lowry *et al*. (1951) in the supernatant of tissue homogenates.

Reagents:

1. Reagent A: 2% Sodium carbonate in 0.1 N NaOH (2 gm Na_2CO_3 in 100 ml of 0.1 N NaOH).
2. Reagent B: 1% Copper sulphate (100 mg of CuSO4 was dissolved in 10 ml distilled water and stored in amber colored bottle).
3. Reagent C: 2% sodium potassium tartarate (200 mg of sodium potassium tartrate was dissolved in 10 ml of distilled water).
4. Reagent D: (Alkaline copper sulphate): Prepared by mixing 49 ml reagent A with 0.5 ml of Reagent B and Reagent C just before assay.
5. Foline-Ciocalteau's phenol reagent (1N): 2 ml Foline-Ciocalteau's phenol reagent was diluted with 2 ml distilled water (prepared freshly).
6. Bovine serum albumin (BAS): 100 mg 0f BSA was dissolved in 100 ml of distilled water and used as standard.

Procedure

The reaction mixture consisted of 20 µl supernatant of homogenate (10%), 980 µl distilled water and 2.5 ml alkaline copper sulphate, which was incubated for 10 min at room temperature. After this foline-Ciocalteau's phenol reagent was added and tubes were shaken immediately. Resultant mixture was again incubated for 30 min at room temperature and absorbance was read at 750 nm. For blank 100 µl distilled water was added instead of tissue homogenates. 10 different BSA standards were prepared by serial dilution of stock solution followed by transfer of 100 µl from respective dilutions into the reaction mixture. The absorbance of standard was used for the preparation of standard curve by plotting concentration vs. OD and values were expressed as mg of protein/ml of tissue homogenate.

3.5.9 Estimation of superoxide anion (O_{2-}) formation

In the liver, kidney and brain O_{2-}generation were estimated indirectly by spectrophotometrically at 540 nm in terms of reduced nitrobluetetrazolium (NBT) which form formazan as an index of superoxide anion generation as per procedure described in the method of Di Wang *et al.* (1998).

Reagents

1. NBT-1mM in distilled water
2. Hydrochloric acid (HCL)- 0.5.

Procedure

Briefly, to the 900 µl of 1mM NBT was added (Final NBT concentration, 100µmol) and reaction mixture was incubated at 37 °C for 90 min. The NBT reduction was stopped by adding 1 ml of 0.5 % HCL. The amount of the superoxide anion generated by the tissue was quantified by measuring the absorbance of the blue formazan spectrophotometrically at 540 nm against blank. Results were expressed as picomole/min/mg protein. The amount of NBT reduced (quantity of formazan), was calculated as follows:

The amount of NBT reduced (pmole/min/mg protein)=$A \times V/ (T \times mgp \times el) \times DF$

Where,

A	=	Absorbance at 540nm; V=Volume of solution
T	=	Time period (90 min) during which homogenates were incubated with NBT
Mgp	=	Milligram protein/g tissue
e	=	Extinction coefficient of blue formazan (i.e. 0.721/mmol/mm)
l	=	Length of the light path
DF	=	Dilution factor

3.5.10 Measurement of NO concentration

Nitrite (NO_{2-}), a stable product of NO, was analyzed in Liver, kidney and brain homogenates by Griess reagent [(0.1% N-(1-naphthyl) ethylenediaminedihydrochloride and 1% sulfanilamide)] as described in the method of Sastry *et al.* (2002).

Reagents

1. HCL- 3N
2. Sulfanilamide: 1% in 3N HCL
3. N-naphthylethylenediamine (NEED): 0.1% in distilled water.

Procedure

In brief, Aliquots (150 µl) of the clear supernatant (tissue homogenates) or sodium nitrite (NaNO2) standards were transferred into the wells of a microplate (96 wells, Polystyrene) in triplicate. Next, components of the Griess reaction were added with gentle mixing each time: 75µl of 1.0% sulfanilamide (prepared in 3 N HCl) and 75µl 0.1% N-naphthylethylenediamine (NEED) (prepared in water). After 10 min the absorbance was measured at 545 nm against a blank containing the same concentrations of ingredients but no biological sample in a microplate reader (Spectra MAX 190, Molecular Devices). Concentration of nitrite (µM/L) was calculated from standard curve and results were expressed as µM nitrite released into the medium per milligram wet weight of the tissues.

Concentration of nitrite (µM/L) = Concentration derived from standard curve × DF

Where,

DF = Dilution factor

3.6 Evaluation of humoral immune response

3.6.1 Serum antibody titer/hemagglutination antibody (HA) titer

3.6.1.1 Collection of sheep blood and separation of sheep red blood cells

Sheep blood was collected in sterile centrifuge tube (50 ml) containing sterile Alsever's solution with help of sterile syringe and needle (18 G) from jugular vein of sheep. The proportion of sheep blood: Alsever's solution was 1: 2.

Composition of Alsever's solution (for 100 ml)

Glucose	2.050 gm
Sodium chloride	0.420 gm
Sodium citrate	0.800 gm
Citric acid	0.055 gm

Distilled water was added to make volume 100 ml and autoclaved at 10 lb pressure for 15 min and then stored at 4°C.

Sheep blood (in Alsever's solution, about 4 ml) was washed thrice in Dulbecco's phosphate buffer saline (DPBS), at 3000 rpm/10 min. RBCs were counted in hemocytometer by diluting 100 times. Final volume of RBCs was adjusted as 1.5x 10^{9} cells/ml.

3.6.1.2 Mice Immunization

For evaluation of HA titre, mice from this experimental set up were immunized by i.p. injection of SRBCs (4.5x10^{8} cells/mice, 300 µl/mice) in saline seven days before completion of treatment period (Elsabbagh and El-tawil, 2001).

3.6.1.3 Titration of haemagglutinating serum antibodies

At the end of experimental period, sera was prepared from peripheral blood sample of each group of immunized mice and decomplemented by heating at 56°C for 30 minutes. These sera samples were then kept at –20°C until estimation of antibody titre. To prevent non-specific agglutination, 1% bovine serum albumin (BSA) was added in DPBS and then this DPBS was used for preparing 1% (v/v) SRBC suspension.

The microtitre HA technique as described by Puri *et al*. (1994) was employed to determine the serum antibody titre. A volume of 50 µl of 0.15 M phosphate buffer saline (PBS, pH 7.2) was placed into all the 96 wells of round bottom microtitre plate (Tarsons Products Ltd., Kolkata, India). Then 50 µl of test serum was added to the first well, mixed thoroughly and transferred serially until last well by double dilution technique and 50 µl of content was discarded from last well. Then 50 µl of 1% (v/v) sheep RBC suspension was added in all the wells. Last row left as SRBC control which contained only PBS and SRBC but no test serum.

Plate was then mixed by tapping and incubated at 37°C for 1.5 to 2 h and then observed for haemagglutination pattern. The reciprocal of the highest dilution giving complete haemagglutination was taken as antibody titre. Results were represented as the log_2 value of the reciprocal of serum dilution giving haemagglutination.

3.6.2 Quantification of mouse IL-4 by ELISA in serum

Serum samples of six mice in each group were quantitatively assayed for IL-4 by sandwich enzyme-linked immunosorbent assay (ELISA), in a 96 well strip plate pre-coated with capture antibody as per the manufacturer's instructions (RayBio® Mouse IL-4 ELISA Kit, Norcross, GA). Briefly, IL-4 standard was reconstituted and serially diluted and assayed. The absorbance was measured at 450 nm on the ELISA reader [TECAN (Infinite F50)]. The absorbance of each dilution was plotted against respective concentrations; straight line graph was drawn to get the regression equation. Then samples were assayed in duplicate, their absorbance was obtained and concentration of IL-4 was quantitatively calculated from the regression equation. The concentration of IL-4 was expressed as pg/ml.

3.7 Evaluation of cell mediated immune response

3.7.1 Delayed type hypersensitivity (DTH) response

The DTH response was assessed by the method of Badgujar *et al.* (2013) using SRBC as antigen.

3.7.1.1 Sensitization of mice

On Day 18th of the exposure period mice were sensitized by s/c injection of SRBCs suspended in Freund's complete adjuvant (FCA) on their back by using 1 ml plastic tuberculin syringe fitted with 26 G needle. SRBCs were separated from blood as mentioned above. To prepare SRBC-FCA emulsion, equal volume of SRBC and FCA (2.5 ml of each, sufficient to be injected for 60 mice) was taken. In a vial containing 2.5 ml of FCA, small quantity of SRBC suspension (100 to 500 µl) of the total 2.5 ml was added and mixed uniformly with the help of 16/18 G needle fitted into 5 ml syringe, until the water in oil emulsion resulted. The emulsion formed was complete as indicated by drop of it not spreading on cold water surface in a beaker.

3.7.1.2 Challenge

After 10 days (i.e. on day 28), sensitized mice were challenged by injecting 50 µl of SRBC in right hind foot pad with the help of 26 G needle mounted on to the tuberculin syringe. Before injecting SRBCs in foot pad mice were lightly anaesthetized by i.p. injection of ketamine HCl (100 mg/kg b. wt).

3.7.1.3 Assessment of reaction

Swelling in the right hind foot pad was measured by pressure sensitive digital vernier calliper (Mitutoyo, Japan), 24 and 48 hours after the challenge. Histopathology of footpad was also performed to study cellular changes in DTH reaction.

3.7.2 Lymphocyte proliferation assay *in vitro*

Lymphocyte proliferation assay was performed by the method of Mosmann, (1983) with some modifications.

3.7.2.1 Preparation of cell culture medium

DMEM was supplemented with sterile 10% fetal bovine serum (FBS), 1 % antibiotics & antimycotics solution (containing 10000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml). Washing of splenic lymphocytes was done with medium containing only antibiotics but no FBS; however, after final washing lymphocytes were suspended in complete medium i.e. medium containing FBS.

3.7.2.2 Isolation of splenocytes

Spleen and thymus cells were harvested as described Badgujar *et al*. (2013). Briefly, spleen was removed aseptically at the time of sacrifice and single-cell suspensions were prepared with the help of a sterile syringe hub and a sterile cell strainer (100 µm, SPL Life Sciences Co. Ltd., Korea). Lymphocytes were washed with DMEM (i.e. respective cell suspension was transferred and centrifuged at 2000 rpm for 5 minutes, 4°C). The supernatant was discarded and splenocyte pellet was suspended with 2 ml of RBC lysis buffer (0.75% NH_4Cl in tris buffer (0.02%, pH 7.2) to omit RBCs. After holding for 5-10 min on ice, the cell suspension was again washed with DPBS. The final cell pellet thus obtained was resuspended in 1 ml of

DMEM and viable lymphocytes counts were obtained by trypan blue (Sigma, USA) exclusion method (as detailed below) using a hemacytometer. Finally, cells were adjusted into different concentrations in complete DMEM (supplemented with 10% fetal bovine serum).

3.7.2.3 Cell viability assay

Cell count and viability was determined in 1:100 dilutions. 5 µl of neat cell suspension (obtained after last washing) was taken in a microcentrifuge tube and to this 445 µl of trypan blue dye (0.4% in PBS) was added and immediately 10 µl was charged in the haemocytometer. It was allowed to stand for 2 min. The slide was examined under light microscope at 40x magnification to count the live (bright) and dead (blue) cells in 64 secondary squares (4 large corner squares). The following calculations were then made.

Average number of viable cells = Total count/4

Total viable cells/ml = (Average number of viable cells) x 10^4 x 100 (dilution factor)

3.7.2.4 Lymphoproliferative response in splenocytes cultures

Two mitogens were used to measure proliferation of splenic lymphocytes. T cell proliferation response was studied with concanavalin-A (con-A), while B lymphocyte proliferation response was studied with lipopolysaccharide (LPS) from E-coli 055:B55. Single spleen cell suspensions without red blood cells were prepared as noted above. After last wash, cells were re-suspended in DMEM containing sterile 10% fetal bovine serum (FBS), 1% antibiotics & antimycotics, i.e. complete DMEM. Cell viability was determined by 0.4% trypan blue dye exclusion test. Finally, the viable cell number was adjusted to 2 x 10^6 cells/ml with complete medium. The test was performed in flat bottom 96 wells tissue culture plates (Grenier Bio-one, Germany). Triplicate cultures of each sample were made with and without mitogen. Lymphocyte cell suspension was added as 100 µl/well and then 100 µl of 5 µg/ml of con-A and 100 µl of 2.5 µg/ml of LPS was added in each of the triplicate cell suspension. To the wells containing no mitogen (cells only control), 100 µl of medium was added. Corner rows and columns were filled with 200 µl of medium which apart from serving medium control, helped in tackling evaporation of well contents. The plate was covered with lid and transferred to humidified CO_2 incubator at 37°C, 5 % CO_2 for 72 h. After 72 h of incubation, number of

proliferating cells was determined with MTT dye. 20 µl of MTT (5 mg/ml in DMEM, thoroughly vortexes and filtered with the help of 0.22 µm syringe filter) was added to each 200 µl of culture. Plates were gently shaken to mix the contents. After additional 3-4 hours of incubation at 37°C in humidified CO_2 incubator, plate was removed and approximately 210 µl of medium was discarded from each well. Formazan crystals formed were dissolved by adding 100 µl of DMSO to each well and absorbance of wells was determined using an ELISA microplate reader at wavelength of 570 nm. Before taking reading, plates were shaken for 60 seconds on plate shaker so as to dissolve crystals uniformly.

Stimulation index (S. I.) was calculated as absorbance of the sample stimulated by con-A/LPS divided by absorbance of the sample without stimulated by mitogen.

3.7.3 Spleen cell cellularity

Spleen single cell preparation was prepared as mentioned above. Spleen cell cellularity was determined by counting these cells with a haemocytometer.

3.7.4 Quantification of mouse IFN-γ by ELISA

Serum samples of six mice in each group were quantitatively assayed for IFN-γ by sandwich enzyme-linked immunosorbent assay (ELISA), in a 96-well strip plate pre-coated with capture antibody as per the manufacturer's instructions (RayBio® Mouse IFN-gamma ELISA Kit, Norcross, GA). Briefly, IFN-γ standard was reconstituted and serially diluted and assayed. The absorbance was read at 450 nm on the ELISA reader [TECAN (Infinite F50)].The absorbance of each dilution was plotted against respective concentrations; straight line graph was drawn to get the regression equation. Then samples were assayed in duplicate, their absorbance was obtained and concentration of IFN-γ was quantitatively calculated from the regression equation. The concentration of IFN-γ was expressed as pg/ml.

3.8 Apoptosis related parameters

3.8.1 DNA fragmentation assay in spleen

3.8.1.1 Extraction of DNA from spleen

Spleen collected for assessment of DNA fragmentation pattern (DNA ladder) was thawed on ice. Apoptotic DNA was isolated from spleen of treated and control group mice

using DNA tissue isolation kit (NucleoSpin® Tissue) (MACHERY-NAGEL GmbH & Co. Germany) as per the manufacturer's instructions. Briefly, about 25 mg of spleen tissue was cut into small pieces, and placed in a 1.5 ml microcentrifuge tube. Samples were first lysed using proteinase K. Buffering conditions were adjusted to provide optimal DNA binding conditions and the lysate was then loaded onto the DNeasy Mini spin column. During centrifugation, DNA was selectively bound to the DNeasy membrane as contaminants were passed through into the collection tubes. Remaining contaminants and enzyme inhibitors were removed in two efficient wash steps and DNA was then eluted in water or buffer, ready for use.

3.8.1.2 Purity and concentration of DNA

The concentration and purity of isolated DNA was determined by NanoDrop™ spectrophotometer (ND-1000, Thermo Fisher Scientific Inc. USA). 1 µl of purified DNA sample was pipette onto the spectrophotometer and the ratio of A_{260}/A_{280} along with concentration of DNA was recorded. Concentration was expressed in ng/µl and samples with $A_{260/280}$ ratio of 1.6 to 1.85 were considered as pure DNA samples and were loaded for agarose gel electrophoresis.

3.8.1.3 Agarose gel electrophoresis

About 2000 ng of DNA was loaded on 0.9-1% agarose gel and electrophoresis was carried out in a submarine horizontal electrophoresis unit (BioRad, USA). The DNA migration and resolution pattern was examined by ethidium bromide staining. Bands/characteristic DNA smearing or laddering pattern were visualized under UV transillumination technique and the picture was documented by photography.

3.9.2 Flow cytometry analysis

All the following assays were carried out on splenocytes. Spleen lymphocytes were collected as described below. The flow cytometric analysis was done on a Becton Dickinson flow cytometer. Cell debris, characterized by a low forward scatter (FSC)/side scatter (SSC) was excluded from analysis. The data was analyzed by Cell Quest software and mean fluorescence intensity was obtained by histogram statistics.

3.9.2.1 Splenocytes preparation

Spleen cells were harvested as described (Badgujar *et al.,* 2013). The procedure was similar to earlier explained point 3.8.2.2; except lymphocytes were washed with DPBS three times instead of DMEM (i.e. respective cell suspension was transferred and centrifuged at 2000 rpm for 5 minutes, 4°C). The final cell pellet thus obtained was resuspended in 1 ml DPBS and viable lymphocytes counts were obtained by trypan blue (Sigma-Aldrich) exclusion method using a haemacytometer. Finally, cells were adjusted into and finally adjusted at a concentration of 2×10^6 cells/ml in DPBS.

3.9.2.2 Flow Cytometric Analysis of Phosphatidyl Serine Externalization in Apoptosis Lymphocytes

To determine the extent of early apoptosis and necrosis in thymus and spleen, cell death was determined by staining cells with annexin-V FITC and PI (Bertho *et al.,* 2000). Positioning of quadrants on annexin-V/PI dot plots was performed and living cells (Annexin V”/PI”), early apoptotic/primary apoptotic cells (Annexin V+/PI”), late apoptotic/secondary apoptotic cells (Annexin V+/PI+) and necrotic cells (Annexin V”/PI+) were distinguished (Vermes *et al.,* 1995). Therefore, the total apoptotic proportion included percentage of cells with fluorescence Annexin V+/PI” and Annexin V+/PI+. For annexin-V/PI analysis, splenocytes (after last wash) were resuspended in 200 µl of annexin V binding buffer at a concentration of 2 x 10^6 cells/ml. An aliquot of 100 µl of each sample was incubated with 5 µl annexin-V–FITC and 10 µl of PI for 15 min in dark at room temperature followed by addition of 400 µl annexin V binding buffer. The FITC and PI fluorescence was measured immediately through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired. At least three independent experiments were performed.

3.9.2.3 Apoptotic DNA (cell cycle) analysis

The splenic lymphocytes with hypodiploid DNA were determined by cell cycle studies. At the end of exposure period, harvested cells were resuspended in PBS at a concentration of 2 x 10^6 cells/ml. 500 µl of this suspension was taken and fixed by transferring this suspension (drop-by-drop addition) with a pasteur pipette into a centrifuge tube containing 4.5 ml of 70% ethanol maintained on ice and stored at 4°C overnight. The ethanol suspended cells were

centrifuged at 300*g* for 5 min and ethanol was decanted. Cell pellet was resuspended in 2.5 ml of PBS and was washed. Finally in a cell pellet, 0.5 ml of PI staining solution was added and tubes were kept in dark at room temperature for 30 min. 250 µl of PI stain (5 mg PI, 50 µl Triton-X (0.1% v/v), 0.1% sodium citrate in 50 ml PBS) and 250 µl of RNase A (100 µg/ml in PBS) and further incubated for 30 min in dark. The PI fluorescence was measured through a FL-2H filter (585 nm) and 10,000 events were acquired (Darzynkiewicz et al., 1992).

3.9.2.4 Mitochondrial transmembrane potential ($\Delta\psi m$) using $DiOC_6$ dye

$DiOC_6$ (3,3-dihexyl-oxacarbocyanine iodide), a lipophilic cationic fluorescent dye transported into mitochondria due to the negative mitochondrial membrane potential and concentrated within the mitochondrial matrix. However, this dye is selective for the mitochondria of viable cells. The mitochondrial membrane potential was quantified by flow cytometric analysis of $DiOC_6$ stained cells.

Stock and working solution preparation:

3'3'Dihexyloxacarbocyanine iodide ($DiOC_6$) dye (Aldrich) 40 µM stock solution was prepared by dissolving 0.23 mg of the dye in 10 ml of dimethyl sulfoxide (DMSO). Working solution (400 nM) was prepared by adding 10 µl of dye in 1 ml PBS. Stock solution was stored -20°C for future use.

Protocol

$\Delta\psi m$ was analyzed according to the method of Castedo *et al.* (2002). 0.5 ml splenocytes cell suspension (2 x 10^6 cells/ml) was taken into the tube and 25 µl of $DiOC_6$ working solution was added (final concentration 20 nM). Cells were incubated at 37°C for 15–20 min, then were returned on ice to be analyzed in flow cytometer through FL1-H (emission: 530 nm).

3.9.2.5 Detection of reactive oxygen species (ROS) by DCFH-DA:

Preparation of stock and working solution of DCFH-DA:

MW= 487.3; 10 mM of DCFH-DA was prepared in DMSO, i.e. (5 mg of DCFH-DA was dissolved in 1 ml DMSO).

DCFH-DA (2', 7'-dichlorofluorescin diacetate) the procedure of Wang *et al*. (1996) was followed. Cell density was adjusted to 1×10^6 cells/ml. 990 µl of this cells suspension was taken in microcentrifuge tube and 10 µl of (0.1 mM) DCFH-DA dye was added (final concentration of 100 µM). Tubes were incubated for 15 min in the dark at room temperature. Flow cytometric analysis for DCF florescence was done as Counts v/s FL-1 and 10000 splenocytes per event were taken. Percent right shift in the fluorescence peak was recorded.

3.10 Sodium dodecyl sulphate polyacrylamide gel electrophorosis (SDS-PAGE) and Western blot analysis for protein expression in tissue lysates

3.10.1 Tissue harvesting

Mice of all groups from all subset of subacute toxicity study were sacrificed at the end of treatment period. Spleen tissue was excised, flash frozen in liquid nitrogen and immediately stored in -80°C until all mice were sacrificed. To prepare spleen homogenates, spleen tissue was thawed and homogenized in ice cold PBS (0.5-0.7 ml) with the help of a homogenizer (Ultra Turrax T10 basic, IKA, Germany). 100-150 µl of homogenate was lysed with 495 µl of lysis buffer (50mM Tris-HCl, pH 7.3; 1% (v/v) Triton X-100; 150mM NaCl and 1mM EDTA) with 5 µl 100x protease inhibitor cocktail (containing 104 mM AEBSF (4-(2- Aminoethyl) benzenesulfonylfluoride); 80µM Aprotinin; 4mM Bestatin; 1.4mM E-64; 2mM Leupeptin and 1.5mM Pepstatin A). Lysates were clarified by centrifugation at 12,000 g for 5 min at 4°C; their aliquots (supernatants) were prepared and stored at -80°C till further processing for western blot. One of the aliquots was used for the determination of protein by the Bradford protein assay method.

3.10.2 Estimation of protein in spleen lysate

One of the aliquots of spleen lysate was used for the determination of protein. The protein concentration of lysate was determined by the Bradford protein estimation kit as per the manufacturer's instructions using bovine serum albumin (BSA) as standard. The concentration of protein for each animals of the each group obtained was then subsequently used for SDS-PAGE.

3.10.3 SDS-PAGE

Stored frozen tissue lysates were thawed on ice. Protein concentration of each sample was adjusted with 5x lamilliae sample loading buffer to give 60 µg protein per lane Then, sample were boiled at 100°C for 5 min and cooled to 4°C and were subjected to SDS-PAGE and western blotting analysis. The separating/resolving gel (15% polyacrylamide gel, pH 8.8) was prepared in between the glass plates using the gel casting assembly spacers (Bio-Rad Laboratories Inc., USA) of 1.5 mm thickness. Stacking gel (5% in Tris buffer of pH 6.8) was poured over it and comb was placed. The gel after polymerization was transferred into an electrophoresis apparatus (Bio-Rad Laboratories Inc., USA) and buffer chambers were filled with gel running buffer. Each sample (in different volume depending upon 60 µg) was loaded into various wells of the gel (two gels of same pore size were prepared at a time; one for internal control protein and second for protein of our interest) and subjected to electrophoresis at 120 V till the tracking dye reached the separating gel. The voltage was then increased to 150-155 V till the dye reached the bottom of the separating gel. Predetermined and pre-stained molecular weight standard (BR Biochem Life Sciences Pvt. Ltd., New Delhi, India) was used as protein marker.

3.10.4 Western blot analysis

After subjecting protein under reducing conditions on polyacrylamide gel, gels were transferred in transfer buffer to equilibrate and fractionated proteins were transferred on a PVDF membrane, 0.45 µm (BioRad Laboratories Inc., USA) by electroblotting at 90 V for 1 h using a Bio-Rad blot transfer apparatus as per the manufacturer's instructions. PVDF membrane used for transfer was equilibrated with ice-cold methanol by soaking in it for 5 min. Membrane pads (BioRad Laboratories Inc., USA) cut to the size of the gel, soaked in the transfer buffer along with membrane were then placed on the anode plate over which the pre-wetted PVDF membrane was placed. The gel was placed carefully over the membrane and membrane pad was placed over the gel. Transfer buffer was poured over it and glass rod (test tube) was rolled over it to exclude excess of air bubbles. Cathode plate was locked with anode plate to seal the cassette which was then immersed in the transfer buffer. The membrane

was blocked for 1 h at room temperature with 5% BSA in PBS to block the non-specific sites on the membrane. Membranes were then washed thrice with phosphate buffer saline-containing 0.5% tween-20 (PBS-T) for 10 min each. Membranes were subsequently incubated with rabbit polyclonal primary antibodies of Fas and Fas-L (each diluted to 1:200) and mouse monoclonal primary β-actin antibody (1:500) for 12 h at 4°C. After washing membranes again thrice with PBS-T, they were incubated for 2 h at 37°C with Horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (diluted to 1:2000) for Fas and Fas-L and HRP conjugated anti-mouse secondary antibody for β-actin (diluted to 1:5000). Following successive washes, target protein bands were developed using the diaminobenzidine (DAB) system (Bangalore, Genei™, India). DAB solution was then immediately poured on the membrane and 10 µl (0.1% v/v) 30% H_2O_2 was added. β-actin was used to normalize the samples. Minimum three blots were performed for every protein analysis.

✍✍✍

Results

4 RESULTS

4.1 Cartap effects on general toxicity parameters

4.1.1 Clinical signs:

Treatment of mice with cartap hydrochloride over a period of 28 days did not show any mortality and overt signs of toxicity during exposure period; however, mice show slight edematous swelling over a forehead and patchy hair loss over the neck which was extensive as the increasing level dose.

4.1.2 Body weight and organ weights

The body weights (gm) of all the mice from different groups were recorded at weekly interval did not show any significant different between control and cartap treated groups except at last week (Table 4; Fig. 2).

There was dose dependent reduction in absolute weight of liver, spleen, heart, lungs, brain of mice treated with three different dose level of cartap as compared to control group (Table 5; Fig. 3). Significant reduction was observed in organ weights with increasing dose level except kidney which did not show any significant weight reduction at the increasing dose level.

Following reduction in absolute organ weights of liver, brain, spleen, heart and lungs, relative organ weight of three organ kidney, liver and spleen were also reduced significantly ($p<0.05$) with increasing dose level of cartap as compared with control group (Table 6; Fig. 4). Likewise, there was no statistical significant change in relative organ weight of any other organ at any dose level of cartap.

4.1.3 Hematological parameters

The values of hematological end points in mice after treatment with cartap are shown in (Table 7 and 7a; Fig. 5 and 6). Significant reduction (p<0.05) in hematological parameters such as PCV, Hb, TEC and TLC were directly proportionate to the dose level of cartap as compared to the control group. Significant variation was observed in DLC, as there was reduction in percent lymphocytes, percent monocytes. However, the percent eosinophils were significantly (p<0.05) increased with increase in the dose of cartap. Neutrophil concentration did not show any significant variation at any dose level of compound.

4.1.4 Serum biochemical parameters

Serum protein level after 28 days exposure to cartap is presented in (Table 8; Fig. 7). There was significant (p<0.05) reduction in total protein (gm/dl) and serum albumin (gm/dl) concentration which were directly proportionate to dose of cartap as compared to the control group. However, significant (p<0.05) change in albumin: globulin (A: G) ratio was observed at medium and high dose of cartap (Table 8; Fig. 7). Globulin level (Table 8a; Fig. 7) reduced significantly (p<0.05) as compared to control group but did not show any significant variation between groups.

Serum ALT, AST and LDH concentrations were found to be increased significantly (p<0.05) at all dose levels of cartap as compared to control group (Table 8a; Fig. 8) after 28 days of oral exposure of compound.

Serum BUN and Creatinine concentration were significantly (p<0.05) increased at all dose levels of cartap as compared to control group (Table 8a; Fig. 9 and 10) after 28 days of oral exposure of compound.

