Physiological Aspects of Buprofezin Toxicity on Albino Mice

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A thesis submitted in partial fulfilment of the requirements for the degree of

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In

Physiology

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Dedication

Every challenging work needs self-efforts as well as guidance of elders especially those who were very close to our heart. My humble effort I dedicate to my;

Sweet and loving

Father and Mother

Whose affection, love, encouragement and prays of day and night make me able to get such success and honor,

&

From them I learned, always do the best job, your reputation is worth more than a quick profit,

Siblings

My soul mates for their eternal love and care,

Myself,

To those who love me,

Along with all hard working and respected Teachers

&

All at end

Dedicated to my beloved Heavenly Abu G

Author's Declaration

I **Ms. Razia Bibi** hereby state that my Ph.D. thesis titled "Physiological Aspects of Buprofezin Toxicity on Albino Mice" is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

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Razia

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LIST OF ABBREVIATIONS

% H	Percentage DNA in Head
% TDNA	Percentage DNA
µg/kg (b.w.)	Microgram/ Kilogram Body Weight
A/min	Absorbance/ minute
ALP	Alkaline phosphatase
ALT	Alanine Amino Transferase
AST	Aspartate Amino Transferase
Balb/c mice	Mouse Strain
BPFN	Buprofezin
CAT	Catalase
CD-1 mice	Mouse Strain
CL	Comet Length
CPF	Chloropyrifos
dH_2O	Distilled water
DNA	Deoxyribo Nucleic Acid
DTNB	5,5-Dithio-bis 2-nitrobenzoic acid
EDTA	Ethylene diamine tetra acetic acid
EFSA	European Food Safety Authority
FNT	Fentrothion
FPN	Fipronil
g/kg (b.w.)	Gram/ Kilogram Body Weight
GPx	Reduced Glutathione
GSH	Reduced Glutathione
GST	Glutathione S transferase
HD	Head Length
LC_{50}	Lethal concentration
LD_{50}	Lethal dose
LDH	Lactate dehydrogenase
LMPA	Low Melting Point Agarose
LPO	Lipid peroxidation
MLT	Malathion

MPT	Methyl parathion
MRL	Maximum residue limit
NMA	Normal Melting Agarose
NRA	National Registration Authority
OTM	Olive Tail Moment
PBS	Phosphate buffer Saline
PMSF	Phenyl methyl sulfonyl fluoride
PND	Pendimethalin
POD	Peroxidase
ROS	Reactive oxygen species
SCGE	Single Cell Gel Electrophoresis
SOD	Super oxide dismutase
SPD	Spinosad
TBA	Thiobarbituric acid
TBARS	Thio barbituric acid
TCA	Trichloroacetic Acid
TL	Tail length
TM	Tail Moment
UNEP	United Nations Environment Programme
WHO	World Health Organization
x*g*	Times gravity
ΔA/min	Change of absorbance/ minute

ABSTRACT

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Buprofezin is a type-1 chitin synthesis inhibitor insecticide used to control hemipteran insects. It is generally considered safe for humans, but its persistent nature can be a health hazard. Adverse effects on mammals are remaining to be explored. The present study investigated broader toxic effects of buprofezin on liver and kidney tissues of mice upon short-term exposure for 24 h. Four groups were formulated (sample size: n = 7). Group-1 control, whereas groups-II, III and IV were treated intraperitoneally with 400, 600 and 800 mg/kg b.w doses of buprofezin respectively. Comparisons were made statistically at $p < 0.05$. Results revealed no pronounced change in body weight and liver, kidneys organ's weight, but increased activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), creatinine and urea, ROS and TBARS (thiobarbutaric acid) in liver and kidney tissues in the experimental groups. Concomitant significant decrease occurred in tissue total protein, antioxidants enzymes, the superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) and non-enzymatic reduced glutathione (GSH). Significantly altered histomorphology of liver and kidney tissues revealed excessive tissue damage. Congestion, hepatocytic necrosis, sinusoidal damage in liver, while in kidneys, glomerular shrinkage, capillary damage, widened Bowman's space and lumens of tubules and collecting ducts and necrosis of tubular epithelial cells were evident. TUNEL assay confirmed apoptosis, while Comet assay demonstrated DNA damage as revealed by an increase in the head length, tail length, comet length, tail moment and olive tail moment. The study concludes that buprofezin is highly toxic for mammalian tissues and warrants further biochemical, molecular, and cellular studies

INTRODUCTION

INTRODUCTION

1.1 Overview of Pesticides

Pesticides are the most widely used synthetic chemical compounds to control undesirable pests and disease-causing vectors to increase food production. Over past few decades, pesticides use has become widespread. Besides being beneficent many adverse effects of pesticides are largely being observed in non-targeted entities predominantly human beings that are of prime importance. World Health Organization (WHO) and the United Nations Environment Programme (UNEP) reports over 26 million pesticide poisonings in human occur worldwide per annum which leads to almost 220,000 deaths (Pimentel, 2002).