4.1.5 Histopathology

Histopathological examination of organs viz. spleen, liver, kidney, brain and lung revealed significant changes in the normal histological architecture.

Table 4 : Effect on body weight (gm) of mice after 28 days oral exposure to cartap

Groups	Dose	Body weight (gm)				
		Days				
		0 day	1st wk	2nd wk	3rd wk	4th wk
I	Control	15.83 ± 0.83	19.50 ± 0.76	23.67 ± 0.67	27.00 ± 0.86	$30.00^{c} \pm 0.30$
II	Cartap 5 mg	15.83 ± 0.83	18.17 ± 0.87	22.83 ± 0.70	26.00 ± 0.77	$27.50^{b} \pm 0.85$
III	Cartap 7.5 mg	15.83 ± 0.83	18.50 ± 0.76	22.33 ± 0.61	25.83 ± 0.60	$25.67^{ab} \pm 0.80$
IV	Cartap 15 mg	16.00 ± 0.52	19.00 ± 0.45	22.83 ± 0.17	25.00 ± 0.26	$23.50^{a} \pm 0.34$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

Table 5 : Effect on absolute organ weight (gm) of mice after 28 days oral exposure to cartap

Groups	Dose	Brain	Kidney	Liver	Lung	Spleen	Heart
I	Control	$0.39^{b} \pm .01$	$0.33 \pm .01$	$1.35^{c} \pm .02$	$0.20^{b} \pm .01$	$0.16^{b} \pm .01$	$0.1233^{b} \pm .02$
II	Cartap 5 mg	$0.38^{b} \pm .01$	$0.33 \pm .01$	$1.13^{b} \pm .02$	$0.21^{b} \pm .02$	$0.16^{b} \pm .01$	$0.1017^{ab} \pm .01$
III	Cartap 7.5 mg	$0.36^{b} \pm .01$	$0.34 \pm .01$	$1.01^{a} \pm .01$	$0.19^{ab} \pm .01$	$0.11^{a} \pm .01$	$0.1067^{ab} \pm .03$
Iv	Cartap 15 mg	$0.31^{a} \pm .01$	$0.30 \pm .01$	$0.95^{a} \pm .01$	$0.17^{a} \pm .01$	$0.09^{a} \pm .01$	$0.0867^{a} \pm .01$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

Table 6 :Effect on Relative organ weight (gm/100gm) of mice after 28 days oral exposure to cartap

Groups	Dose	Brain	Kidney	Liver	Lung	Spleen	Heart
I	Control	1.29 ± 0.04	$1.09^{a} \pm 0.04$	$4.51^{b} \pm 0.08$	0.68 ± 0.03	$0.54^{b} \pm 0.02$	0.41 ± 0.01
II	Cartap 5 mg	1.38 ± 0.03	$1.19^{ab} \pm 0.03$	$4.13^{a} \pm 0.12$	0.77 ± 0.04	$0.59^{b} \pm 0.03$	0.37 ± 0.02
III	Cartap 7.5 mg	1.41 ± 0.04	$1.32^{b} \pm 0.05$	$3.92^{a} \pm 0.08$	0.77 ± 0.05	$0.43^{a} \pm 0.02$	0.42 ± 0.02
IV	Cartap 15 mg	1.33 ± 0.04	$1.29^{b} \pm 0.03$	$4.06^{a} \pm 0.08$	0.71 ± 0.03	$0.38^{a} \pm 0.02$	0.37 ± 0.03

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

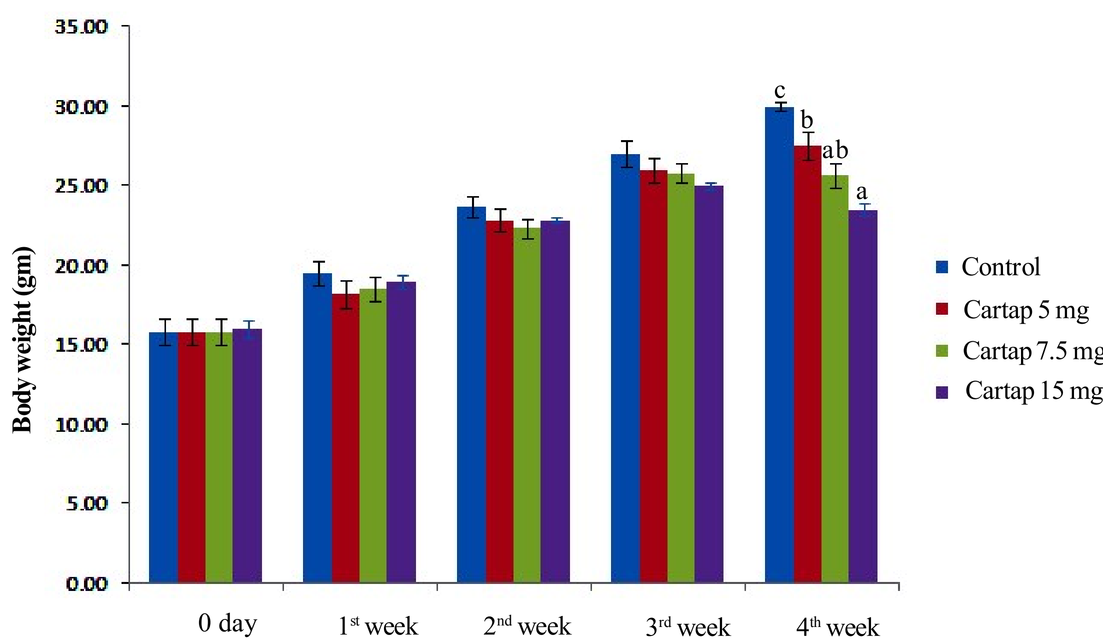

Fig. 2: Effect on body weight (gm) of mice after 28 days oral exposure to cartap

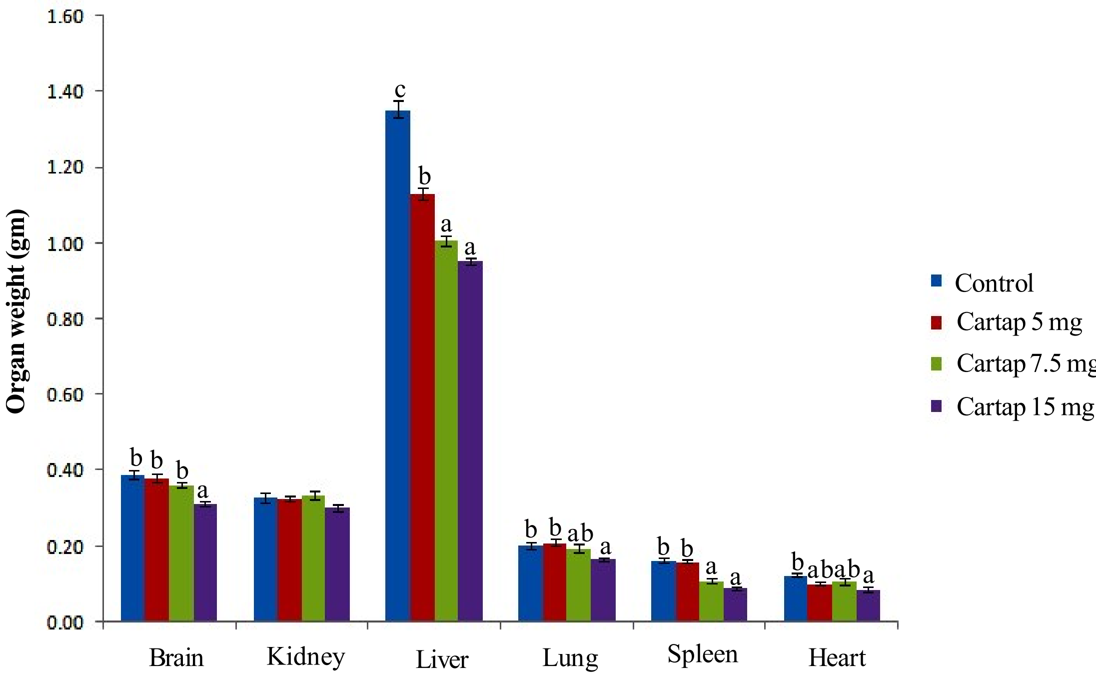

Fig. 3: Effect on absolute organ weight (gm) of mice after 28 days oral exposure to cartap

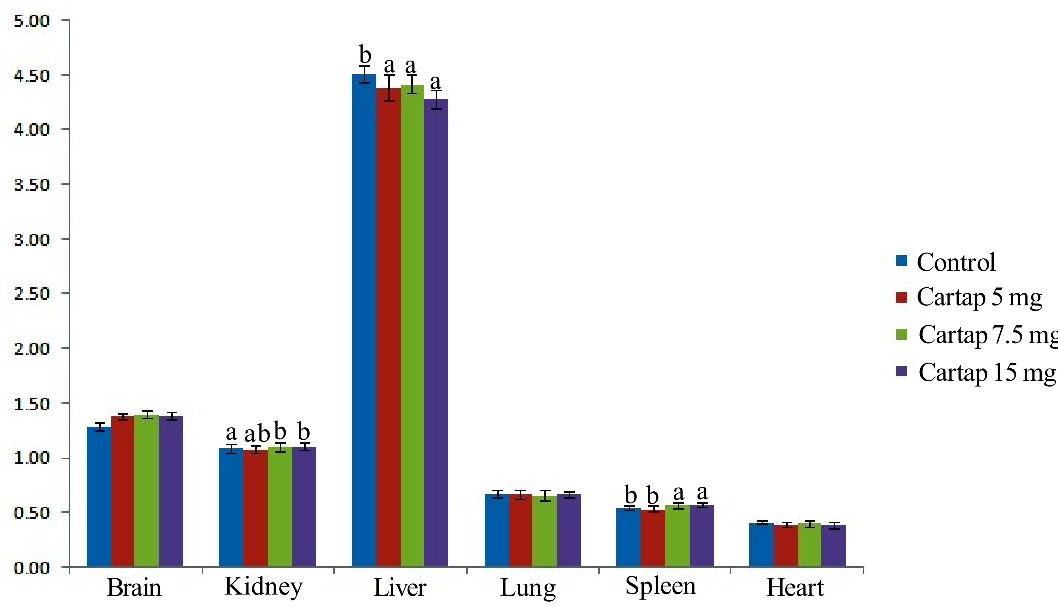

Fig. 4: Effect on Relative organ weight (gm/100gm) of mice after 28 days oral exposure to cartap

Table 7: Haematological parameters of mice after 28 days oral exposure to cartap

Groups	Dose	Haematological parameters		
		PCV (%)	Hb (gm/dl)	TEC ($x10^6/\mu l$)
I	Control	$46.08^d \pm 0.32$	$15.35^d \pm 0.11$	$7.68^d \pm 0.05$
II	Cartap 5 mg	$44.17^c \pm 0.22$	$14.73^c \pm 0.08$	$7.36^c \pm 0.04$
III	Cartap 7.5 mg	$41.47^b \pm 0.19$	$13.85^b \pm 0.06$	$6.91^b \pm 0.03$
IV	Cartap 15 mg	$39.11^a \pm 0.23$	$13.03^a \pm 0.08$	$6.52^a \pm 0.04$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;

Table 7a : Haematological parameters of mice after 28 days oral exposure to cartap

Groups	Dose	TLC ($x10^3/\mu l$)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Eosinophil (%)
I	Control	$5.42^d \pm 0.02$	$74.67^d \pm 0.67$	$4.33^b \pm 0.67$	15.67 ± 0.42	$5.33^a \pm 0.61$
II	Cartap 5 mg	$5.22^c \pm 0.02$	$70.17^c \pm 0.60$	$2.00^a \pm 0.37$	15.50 ± 0.89	$12.33^b \pm 0.80$
III	Cartap 7.5 mg	$4.48^b \pm 0.02$	$67.50^b \pm 0.43$	$1.83^a \pm 0.40$	15.17 ± 0.60	$15.50^{bc} \pm 0.34$
IV	Cartap 15 mg	$3.56^a \pm 0.02$	$65.00^a \pm 0.73$	$1.33^a \pm 0.21$	14.83 ± 1.14	$18.83^c \pm 1.35$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

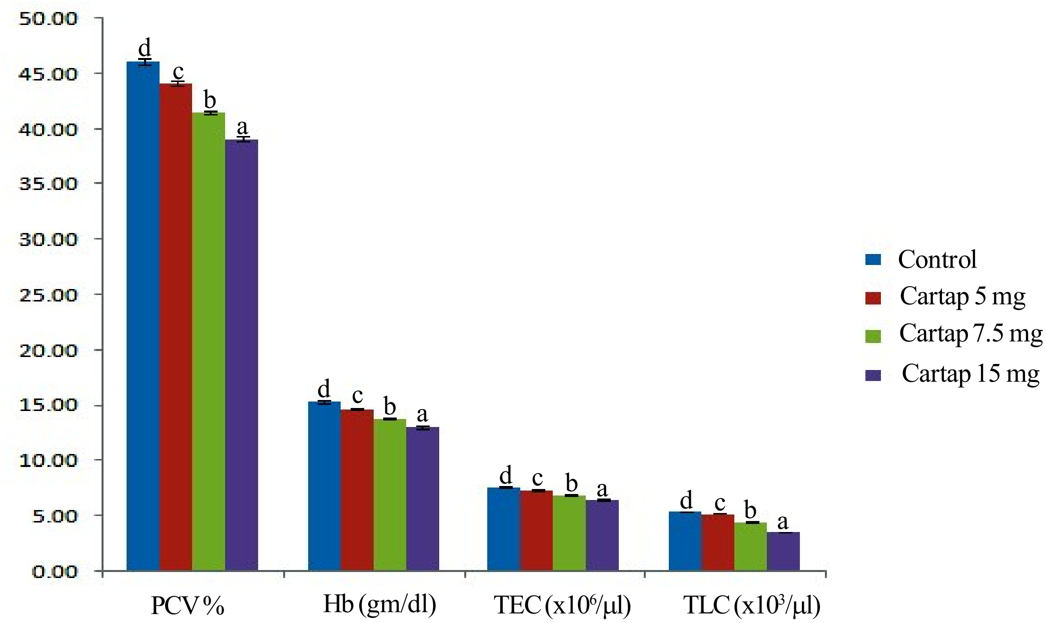

Fig. 5: Haematological parameters of mice after 28 days oral exposure to cartap

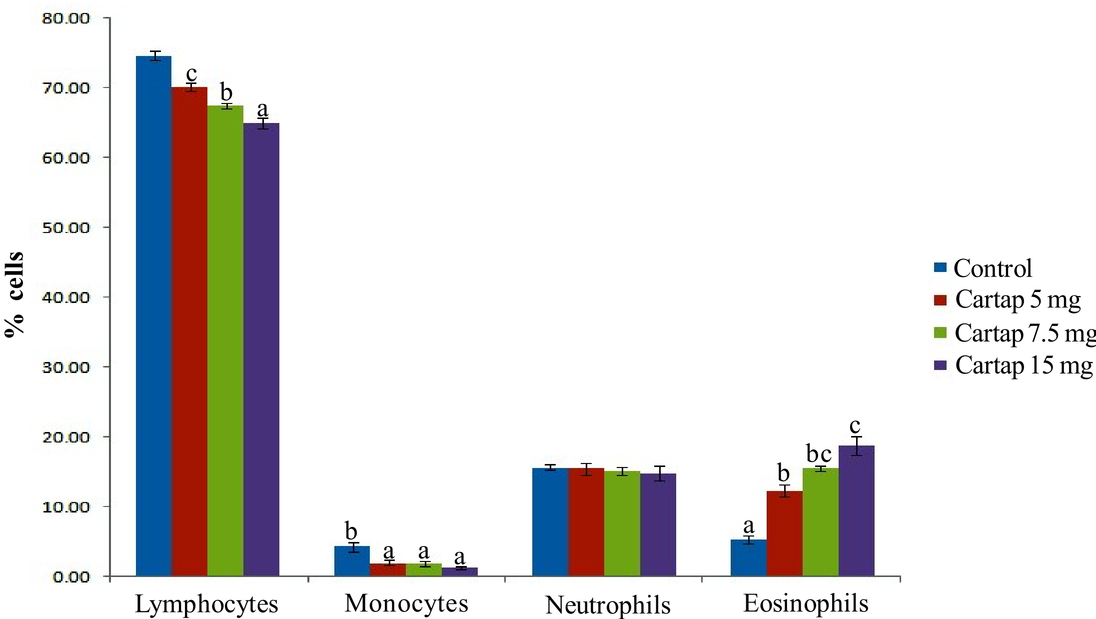

Fig. 6: Haematological parameters of mice after 28 days oral exposure to cartap

Table 8 : Serum biochemical parameters of mice after 28 days oral exposure to cartap

Groups	Dose	Total protein (gm/dl)	Albumin (gm/dl)	Globulin (gm/dl)	Albumin: Globulin
I	Control	$6.36^{d} \pm 0.05$	$3.26^{d} \pm 0.03$	$3.10^{b} \pm 0.05$	$1.06^{b} \pm 0.02$
II	Cartap 5 mg	$5.64^{c} \pm 0.07$	$2.80^{c} \pm 0.01$	$2.84^{a} \pm 0.06$	$0.99^{b} \pm 0.02$
III	Cartap 7.5 mg	$5.14^{b} \pm 0.04$	$2.31^{b} \pm 0.04$	$2.83^{a} \pm 0.07$	$0.82^{a} \pm 0.04$
IV	Cartap 15 mg	$4.75^{a} \pm 0.06$	$2.03^{a} \pm 0.02$	$2.73^{a} \pm 0.07$	$0.75^{a} \pm 0.02$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

Table 8a : Serum biochemical parameters of mice after 28 days oral exposure to cartap

Groups	Dose	BUN (mg/dl)	Serum Creatinin (mg/dl)	ALT (Unit/l)	AST (Unit/l)	LDH (Unit/l)
I	Control	$13.06^{a} \pm 0.10$	$0.79^{a} \pm 0.03$	$24.91^{a} \pm 0.74$	$37.99^{a} \pm 0.56$	$33.66^{a} \pm 2.72$
II	Cartap 5 mg	$22.12^{b} \pm 0.62$	$1.10^{b} \pm 0.01$	$49.27^{b} \pm 0.93$	$88.08^{b} \pm 1.35$	$66.93^{b} \pm 2.10$
III	Cartap 7.5 mg	$26.77^{c} \pm 0.41$	$1.31^{c} \pm 0.02$	$76.87^{c} \pm 1.32$	$135.81^{c} \pm 2.63$	$92.17^{c} \pm 1.82$
IV	Cartap 15 mg	$35.19^{d} \pm 0.42$	$1.67^{d} \pm 0.03$	$105.39^{d} \pm 0.63$	$271.91^{d} \pm 4.18$	$112.61^{d} \pm 1.86$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

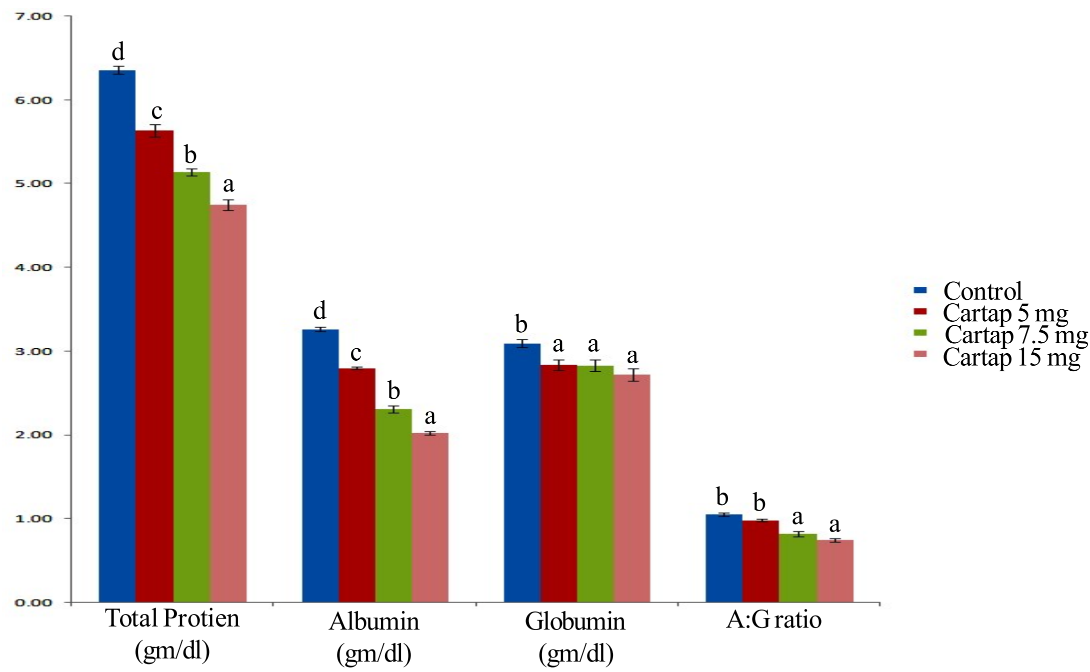

Fig. 7: Serum biochemical parameters of mice after 28 days oral exposure to cartap

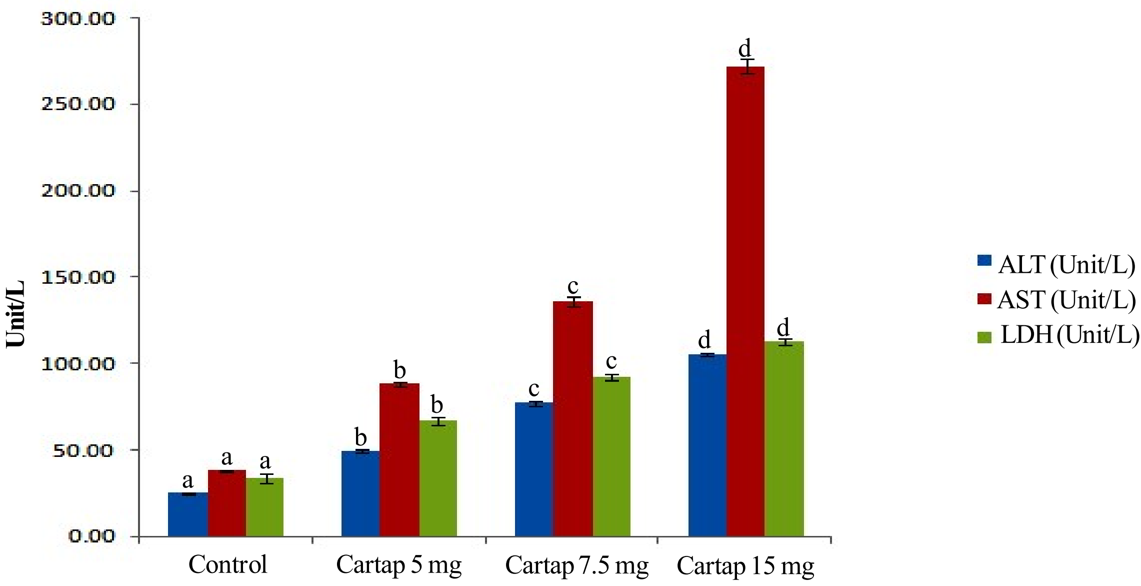

Fig. 8: Serum biochemical parameters of mice after 28 days oral exposure to cartap

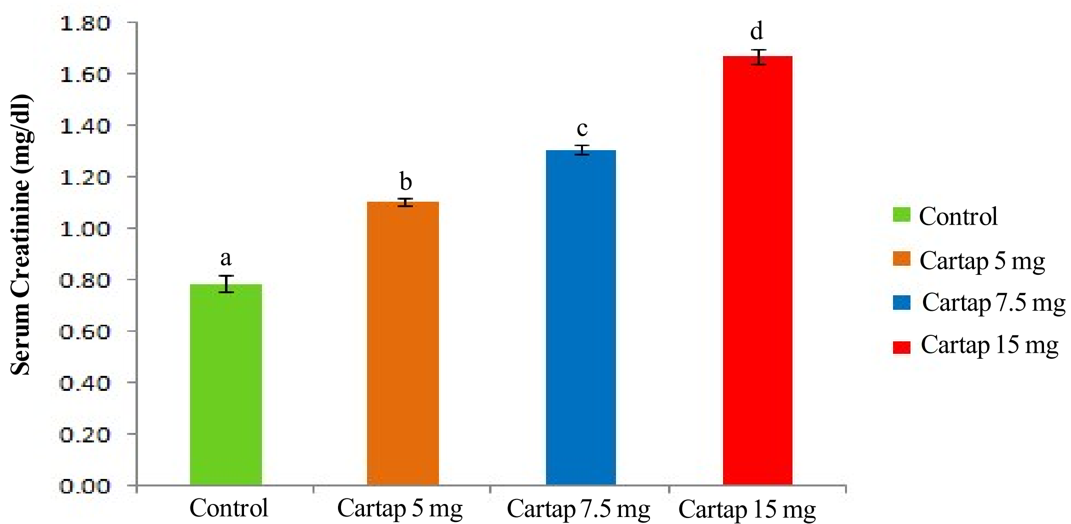

Fig. 9: Serum creatinine of mice after 28 days oral exposure to cartap

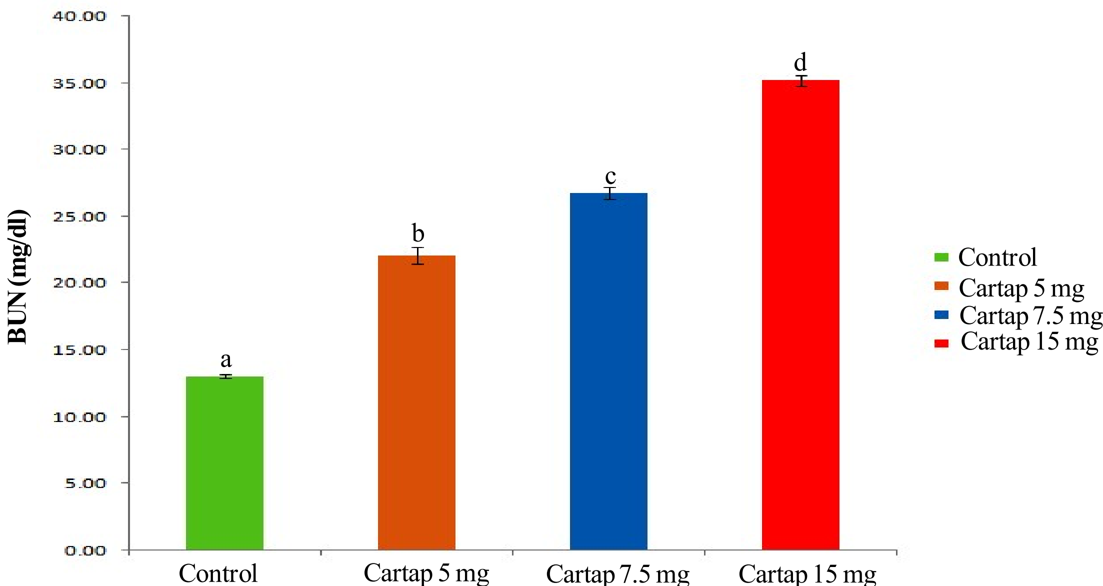

Fig. 10: Blood Urea Nitrogen of mice after 28 days oral exposure to cartap

Spleen

Spleen of control group mice showed normal histological architecture with abundant lymphocytes in lymphoid follicles in the white pulp. Spleen of medium and high doses treated mice showed characteristic atrophic changes in white pulps characterized by decreased size of lymphoid follicles and peri arteriolar lymphoid sheath (PALS) in comparison with control group (Fig. 11-14).

Liver

The liver of control group mice showed normal histological architecture with intact nucleus and cytoplasm. Focal area of hepatic degeneration with mild congestion of portal area at low dose, vascular degenerative changes in cytoplasm with focal infiltration of inflammatory cells at medium dose and multifocal infiltration of mononuclear cells in hepatocytes showing maldegenerative changes in hepatic lobule observed infiltration of inflammatory cells with congestion of portal area were at high dose level of cartap (Fig. 15-18).

Kidney

28 days exposure to cartap induced marked histopathological alteration in kidney. Characteristic changes were, degenerative changes in renal tubular cells, hemorrhage in medulla and cortex, mild degenerative changes in convoluted tubules with hemorrhage and degenerative changes in glomerular area at increasing dose levels as compared to the control group mice which showed normal architecture (Fig. 19-22).

Lungs

Lungs of mice treated with low dose of cartap showed nearly normal appearance like normal lungs of control group mice. At higher doses of cartap lung showed infiltration of inflammatory cells in alveoli, alveolar septum, accumulation of pinkish edematous fluid in bronchi and capillary congestion with thickening of interstitial septa (Fig. 23-26).