A "pesticide" is described as a chemical or a combination of synthetic chemicals prepared with the intension to destroy and prevent pests, weeds, insects, ticks, rodents, snails, termites etc. In broader sense, these include many sets of chemicals such as herbicides, nematicides, insecticides, fungicides, molluscicides, and rodenticides as well as growth regulators for plant (US EPA, 2014; Kim et al., 2017).

Pesticides have an importance as they are involved in supporting food production by protection the agricultural crops from vector borne diseases as well as pest/weeds attacks (Adhikari et al., 2004; Abhilash and Singh, 2009). Therefore, pesticides are one of the main constituents that updated modern agrarian expertise. Pesticides have been extensively used throughout the biosphere to protect yields as of detrimental nuisances those then abolish yields and subsidize in financial benefit. Ohkawa et al. (2007) and others explained that even nevertheless the pesticides have been very effective but most likely hazardous and their widespread consumption has elevated many health hazards not only to humans but to whole environment. Similarly, Wang et al., (2017) described the persistence of some of the pesticides for longer duration is still unidentified in the environment and their effects on native fauna. Generally, these pesticides/insecticides are noxious wastes of aquatic environment destroying non targeted organisms like fish, birds, animals and humans which feed on them (Nolletk and Rathore, 2010). Above 0.1 million deaths in China each year are caused by 0.5 million human pesticide poisonings (Zhang et al., 2011). Thus, the random use of such poisonous chemicals has led to contamination of groundwater

reservoirs and running water indiscrimination via agricultural runoff. These toxins would cause serious wellbeing hazards to all marine creatures comprising fish (Murthy et al., 2013; Gibbons et al., 2015). These chemicals not only influence surface water but seeps into groundwater through heavy rainfall or runoff from agriculture fields that made their route to groundwater level thus contaminating the existing water storage basins. That again produces a shocking condition for human health as their exposure (direct/ indirect) might cause numerous cancers (Cok et al., 2011; Kim et al., 2017).

1.2 Commonly used insecticides

Additional important class of pesticides are the insecticides, since the insecticides protect many crop varieties used in almost all farming efforts. Those are mainly being practiced as plant growth promotors and help to curtail agricultural yield harms initiated by insects (Ecobichon, 2000). Similarly, many diseases (vector-borne) are caused by insects and parasites now a days by use of insecticides are largely being prevented (Rivero et al., 2010). Insecticides are used to avoid insects and ectoparasites in aquaculture and nurseries (Bolognesi and Holland, 2016).

Pyrethroid is an organic compound like natural pyrethrin that is formed by pyrethrum's flower. Maximum of the commercially used insecticides create pyrethroids (Metcalf and Robert, 2000). Pyrethroids are also poisonous to useful insects, bees, gadfly mayflies and some invertebrates which affect food webs i.e. terrestrial and aquatic (Zaveri and Mihir, 2010). Pyrethroids are toxic even at extremely small concentrations such as 4 parts per trillion to fish and other aquatic organisms (Pyrethroids fact sheet, 2007; Thatheyus et al., 2013).

The pyrethroids are lethal to cats as they lack glucuronidase enzyme required in hepatic detoxifying metabolic pathways (Jim et al., 2009). Earlier studies suggested that mice exposed to pyrethroid in developmental stage are more susceptible to neurological and behavioral changes resembling attention-deficit and hyperactive disorder in humans (Richardson et al., 2015). Pyrethroids are axonic excitotoxins, their noxious effects are mediated by preventing sodium channels closure in axonal membrane. These chemicals keep the channels open, prevent nerve repolarization and thus leave axonal

membrane as permanently depolarized by paralyzing the targeted organisms (Soderlund et al., 2002).

Another synthetic, compound cypermethrin, applied as an insect repellent in commercial agriculture and domestic applications. It acts as a fast-acting neurotoxin for insects and highly toxic to bees, aquatic insect even fish and cats, and is added to many household cockroach and ant killers.

Cypermethrin has its application in agriculture as control agent against ectoparasites that infect poultry, cattle and sheep (FAO). Moreover, in veterinary practices this insecticide is effectively used for dogs to control ticks (Somasani and Ayodhya, 2014; Merck Veterinary Manual). It causes irritation in eyes and skin through ingestion or direct skin contact being moderately toxic to humans. Such dermal exposure results itching, numbness, tingling and burning while long term effects include incoordination or loss of bladder control and in some cases death (Ecobichon and Donald, 1993). Research reports highlighted cypermethrin hilarious effects on reproductive system in male rats (Hu et al., 2011). Cypermethrin residue persist in the air and on floor, walls, furniture for more than 84 days have been depicted from similar studies (Martinez-Cabrillo et al., 1991).