Brain

In present study, brain of mice after exposure to cartap for 28 days showed mild meningitis with infiltration of inflammatory cells in meninges, focal cerebral gliosis with vascular neurodegenerative changes in the mid brain and infiltration of glial cells in cerebral cortex (Fig. 27-30).

4.2 Oxidative stress related biochemical parameters

4.2.1 Effect on lipid peroxidation (LPO) level in liver, kidney and brain

LPO was measured in terms of the malondialdehyde (MDA) produced in liver, kidney and brain of mice exposed to cartap for 28 days (Table 9; Fig. 31). MDA formed in liver was significantly ($p<0.05$) increased at medium (7.5 mg) and high dose (15 mg) level of cartap as compared to the control and low dose of cartap (5 mg) group, but production of MDA in kidney and brain increased significantly ($p<0.05$) at all three dose level of cartap as compared to control group.

4.2.2 Superoxide anion generation:

Super oxide anion generation expressed as amount of NBT reduced (pmol/min/mg of protein) in liver, kidney and brain of mice after 28 days oral exposure of cartap (Table 10; Fig. 32). Amount of NBT reduced in liver was significantly ($p<0.05$) increased at medium (7.5 mg) and high (15 mg) dose level of cartap as compared to the low dose of cartap (5 mg) and control group; however significant ($p<0.05$) reduction of NBT was directly proportionate to the dose level of cartap in kidney and brain as compared to the control group.

4.2.3 Nitrite production:

The results of nitrite estimation in liver, kidney and brain are expressed in terms of µM/l of tissue homogenates and were significantly ($p<0.05$) increased at all three dose levels of cartap as compared to the control group (Table 11; Fig. 33).

4.2.4 Effect on non-enzymatic anti-oxidants in liver, kidney and brain

A. Reduced glutathione (GSH)

GSH concentration in liver, kidney and brain tissue homogenates were significantly ($p<0.05$) reduced at all three dose levels after 28 days exposure to cartap as compare to the control group (Table 12; Fig. 34).

B. Total thiol (T-SH)

Total thiol concentrations in the liver, kidney and brain are shown in (Table 13; Fig. 35). In liver there was significant ($p<0.05$) dose dependant reduction in T-SH concentration

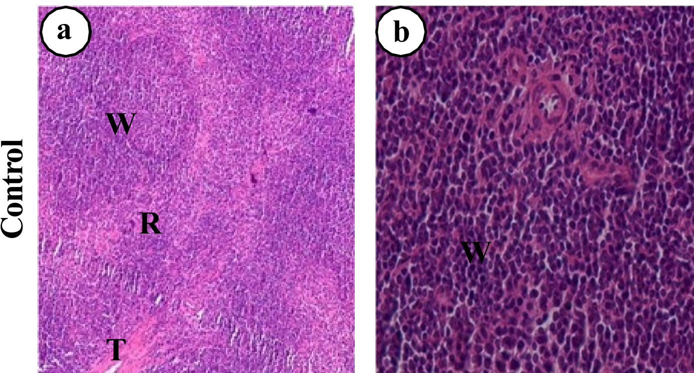

Fig. 11: Histopathological architecture of spleen of control mice (H&E). a: 100x; b: 400x; W: White pulp; R: Red pulp; T: Trabaculae

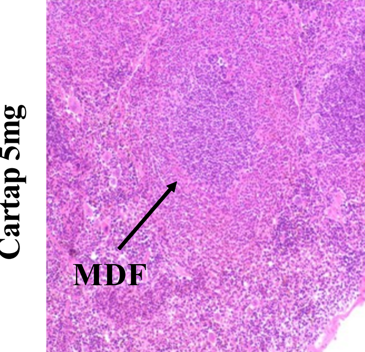

Fig. 12: Histopathological architecture of spleen after 28 days oral exposure to cartap 5mg (H&E). 100x; MDF: Mild depletion in lymphoid follicular area

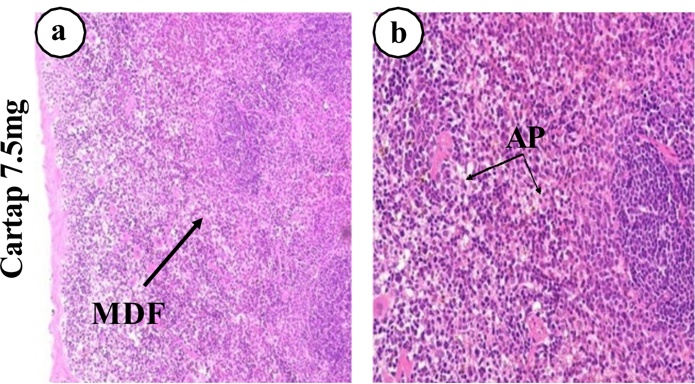

Fig. 13: Histopathological architecture of spleen after 28 days oral exposure to cartap 7.5mg (H&E). a: 100x; b: 200x; MDF: Mild depletion in lymphoid follicular area; AP: Apoptotic bodies

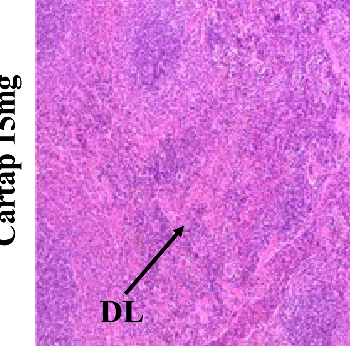

Fig. 14: Histopathological architecture of spleen after 28 days oral exposure to cartap 15mg (H&E). 100x; DL: Depopulation of lymphocytes in white pulp

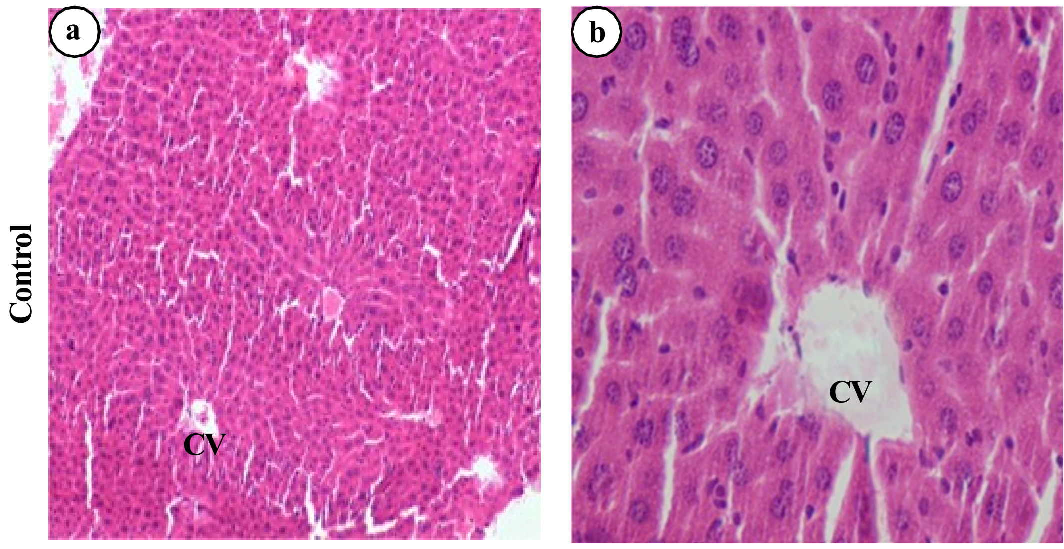

Fig. 15: Histopathological architecture of liver of control mice (H&E). a:100x; b: 400x; CV: Central vein

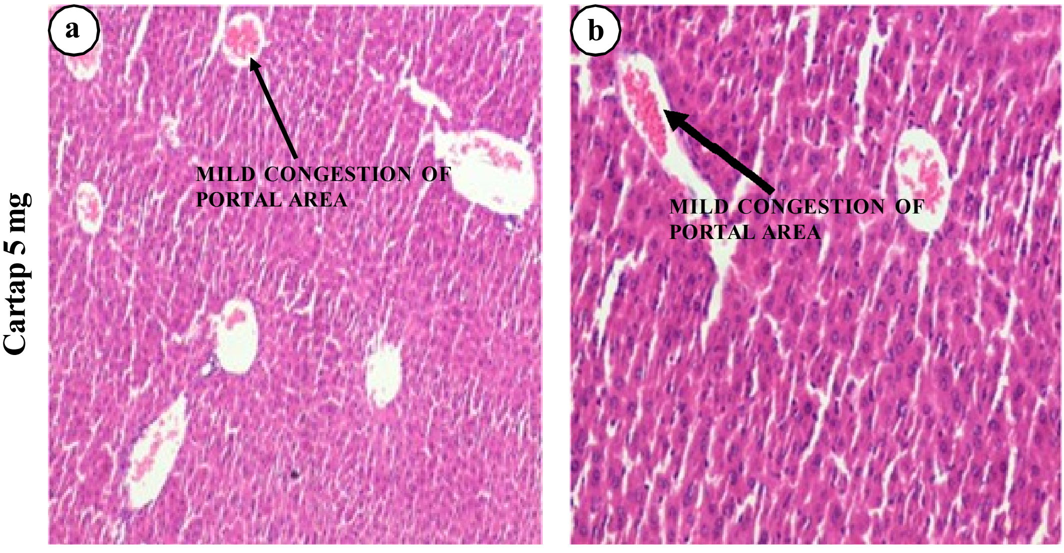

Fig. 16: Histopathological architecture of liver after 28 days oral exposure to cartap 5mg (H&E). a: 200x; b: 400x

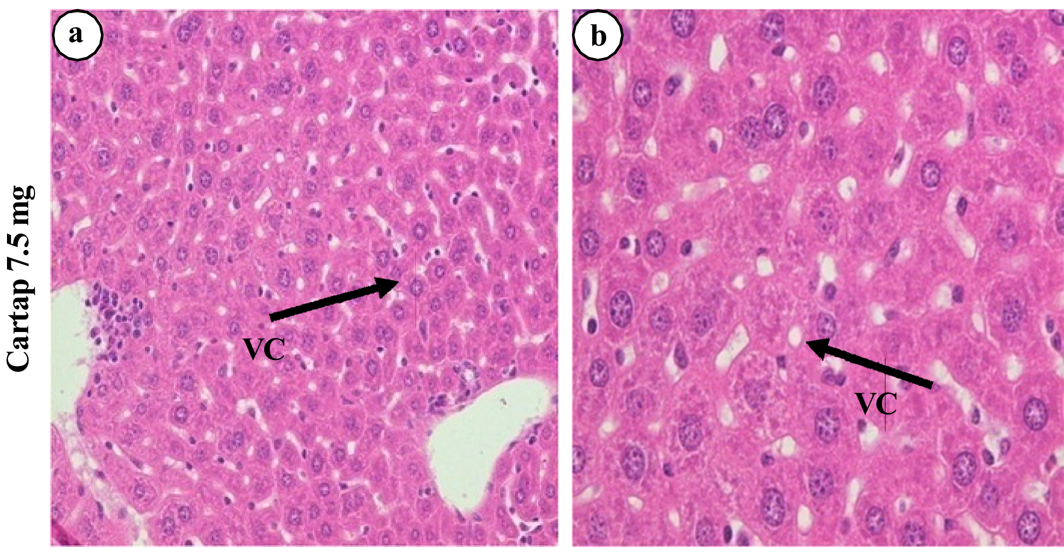

Fig. 17: Histopathological architecture of liver after 28 days oral exposure to cartap 7.5mg (H&E).
a: 200x; b: 400x; VC: Severe vacular degeneration in hepatocytes

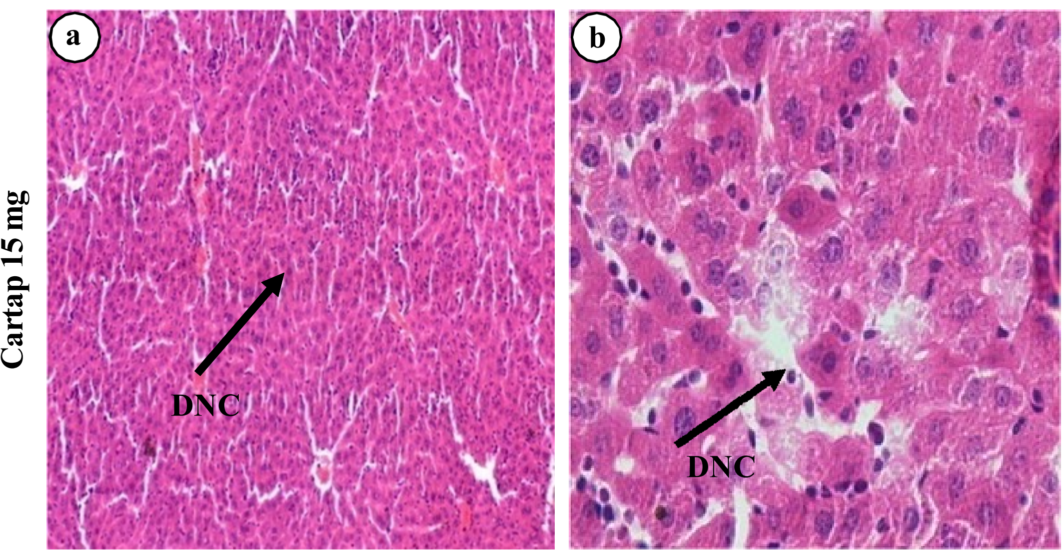

Fig. 18: Histopathological architecture of liver after 28 days oral exposure to cartap 15mg (H&E).
a: 100x; b: 400x; DNC: Degenerating and necrotic changes in hepatocytes

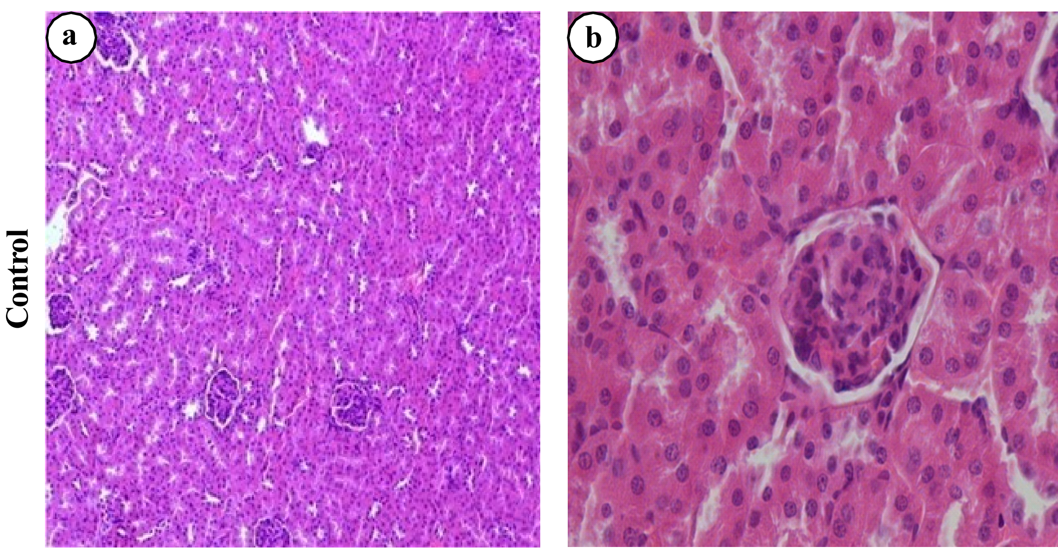

Fig. 19: Histopathological architecture of kidney of control mice (H&E). a: 100x; b: 400x;

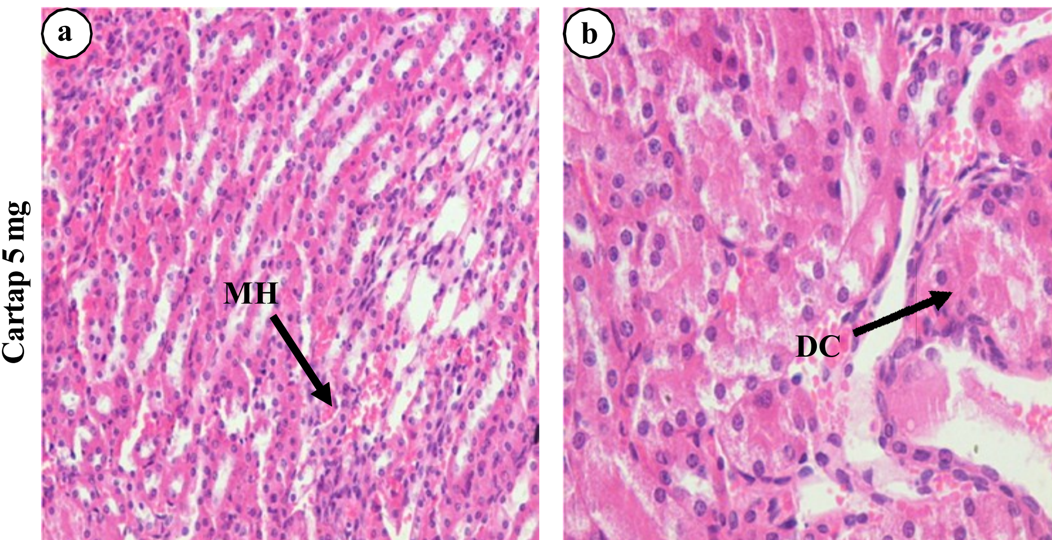

Fig. 20: Histopathological architecture of kidney after 28 days oral exposure to cartap 5mg (H&E). a: 200x; b: 400x; MH: Medullary haemorrhage, DC: Degenrating changes in renal tubule

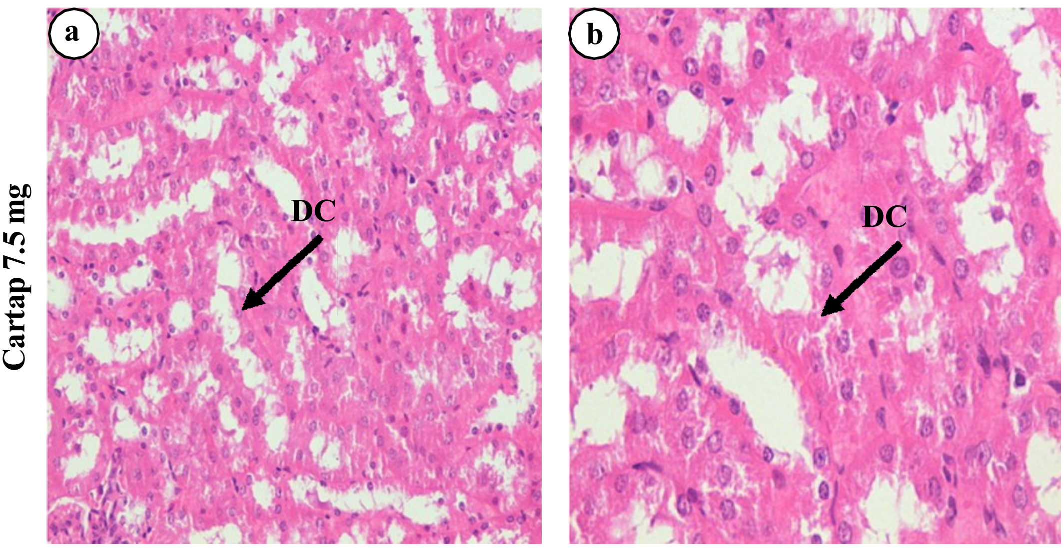

Fig. 21: Histopathological architecture of kidney after 28 days oral exposure to cartap 7.5mg (H&E) a: 200x; b: 400x; DC: Degenrating changes in proximal renal tubule

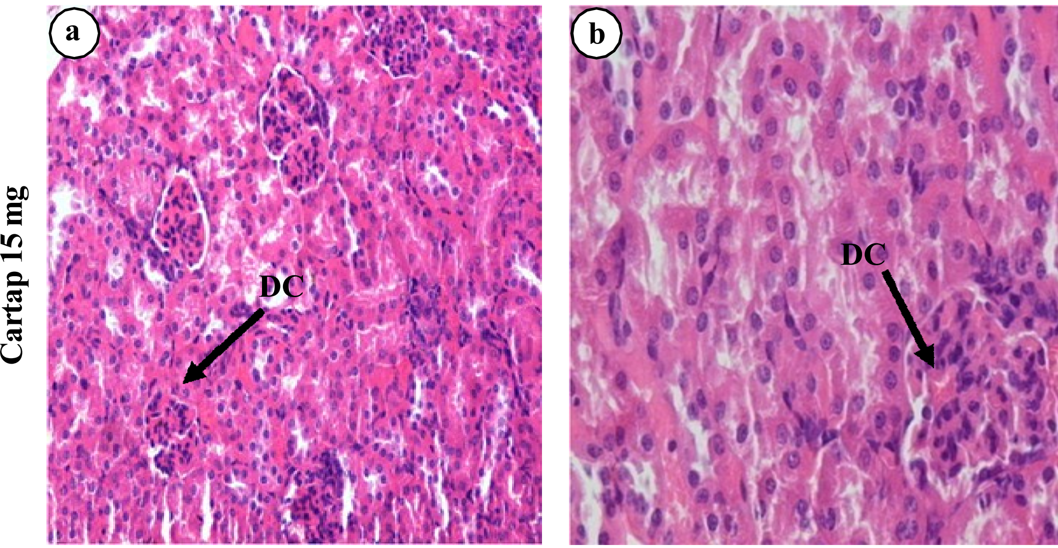

Fig. 22: Histopathological architecture of kidney after 28 days oral exposure to cartap 15mg (H&E). a: 100x; b: 400x; DC: Degenrating changes in glomerular area

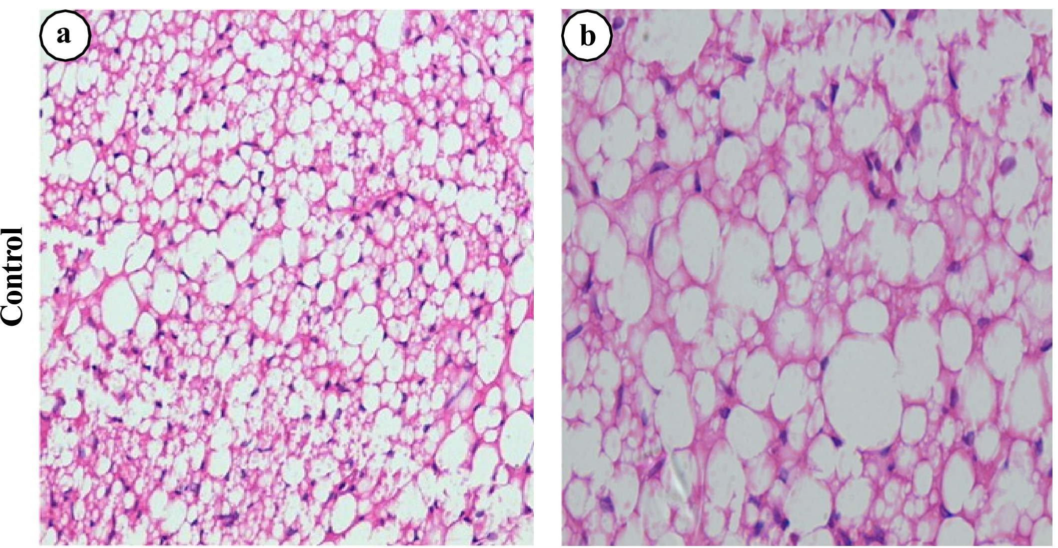

Fig. 23: Histopathological architecture of lung of control mice (H&E). a: 100x; b: 400x

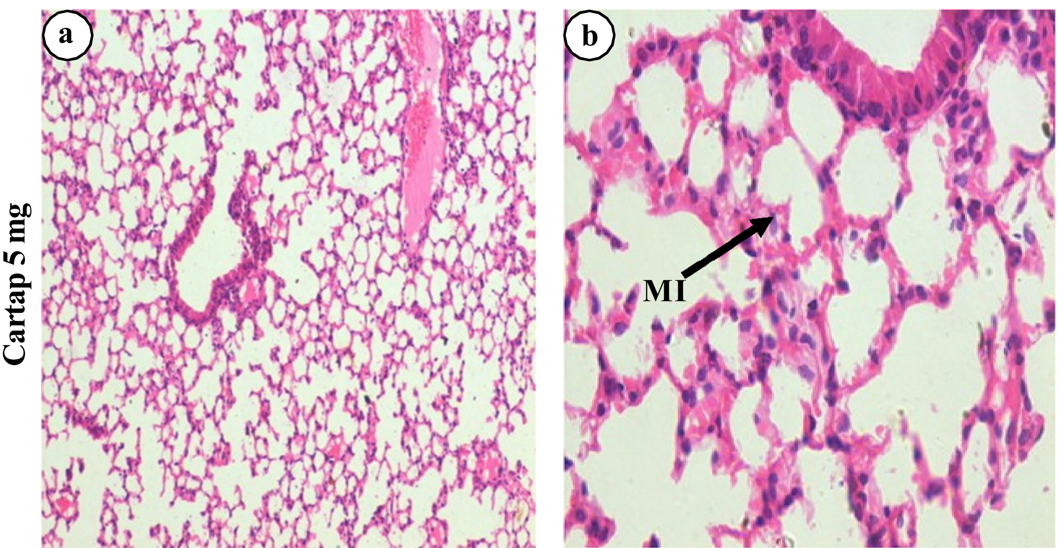

Fig. 24: Histopathological architecture of lung after 28 days oral exposure to cartap 5mg (H&E). a: 100x; b: 400x; MI: Mild infiltration of inflammatory cells in alveolar septa

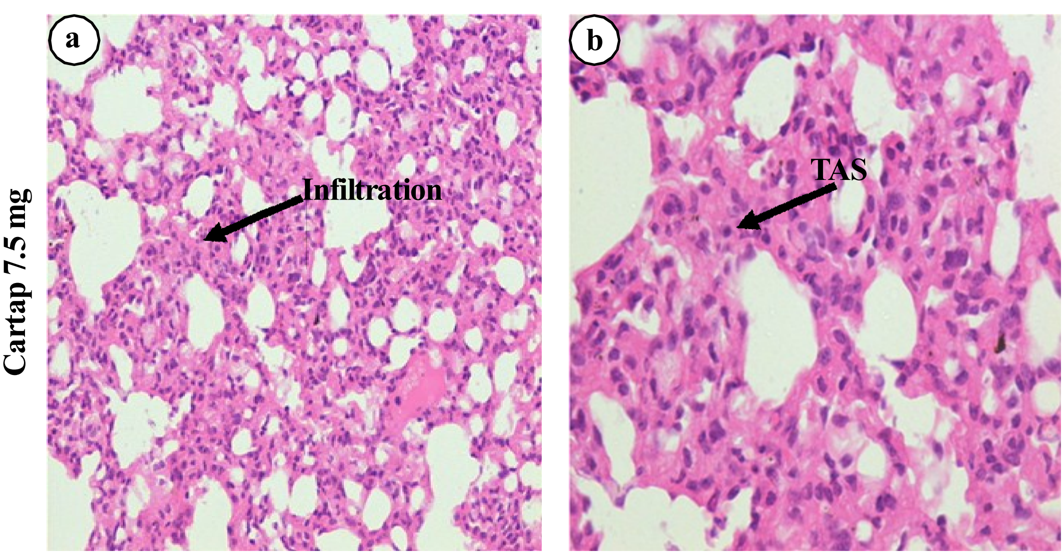

Fig. 25: Histopathological architecture of lung after 28 days oral exposure to cartap 7.5mg (H&E). a: 200x; b: 400x; TAS: Thickning of alveolar septum with neutrophil infiltration

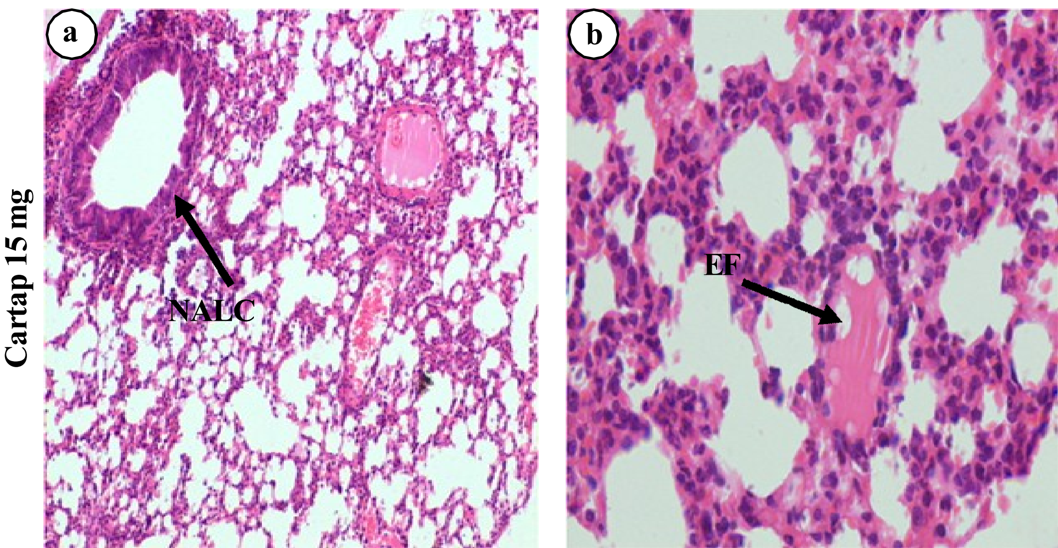

Fig. 26: Histopathological architecture of lung after 28 days oral exposure to cartap 15mg (H&E). a: 100x; b: 400x; NALC: Necrosis of alveolar lining, EF: Pinkish edematous fluid