Chloropyrifos (CPS) is an organophosphate pesticide. It was introduced by Dow Chemical Company in 1965 to be used on crops, animals, and buildings to kill pests, worms and insects. Chloropyrifos by inhabiting acetylcholinesterase enzyme activity, act on insect nervous systems. This chemical was used for the first time as a nerve gas in 2nd World War (California Department of Pestidicide Regulation, Chlorpyrifos, Product List. May, 2015). Based on its acute toxicity, chloropyrifos is considered moderately toxic for humans (WHO 2010). Its recommended levels for use have been associated with autoimmune, persistent developmental disorders and neurological effects. Chloropyrifos may affect children mental development, since pregnant mothers that are exposed to it. So chlorpyrifos home uses were banned in the U.S. since 2001 (Scientific American, 2012).

Chlorpyrifos and other organophosphate pesticides upon acute exposure of bees interfere with cholinergic signaling and transmission of impulses (Christensen et al.,

2009). Aquatic (freshwater) organisms i.e., insects, crustaceans are more sensitive to chloropyrifos than fish (Giddings et al., 2014).

Crystalline chemical dichloro-diphyenyl-trichloroethane DDT, a tasteless, ordorless organochlorine was firstly developed as an insecticide but ultimately become unauthorized due to its environmental impacts (Pedra et al., 2004). DDT opens sodium ion channels in insect's neurons, triggering them to fire instinctively, which leads to contract and ultimate death (DeCarvalho Anderson and Juliana, 2013).Insects are resistant to DDT and similar insecticides with certain mutations in their sodium channel gene.

In some insect species, DDT resistance also conversed up-regulation of genes expression by cytochrome P450 genes (Denholm et al., 2002), as larger measures of some enzymes of this group speed up the contaminant's metabolism into low metabolites. In the *Drosophila melanogaster*, genomic studies showed that elevated level DDT resistance is polygenic, including multiple resistance mechanisms (Pedra et al., 2004).

In most developed countries agricultural use was banned, in the 1970s and 1980s, beginning with Hungary in 1968 (Archives of DDT from the original, 2009) followed by Norway and Sweden in 1970, West Germany and the United States in 1972, but not in the United Kingdom until 1984. By 1991 total bans, comprising for disease control, was imposed in at least 26 countries; like Cuba in 1970, the US in the 1980s, Singapore in 1984, Chile in 1985 and the Republic of Korea in 1986 (FAO/UNEP, 1991).

The word "organophosphates", when act in communications, in regions including human and animal health, agriculture and environment, very frequently referred to a group of insecticides (pesticides) that acted on enzyme acetylcholinesterase. Currently, organophosphates contributed about 50% of the killing agents in chemical insecticides (Coats, 1990).

Organophosphate pesticides (OPPs), comparable to some nerve agents, prevent this neuromuscular enzyme, is largely necessary for normal function in insects,

however also in many other animals and humans (Robert and Metcalf, 2002). OPPs affect acetylcholinesterase in different ways, but principally through irreversible covalent inhibition (Connell et al., 1999).

In many applications, organochlorides pesticides are very valuable compounds, but some are of thoughtful environmental importance (Rossberg et al., 2006). Some types of organochlorides have substantial toxicity to plants or animals, including humans. Dioxins, produced when carbon-based matter is burned in the presence of chlorine, are determined organic pollutants which pose dangers when they are released into the environment, as are some insecticides DDT. For example, DDT in the mid - 20th century, was generally used to control insects, it also accumulates in food chains, equally do its metabolites DDE and DDD, and in certain bird species, causes reproductive complications (for example, eggshell thinning), (Connell et at., 1999).

1.3 Impact on environment

Unreasonable use of pesticides may harmfully affect the vegetation and wildlife comprised ecosystem (Kumaraguru and Beamish, 1986). Agrarian overspill as well as addition of likely toxicants into water reservoirs such as oceans, rivers lakes, and ponds where they threatened the fish as well as other inhabitants while slowly accumulated in the form of bodily creatures (John and Prakash, 2003). Jayarai et al. (2016) and others describe that noxious agents as well as in the long run are transmitted to hominids (Stadlinger et al., 2016).

Bolognesi and Holland, (2016) explained, for instance simple background of entirely different creatures was silently comparable hence, specificity in the mode of action of these insecticides is being challengeable completion. Accordingly, the widespread variety of pesticides become discrete as well as could cause noxiousness in undifferentiated individuals of fauna via subsequent continuous exposure to pesticides such as, humans, fishes and birds. Pesticides severely affect the bodily creatures, while their applications surpass the onset level of the bodies of creatures or their concentration if in the environment are above tolerable stages (Mossa et al., 2018).