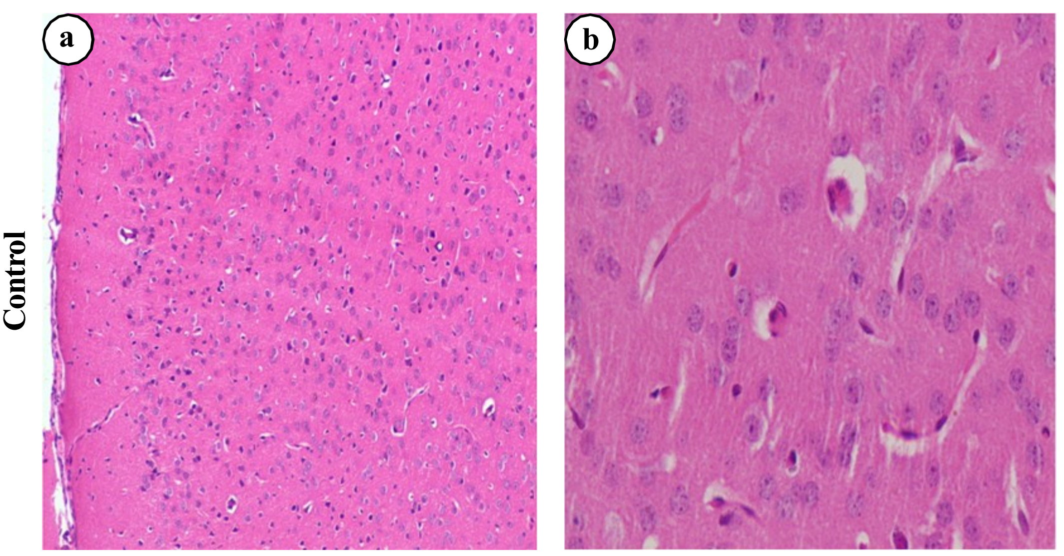

Fig. 27: Histopathological architecture of brain of control mice (H&E). a:100x; b: 400x

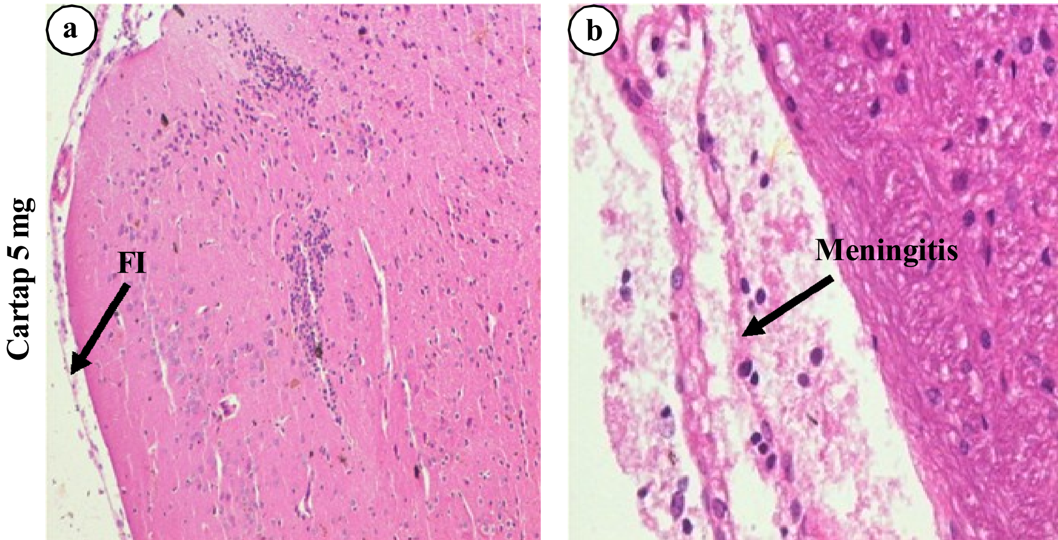

Fig. 28: Histopathological architecture of brain after 28 days oral exposure to cartap 5mg (H&E). a: 100x; b: 400x; FI: Focal infiltration of inflammatory cells in meningis

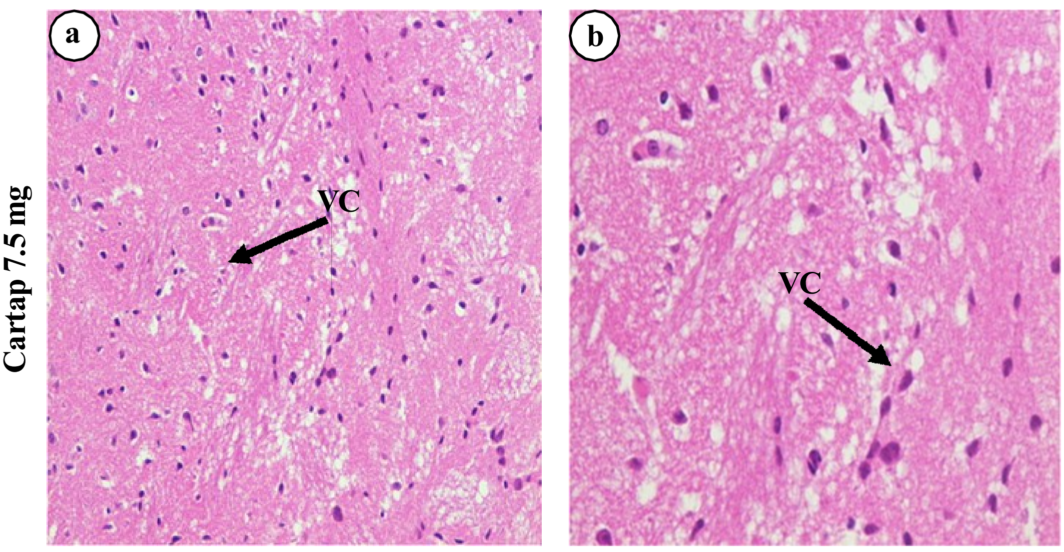

Fig. 29: Histopathological architecture of brain after 28 days oral exposure to cartap 7.5mg (H&E).
a: 200x; b: 400x; VC: Degenrating changes in mid brain

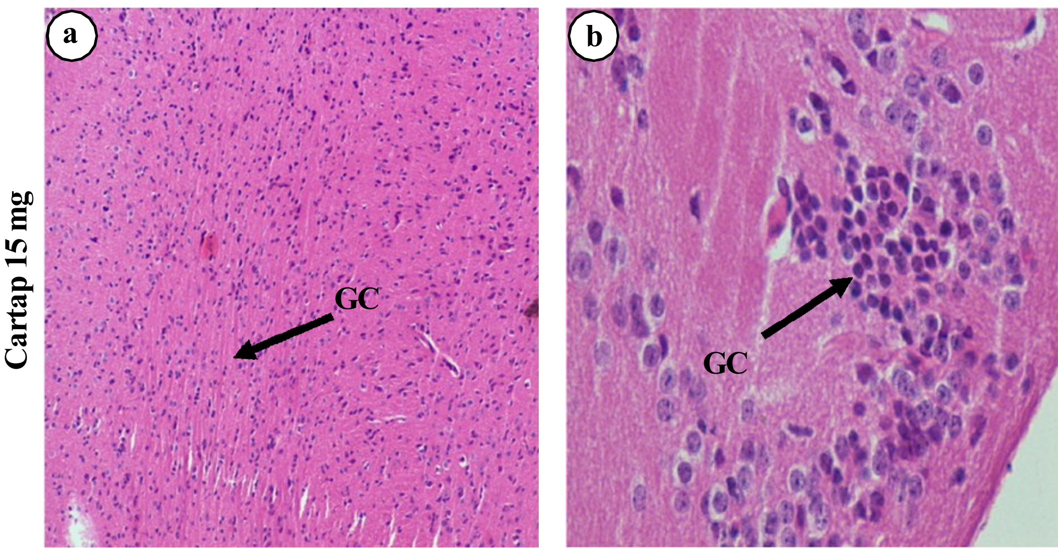

Fig. 30: Histopathological architecture of brain after 28 days oral exposure to cartap 15mg (H&E).
a: 100x; b: 400x; GC: Focal infiltration of glial cells

Table 9 : Effect on lipid peroxidation (LPO) level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$51.22^{a} \pm 0.65$	$45.03^{a} \pm 0.83$	$92.24^{a} \pm 1.87$
II	Cartap 5 mg	$53.21^{a} \pm 0.42$	$51.22^{b} \pm 0.65$	$112.60^{b} \pm 1.23$
III	Cartap 7.5 mg	$80.29^{b} \pm 0.83$	$62.44^{c} \pm 0.85$	$126.67^{c} \pm 0.86$
IV	Cartap 15 mg	$95.32^{c} \pm 1.38$	$82.34^{d} \pm 0.73$	$148.30^{d} \pm 1.29$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;
LPO expressed as nmol MDA formed/gm tissue.

Table 10: Effect on Super oxide anion generation (O_2) in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$10.33^{a} \pm 0.23$	$4.66^{a} \pm 0.43$	$0.53^{a} \pm 0.03$
II	Cartap 5 mg	$11.34^{a} \pm 0.20$	$5.36^{ab} \pm 0.33$	$0.90^{b} \pm 0.04$
III	Cartap 7.5mg	$13.31^{b} \pm 0.33$	$6.39^{b} \pm 0.26$	$1.30^{c} \pm 0.13$
IV	Cartap 15 mg	$16.85^{c} \pm 0.61$	$10.13^{c} \pm 0.44$	$2.07^{d} \pm 0.06$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;

Super oxide anion generation expressed as amount of NBT reduced (pmol/min/mg of protein).

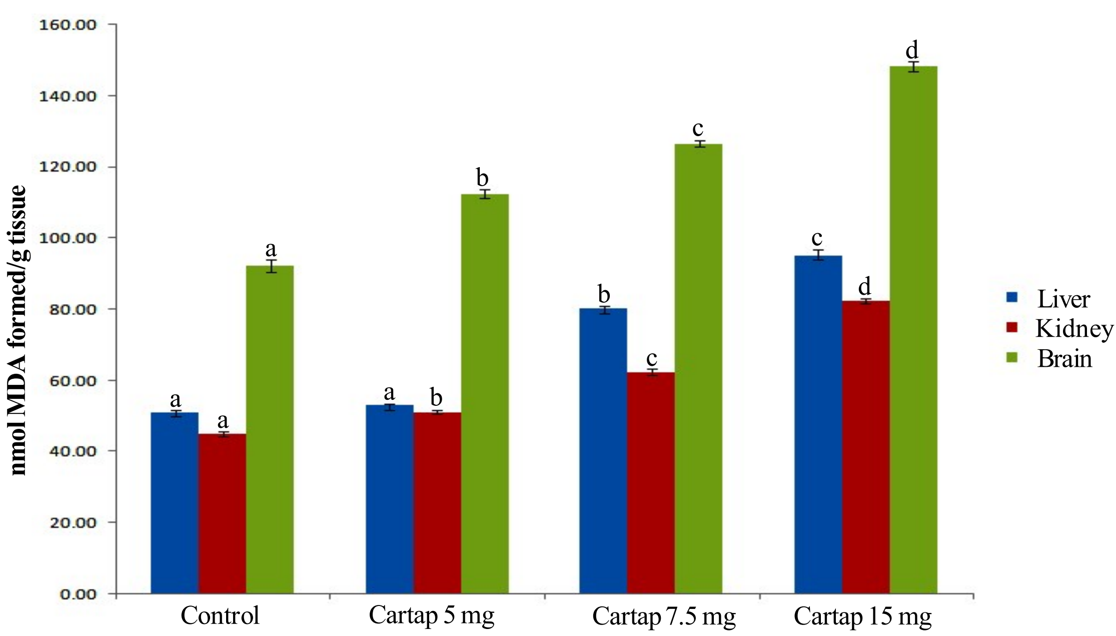

Fig. 31: Effect on lipid peroxidation (LPO) level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

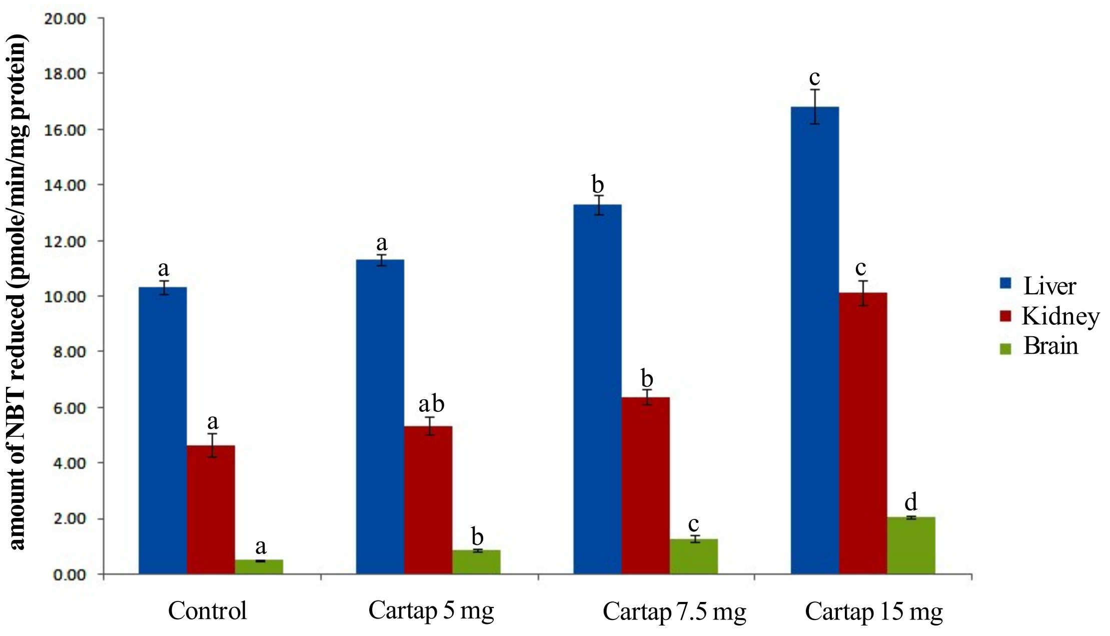

Fig. 32: Effect on superoxide anion generation (O_2^{-}) in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Table 11 : Effect on nitrite level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$15.52^{a} \pm 0.33$	$13.54^{a} \pm 0.14$	$10.36^{a} \pm 0.18$
II	Cartap 5 mg	$20.99^{b} \pm 0.34$	$14.43^{b} \pm 0.12$	$12.30^{b} \pm 0.19$
III	Cartap 7.5 mg	$24.04^{c} \pm 0.11$	$16.01^{c} \pm 0.15$	$14.16^{c} \pm 0.26$
IV	Cartap 15 mg	$31.26^{d} \pm 0.43$	$29.22^{d} \pm 0.31$	$18.94^{d} \pm 0.29$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;

Nitrite level expressed as μM/L of tissue homogenate.

Table 12: Reduced glutathion (GSH) in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$22.67^{d} \pm 0.07$	$13.43^{d} \pm 0.08$	$14.63^{d} \pm 0.07$
II	Cartap 5 mg	$21.44^{c} \pm 0.12$	$12.44^{c} \pm 0.05$	$13.57^{c} \pm 0.06$
III	Cartap 7.5 mg	$16.67^{b} \pm 0.30$	$9.84^{b} \pm 0.05$	$9.65^{b} \pm 0.15$
IV	Cartap 15 mg	$12.44^{a} \pm 0.15$	$5.66^{a} \pm 0.10$	$4.57^{a} \pm 0.08$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;
GSH is expressed as μmol GSH/gm tissue

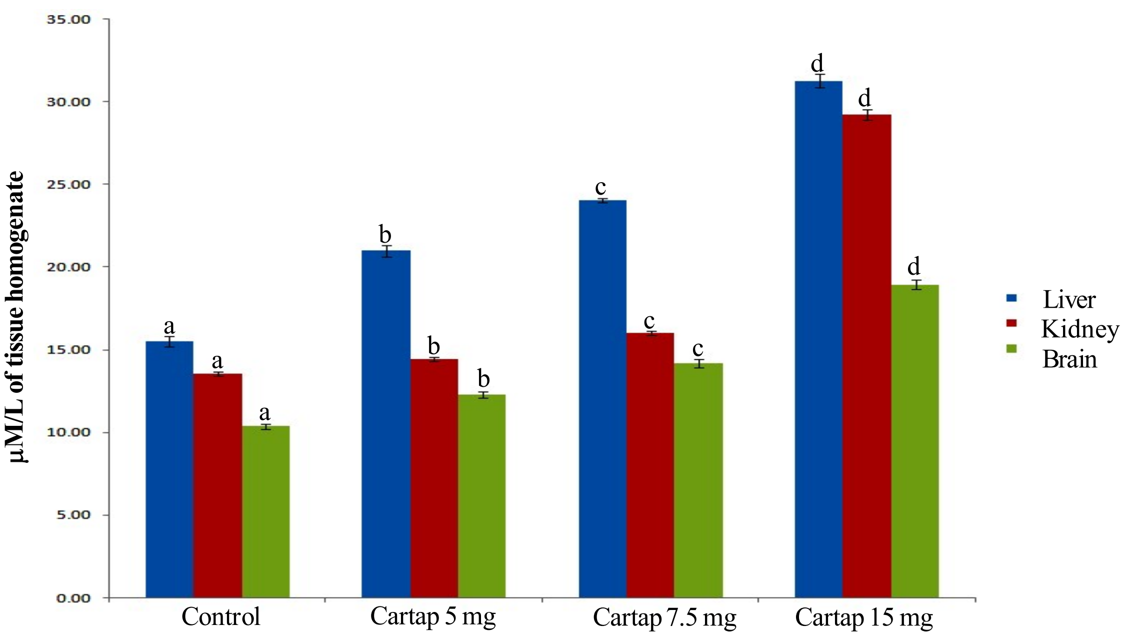

Fig. 33: Effect on nitrite level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

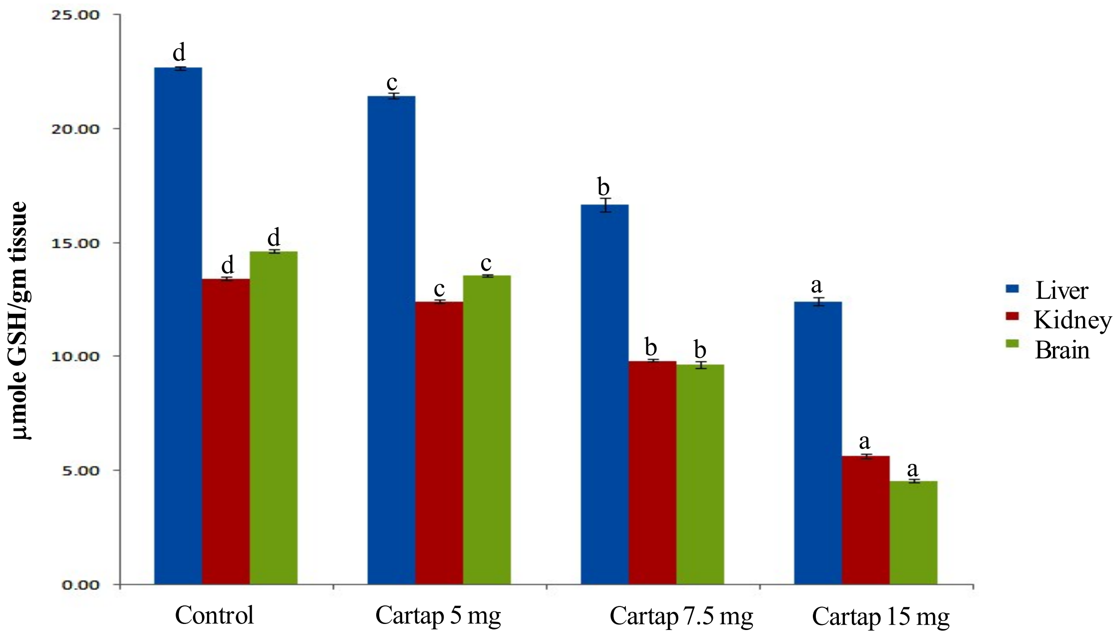

Fig. 34: Reduced glutathion (GSH) in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Table 13: Effect on total thiol (T-SH) level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$0.527^{d} \pm 0.007$	$0.537^{c} \pm 0.015$	$0.385^{c} \pm 0.006$
II	Cartap 5 mg	$0.422^{c} \pm 0.005$	$0.507^{bc} \pm 0.008$	$0.378^{c} \pm 0.005$
III	Cartap 7.5 mg	$0.342^{b} \pm 0.003$	$0.472^{b} \pm 0.004$	$0.358^{b} \pm 0.003$
IV	Cartap 15 mg	$0.278^{a} \pm 0.005$	$0.427^{a} \pm 0.006$	$0.328^{a} \pm 0.003$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;

Total thiol expressed as µmol T-SH/ml tissue homogenate.

Table 14: Effect on Super oxide dismutase (SOD) level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$111.01^{d} \pm 2.58$	$77.64^{c} \pm 0.91$	$72.95^{d} \pm 2.93$
II	Cartap 5 mg	$94.87^{c} \pm 0.78$	$75.41^{c} \pm 0.84$	$65.95^{c} \pm 1.12$
III	Cartap 7.5 mg	$87.33^{b} \pm 1.36$	$64.32^{b} \pm 1.59$	$49.64^{b} \pm 1.15$
IV	Cartap 15 mg	$73.89^{a} \pm 0.85$	$54.39^{a} \pm 0.68$	$33.08^{a} \pm 0.83$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;
SOD expressed as Units/mg of protein.

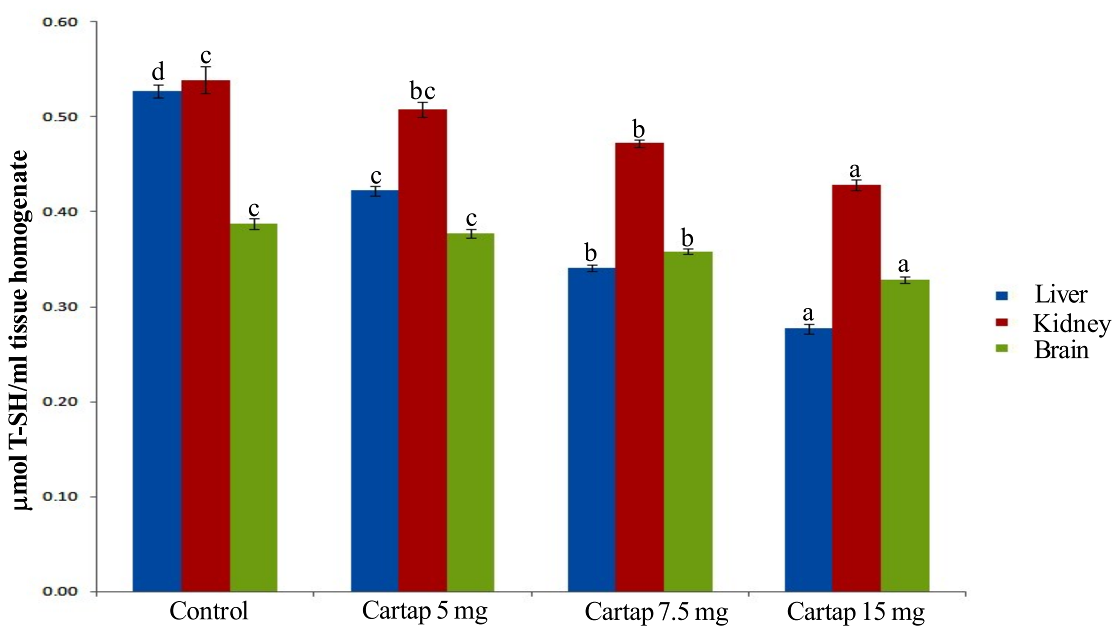

Fig. 35: Effect on total thiol (T-SH) level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

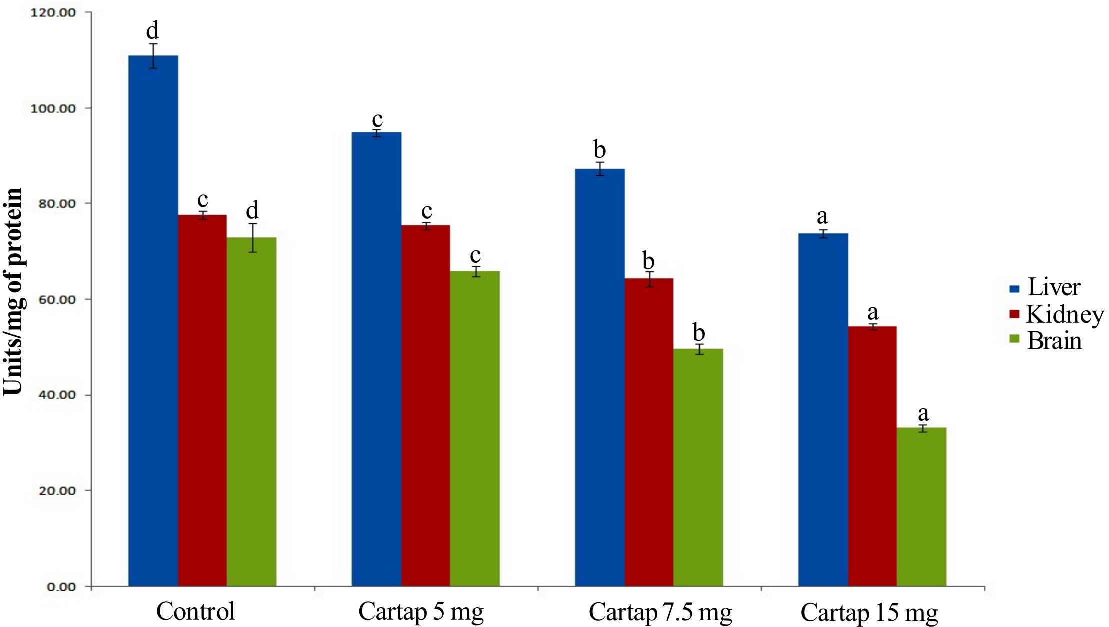

Fig. 36: Effect on superoxide dismutase (SOD) level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

as compared to the control group; however, concentration of T-SH in kidney and brain reduced significantly (p<0.05) only at medium (7.5 mg) and high (15 mg) dose level of cartap as compared to the low dose (5 mg) and control group but level of T-SH between low (5 mg) and control was statistically non- significant (p>0.05).

4.2.5 Effect on enzymatic antioxidants in liver, kidney and brain

A. Superoxide dismutase (SOD)

Superoxide dismutase level in liver, kidney and brain are given in (Table 14; Fig. 36). In liver and brain reduction of SOD concentration was directly proportionate to the dose of cartap as compared to the control group; however, concentration of SOD in kidney was reduced significantly (p<0.05) at medium (7.5 mg) and high (15 mg) dose level of cartap as compared to the low dose (5 mg) and control group. But level of SOD between low dose (5 mg) and control group was statistically *non*- significant (p>0.05).

B. Glutathione S- transferase (GST)

Level of GST in liver, kidney and brain are presented in (Table 15; Fig. 37). In brain there was dose dependent reduction in GST concentration was observed compared to the control group ($p<0.05$). However, concentration of GST in liver and kidney reduced significantly ($p<0.05$) at medium (7.5 mg) and high (15 mg) dose level of cartap as compared to the control group but level of GST between low dose (5 mg) and control group was statistically *non*-significant (p<0.05).

C. Catalase

Level of catalase in liver, kidney and brain are shown in (Table 16; Fig. 38). In liver and kidney decrease in concentration of catalase was directly proportionate to the increasing dose of cartap as compared to the control group (p<0.05); however, concentration of catalase in liver decreased significantly (p<0.05) only at medium (7.5 mg) and high (15 mg) dose level of cartap as compared to the control group but level of catalase between low dose (5 mg) and control group was statistically *non*- significant (p>0.05).

4.3 Evaluation of humoral immune response

4.3.1 Serum antibody titer/ haemagglutination antibody (HA) titer

Haemagglutination titers (HA) after 28 days exposure of cartap in mice are given in (Table 17; Fig.39). There was *non*-significant (p>0.05) serum HA titer decrease in at low dose (5 mg) of cartap as compared to the control group HA titers; however, were decreased significantly (p<0.05) decreased at medium (7.5 mg) and high dose (15 mg) of cartap as compared to the control group (Table 17; Fig. 39).

4.3.2 Spleen cell cellularity

Spleen cell cellularity in terms of both total and viable cell count decreased significantly (p<0.05) at all dose levels of cartap as compared to the control group after 28 days of oral exposure (Table 18; Fig. 40).

4.3.3 Quantification of mouse IL-4 by ELISA

A significant (p<0.05) and dose dependant reduction in IL-4 concentration in mouse serum (Table 19; Fig. 41) at all the dose level of cartap after 28 days exposure as compared to the control group.

4.4 Evaluation of cell mediated immune response

4.4.1 Delayed type hypersensitivity (DTH) response

Gross evaluation of DTH response to SRBC was characterized by intense local inflammatory reactions in terms of erythema, edema, vesiculation and swelling in foot pad of control group mice. In 28 days cartap treated mice these local inflammatory reactions were inversely related to the dose of cartap administered, i.e. at high dose level group only mild inflammatory reactions were observed as compared to the control group, low dose group mice showed nearly similar cellular infiltration to the control group.

The DTH response (% increase in paw thickness at a given time) was significantly (p<0.05) decreased as compared to the control group (Table 20; Fig. 42); however, paw thickness decreased *non*- significantly between at low dose (5 mg) and medium (7.5 mg) dose level of cartap treated group.

Table 15: Glutathione S- tranferase activity (GST) in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$0.360^{c} \pm .013$	$0.302^{c} \pm .005$	$0.387^{d} \pm .008$
II	Cartap 5 mg	$0.348^{c} \pm .004$	$0.285^{c} \pm .004$	$0.330^{c} \pm .005$
III	Cartap 7.5 mg	$0.317^{b} \pm .002$	$0.250^{b} \pm .003$	$0.258^{b} \pm .003$
IV	Cartap 15 mg	$0.273^{a} \pm .007$	$0.212^{a} \pm .008$	$0.198^{a} \pm .008$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;

GST activity expressed as µmol/min/mg of protein.

Table 16: Effect on Catalase level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Groups	Dose	Liver	Kidney	Brain
I	Control	$102.19^{d} \pm 0.37$	$90.63^{d} \pm 0.73$	$86.62^{c} \pm 1.89$
II	Cartap 5 mg	$95.68^{c} \pm 0.90$	$79.89^{c} \pm 1.14$	$83.74^{c} \pm 0.76$
III	Cartap 7.5 mg	$87.39^{b} \pm 1.06$	$63.84^{b} \pm 1.50$	$74.54^{b} \pm 1.70$
IV	Cartap 15 mg	$76.35^{a} \pm 0.84$	$48.93^{a} \pm 0.81$	$62.06^{a} \pm 1.33$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;
Catalase expressed as mmol H_2O_2 utilized/min/mg protein.

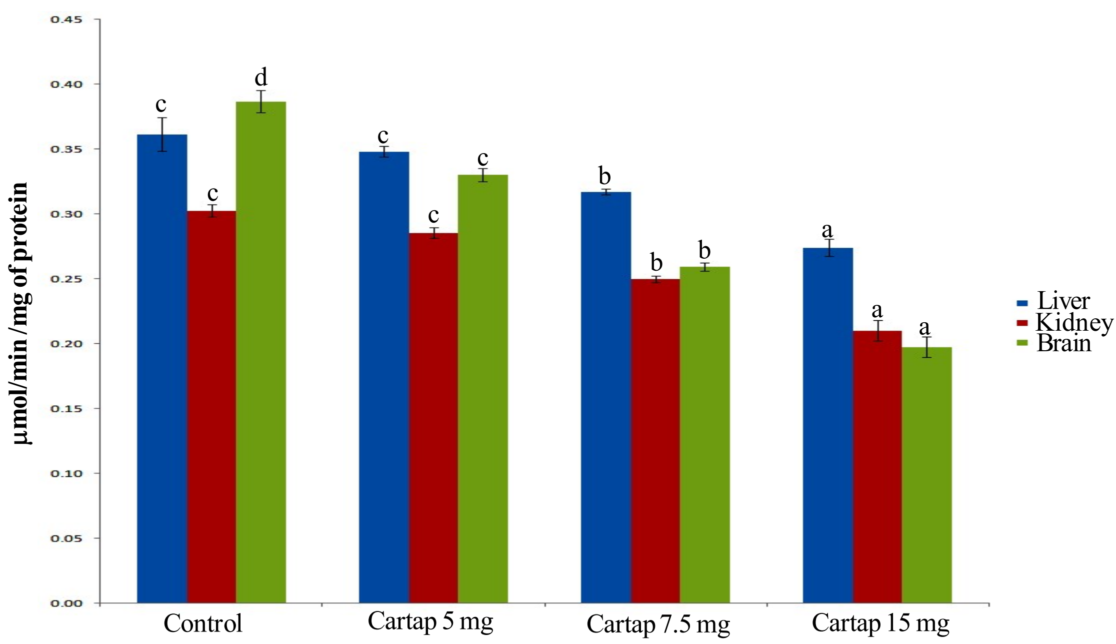

Fig. 37: Glutathione S- tranferase activity (GST) in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

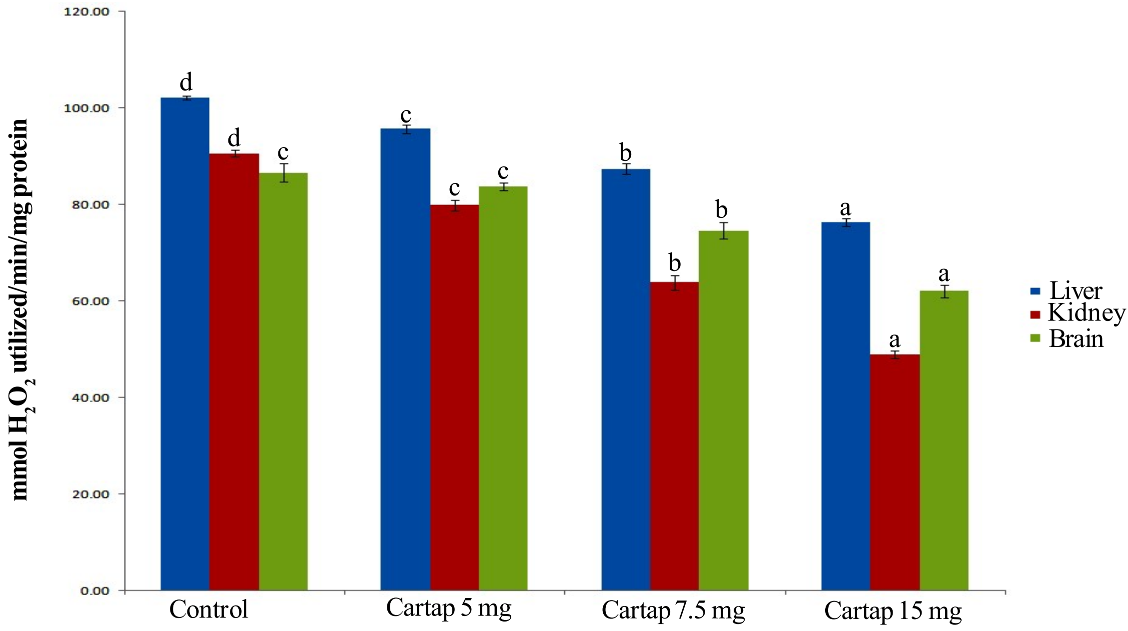

Fig. 38: Effect on catalase level in mice liver, kidney and brain tissue homogenates after 28 days oral exposure to cartap

Table 17 : Effect on haemagglutination titer (HA) in mice after 28 days oral exposure to cartap

Groups	Dose	Log_2 antibody titer
I	Control	$8.17^{c} \pm 0.31$
II	Cartap 5 mg	$7.17^{c} \pm 0.31$
III	Cartap 7.5 mg	$5.33^{b} \pm 0.21$
IV	Cartap 15 mg	$3.67^{a} \pm 0.33$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

Table 18 : Effect on splenic cell cellularity (Total and viable cell) in mice spleen after 28 days oral exposure to cartap

Groups	Dose	Total cell X 10^7/µl	Viable cell X 10^7/µl
I	Control	$8.71^{d} \pm 0.06$	$2.18^{d} \pm 0.02$
II	Cartap 5 mg	$5.48^{c} \pm 0.07$	$1.37^{c} \pm 0.02$
III	Cartap 7.5 mg	$4.48^{b} \pm 0.09$	$1.12^{b} \pm 0.02$
IV	Cartap 15 mg	$2.41^{a} \pm 0.13$	$0.60^{a} \pm 0.03$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

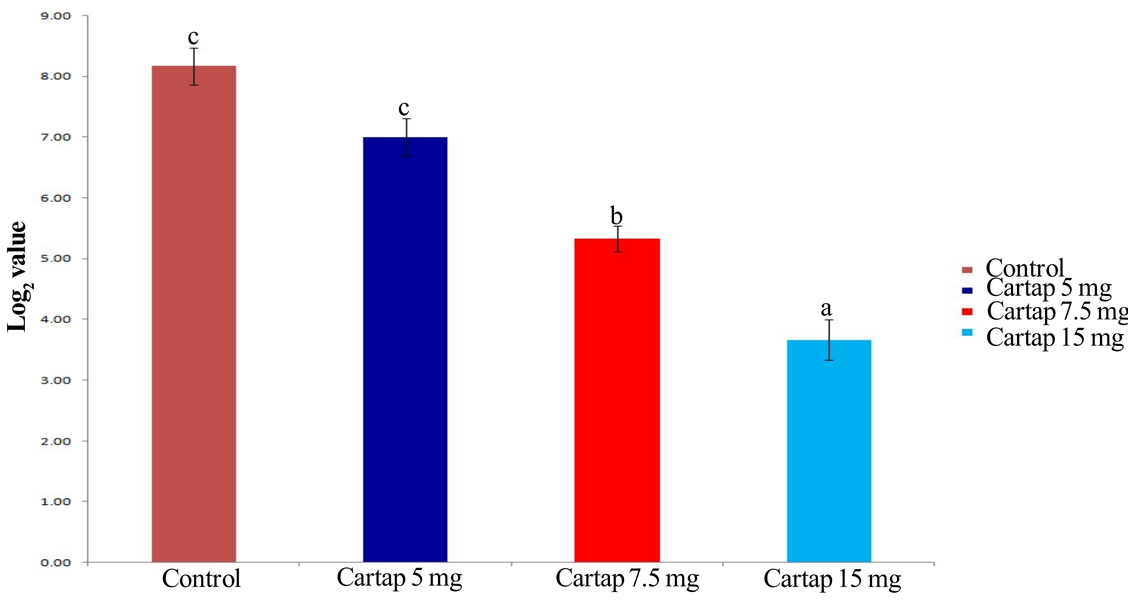

Fig. 39: Effect on haemagglutination titer (HA) in mice after 28 days oral exposure to cartap

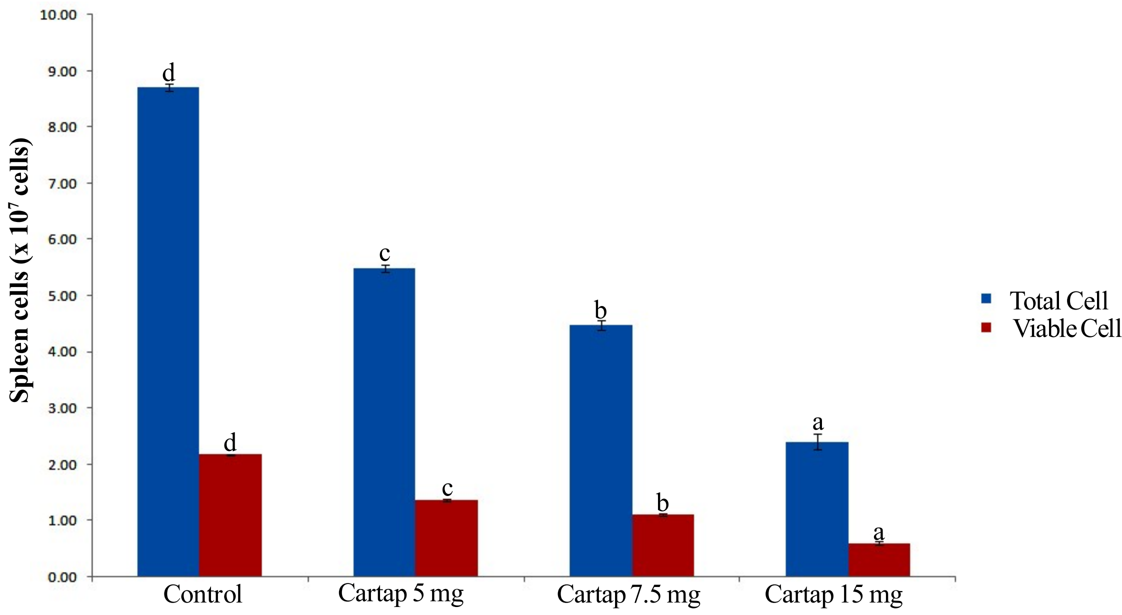

Fig. 40: Effect on splenic cell cellularity (total and viable cell) in mice spleen after 28 days oral exposure to cartap

Table 19: Effect on cytokine of cellular (IFN-γ) and humoral (IL-4) immune response of mice, quantified by ELISA after 28 days oral exposure to cartap

Groups	Dose	IFN-γ (pg/ml)	IL-4 (pg/ml)
I	Control	$1239.18^{c} \pm 42.23$	$70.44^{d} \pm 1.30$
II	Cartap 5 mg	$469.53^{b} \pm 9.95$	$41.39^{c} \pm 0.34$
III	Cartap 7.5 mg	$132.18^{a} \pm 11.34$	$28.59^{b} \pm 0.85$
IV	Cartap 15 mg	$60.14^{a} \pm 6.16$	$7.88^{a} \pm 0\ .72$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test.

Table 20 : Effect on delayed type hypersensitivity (DTH) response in mice after 28 days oral exposure to cartap

Groups	Dose	% DTH response		
		0 hr	48 hr	72 hr
I	Control	100.00	$143.94^{c} \pm 2.41$	$122.92^{c} \pm 1.97$
II	Cartap 5 mg	100.00	$131.58^{b} \pm 1.36$	$116.67^{b} \pm 1.62$
III	Cartap 7.5 mg	100.00	$127.20^{b} \pm 0.88$	$111.41^{ab} \pm 0.88$
IV	Cartap 15 mg	100.00	$118.42^{a} \pm 1.18$	$106.14^{a} \pm 0.88$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;

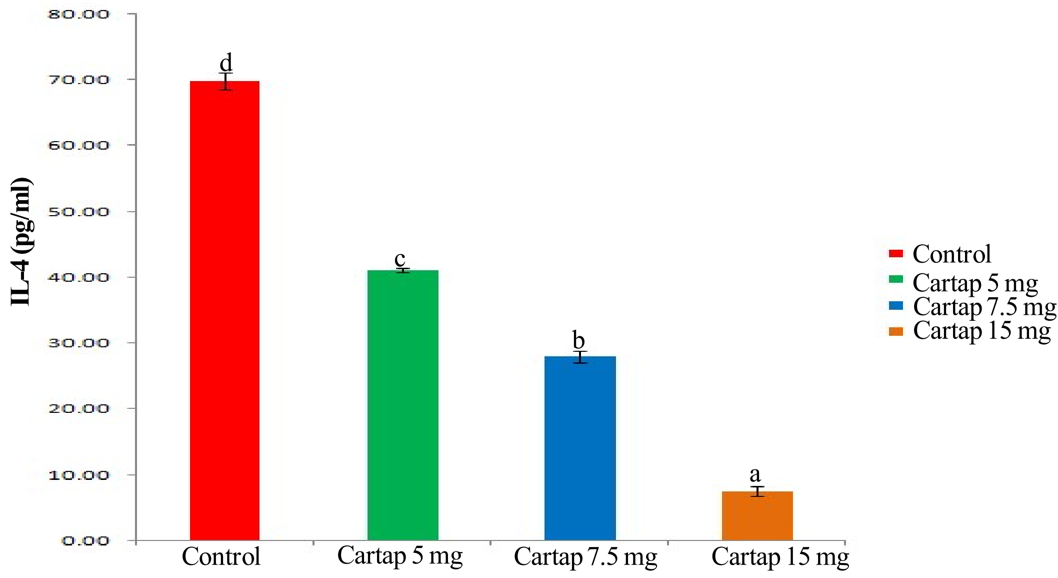

Fig. 41: Effect on cytokine of humoral (IL-4) immune response of mice, quantified by ELISA after 28 days oral exposure to cartap

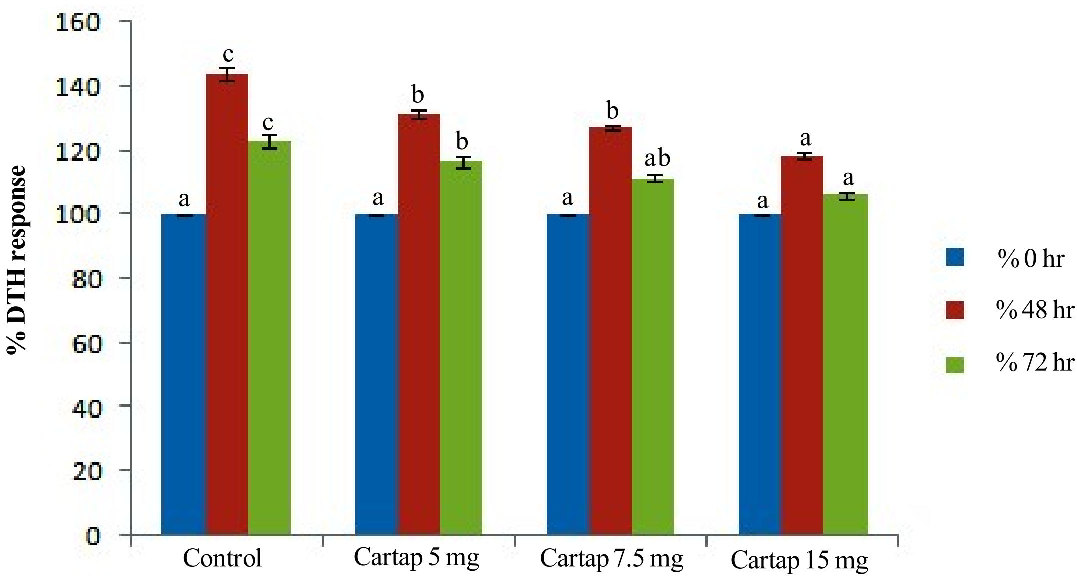

Fig. 42: Effect on delayed type hypersensitivity (DTH) response in mice after 28 days oral exposure to cartap

Histopathological finding of foot pad:

Foot pad sections from control group mice (72 h after challenge with antigen) revealed marked local inflammatory reactions characterized by presence of huge infiltration of mononuclear cells in subcutaneous tissue with hyperkeratosis, acanthosis and separation of dermal layer (Fig. 43-46). Only mild inflammatory reactions with infiltration of very few inflammatory cells were observed in foot pad sections of mice treated with higher dose of cartap suggesting marked suppression of DTH response. In case of mice treated with medium dose of cartap showed moderate type of reactions while low dose treated mice group showed reaction nearly similar to the control group mice.

4.2.2 Lymphocyte proliferation assay *in vitro*

Lymphocyte proliferation of T cells and B cells are given in (Table 21; Fig. 47). T cells (against mitogen Con-A) proliferation was significantly ($p<0.05$) decreased at medium (7.5 mg) and high (15 mg) doses after 28 days exposure of cartap, but proliferation at low dose (5 mg) is *non*-significant ($p>0.05$) as compared to the control group. B cells (against mitogen LPS) proliferation was significantly ($p<0.05$) decreased at all three doses level of cartap as compare to the control group.

4.2.3 Quantification of mouse IFN- γ by ELISA in serum

There was significant ($p<0.05$) and dose dependant reduction in mouse IFN- γ concentration in serum of mouse (Table 19; Fig. 48) after 28 days of cartap as compared to the control group; however, the decrease in IFN- γ concentration was *non*-significant ($p>0.05$) between medium (7.5 mg) and high (15 mg) dose level of cartap.

4.5 Apoptosis related parameters:

4.5.1 DNA fragmentation assay in spleen

DNA damage was evaluated by electrophoresis of DNA against 100bp ladder and stained by ethidium bromide. DNA was isolated from mouse spleen after 28 days exposure to increasing dose level i.e. control (distilled water), 5mg, 7.5 mg and 15 mg of cartap. Smearing of DNA (Fig. 49) was evident in cartap treated group as compared to the control group.

4.5.2 Flow cytometry analysis

4.5.2.1 Apoptotic DNA (cell cycle) analysis

Percent lymphocytes in spleen containing haplodiploid DNA with apoptotic DNA (Table 22; Fig. 50 and 50a) was evaluated by propidium iodide (PI) dye. There was significant ($p<0.05$) increase in apoptotic DNA at medium and high dose of cartap and *non*-significant ($p>0.05$) increase at low dose of cartap as compared to the control group.

4.5.2.2 Flow cytometric analysis of phosphatidyl serine externalization in apoptotic lymphocytes

The results of Annexin-V/PI staining are presented in (Table 22; Fig. 51, 51a and 51b). Numbers of apoptotic cells were increased with increase in the dose of cartap. Percentage of apoptotic lymphocytes (early apoptotic and late apoptotic) and necrotic cells were significantly ($p<0.05$) increased at all three dose level of cartap as compare to the control group following staining with FITC Annexin-V and PI dye.

4.5.2.3 Mitochondrial membrane potential ($\Delta\psi m$) using $DIOC_6$ dye

There was significant ($p<0.05$) and dose dependant loss of $\Delta\psi$ m of lymphocytes from different groups of mice treated with cartap as compare to the control group (Table 22; Fig. 51c).

4.5.2.4 Measurement of reactive oxygen species (ROS) by DCFH-DA

The results of detection of ROS by DCFH-DA are presented in (Table 22; Fig. 51). The fluorescence proportionate to the ROS level in the cells was analyzed in the spleen lymphocytes. There was significant ($p<0.05$) increment in the % of cells showing ROS generation at increasing dose of cartap after 28 days exposure as compare to the control group.

4.6 SDS-PAGE and western blot analysis for the expression of β-actin, Fas and Fas-L protein in supernatant of spleen lysates

The representative bands of β-actin, Fas and Fas-L at 42, 48 and 42 kDa are represented in Fig. 52, 53 and 54 respectively.

Increased relative protein expression of Fas and Fas-L as evident in cartap treated group comapred to control as shown in Fig. 53 and 54.

✍✍✍

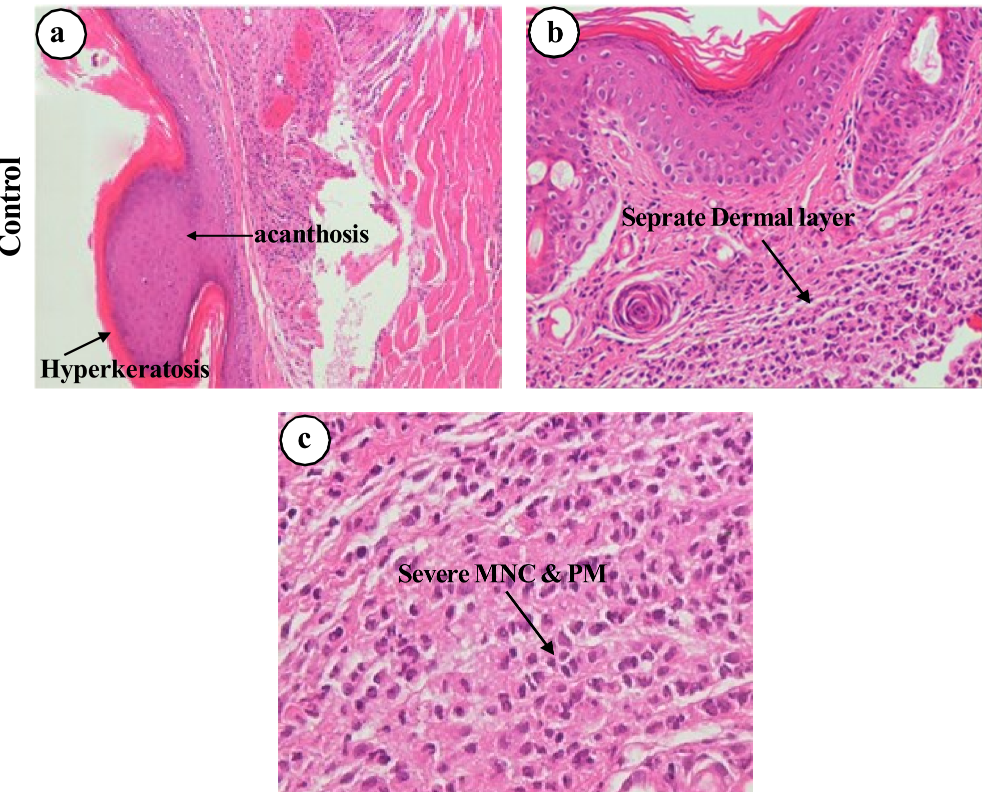

Fig. 43: Histopathological architecture of food pad of control group mice (H&E). a- 100x; b- 200x; c- 400x. MNC: Mononuclear cells (lymphocytes & macrophages) and PM: Polymorphs

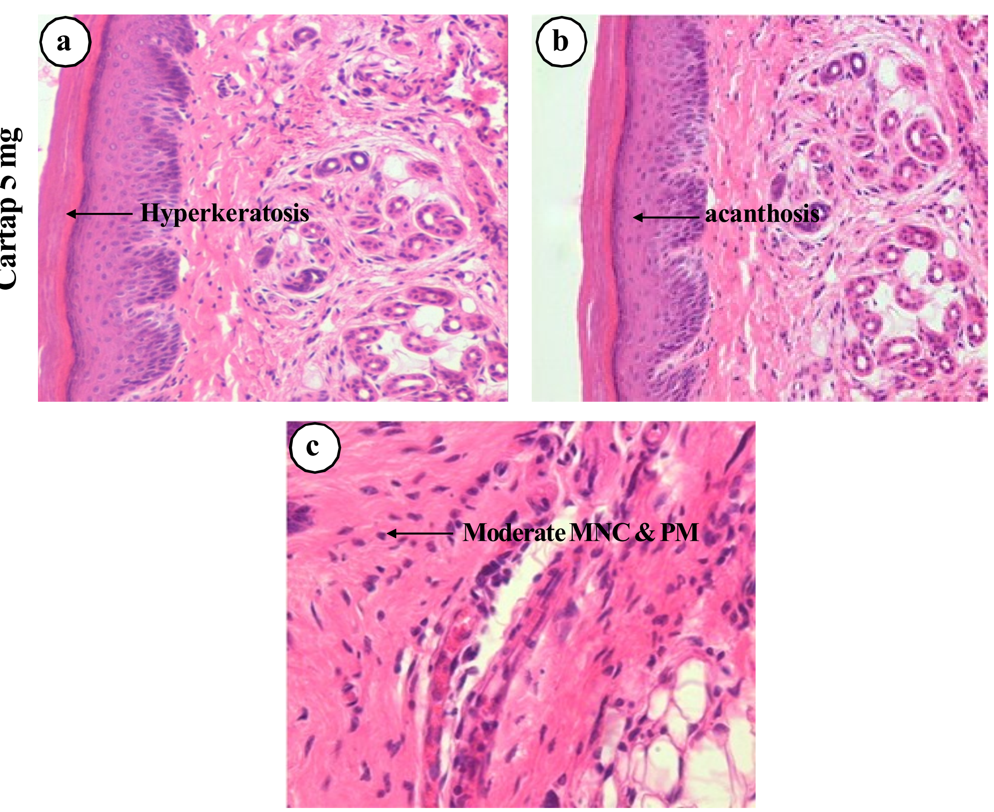

Fig. 44: Histopathological architecture of food pad after 28 days oral exposure to cartap 5mg (H&E). a- 100x; b- 200x; c- 400x. MNC: Mononuclear cells (lymphocytes & macrophages) and PM: Polymorphs