Ecosystems of the world are under constant threat from extensive contamination of air, soil, and water. Inconsistent insecticide practices in farming as well as in cities

increased inhabitant proportion has undoubtedly affecting the environment and industrial wastes. The main cause of air, soil as well as water pollution eventually put veracity at great risk of the ecosystems are the pesticides (Rusch et al., 2016).

van der Werf, (1996) described that almost 2.5 million tons of insecticides have been applied in agrarian fields per year all over biosphere. Only 0.3 %, from great expense, holding out selected creatures, whereas the maximum 99.7 % is going into nearby atmosphere thus pollute, air, soil as well as water.

In wildlife, acute poisoning had reduced in number during recent decades, the problem of long-term pesticide harmfulness had moved into the focus of scientific interest at least in developed countries. Pesticide application is limited, wherever wildlife vertebrates currently are measured dubious to be exposed to pesticide levels, that are acutely toxic with the exemption of some instances of high levels of acute toxicity standards in aquatic systems (Starner, 2011; Starner and Goh, 2012), and anticholinesterase poisoning of birds (Fleischli et al., 2004).

Chronic toxicity, for all pesticides are taken into explanation as applied at permanent timing, predominantly which would be greatly determined, like organochlorines. Inclusion to their severe noxiousness, that had sporadically controlled to mass deaths in the earlier years, this group of insecticides (including DDT and its metabolite DDE, an androgen receptor antagonist) well known to chronically act as endocrine disruptors (Turusov et al., 2002), employing estrogenic and/or androgenic effects in fish, birds, and rats (Bruker-Davis, 1998). To date, additional 120 endocrine disrupting insecticides are known to cover because of numerous chemical assemblages (Mckinlay et al., 2008). Organochlorines, thiocarbamates organophosphates, pyrethroids, triazoles, carbamates, triazines, all show thyroid disorder in fish, tetrapods, fowls and rodents (Turusov et al., 2002).

Immunotoxicity, due to organophosphates had described, that toxicity was initiated by disruption of hormone production like esterases or serine hydrolases, oxidative stress impairment, and disparity of pathway those describe the signal transduction (Galloway and Handy, 2003). The interaction with resistant system of

chordates were found in close association to family of atrazine, organochlorine, carbamates and the phenoxy herbicide (Galloway and Depledge, 2001).

In vertebrates, carbamates and organophosphates harm organic processes necessary for life, such as thirst or hunger, performance, thermoregulation (activity, learning ability and foraging time). Additional significances are weight reduction, disabilities, hatching success and ultimately reduced reproduction (Story and Cox, 2001).

Particularly in aquatic biota, those have discovered an extensive variety of insecticides demonstrating wide-range of elements to induce abnormal juvenile development in non-target invertebrates, in amphibian, which result in organ impairments, embryonic mortality and ultimately caused growth suppression (Paskova et al., 2011). Nearly, these above-mentioned insecticide properties alone or at group levels understudy was considerably or reasonably linked to the significances within inhabitants (Köhler and Triebskorn, 2013) (Fig. 1).