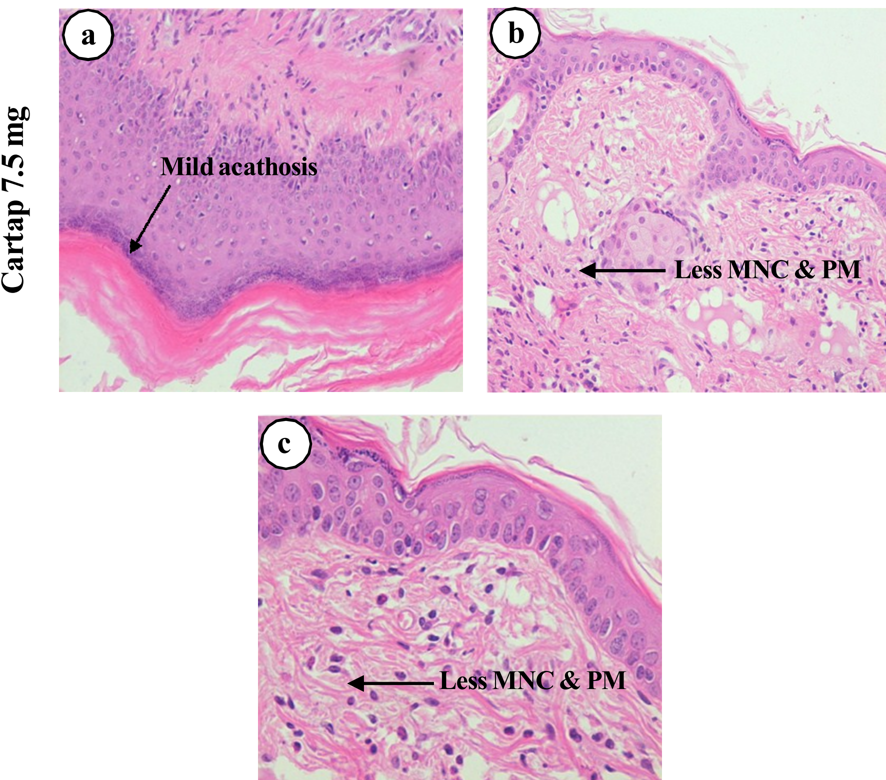

Fig. 45: Histopathological architecture of food pad after 28 days oral exposure to cartap 7.5mg (H&E). a- 100x; b- 200x; c- 400x. MNC: Mononuclear cells (lymphocytes & macrophages) and PM: Polymorphs

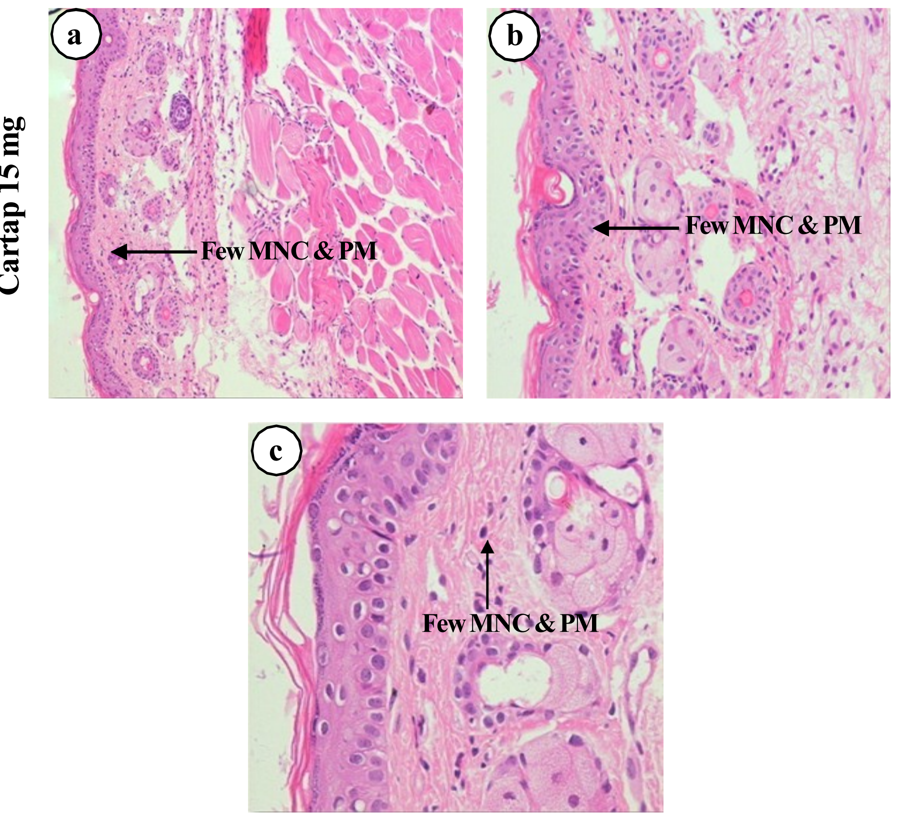

Fig. 46: Histopathological architecture of food pad after 28 days oral exposure to cartap 15mg (H&E). a- 100x; b- 200x; c- 400x. MNC: Mononuclear cells (lymphocytes & macrophages) and PM: Polymorphs

Table 21- Effect on lymphocyte proliferation (T and B cell) in mice after 28 days oral exposure to cartap

Groups	Dose	Stimulation index- ConA (T cell)	Stimulation index- LPS (B cell)
I	Control	$1.00^{c} \pm 0.012$	$1.008^{d} \pm 0.012$
II	Cartap 5 mg	$0.97^{bc} \pm 0.007$	$0.96^{c} \pm 0.008$
III	Cartap 7.5 mg	$0.95^{b} \pm 0.004$	$0.79^{b} \pm 0.009$
IV	Cartap 15 mg	$0.84^{a} \pm 0.013$	$0.68^{a} \pm 0.004$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test;

Table 22 : Effect on apoptosis related end point parameters analyzed by flow cytometry in mice after 28 days oral exposure to cartap

Groups	Dose	% apoptotic cells (FITC Annexin-V)	% necrotic cells (PI)	% lymphocytes with loss of DDm[2]	% cells showing ROS generation[3]	% lymphocytes with apoptotic DNA[1]	
						MI	M2
I	Control	$3.48^{a} \pm 0.35$	$6.60^{a} \pm 0.22$	$76.31^{a} \pm 0.70$	$15.41^{a} \pm 0.34$	$14.08^{a} \pm 0.55$	$3.21^{a} \pm 0.15$
II	Cartap 5 mg	$7.10^{b} \pm 0.04$	$8.71^{b} \pm 0.23$	$81.81^{b} \pm 0.31$	$22.18^{b} \pm 0.29$	$16.12^{a} \pm 0.51$	$4.15^{a} \pm 0.28$
III	Cartap 7.5 mg	$14.90^{c} \pm 0.17$	$14.83^{c} \pm 0.44$	$93.99^{c} \pm 0.81$	$27.77^{c} \pm 0.17$	$21.80^{b} \pm 0.80$	$7.81^{b} \pm 0.37$
IV	Cartap 15 mg	$27.68^{d} \pm 1.03$	$22.86^{d} \pm 0.34$	$97.23^{d} \pm 0.52$	$39.15^{d} \pm 0.25$	$25.56^{c} \pm 0.84$	$8.28^{b} \pm 0.30$

Values (Mean ± SE, n = 6 mice/group) bearing different superscript in the same column differ significantly between groups at $p < 0.05$ in Tukey's multiple comparison post hoc test. 1, 2, 3 superscripts, denates mouse lymphocytes analyzed with propidium iodide, $DIOC_6$, DCFH-DA stain respectively in a flow cytometer.

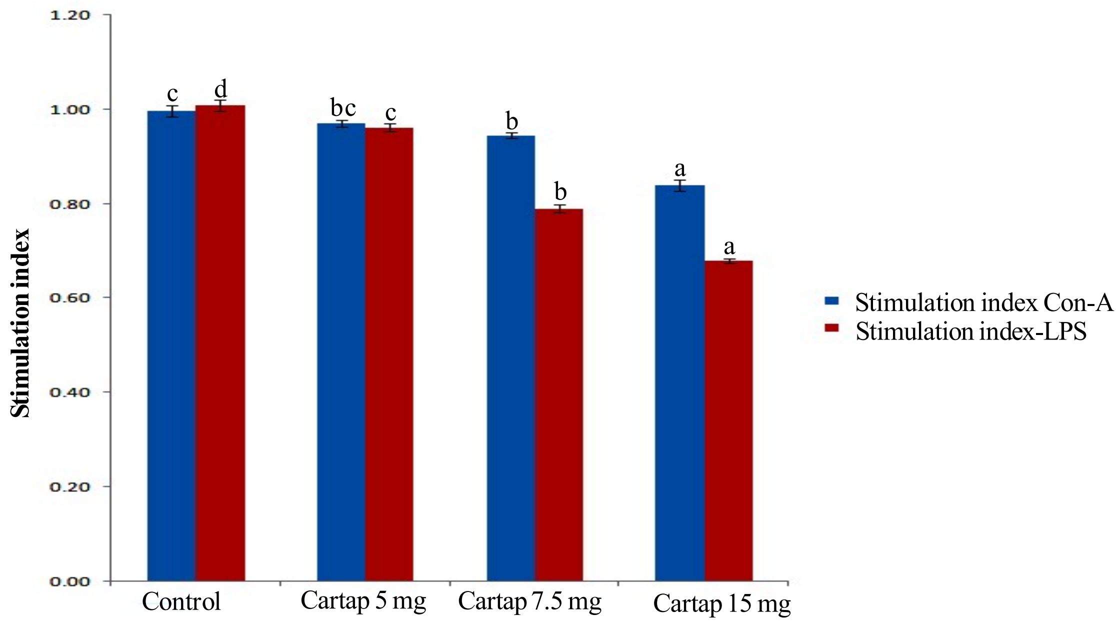

Fig. 47: Effect on lymphocyte proliferation (T and B cell) in mice after 28 days oral exposure to cartap

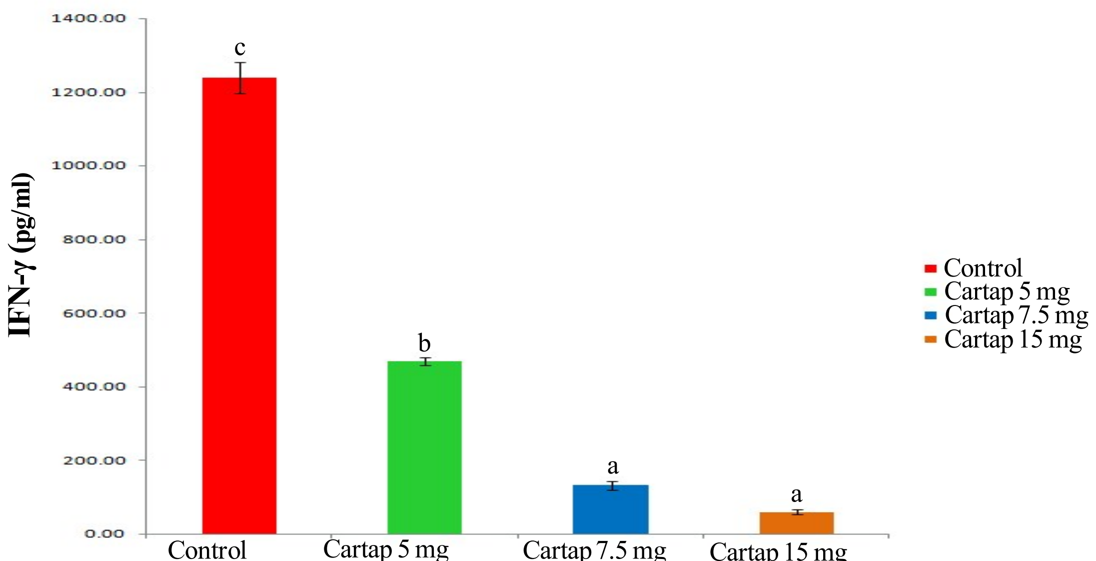

Fig. 48: Effect on cytokine of cellular (IFN-γ) immune response of mice, quantified by ELISA after 28 days oral exposure to cartap

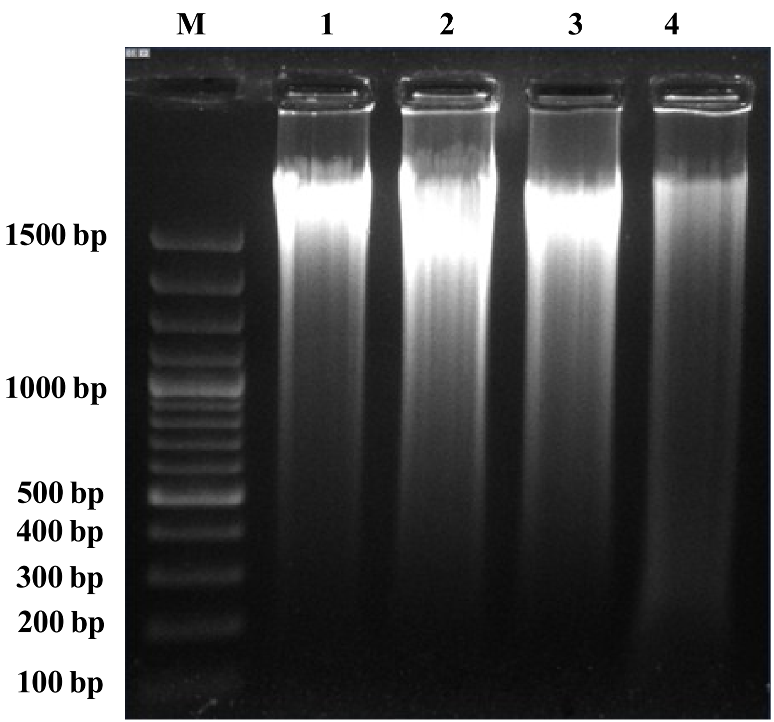

Fig. 49: Representative image of DNA fregmentation assay in spleen of mouse after 28 days oral exposure to cartap

Lane M : DNA ladder- 100bp

Lane 1 : Control

Lane 2 : Cartap 5 mg

Lane 3 : Cartap 7.5 mg

Lane 4 : Cartap 15 mg

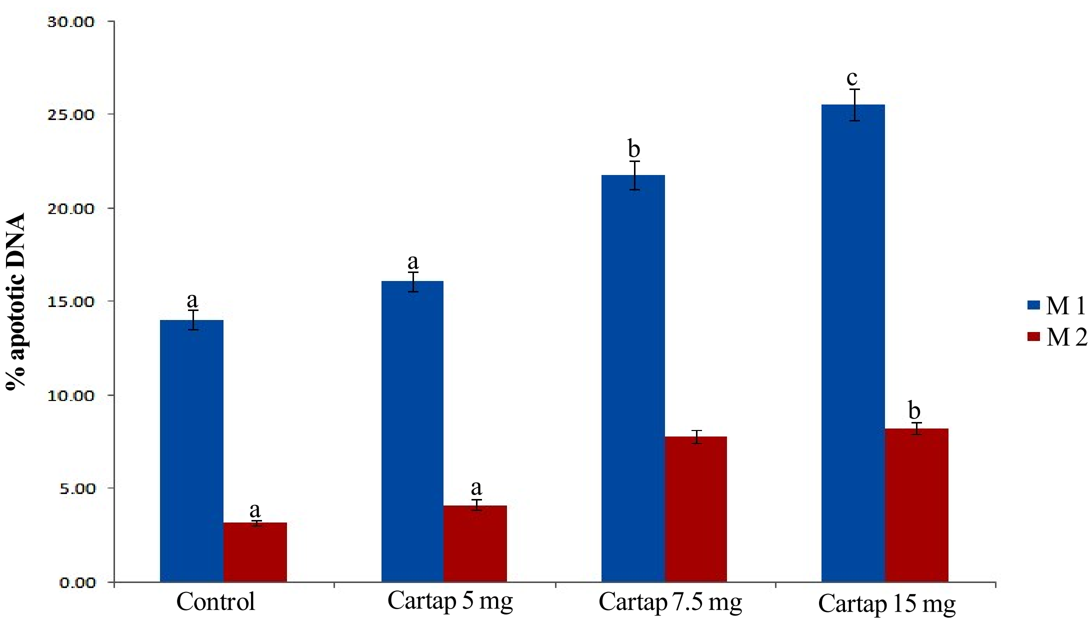

Fig. 50: Effect on apoptotic DNA analyzed by flow cytometry in mice after 28 days oral exposure to Cartap

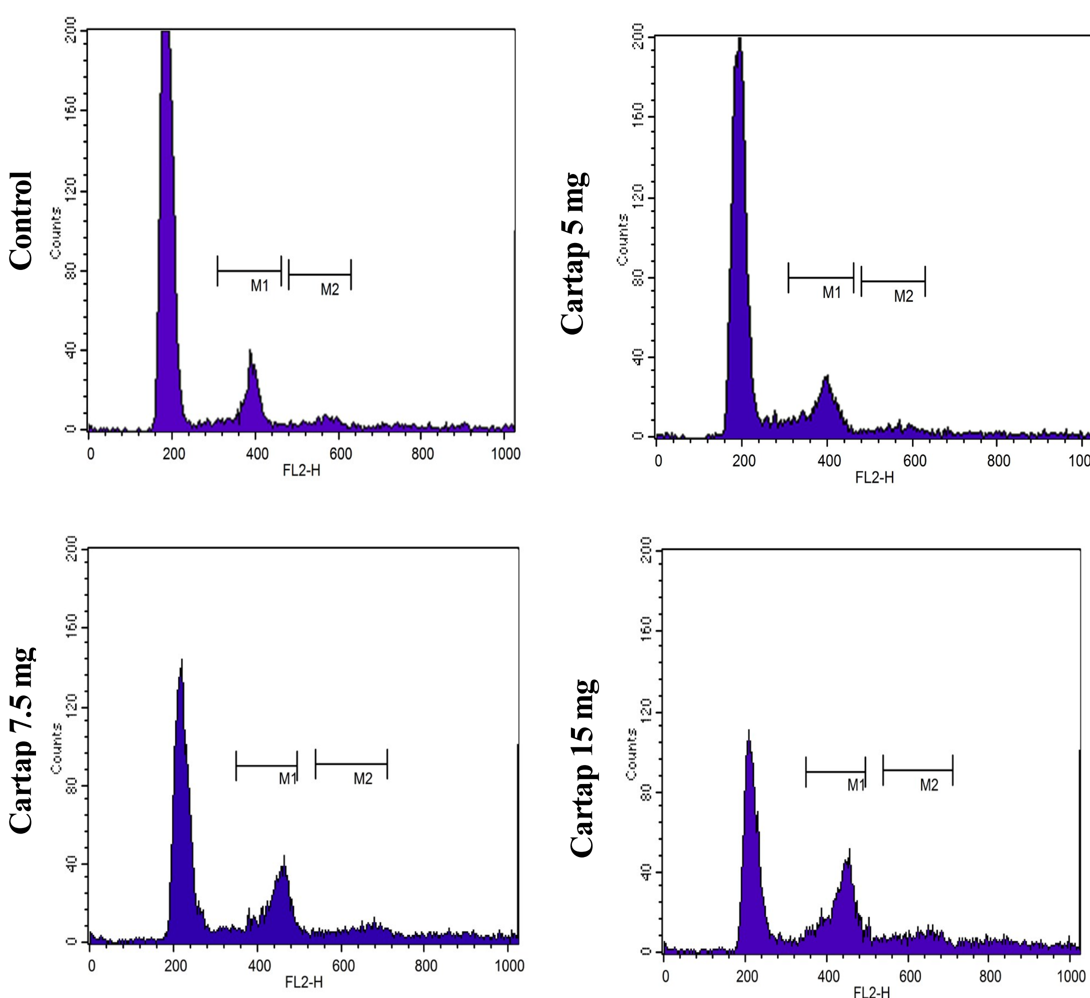

Fig. 50a: Representative histograms of flow cytometry showing percent lymphocytes containing apoptotic DNA (PI staining) in mouse spleen after 28 days oral exposure to cartap

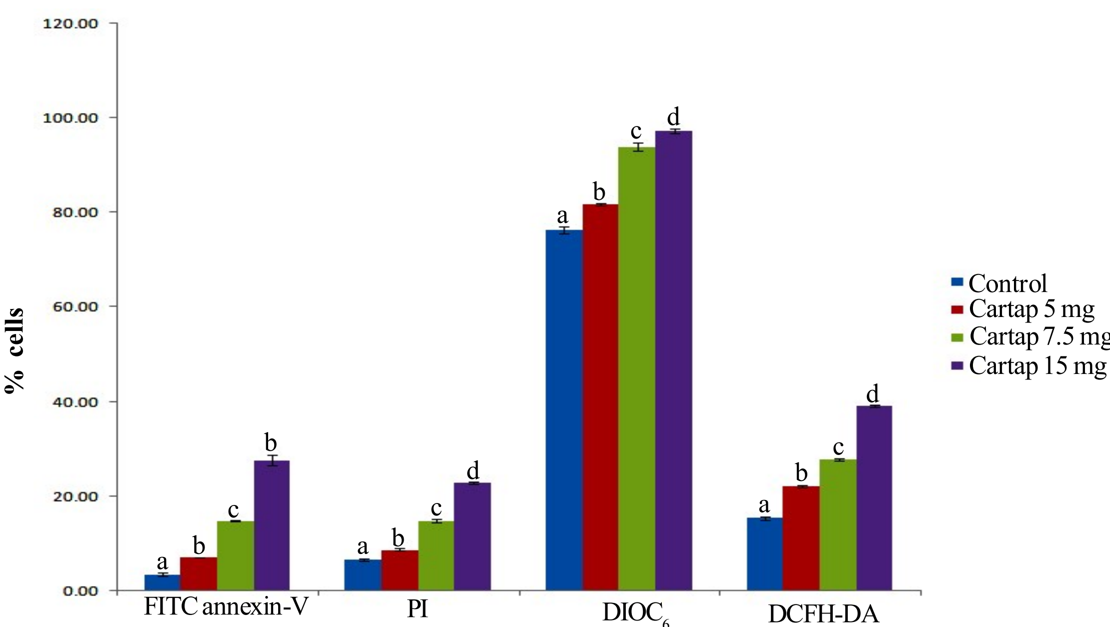

Fig. 51: Effect on apoptosis related end point parameters analyzed by flow cytometry in mice after 28 days oral exposure to cartap

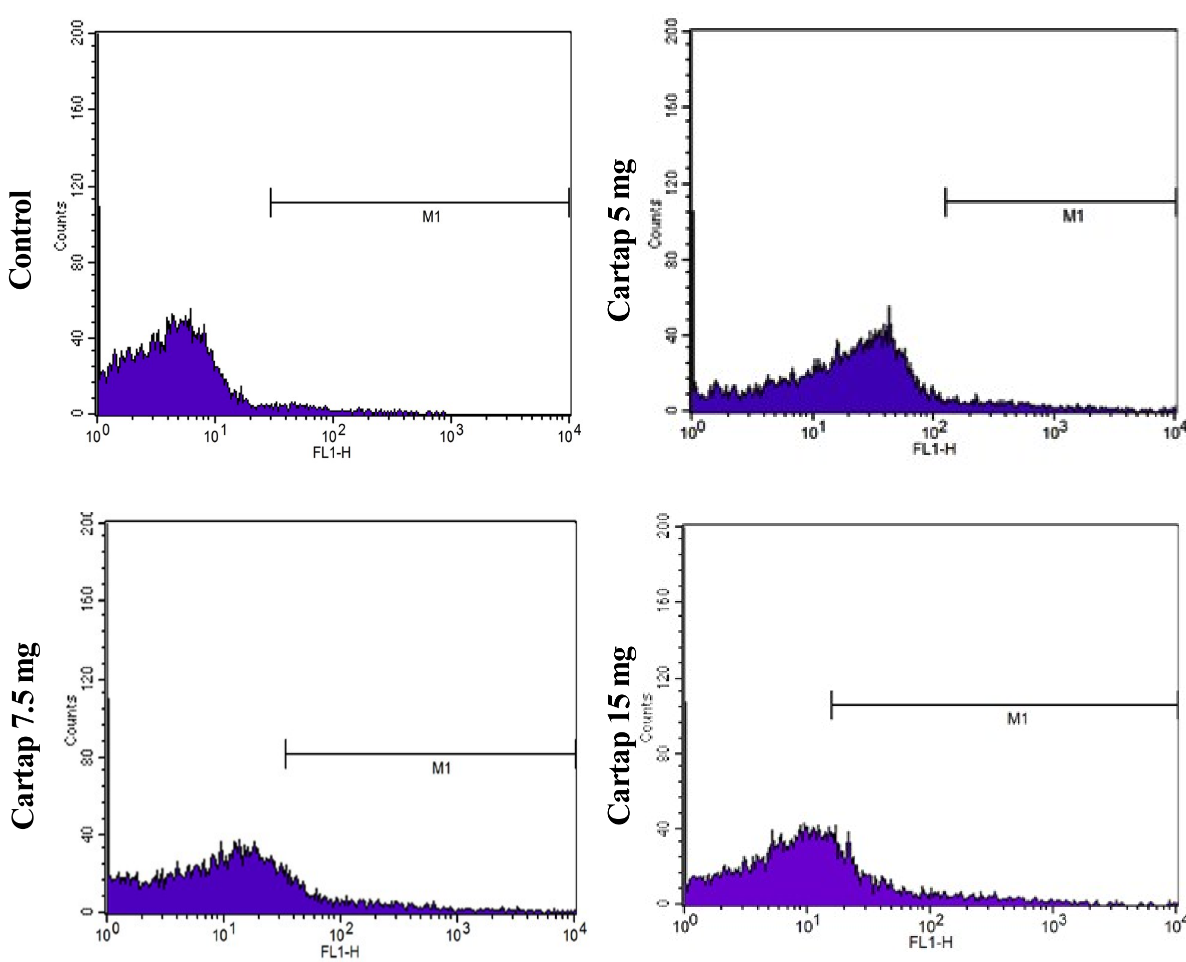

Fig. 51a: Representative histograms of flow cytometry showing percent apoptotic lymphocytes (FITC annexin- V/PI staining) in mouse spleen after 28 days oral exposure to cartap

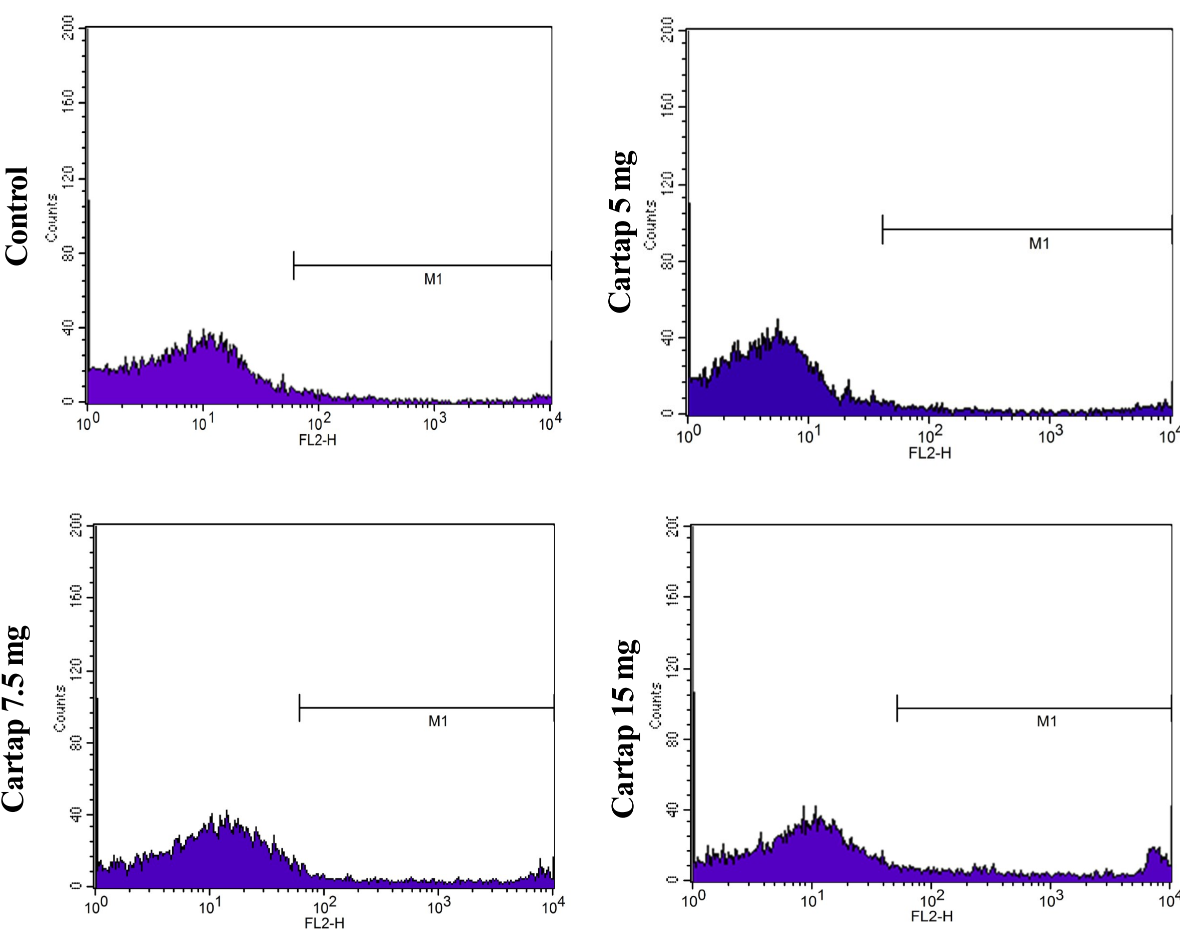

Fig. 51b: Representative histograms of flow cytometry showing percent necrotic lymphocytes (FITC annexin- V/PI staining) in mouse spleen after 28 days oral exposure to cartap