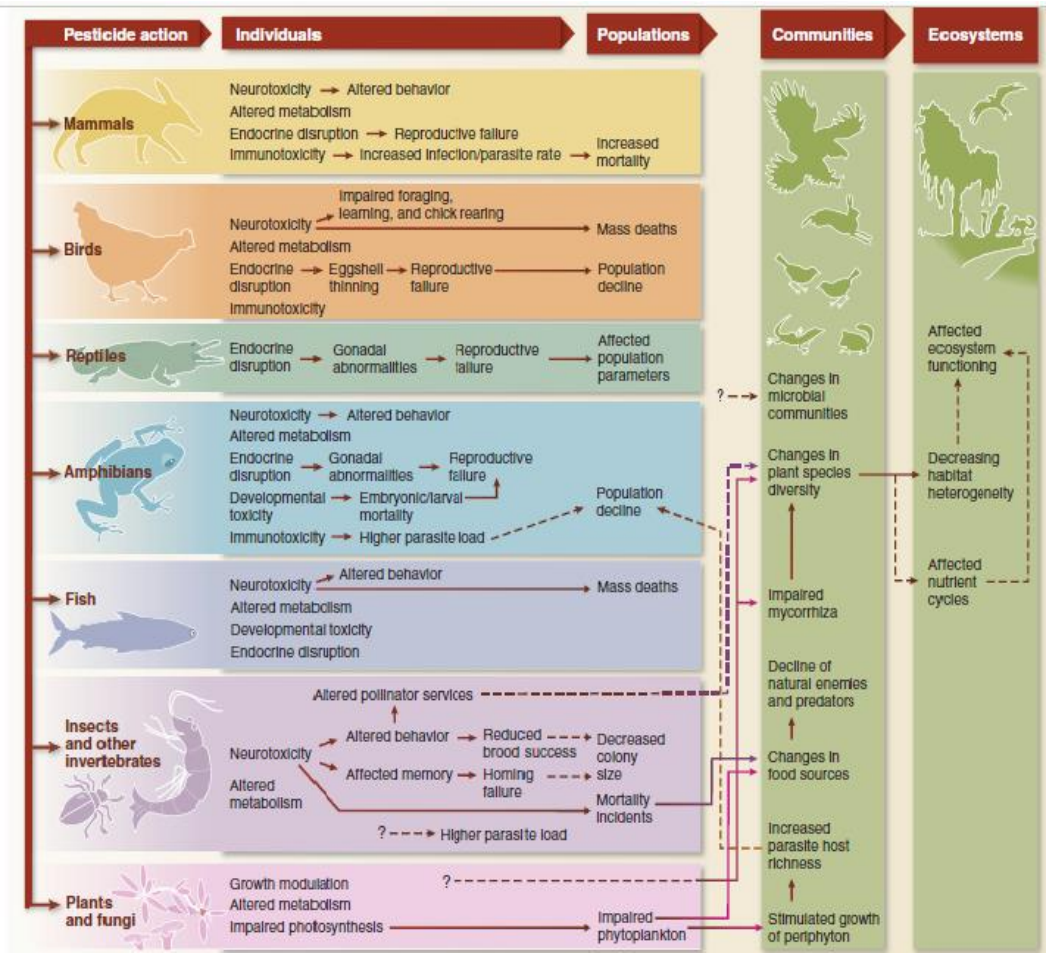


Fig. 1.1 General effects of pesticides on the ecosystem (Köhler and Triebskorn, 2013).

The harmful effects of pesticides toxic waste on undifferentiated water related species can be surveyed in noticing variations in species at their biological or microscopic stages, developmental and physiological levels, which can be responsible for biomarker tools in perceiving environmental eminence (NRA, 2001; EFSA, 2010).

Prolonged administration to insecticides sources merely environmental contamination, nevertheless had engaged to harmful consequences on arthropods existance. Mostafalou and Abdollahi, (2013) and others have reported pesticide administration consequences to numerous human impact which disturbed the function of chief organs comprising the body systems like the respiratory, circulatory, immune, nervous systems and similar other bodily systems (Calvert, 2016; Alberto et al., 2017). Maximum insect repellents are synthesized to block the activities of insect

neurotransmitters or to block the neurotransmitter degradation or reuptake (Xiao et al., 2017). Song et al. (2016) explained that insect repellent not merely showed action on hormone receptors and neurotransmitter nevertheless brain get disrupted (Parron et al., 2014; Aminov et al., 2016). Some deadly human infections related to insecticide administration, like malignant cancer, kidney and heart diseases, melanoma, nervous ailments (Lebov et al., 2015; Cowie et al., 2017).

1.4 Buprofezin

The synthetic agrochemical *EZ*-2-tert-butylimino-3-isopropyl-5-phenyl-1,3,5-thiadiazinan-4-one named as 'Buprofezin' has the following structural formula (Ishaaya and Horowitz, 1998) (Fig. 1.2).

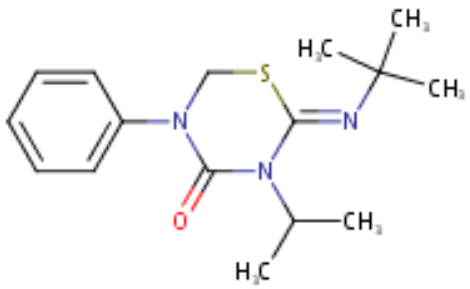


Fig. 1.2: Buprofezin: Structural formula (Ishaaya and Horowitz, 1998)

Buprofezin is a renowned insecticide, goes to thiadiazine and acts as exuviate embarrass for nuisances like whiteflies, leafhopper and mealybug (Liu and Chen, 2000). It was first manufactured by Nihon Nohyaku in 1981 and ensured a specific mode of action that controlled the insect growth (Chen et al., 2011).

Buprofezin obstructs cuticle removal at early stages of development in insects. In agriculture it is widely used on insects infesting tomato, lettuce and citrus crops. Buprofezin have been occupied itself while presenting a significant part in monitoring pests and extracting pests later 1980s. (Authority EFSA, 2010). It could be cast-off to

resist dengue mosquito, *Aedes aegypti,* juveniles were noticeable from current study in Pakistan (Jahan and Jan, 2011).

Ideal pesticide administration at domestic level has been initiated in the south-western region of Vietnam. Maximum absorption of it were documented in deposits component 521 µg/kg dm. Van Toan et al., (2013) Quantification rate 58.7%, average absorption 0.19 µg/L in aquatic models were found. Buprofezin 0.04 µg/L was found 4.8% of drinking water of composed model.

1.5 Buprofezin Mechanism of action

Holmstrup et al. (2010) and others demonstrated that it acts as moulting inhibitor insecticide that prevents cuticle removal at early stages of development in some whiteflies, leafhoppers, hemipteran insects and coleopteran pests (Chang et al., 2015). It also suppresses egg lying in female adults by preventing production of groth hormones affect moulting in larvae by inhibition of hormone secretion (Authority EFSA, 2010). Prabhaker and Toscano, (2007) as well as others revealed, it prevents chitin production in early developmental stages of lady bird, *Henosepilachna vigintioctopunctata Fabricius* whereas in grown-up stage it transforms their egg lying (Izawa et al., 1986). Similar consequences were detected in *Nilaparvata lugens* subsequent buprofezin exposure (Asai et al., 1985; Wu et al., 2018).

Ishaaya and Degheele, (2013) and other described that during the time of moulting, only hostile effects are detected. Owing to chitin deficit exuvial fluid applies pressure on early formed cuticle, and for instance the early formed cuticle dislocates that prevent molting over pest. Juvenile phases could not execute it's exuviates that causes darkening and water loss and ultimate mortality in exuviate phase (Ishaaya and Horowitz, 1998).

Several outdated insecticides had forbidden then exchanged by harmless insecticides, although since the conservational and well-being hazards of insecticides. Insect growth regulators seem to be environmentally friendly firstly, due to pesticide pest definite means of act and consequently due to less harmfulness over unmarked creatures except the foreseeable insect repellent, which are chiefly use to halt chitin production. At sub lethal concentrations, chitin synthesis inhibitors on mark and

unmarked organisms as well may exert toxic effects. In certain instars of insects, sub lethal dosages of hexaflumuron were shown to delay normal carbohydrate metabolism (Zhu et al., 2012; Tan et al., 2014).

It is a distinctive chitin synthesis inhibitor and moulting insecticide that acts explicitly on juvenile phases of hemipteran nuisances by preventing in corporation as regards to a compound (N-acetyl-[D-H3] glucosamine) detected in the exoskeleton and consequently disrupting the carapace synthesis, this results into larval death in course of moulting (Kanno and Maekawa, 1981; Ishaaya and Horowitz, 1998). Buprofezin also shows lethal activity beside the brunet rice plant hopper, *Nilaparvata lugens*, and the conservatory whitefly, *Trialeurodes vaporariorum* (Hatakoshi, 1992; Toscano et al., 2001).

1.6 Properties of Buprofezin and its metabolites

Buprofezin in acidic and alkali environment is very stable. Li et al. (2012) explained that being hydrophobic buprofezin gets freely adsorbed to soil. Since the attributes, mud preserved it extended besides somewhat additional insect killer (Funayama et al., 1986). The 50 % decaying of buprofezin under aerobic conditions was about 50 - 70 days nevertheless its 50 % decaying was about 36 - 104 days, in flooded field regions (Li et al., 2012).

Equally, be commonly cast-off globally on citrus plants, luxuriant yields, fruitlet yields its metabolites and deposits may pose latent hazard to close surroundings, and MRLs (maximum residue limits) were for citrus as well as tomatoes (EFSA, 2007).

Researchers reported that absorption of buprofezin deposits in Spain, Australia, Italy, Portugal, New Zealand differ as of 0.05 to 0.69 mg kg^{-1}on mandarins, lemons, and oranges (Valverde-Garcia et al., 1993).

Buprofezin within earth could be deteriorated towards dissimilar metabolites by soil microbes. Chen et al. (2011) designated buprofezin conversion routes in contaminated soil in China by Pseudomonas sp. DFS35-4, a type of bacterial species that break down of buprofezin, was originated. Strain YL-1 identified as *Rhodococcus* sp., originates from rice field, and was accomplished by degrading buprofezin in following types of metabolites namely 2-isothiocyanato-propane, 2-isothiocyanato-2-

methyl-propane, 2-tert-butylimino-3-isopropyl-1, 3, 5-thiadiazinan-4-one and N-tert-butyl- thioformimidic acid formylaminomethyl ester as described by Li et al., 2012.

One of the effects of buprofezin dreadful conditions was buprofezin sulfoxide, is liberally found in water-logged soil which gives an indication that buprofezin present in drowned and upland soils can be disintegrated by microbes (Funayama et al., 1986).