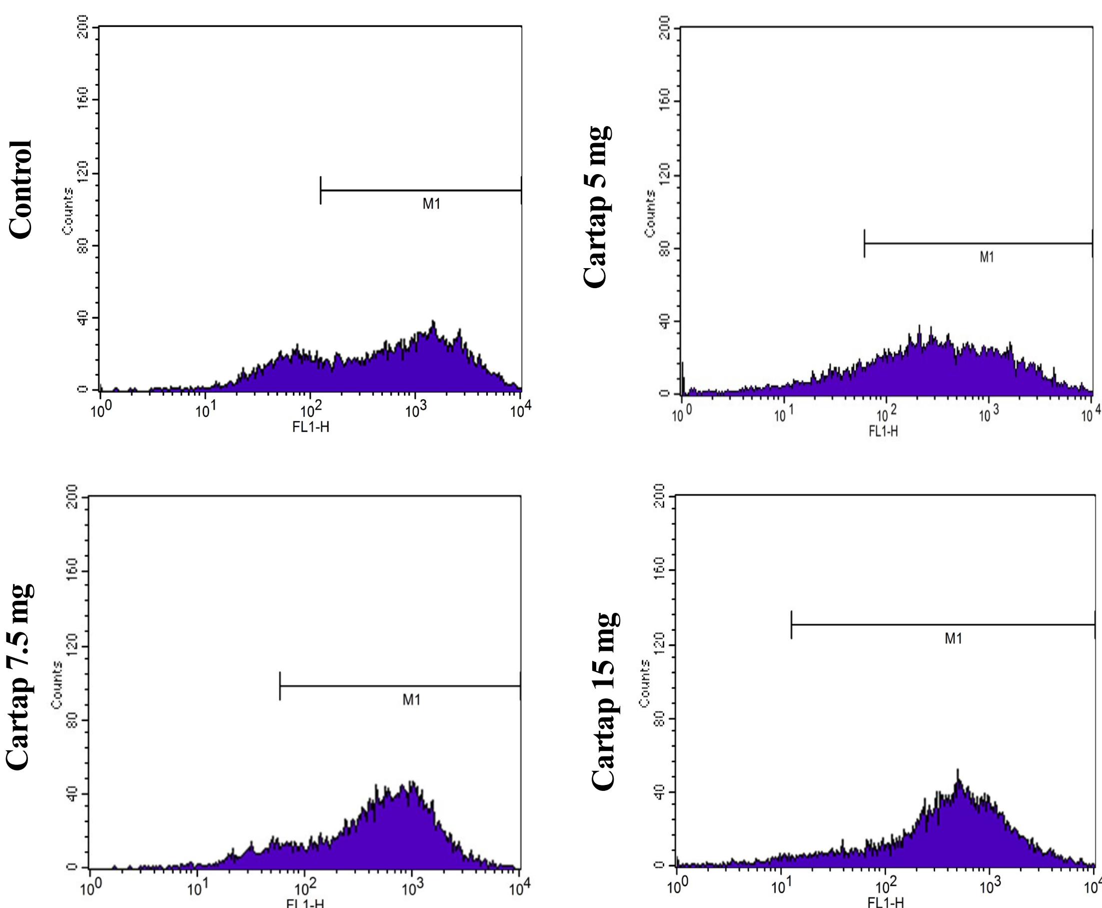

Fig. 51c: Representative histograms of flow cytometry showing percent lymphocytes with loss of mitochondrial transmembrane potential ($DiOC_6$ staining) in mouse spleen after 28 days oral exposure to cartap

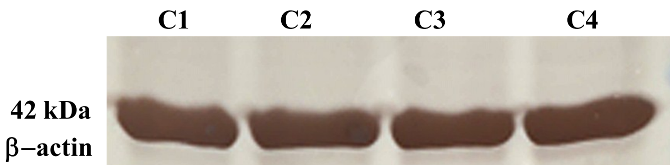

Fig. 52: Representative western blot showing protein expression of β-actin in spleen of mice after 28 days exposure to cartap

Lane C1 : Control
Lane C2 : Cartap 5 mg
Lane C3 : Cartap 7.5 mg
Lane C4 : Cartap 15 mg

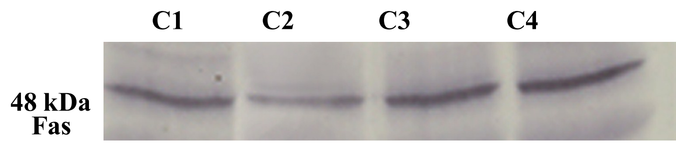

Fig. 53: Representative western blot showing protein expression of Fas in spleen of mice after 28 days exposure to cartap

Lane C1 : Control
Lane C2 : Cartap 5 mg
Lane C3 : Cartap 7.5 mg
Lane C4 : Cartap 15 mg

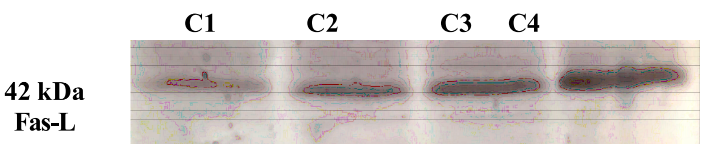

Fig. 54: Representative western blot showing protein expression of Fas-L in spleen of mice after 28 days exposure to cartap

Lane C1 : Control
Lane C2 : Cartap 5 mg
Lane C3 : Cartap 7.5 mg
Lane C4 : Cartap 15 mg

Discussion

5 DISCUSSION

Cartap [*S, S2* -(2-dimethylaminotrimethylene) bis (thiocarbamate) hydrochloride] is an organonitrogenous thiocarbamate insecticide that has been recognized as a nereistoxin analogue. It is a derivative of nereistoxin which is a naturally occurring insecticidal substance isolated from the marine segmented worms *Lumbrinereis heteropoda* and *L.brevicirra*. It is primarily used to control rice stem borer and leaf folder pests in rice paddy field and diamond black moth and aphids in cabbage and cauliflower crops. Cartap hydrochloride is rapidly absorbed, metabolized and excreted in urine both in rats and mice. In urine, following a single acute oral administered dose 94% and 89% is excreted within 24 h in rats and mice, respectively (Fujita *et al*., 1971). Nagata *et al*. (1997) by using single patch clamp technique on pheocytochroma cells of rat showed that cartap hydrochloride act on the nicotinic acetylcholine receptor (nAchR) in open state and cause burst of sub-conductance of action potential. Fleckenstein (1983) suggested that cartap induced muscular contractions are primarily due to influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular pool by inhibiting the Ca^{2+}-ATPase pump (Hosey and Lezduuski, 1988; Lai *et al*., 1988) resulting increase in the intracellular Ca^{2+} level by unloading of Ca^{2+} from sarcoplasmic reticulum vesicles.

The exposure of animal to residual concentration of pesticides can lead to immunosuppression either by directly or indirectly by participating oxidative stress mechanism. Long term exposure with the pesticides can harm human life and disturb the various body functions of different organs including nervous, endocrine, immune, reproductive, renal, cardiovascular and respiratory systems. Strength of this statement of toxic effect by giving evidence on the link of exposure of pesticides and increased incidence of various human

chronic diseases like cancer, Parkinson's, Alzheimer, multiple sclerosis, diabetes, ageing, cardiovascular and chronic kidney disease etc. (Abdollahi *et al*., 2004; De Souza *et al*., 2011; Mostafalou and Abdollahi, 2012). A number of available data focused toward the immune system may be target to the toxic effect of several pesticides and evidence suggests that children particularly are most susceptible to the adverse effect to pesticides exposure (Repetto and baliga, 1996; Voccia *et al*., 1999; Wigle *et al*., 2008; Corsini *et al*., 2008). Cartap is quite stable in acidic medium of environment including soil and water. Thus, exposure to cartap in terms of feed residue present in cereals, various green vegetables and fruits (Koyama *et al*., 1975) as well as occupational exposure to the human beings cause various health implications too. Therefore, immunomodulatory effect of cartap and various mechanisms underlaying involved in such modulation were evaluated in the present investigation.

To evaluate the toxic effect of the cartap on immune system of swiss albino mice after 28 days exposure by oral gavage, a tier approach was used as recommended by De Jong and Loveren (2007). In tier 1, general toxicity study was conducted with emphasis on evaluation of effects on organ belonging to the immune system and includes body weights, absolute organ weights, relative organ weights, hematological parameters, serum biochemical analysis, histopathology of various organs such as liver, kidney, brain, heart, spleen and lung, estimation of oxidative stress in liver, kidney and brain which may focus toward direct or indirect mechanisms involved in the immune toxic effects of the cartap. In tier 2, studies were done to investigate the direct immunotoxic effect of cartap on cell mediated immune response, including delayed type hypersensitivity (DTH) response, T and B lymphocyte proliferation assay, IFN-Y estimation and western blot analysis for the expression of Fas and Fas-L proteins. In tier 3, the immunotoxic effects of cartap on humoral immune response, including serum antibody tire (HA), quantization of IL-4 and apoptosis related toxic effect on splenocytes (Flow cytometric analysis) and DNA fragmentation analysis were investigated. These studies were conducted as described in OECD (Organization for Economic Co-operation and Development, Paris, France) guidelines 407 of the year 1995.

5.1 General toxicity

In present study, the oral LD_{50} of cartap as determined in the acute toxicity test, was calculated to be 156 mg/kg b.wt. in mice. This has been in agreement with the Ray (1991) and

Tomlin (1997) who reported it as reported 150-225 mg/kg b.wt. in mice. Based on LD_{50} value of cartap subsequently, three sublethal doses of cartap i.e. 5 mg, 7.5 mg and 15 mg/kg b.wt. were selected for this study. In present study, treatment of mice with cartap over a period of 28 days by oral gavage did not show any mortality and overt signs of toxicity in any of the treatment groups. However, mice showed slight edematous swelling over a forehead and patchy hair loss over the neck which was extensive with increasing the dose level. A significant reduction in body weights of mice treated with cartap were observed only at 4th week of treatment as compared to the control group. These results are in agreement with the findings of (Tsubura *et al*., 1976; Rivett *et al*., 1972; Hunter *et al*., 1974; Hunter *et al*., 1975). Significant diminuation of spleen weight as reported in present study at medium and higher doses of cartap as compared to the control group mice could be associated with the reduction in splenic cells count. Earlier studies with pesticides, such as lambda cyhalothrin, imidacloprid, atrazine, primiphos-methyl and simazine have also reported reduction in spleen and thymus weight (Morgan *et al*., 2007; Badgujar *et al*., 2013; Zhang *et al*., 2011; Kim *et al*., 2007; Ren *et al*., 2013). In the National toxicology program study (NTP study), thymus and spleen weights exceeding the normal reference range were considered important indicators of potential immunotoxicity (Luster *et al*., 1992). The most convincing explanations for these observations are inhibition of lymphocyte proliferation and induction of the lymphocytes apoptosis in thymus and spleen (Kamnath *et al*., 1997; Vandebriel *et al*., 1999).

5.2 Hematological parameters

The results of present investigation indicated that exposure of mice to cartap for 28 days resulted in significant reduction in PCV, Hb, TEC, TLC and DLC values. These finding suggest suppressive effect of cartap on immune system. It was observed that there was significant reduction in total leukocytes count and differential leukocytes count (in terms of percent lymphocytes) at all the dose levels of cartap. Significant decrease in percent monocytes count at higher dose, although reduction in percent of neutrophils was statistically *non*-significant but percent eosinophils count increased significantly at medium and high doses after exposure to cartap as compared to the control group. On the Similar lines dose dependant reduction in percent lymphocytes, RBCs and Hb concentration in rats on an exposure to endosulfan,

chlorpyrifos-ethyl, chlorpyrifos-methyl and methomyl (Mossa and Abbassy, 2012). Capcarova *et al*. (2012) reported effects on blood parameters of rabbits with significant reduction in WBCs, RBCs and Hb concentration on exposure to bendicarbamate. In rats dose dependant reduction in TLC on exposre to Basalin was reported by Gupta *et al*. (1983). Elsabbagh *et al*. (2001) reported reduced total leucocytes count and increased neutrophilic count in a study performed to investigate the immunotoxic effect of Cupravit and Pervicur fungicide in mice. Sankar *et al*. (2012) observed lymhocytopenia and neutrophilia in cypermethrin treated rats.

5.3 Biochemical parameters

Significant reduction in absolute and relative organ weight of liver and kidney in cartap treated groups as compared to the control group indicated that toxic nature of cartap. The toxic effect of cartap on liver was confirmed by significant increase in the classical biomarkers of liver damage, i.e. AST, ALT, LDH and reduction in total protein/albumin concentration following exposure of even low dose of cartap as compared to the control group and the extent of damage was increased when dose of cartap was increased in present investigation. It was reported that there was significant elevation of ALT, AST and reduction in total protein concentration in rat serum after exposure to mixture of chlorpyrifos-ethyl, chlorpyrifos-methyl and methomyl (Mossa and Abbassy, 2012). A significant decrease in serum albumin of cypermethrin treated rats has been reported by (Saxena and Saxena, 2010). This perturbation of liver enzyme may be due to liver damage or alterations in the permeability of cell membrane and increased synthesis or decreased catabolism of aminotransferases (Alsahhah *et al*., 2006; Kalender *et al*., 2010). Mishra *et al*. (2011) reported raised ALT and AST level in rat serum with flumethrin exposure. Profenofos exposure in male wister rats caused increase in LDH and GGT level in serum (Mansour *et al*., 2009).

In this study, renal damage was indicated by significantly elevated levels of blood urea nitrogen (BUN) and serum creatinine (marker of glomerular injury) at all dose levels of cartap as compared to the control group. Elevated BUN is correlated to the increased protein catabolism in mammals or the conversion of ammonia to urea as a result of increased synthesis of arginase enzyme involved in urea production. It has been shown that cypermethrin treatment

resulted in significant increase in serum urea, BUN and uric acid in rats and serum creatinine levels were declined after acute treatment; however, it was increased after 14 and 21 days exposure to cypermethrin (Saxena and Saxena, 2010).

5.4 Histopathology

In present study, hepatic degeneration with mild congestion of portal area, vascular degenerative changes in cytoplasm of hepatocytes and degenerative changes in hepatic lobule showing infiltration of inflammatory cells with congestion of portal area explain the degree of damage which was corroborated with increased levels of ALT, AST and LDH in cartap treated mice groups. Awad *et al*. (1998) found that cell damage exhibited good correlation with the enzyme leakage. Cartap exposure to mice lead to severe degenerative changes of renal tubule along with swelling and vacuolization, hemorrhage in cortex/medulla and distorted glomerulus with reduced bowman's space related with the elevated level of BUN and serum creatinine level. These changes may be due to increased lipid peroxidation in kidney as a consequence of excessive free radicals accumulation. Analogous to liver and kidney, detailed histopathological evaluation of spleen of mice treated with cartap revealed significant toxico-pathological alterations. In spleen, depletion of lymphocytes in lymphoid follicles showed characteristic atrophic changes in white pulps. These distinct histopathological findings strongly substantiate decrease in absolute and relative weights of spleen along with reduced spleen cells count in cartap treated mice. These findings are parallel with several researchers who have reported atrophic changes, depletion of lymphocytes with presence of apoptotic bodies in spleen of animal treated to pesticides (Gao *et al*., 2008; Zhang *et al*., 2011; Ren *et al*., 2013; Badgujar *et al*., 2013). Lungs of cartap treated mice showed accumulation of pinkish edematous fluid in bronchi, capillary congestion which showed infiltration of inflammatory cells in alveoli, alveolar septum and thickening of interstitial septa. Meningitis with infiltration of inflammatory cells in meninges, focal cerebral gliosis with vascular neurodegenerative lesions in brain sections reflect potential of cartap to induce neurotoxicity. These findings were parallel to the findings of (Luty *et al*., 1998).

In present study, histopathological findings together with serum biochemical observations suggested potentially histotoxic nature of cartap.

5.5 Oxidative stress

Oxidative stress through generation of free radicals, lipid peroxidation, alteration in enzymatic and non enzymatic antioxidants mechanism due to pesticides exposure contribute directly or indirectly to the toxicity (Abdollahi *et al*., 2004; Broznic *et al*., 2008). Generation of excessive free radicals cause damage to DNA, protein and liver causing injury to the various vital organs such as liver, kidney and brain (Banerjee *et al*., 1999; Khan *et al*., 2005). Increased level of free radicals cause oxidative damage to cell membrane and cell disruption lead to increased susceptibility to LPO (Kapoor *et al*., 2009). The damaged membrane lipids, protein and DNA are the end point biomarkers of oxidative stress induced after exposure to pesticides (Tuzmen *et al*., 2008).

In present investigation, 28 days treatment of mice with cartap through oral gavaging caused significant increase in level of malondialdehyde (MDA) in hepatic, kidney and brain tissues. LPO of lipid membrane is regulated by the availability of substrate in the form of polyunsaturated fatty acid (PUFA), availability of inducer such as free radicals and excited state of molecules to initiate propagation, internal antioxidants defense mechanism present in the environment and the physical state of membrane lipid (Pepicelli *et al*., 2005). Cartap hydrochloride primarily metabolized by hydrolysis of the carbonyl carbon and oxidation of the sulphur atom to the sulfoxide (SO), sulfone (SO_2) and ultimately the sulfate (SO_3H) causing oxidative damage indirectly (Kamesaki *et al*., 1976). These radicals attack on cell membrane and lead to destabilization and disintegration of cell membrane resulting peroxidation of membrane lipid (Stajn *et al*., 1997).

It was observed that the superoxide anion ($O_2^{\cdot-}$) generation was significantly increased in liver and kidney of mice exposed to medium and high dose of cartap; however, in brain $O_2^{\cdot-}$ generation was increased at all dose levels of cartap as compared to the control mice. Cellular damage resulting from superoxide anion generation is implicated in the pathophysiology of many diseased conditions like stroke, ischemia, asthma, atherosclerosis and several neurodegenerative diseases (Hancock *et al*., 1997; Mc Cord *et al*., 2002). It has been shown that in paraquat induced nephrotoxicity Superoxide plays an important role (Krall *et al*., 1988).

Further, it was also investigated whether nitric oxide (NO) could be induced by cartap, since SOD activity could be altered by peroxinitrite, the most harmful NO derivative (Yamakura *et al*., 2005). The excessive production of reactive nitrogen species (RNS) resulting excess or deregulated NO production which react with ROS. NO is a signaling molecule that reacts with superoxide radicals to generate RNS, including peroxinitrite, which is very reactive molecule that can modify biomolecules i.e., DNA, lipid and proteins (Barnes *et al*., 2003). In present study, the nitrite level in liver, kidney and brain were significantly raised in cartap treated mice as compared to the control group mice. Role of nitric oxide induced nigro-striatal injury and decrease in striatal dopamine levels after 40 days exposure to rotenone in rats has been explored by (Yi *et al*., 2003).

Glutathione (GSH) is the most abundant non-protein thiol in organism and plays a key role in intracellular defense against toxic compounds, such as reactive oxygen intermediates and other free radicals (Andersons and Luo, 1998). GSH plays an important role in antagonizing the oxidative action of herbicides or insecticides (Parke and Poitrowski, 1996). In present study, GSH levels were significantly decreased in cartap treated mice as compared to the control group mice. It has been reported that tissue which have depleted GSH level are highly susceptible to toxicity (Bus *et al*., 1976). GSH with T-SH group functions as a catalyst for disulfide exchange reactions and contributes in H_2O_2 detoxification. Thiols together with non-enzymatic and enzymatic factors regulate the intracellular metabolism defending various biological structure and functions from noxious attack by ROS (Di Simiplicio *et al*., 1998). Moreover, depleted level of T-SH suggested that the non-protein sulfhydryl compounds and protein bound sulfhydryl groups could alter each other through thiol/disulfide exchange. In fact, in oxidative reactions, GSH is readily oxidized to GSSG, which may react with the –SH group of proteins to form mixed disulfide through thiol/disulfide exchange. Thiol (-SH) are recognized to play a fundamental antioxidant role by protecting the cellular and extracellular functions against oxidative stress. In this context, it was observed that the cartap treatment of mice significantly decreased the levels of T-SH indicating that compound adversely affect the non-enzymatic antioxidants defense mechanism. The synthetic pesticides decreases the cellular content of reduced –SH with a consequently increased the levels of reactive oxygen species (ROS) (Liu *et al*., 2005;

Fetoui *et al*., 2009). Mammalian cells from different tissues, including brain possess a mechanism that regulates the redox status of cellular –SH and protect –SH containing proteins from excessive oxidation. It includes low molecular weight donar of –SH groups and enzymes which can catalyse the reduction in –SH groups in proteins and detoxify pro-oxidants by conjugation with GSH (Zugno *et al*., 2008).

Antioxidants enzymes such as superoxide dismutase (SOD) and catalase act as preventive antioxidant and plays protective role against deleterious effects of lipid peroxidation by scavenging superoxide anion and hydroxyl ions. Catalase and SOD constitute a mutually protective set of enzymes and this cooperative interaction is distinct from the one which derives from prevention of hydroxyl radical produced in the iron-catalyzed interaction of O_2^{-} with H_2O_2 (Kono and Fridovich, 1982). SOD catalyzes the dismutation of superoxide radical to H_2O_2 and O_2^{-} (Matsumoto and Fridovich, 2001). Catalase is a ubiquitous enzyme and a primarily major antioxidant defense component along with glutathione peroxidase (GPx) that catalyzes the metabolism of H_2O_2 to H_2O (Cheng *et al*., 1981). Decreased catalase activity implies Fenton reaction mediated conversion of more H_2O_2 to ultimate toxicant, the hydroxyl radical (Klassen, 1996). The effort of the endogenous antioxidant enzymes is to remove continuously generated free radicals from cells, initially their activity increases due to an induction but with chronic exposure of toxicant causing enzyme depletion resulting in oxidative damage (Kalra *et al*., 1994). In present study, level of catalase and SOD were significantly reduced in cartap treated mice groups as compared to the control mice group in different organ i.e. liver, kidney and brain, suggesting that cartap treatment resulted in disturbed internal antioxidant defense mechanism. Similarly, Significant changes in SOD and catalase activities in different organs of mice/rat exposed to various pesticides/insecticides have been reported by several workers (El-Demerdash *et al*., 2013; Sankar *et al*., 2012; Goel *et al*., 2005; Ozden *et al*., 2009).

Furthermore, alteration in GST activity has also been recorded in the current investigation. Glutathione –S-transferases are detoxifying enzymes that catalyse the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing less toxic forms (Finkel, 2003). Thus, the inhibition of enzymes involved in free radical scavenging led to accumulation of peroxide, which promotes lipid peroxidation, modulation of DNA, altered

gene expression resulting cell death (Stohs and Bagchi, 1995; Calviello *et al*., 2006). Significantly reduced GST activity as observed in liver, kidney and brain of mice exposed to cartap could be probably related to the decreased GSH levels.

In our present study, 28 days exposure of mice to cartap significantly reduced the level of antioxidant enzymes such as SOD, catalase, and GST in liver, kidney and brain tissue. These finding indicates that cartap produce adverse effects on endogenous antioxidant defense mechanism through oxidative stress/ROS generation. ROS generation was also confirmed by flow cytometry with the help of DCFH-DA dye in the splenocytes of mice treated with cartap and compared with the control mice.

5.6 Immunotoxicity

Subacute oral exposure to cartap inhibited *in-vitro* T and B cell proliferation as evident by MTT lymphocyte proliferation assay. Con-A (a T lymphocyte mitogen) and LPS (a B lymphocyte mitogen) failed to produce proliferation of B lymphocytes as compared to the T lymphocytes in cartap treated mice at medium and high dose as compared to the control group mice. The decreased mitogen response may be due to decrease in the expression of mitogen receptors on lymphocytes (Chakrabarti *et al*., 1994). These findings are parallel with the findings of other researchers who have reported significant inhibition of B and T cells proliferation after treatment with pesticides/chemicals such as imidacloprid, simazine, primiphos methyl, dibromoacetic acid, diazinon and captan (Ren *et al*., 2013; Badgujar *et al*., 2013; Kim *et al*., 2007; Gao *et al*., 2008; Neishabouri *et al*., 2004; Lafarge and Decloitre, 1982). Hence, it could be concluded from these results that cartap exposed mice showed reduced proliferative abilility of the lymphocytes.

Diminished proliferative ability of T and B Lymphocytes on exposure to cartap was further confirmed by significant reduction in the haemagglutination titer against T-cells dependent antigens, SRBC in mice at medium and high dose level. Banerjee *et al*. (1996) reported the significant influence of humoral immunity in mice with pronounced suppression of both primary and secondary antibody response to SRBC on subchronic exposure to lindane. Suppression of the humoral immune response following oral administration of nearly lethal doses pesticides

i.e. Carbaryl, DDT, Parathion, Chlordneform and Ametryne to mice was reported by (Willtrout *et al*., 1978). It has been shown that Imidacloprid exposure to Balb/C mice for 28 days caused significant reduction in anti SRBC HA titer (Badgujar *et al*., 2013). In present study, a significant decline of serum HA titer in cartap exposed mice at medium and higher dose level was observed. Since T-helper (T_h) cells are involved in the generation of B cell responses (T and B cell co-operation for antibody synthesis) leading to production of antibodies against T-cell dependent antigens such as SRBC, the noted suppression of HA titer (antibody response) as observed with cartap treatment could be attributed to the impairment of T_h cell activity alone or in conjunction with pesticides effects on intrinsic B-cells functions. Further, proliferative ability of B-cells was found to be significantly decreased in mice treated with cartap. Thus, the reduction in lymphocyte proliferation along with serum HA titer reflect that cartap caused severe impairment in T and B cell functional activities.

Effect of 28 days oral exposure of cartap on T-cell function was further evaluated by measurement of DTH response. DTH response is one of the best validated biomarker for assessment of cell mediated immune response. The results of present study showed significant decrease in DTH response after 48 and 72 hrs of antigen (SRBC) challenge and marked alterations at cellular level in foot pad were observed in histopathological examination. Comparative histopathological evaluation of foot pad section of cartap treated mice to control mice confirmed the DTH reactions since there was decrease in paw thickness in cartap treated mice with increasing the dose level. Such, histopathological studies corroborate gross findings of the DTH reactions and are of upmost importance while evaluating pesticides for immunotoxicity. Suppression of cell mediated immune response as indicated by significant reduction in DTH reaction was observed in sheep exposed to fenvalrate along with lindane and carbofuran (Khurana *et al*., 1999). Singh *et al*. (2001) reported significant reduction in total leukocyte count, absolute lymphocyte count and DTH reaction in chicken exposed to fenvalerate (20 ppm) for a period of 6 months. In buffalo calves a significant reduction of haemagglutination titers against sheep red blood cells (SRBC) and skin thickness in DTH response following single administration of fenvalerate have been reported by (Singh *et al*., 2003). Similar findings are also observed in present investigation that focused on the

immunotoxic effects of cartap. Significant reduction in DTH reactions to SRBC, a T-cell dependent antigen, often is indicative of reductions in cell mediated immunity. Alteration in the magnitude of DTH reactions, symptomatically and/or at a histopathological level, is usually indicative of an impairment of Th1 effector cells (Badgujar *et al*., 2013). Th1 effector cells (also termed T_{DTH} cells) are responsible for the DTH reaction. Specifically, following interaction with a specific antigen, the Th1 cells produce cytokines that evoke mononuclear cells infiltration, cell interaction and increased vascular permeability in the vicinity of stimulus (Luster *et al*., 1982). Histopathological findings in the present study, indicating reductions in mononuclear cells involvement at the injection site (foot pad) and a generalized lower inflammatory reactions could be explained by cartap induced cytotoxic effect on Th1 cells in particular and/or their capacity to evoke the physiologic outcomes noted above (by still unknown mechanism). Hence, results of important indices of immune system assessed in the present study emphasized suppression of humoral and cellular immunity after 28 days oral exposure of mice to cartap as compare to the control mice.