It was extensively cast-off insecticide which had initiated conservational effluence within several regions. Nonetheless, degradation of buprofezin at uncontaminated cultures had not largely considered, and the revolutionary channels of buprofezin remains indistinct. The study shows, buprofezin simultaneous degradation stress of Pseudomonas sp. DFS35-4 is inaccessible as of buprofezin-polluted mud in China. The DFS35-4 degenerated above 70 % of 50 mg/L buprofezin in 3 days, where 2.0 g/L sodium citrate, is added in the soil. The microbial sp. DFS35-4 robustly degenerated buprofezin at pH range of 5.0 - 10.0 and at temperatures between 20 and 30 °C, into its three metabolites, 2-imino-5-phenyl-1,3,5-thiadiazinan-4-one, 2-imino-5-phenyl-3-(propan-2-yl)-1,3,5-thiadiazinan-4-one, and methyl(phenyl) carbamic acid, by using the following techniques: gas chromatography-mass spectrometry (GC-MS) and tandem mass spectrometry (MS/MS). Limited revolutionary pathway of buprofezin in Pseudomonas sp. DFS35-4 was recommended by Chen et al., 2011 based on these metabolites (Fig. 1.3).

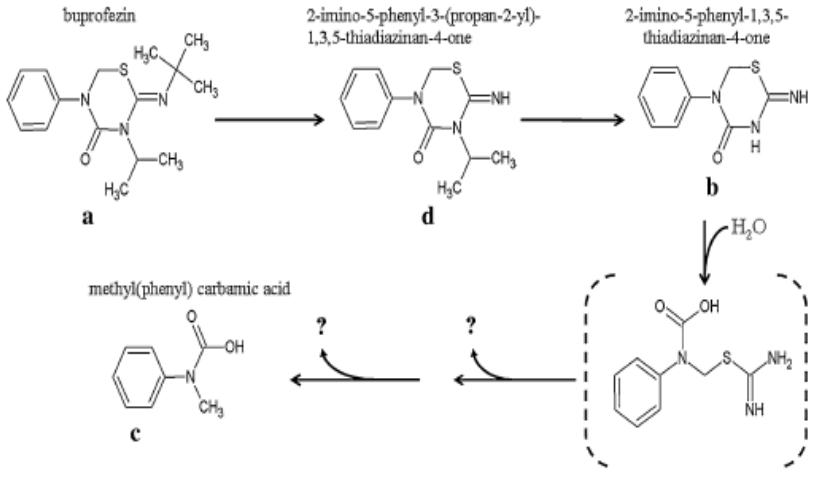


Fig 1.3: Buprofezin degradation pathway, by pseudomonas (Chen et al., 2011)

1.7 Toxicity of Buprofezin

Although simple buprofezin noxiousness has been described to be reduced in osprey, earthworm, and creatures, nevertheless it had been originated definite noxious insecticide for marine animals. As buprofezin stores in the aquatic atmosphere, the aquatic biomes are at greater hazard due to its strong interaction. Critical administration of buprofezin to *Daphnia magna* caused rigidity, whereas prolonged administration up till 14 days affected the growth and reproduction of daphnids (Liu et al., 2012).

Buprofezin is extensively cast-off development control however details associated to its long-term harmfulness in marine creature are very limited. Its noxiousness has been reported to be comparatively low in animals. For oral treatments, LC_{50} to skin contact is 4.57 mg/L air /4 h and did not infuriate skin, eyes while > 2000 mg/kg b.w. was measured as LD_{50} (EFSA, 2010).

Amongst insecticides, it was generally cast-off in arenas for controlling insect nuisances. This group of compounds could locate their ways into aquatic reservoirs, streams, rivers, therefore creating a contrary effect taking place marine collections (Svensson et al., 1994; Marimuthu et al., 2000; Marimuthu et al., 2001; John et al., 2003; Haniffa et al., 2003; Haniffa et al., 2004).

Culleres et al. (2007) reported that it was categorized as a low-toxicity insecticide, according to the National Pesticide Toxicity Grading Standards Organization of China. The present-day buprofezin manufacture in China is nearly 4500 tons per year, buprofezin deposits persist in atmosphere and floras, owing to its long half-life (Liu et al., 2012). The literature reveals that the deposits of buprofezin in samples of rice from 0.07 to 2.5 mg/kg in Korea, while deposits of buprofezin on oranges and lemons range from 0.05 to 0.69 mg/kg in Spain, Italy, Portugal, Australia and New Zealand. Furthermore, buprofezin deposits have been normally discovered in water and soil (Valverde-Garcia and Gonzalez-Pradas 1993: Nguyen, 2008).

It has been stated that exposure to buprofezin different tissues greatly affected harmfully, instead of bringing about tissue pathology deviations over the affected objects nonetheless also change medical interaction of tissues like liver and thyroid. Inagaki, (2006) described in the study that following oral route for two days of its

treatment carries small nuclei in the red blood cells of mouse bone marrow. It is noticeable buprofezin was not cancer-causing insecticide in mice besides did not indicate approximate signs of generative toxicity neither brought neurotoxicity established animals (EFSA, 2008).