5.7 Role of apoptosis in cartap induced immunosuppression

The most possible explanation for decreased lymphocytes proliferation and splenic cell cellularity as observed in present investigation appear to involve three fundamental (interlinked) mechanisms/pathways.

a) Apoptosis of lymphocytes

b) Generation of reactive oxygen species leading to oxidative stress

c) Participation of cytokines

These possibilities were evaluated in details in present investigation. Cartap induced histopathological observations in the spleen evinced presence of apoptotic bodies; this also partially corroborates suppression of proliferative response to mitogens.

Apoptosis of lymphocytes

Apoptosis of lymphocytes was confirmed by variety of markers:

1) Studying DNA damage in spleen by agarose gel electrophoresis

2) FITC annexin-V/PI assay in splenic lymphocytes by flow cytometry to determine extent of apoptosis and necrosis
3) Apoptotic DNA (cell cycle) analysis by flow cytometry
4) Mitochondrial transmembrane potential ($\Delta\psi m$) using $DIOC_6$ dye
5) Detection of reactive oxygen species (ROS) by DCFH-DA
6) Western blot analysis of apoptotic proteins, Fas and Fas-L. These proteins are upstream regulators of death receptor pathway.

Administration of cartap to mice for 28 days resulted in splenocytes DNA damage which was evaluated by agar gel electrophoresis. Smearing/shearing of DNA was observed in cartap treated mice as compared to the control animal although cartap did not induced classical DNA ladder pattern seen in apoptosis; however, shearing of DNA also indicates damage. These finding are in accordance with the other researchers who have also reported shearing of DNA instead of DNA laddering and attributed to a fewer number of apoptotic cells or the possibility of laddering being masked by necrotic cell death (Eldadah *et al*., 1996; Singh *et al*., 2008).

Flow cytometry assay with annexin-V/PI staining is based on the observation that dying cells undergo the apoptotic stages show phagocytic molecules, such as phospatidylserine (PS) ligand of annexin-V on the cell surface (Li *et al*., 2003). The results of annexin-V/PI staining revealed exclusive apoptotic effect of cartap on splenic lymphocytes. Annexin-V is a member of the annexin family of intracellular proteins that binds to PS in a calcium dependant manner. PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane symmetry lost and PS translocates to external leaflet. Fluorochrome-labelled (FITC) annexin-V can then be used to specifically target and identified apoptotic cells. To distinguish between necrotic and apoptotic cells, PI is used. Early apoptotic cells exclude PI, while late apoptotic cells and necrotic cells stain positively, due to passage of these dyes into the nucleus where they bind to DNA. Significant increase in the % apoptotic lymphocytes was due to arrest in sub G_1- phase of cell cycle. Splenic lymphocytes of mice treated with cartap were predominantly found in G_1- phase following PI staining and flow cytometric analysis. Apoptotic cells often have fractional DNA content due to the fact

that the fragmented (low molecular weight) DNA undergoes extraction during the staining procedure. Some cells also loose DNA (chromatin) by shedding apoptotic bodies; thus, only a fraction of DNA remain within apoptotic cells they are then represented on the DNA content frequency histograms by sub-G_1 phase peak. In present study, cartap at medium (7.5 mg) and higher (15 mg) dose significantly increased the number of lymphocytes with apoptotic DNA.

Mitochondrial membrane potential, in situ, is an important indicator of mitochondrial function and dysfunction. Several studies have shown that mitochondria are the main target for oxidative damage by ROS which lead to mitochondrial dysfunction and apoptosis and necrosis of cells (Arai *et al*., 1999; Kowaltowski and Vercesi, 1999; Kowaltowski *et al*., 1998; Melendez and Davies, 1996). Early drop in mitochondrial transmembrane potential ($\Delta\psi m$) occur during intrinsic apoptosis which can be analyzed by uptake of lipophilic cationic fluorescent dye, like $DIOC_6$. Splenic lymphocyte isolated from cartap treated mice showed prominent loss in Δpm, which indicates that these cells are undergoing apoptosis.

Excessive generation of reactive oxygen species (ROS) cause oxidative stress by targeting genome, protein, structural carbohydrate and lipid within cells (Romero *et al*., 1998). In present study, cartap exposure caused significant excessive production of ROS in cartap treated mice which was detected through increased number of dichlorofluorescin (DCF) positive cells as a result of breakdown of 2’7’-dichlorofluorescin diacetate (DCH-DA) by ROS to DCF a fluorochrome in splenic lymphocytes. Excessive ROS generation can ultimately lead to apoptosis and since accumulation of ROS targets the mitochondrial membrane disruption and loss of MTP (Gupta *et al*., 2003; Shih *et al*., 2003). So that, it is reasonable to assume that mitochondria play a crucial role in pesticide induced toxicity.

Thus, it is clear that cartap exposure caused significant increased in the percents of apoptotic lymphocytes with simultaneous increase in percents apoptotic DNA due to increased production of ROS which in turn led to decrease in the mitochondrial membrane potential, these all factors culminated in apoptosis of lymphocytes. Now, the question arises that in which pathway apoptosis was triggered. Apoptosis is mainly triggered through two pathways i.e. the death receptor pathway and the mitochondrial pathway. In the present study, it was

tried to discern one of the pathway of cartap induced apoptosis in mouse lymphocytes. However, there is evidence that the two pathways are interlinked and the molecule of one pathway can influence the other (Igney and Krammer, 2002). The death receptor pathway involves two receptors that are members of the TNFR3 superfamily: TNFR1 and CD95 (Fas/APO-1) and the TRAIL receptors DR-4 and DR-5 (Ashkenazi, 2002). The ligand for TNFR1 is TNF, which is pleotropic cytokine with various biological activities. In contrast, the ligand for Fas (Fas-L) is mainly expressed on T-lymphocytes. The binding of Fas ligand to Fas receptor resulting in the binding of the adapter protein FADD; then FADD associated with procaspase-8 via dimerization of the death receptor domain. At this point, a death-inducing signaling complex (DISC) is formed; resulting in the apoptosis of cells expressing Fas, and this process plays an important role in the maintenance of immunological homeostasis (Nagata and Golstein, 1995; Dhein *et al*., 1995). Fas protein is ubiquitously expressed in a variety of normal cells, including activated T and B-cells (Suda *et al*., 1995).

In the present study, expression of Fas and Fas-L were determined in spleen by semi-quantitative western blot. Western blot analysis for Fas and Fas-L demonstrated increased expression of these proteins in the spleen resulting in activation of the death receptor pathway because the peripheral lymphocytes were activated following stimulation with a foreign antigen, i.e., cartap and the death receptor pathway activated. From these results it is evident that cartap might mediate cell death in the spleen through the death receptor pathway with the involvement of the Fas and Fas-L interaction.

Participation of cytokines of adaptive immunity:

Cell mediated immune responses are generally associated with Th1 type of helper T-cells, while humoral immune response is associated with Th2 type cells (Mosmann and Coffman, 1989). These Th cells further subdivided into Th1 cells, which are involved in cellular immunity and Th2 cells which regulate humoral immune responses (Romagnani *et al*., 2009). These two different subsets of helper T-cells are defined by their profile of cytokine production and resulting activation of different immune responses. Th1- type cells predominantly produce interleukin-2 (IL-2), IFN-γ and tumor necrosis factor-β (TNF-β). Conversely, Th2-type cells produce IL-4, interleukin-5 (IL-5), IL-6 and IL-10.

Thus, another credible mechanism that could have lead to decrease in the proliferative capacity of T and B lymphocytes in the suppression of Th1 and Th2 type cytokines respectively. Level of both IFN-y and IL-4 were remarkably suppressed by cartap treatment. Th1 cytokine, IL-2 and IFN-γ are involved in lymphocytes proliferation, behaves as hormone-like growth factor which stimulates proliferation of activated T-lymphocytes (Pacifici *et al*., 2003). The differential suppression in the cytokines of adaptive immunity by cartap could have resulted in the disruption of the Th1/Th2 balance and caused suppression of immune response.

The susceptibility of the immune system to toxic damage can result from many factors. Host resistance to infectious agents and spontaneous neoplasms depends on immunocompetant cells, which are subject to continuous proliferation and differentiation and because of that they become excessively susceptible to variety of agents. The immune systems is known for highly organized co-operation and regulation of various cells, which is ensured on one hand by soluble mediators (immunoglobulin, immunohormones and cytokines) and on the other hands by intercellular interactions on the level of membrane receptors and anti-receptors. All agents that affect the fine balance mechanisms mentioned can cause agent specific or species specific immunity damage which in majority of cases results in immunosuppression (Kacmar *et al*., 1999). Together, results of lymphocyte proliferation assay, HA titer and cytokine quantification confirmed an immunosuppressive nature of cartap.

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Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

Cartap, a new 3rd generation type II synthetic thiocarbamates, is being extensively used in controlling a wide range of insects and pests, mosquitoes, fleas, cockroaches, aphids, moths in agriculture and public health programme. The present study was conducted to evaluate immunotoxic potential of cartap exposure in mice following subacute exposure and to study the role of oxidative stress and apoptosis in cartap induced immunotoxicity in mice. The study was conducted in swiss albino mice following 28 day oral exposure to cartap according to OECD guideline 407. Young swiss albino male mice (4-6 weeks) were randomly divided into 4 different groups comprising of 6 animals each. Group I was kept as vehicle control (water). Group II, III, and IV were administered cartap at three dosage based on oral LD_{50}, i.e. $1/10^{th}$ (15 mg/kg b.wt.), $1/20^{th}$ (7.5 mg/kg bw) and $1/40^{th}$ (5 mg/kg b.wt.) of LD_{50} respectively.

Parameters related to general toxicity like weekly body weight, absolute and relative organ weights and detailed hemato-biochemical alterations were evaluated after 28 days of oral exposure. Effect on Humoral immunity was estimated by serum antibody (anti-SRBC) titre, quantitation of Th2 cell cytokine (mouse IL-4) by sandwich ELISA and splenic cell cellularity. Cell-mediated immunity was determined by delayed type hypersensitivity (DTH) response, Lymphocyte proliferation response in splenocytes and quantitation of Th1 cell cytokine (mouse IFN-γ) by sandwich ELISA. Effect of cartap on apoptosis was estimated by DNA fragmentation assay (ladder formation), mitochondrial trans-membrane potential (with $DiOC_6$ dye) by flow cytometry and analysis of apoptotic lymphocytes by annexin-V and PI by of flow cytometry and cell cycle analysis. To assess the role of oxidative stress marker in cartap induced immunotoxicity, oxidative stress related end points in liver, kidney and brain were

evaluated such as LPO, SOD, Catalase, Nitrite, GSH, GST, Superoxide anion generation and total thiol. ROS generation was evaluated by flow cytometry. The silent findings are given below:

6.1 Effect on General toxicity parameters

1. Treatment of mice with cartap over a period of 28 days did not show any mortality in swiss albino mice.

2. Cartap treatment did not caused marked reduction in body weight of mice except at last week of exposure.

3. Statistically significant reduction in absolute and relative organ weights of liver, spleen, brain, heart and lung were observed in mice treated with cartap. There was statistically significant change in the relative weights of the organs liver, kidney and spleen in cartap exposed mice groups.

4. Significant reduction in DLC and platelet count in cartap treated groups was observed. In DLC significant decrease ($p<0.05$) in the % lymphocytes was observed at all dose levels of cartap.

5. Cartap treatment caused significant ($p<0.05$) decrease in the total protein and albumin levels in mice. Serum ALT, AST and LDH concentrations were found to be increased significantly ($p<0.05$) at all dose levels of cartap. Serum BUN and Creatinine concentration were significantly ($p<0.05$) increased in all dose levels of cartap.

6. Histological observations like depletion of lymphocytes, very few numbers of megakaryocytes in the spleen leading to atrophy indicated severe toxico-pathological alterations induced by cartap corroborated findings of reduction in the spleen weight.

Conclusions:

Subacute exposure to cartap significantly decreased general toxicity related parameters suggesting its toxic nature. Histopathological findings of spleen also indicate insult of immune organs after cartap treatment. Reduction in these parameters are suggestive of direct

immunotoxicity and thus in the present study, decrease in these parameters gave a signal that cartap may be immunotoxic.

6.2 Effect on humoral and cellular immune response

1. Medium and high dose of cartap caused significant reduction ($p<0.05$) in the antibody titer in mice as compared to control.
2. Cartap treatment caused significant ($p<0.05$) decrease in the spleen cell cellularity.
3. Cartap exposure caused severe and significant ($p<0.05$) decrease in the mouse serum IL-4 level as compared with the control group.
4. In cartap treated mice, mild inflammatory reaction with mild edema and erythema were noted. The DTH response (i.e., percent increase in paw thickness at a given time point) was significantly ($p<0.05$) decreased at all dose levels as compared to control.
5. Cartap treatment of mice showed significant ($p<0.05$) decrease in the T and B lymphocyte proliferation (against mitogens con-A and LPS).
6. Cartap exposure caused significant ($p<0.05$) decrease in the serum IFN-γ level as compared to control.

Conclusions:

Results of lymphocytes proliferation assay, DTH response and HA titer demonstrated that cartap induces severe impairment in T and B cell activity, wherein their ability to co-operate (T and B cell co-operation) and respond to foreign antigen (SRBC) was remarkably decreased. The differential suppressions in the cytokines of adaptive immunity by cartap could have resulted in the disruption of the Th1/Th2 fine balance leading to remarkable suppression of humoral and cellular immune response. All the interlinked functions of the immune response assessed after 28 days treatment with cartap indicate its potential immunotoxic nature.

6.3 Mechanism of cartap induced suppressed humoral and cellular immune response

1. DNA damage in the splenic lymphocytes evaluated by electrophoresis revealed dose dependent increase in shearing of DNA.

2. There was significant increase in the % lymphocytes containing apoptotic DNA (Sub G1 phase) after treatment with both medium and high dose of cartap (in dose related fashion), which was assessed by propidium iodide.

3. The results of FITC annexin-V/PI staining showed significant increase in the percentage of apoptotic lymphocytes (early and late apoptotic cells) at all dose levels of cartap.

4. Significant loss ($p<0.05$) in the $\Delta\psi m$ of lymphocytes treated with various dose of cartap was evident from less uptake of DiOC6 in the mitochondrial matrix.

5. There was significant increase in the % of cells showing ROS generation, after treatment of mice with cartap.

6. Cartap significantly ($p<0.05$) increased the relative protein expression of CD95/Fas and Fas-L in the spleen lysates of mice as compared to control.

Conclusion:

Cartap exposure caused increase in % of apoptotic lymphocytes with simultaneous increase in % apoptotic DNA due to increased production of ROS which in turn led to decrease in the mitochondrial membrane potential. Often, an early drop in the mitochondrial transmembrane potential ($\Delta\psi m$) occurs during intrinsic apoptosis. Further, western blot analysis for the expression of CD95/Fas and Fas-L protein revealed that cartap mediated cell death in the spleen occur through the death receptor pathway with possible involvement of Fas/Fas-L interaction.

6.4 Effect on Oxidative stress related end points

1. The MDA formed (LPO) was significantly ($p<0.05$) increased at all dose levels of cartap in kidney and brain, while in liver only medium and higher dose caused significant increase in MDA level as compared to control group.

2. The amount of NBT reduced (superoxide anion generation) was significantly (*p*<0.05) increased in liver and kidney tissues in medium and high dose, while in brain only at all dose levels of cartap as compared to control group.

3. The nitrite level in Liver, Kidney and Brain was significantly increased after cartap treatment.

4. Cartap treatment significantly decreased the GSH and total thiol level in liver, kidney and brain tissues.

5. Administration of cartap for 28 days caused significant decrease in the level of enzymatic anti-oxidants such as SOD, catalase and GST in liver, kidney and brain.

Conclusion:

Alterations in the parameters of oxidative stress and ROS generation in the liver, kidney, brain and spleen of mice indicates deleterious effect of cartap on endogenous anti-oxidant status. Since at the low dose of cartap, (5 mg/kg b.wt.) most of the parameters remaind unaltered, so it could be considered as NOAEL of cartap for immunotoxicity. Thus, lowest observed adverse effect level (LOAEL) of cartap for immunotoxicity could be considered as 5 mg/kg. More extensive studies are needed to assess the possible risks to human and animal health caused by cartap hydrochloride.

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Mini Abstract

7 MINI ABSTRACT

Cartap is an organonitrogenous thiocarbamates insecticide that has been recognized as a nereistoxin analogue. Cartap normally used as a hydrochloride; has broad spectrum activity against insects and first choice to controlling the chewing and sucking pests (e.g. weevil and caterpillar in paddy and diamond black moth in cabbage field). Despite overt use of cartap in agriculture, reports are lacking on detailed study of immunotoxicity of thiocarbamates groups of pesticide to which cartap belongs. It is possible that residues of this compound in vegetables and fruits continue to cumulate and affect the general population on their long term exposure. The present study was undertaken to examine immunotoxic potential of cartap in mice. Administration of cartap at different dose levels (5, 7.5 and 15 mg/kg, orally for 28 days) caused significant decrease in the absolute weight of spleen along with reduction in serum proteins (i.e. total proteins, albumin and globulin) at all dose levels. Further, markers of liver (AST, ALT and LDH) and kidney (serum creatinine and BUN) injury were also increased in the serum after cartap exposure. Histopathological alterations in the spleen, liver and kidney confirmed these findings. Results of lymphocyte proliferation assay, DTH response and HA titer demonstrated that cartap induces severe impairment in T and B lymphocytes activity. The suppression in the cytokines of adaptive immunity (IFN-γ and IL-4) by cartap suggested disruption of the Th1/Th2 fine balance which led to remarkable suppression of humoral and cellular immune response. All these inter-linked functions of the immune response were assessed after 28 day treatment with cartap indicate its immunotoxic potential. Cartap treatment also increased lipid peroxidation and ROS generation with concomitant decrease in the glutathione and activities of antioxidant enzymes. The mechanism of immunosuppression caused by cartap is attributed to cartap-induced oxidative stress and DNA damage in spleen along with apoptosis of lymphocytes as revealed by increased levels of apoptotic markers. Further, increased expression of apoptotic proteins, Fas and Fas-L in spleen showed cartap induced apoptosis in splenocytes mediated by Fas/Fas-L death receptor pathway.

लघु सारांश

8 लघु सारांश

कारटेप एक ऑरगेनिक नाइट्रोजिनस थायोकार्बामेट कीटनाशक है, जोकि नीरिटॉक्सिन एनालॉग के रूप में स्वीकार किया गया है। सामान्य रूप से यह हाइड्रोक्लोराइडत्र के रूप में चबाने और चूसने वाले कीट (जैसे–धुन और कमला कोधान के खेत तथा हीरे काले कीट को गोभी के खेती में)। कृषि क्षेत्र में कारटेप के व्यापक उपयोग के बावजूद थायोकार्वामेट समूह का प्रतिरक्षा तंत्र पर प्रभाव का विस्तृत अध्ययन नहीं हो सका है जिस समूह से कारटेप संबंध रखता है। संभव है कि कारटेप के अवशेष फलोऔर सब्जियों में एकत्र होकर लंबी अवधि तक प्रयोग करने पर सामान्य आबादी को प्रभावित करने की क्षमता रखता है। वर्तमान अध्ययन चूहों में कारटेप का प्रतिरक्षा तंत्र पर संभावित प्रभाव की जांच करने के लिए किया गया था। चूहों के विभिन्न समूहों में कारटेप की अलग–अलग मात्रा में 28 दिन खुराक (5, 7.5 एवम् 15 मिग्रा/किलो) देने से प्लीहा तथा रक्त जल प्रथिनोंमें कमी पाई गई। इसके साथ ही यकृत तथा गुर्दे के रसायनों के स्तर में वृद्धि पाया गया। इन्ही अंगों में विभिन्न ऊतकीय विकृति विज्ञानी परिवर्तन पाये गये। कारटेप अविषता से चूहों के प्रतिरक्षा पिण्ड व कोशिकीय प्रकार की प्रतिरक्षा क्षमता का ह्रास हुआ। प्रयोग के 28 दिन बाद यकृत, गुर्दे एवम् मस्तिष्क में ऑक्सीकारक तनाव (जी.एस.एच., एल.पी.ओ., एस.ओ.डी. एवम जी.एस.टी.) में सार्थक परिवर्तन पाये गये। इसी के साथ प्लीहा के लिम्फ कोशिकाओं में स्वयं कोशिका मृत्यु दर में वृद्धि देखी गई। वर्तमान अध्ययन के परिणामों से इंगित होता है कि उपतीव्र कारटेप से प्रेरित चूहों के प्रतिरक्षा प्रणाली में सार्थक परिवर्तन (दुष्परिणाम) होते है तथा उसमें ऑक्सीकारक तनाव एवम् स्वयंकोशिका मृत्यु दर अहम भूमिका निभाता है।

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Appendix

APPENDIX-I

General Buffers and Reagents.

A. Phosphate buffered saline (PBS) pH 7.4

Sodium chloride	8.0g
Potassium Chloride	0.2 g
Potassium dihydrogen phosphate	0.2g
Dipotassium hydrogen phosphate	2.17g
Distilled water to make the volume to	1000 ml

B. 10 N Sodium hydroxide (NaOH)

Sodium hydroxide	40g
Distilled water to	100ml

C. 5 N Hydrochloric acid (HCl)

Concentrated hydrochloric acid	42.20 ml
Distilled water to	100ml

Buffers and solutions for isolation of DNA

A. 1 M Tris-Cl, pH 8.0, 100 ml

Tris-HCl	15.7g

Add DW up to 80 ml volume, adjust the pH to 8.0 and make the volume to 100 ml

B. 0.5 M EDTA, pH 8.0, 100 ml

EDTA	18.61 g
Distilled water up to	80 ml

Stir vigorously on a magnetic stirrer; adjust the pH with 10 N NaOH. Make the volume to 100 ml with distilled water.

C. Cell lysis buffer (10 mM Tris-Cl, 1 mM EDTA, 0.1% w/v SDS) pH 8.0

Tris-HCl	1.0 ml
0.5M EDTA (pH 8.0)	0.2 ml
SDS	0.1g

Add autoclaved DW and make the volume to 100 ml, store at RT. Chill it before use.

D. DNase free RNase (4 mgml^{-1})

DNase free RNase	0.004g
Add sterile DW upto	1 ml

E. Proteinase K (20 mgml^{-1})

Proteinase K	0.020 mg
Add sterile DW upto	1 ml

Buffers for agarose gel electrophoresis

A. Tris Acetate EDTA (TAE) stock 50X

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Distilled water to	1000ml

B. Tris Acetate EDTA (TAE) working 1X

50X TAE stock	20 ml
Distilled water upto	1000 ml

C. 1% Agarose (60 ml)

Agarose low EEO	0.6 g
TAE 1X upto	60ml

D. Ethidium bromide (10 mg ml^{-1})

Ethidium bromide	100mg
Distilled water	10 ml

The solution was prepared by vigorous vortexing till complete dissolution in a 15 ml sterile tube and wrapped with aluminium foil and kept at 4°C.

E. Ethylene diamine tetra acetic acid (EDTA), pH 8.0, 0.5M

EDTA	18.61 g
Distilled water upto	80 ml

Stir vigorously on a magnetic stirrer; adjust the pH with 1 N NaOH. Make the volume to 100 ml with distilled water.

Buffers and reagents for Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

A. Acrylamide/Bis-acrylamide solution (30%)

Acrylamide	87.6g	(29.2 g/ 100ml)
Bis-acrylamide	2.4 g	(0.8 g/ 100 ml)

Filter and store at 4^0C in dark

B. Sodium dodecyl sulphate (SDS), 10% (w/v)

SDS 10g in 90 ml water

Mix well by stirring till SDS dissolves

Add water to 100ml

C. 1.5 M Tris-HCl, pH 8.8 (Resolving buffer)

Tris base 18.15g

Add distilled water to dissolve Tris-base by stirring till 90 ml volume. Adjust the pH to 8.8 with 6N HCl.

Add distilled water till 100 ml.

D. 0.5 M Tris-HCl, pH 6.8 (Stacking buffer)

Tris base 6g

Add distilled water to distilled water to 60 ml and adjust the pH to 6.8 with 6N HCl

Make the volume to 100 ml with distilled water

E. Sample buffer 2X (9.5 ml)

Distilled water	3.55 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
Glycerol	2.5 ml
SDS (10% w/v)	2.0 ml
Bromophenol blue (0.5% w/v)	0.2 ml

Add 0.05 ml β-mercaptoethanol to 0.95 ml sample buffer fresh before working

F. Sample buffer 5X

0.5 M Tris-HCl (pH 6.8)	1.0 ml
10% SDS	1.6 ml
β-mercaptoethanol	1.0 ml

Glycerol	0.80 ml
Bromophenol blue (0.5% w/v)	2 ml
Distilled water	4.0 ml

Mix well and store at room temperature or $4^{O}C$

G. SDS-PAGE running buffer (electrode buffer), pH 8.3

Tris-base	30.3 g
Glycine	144g
SDS	10g

Add distilled water to dissolve the components and make the volume to 1000ml.

Donot adjust the pH of the buffer and store at 4^{0}C.

H. 10% Ammonium persulphate (APS)

Ammonium persulfate	100mg

Add distilled water to 1 ml. Use freshly made.

I. 6 N Hydrochloric acid

Concentrated hydrochloric acid	50.64 ml
Add distilled water to	100 ml

J. Bromophenol blue solution (0.5%)

Bromophenol blue	0.005g
Glycerol	0.2 ml
Distilled water to	1 ml

K. Coomassie Brilliant Blue R-250 (CBB R-250) staining solution

Coomassie brilliant blue R-250	2.5g
Methanol	500ml
Glacial Acetic acid	100ml
Distilled water 400ml to make the volume to	1000ml

L. Destaining solution

Same composition as the staining solution, but without CBB R-250

Buffers and reagents for Western Blot

A. Transfer buffer, 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3

Tris base	3.03 g
Glycine	14.4 g

Methanol	200 ml
Add distilled water to	1000 ml.

Donot adjust pH, if pH is below 8.0; remake the buffer. Store the buffer at 4°C. preferably use fresh buffer for every transfer.

B. Tris Buffered Saline (TBS); 10 mM Tris chloride (Tris-Cl), 150 mM sodium chloride (NaCl), pH 7.5 for Ni/NTA blot

Tris-chloride	1. 57 g
NaCl	8.766g

Distilled water to 950ml, adjust the pH with 1N NaOH/ HCl and make the volume to 1000 ml, store at 4°C.

C. Tris Buffered Saline-Tween (TBS-Tween); 20mm Tris-Cl; 500mM NaCl, 0.05% (v/v) Tween-20 pH 7.5 for washing for Ni/NTA blot

Tris-chloride	3.14 g
NaCl	29.22 g

Distilled water to 950ml, adjust the pH with 1N NaOH/ HCl and make the volume to 1000 ml. Add 0.5 ml Tween-20, mix nicely and store at 4°C.

D. Phosphate buffered saline 10X (PBS), pH 7.4

Sodium chloride	50g
Potassium chloride	2.5g
Anhydrous disodium hydrogen phosphate	14.2g
Anhydrous potassium dihydrogen phosphate	2.5g

Add distilled water, adjust the pH to 7.4 with 1N NaOH/ HCl and make the volume to 1000 ml.

E. Phosphate buffered saline 1X (PBS), pH 7.4

10X PBS, pH 7.	4 100ml

Add de-ionised/ distilled water 900 ml, to make volume upto 1000 ml.

F. Phosphate buffred saline-Tween 20 (PBS-T), pH 7.4

10X PBS, pH 7.4	100ml
Tween-20	0.5 ml

Add de-ionised/ distilled water 900 ml, to make volume upto 1000 ml.

G. Blocking buffer, 5% bovine serum albumin (BSA)

BSA	0.5 g
TBS upto10 ml **OR**	

H. Blocking buffer, 5% skimmed milk (skimmed milk)

Skimmed milk	0.5 g
PBS upto	10 ml

Add any other heterologous serum (serum of species, other than against which the conjugate is being used) at the concentration of 5 – 10%. Prepared fresh before use.

I. Dog Anti-mouse HRPO conjugate (1:20000) (for β-actin)

Goat anti-chicken HRPO conjugate	0.0005 ml
TBS-Tween (pH 7.5)	10 ml

J. Goat Anti-rabbit HRPO conjugate (1:3000)

Goat anti-rabbit HRPO conjugate	0.0034 ml
TBS-Tween (pH 7.5)	10 ml

K. DAB staining solution

DAB tablet one (5 mg DAB/ 240 mg tablet)	
PBS (pH 7.4)	10 ml

Dissolve the tablet by vigorous vortexing; add 0.010 ml hydrogen peroxide (30% v/v) just before use. Nickel chloride (8%, w/v) 0.010 ml may be added to the solution for strong colour development as an option.

L. Ponceau-S staining solution (0.1% w/v Ponceau in 1% v/v acetic acid)

Distilled water	10 ml
Glacial acetic acid	0.3 ml
Ponceau-S powder	0.033g

Add DW to make the volume upto 30 ml and store the solution at RT.

VITAE

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