It had been reported that buprofezin did not aggravate skin nevertheless somewhat irritable to eyes of rabbits. The general buprofezin toxicity as studied in dogs for 13 weeks and two years revealed liver cell accumulation, bile-duct and increased liver and thyroid weight and mammary gland deformity (EFSA, 2007).

It is recognized for its conservational care and broad-spectrum achievement. The fame of buprofezin insecticide has upraised apprehensions about its possibly opposing effects on human health and hazard to atmosphere. As a chief detoxifying organ, core place of act of buprofezin is the liver. It has been demonstrated that buprofezin get stored in liver, eventually derived cumulative damage by free radicals. It was identified in this study, that the liver as one of the major organs in which buprofezin accumulated, and was identified a severe oxidative stress reaction, sublethal concentrations of buprofezin promoted the conversion of energy metabolism from the oxidative phosphorylation to anaerobic glycolysis and aerobic tricarboxylic acid (TCA) cycle. Significantly, reactive oxygen species (ROS) generation moderately accounted for the shifting of the energy metabolism through the buprofezin-mediated inhibition of cytochrome c oxidase activity.

ROS openly perturbed the activities of numerous key TCA cycle enzymes, stimulated glycolysis, and ultimately disturbed the activity of the respiratory chain complex by altering mitochondrial DNA (mtDNA). These results elucidate the prospective mechanisms of buprofezin toxicity and provide biomarkers for buprofezin-mediated hepatotoxicity at sub lethal concentrations (Ji et al., 2016) (Fig. 1.4).

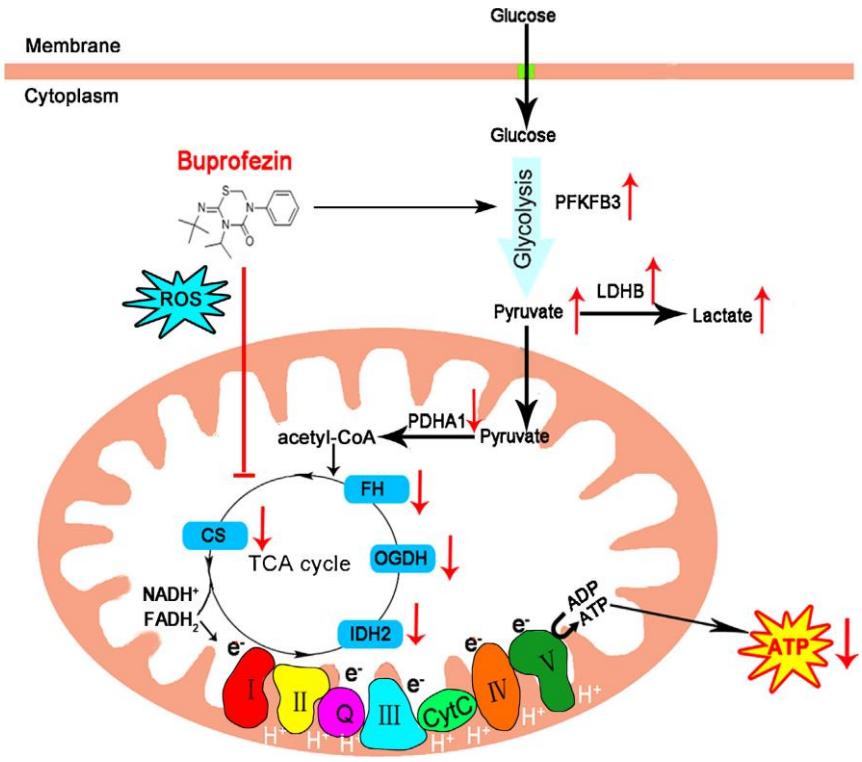


Fig 1.4: Interruption of energy metabolism by buprofezin administration (Ji et al., 2016)

The oxygen-derived pro-oxidants could cause harm to natural targets like lipids, proteins and DNA, cell defensive systems, comprised of enzymes or antioxidants. Overall, these pro-oxidants were discussed by means of ROS which could categorize two sets of combinations, radicals, non-radicals. The radical group, inaccurately called free-radical, contained combination of radicals like superoxide ion, nitric oxide, hydroxyl (OH^-), alkoxyl (RO^-) and peroxyl (ROO^-), and one form of singlet oxygen ($O^-{}^1$). The above said radicals are capable of independent, as contained 1 unpaired electron around the atomic nucleus (Kohen and Nyska, 2002).

Buprofezin chronic toxicity and carcinogenicity illustrated liver was the main target of noxiousness in rats and mice. Yoshida, (1990) demonstrated in two years study of mice obtained body weight of female as well as male mice reduced from 9 week (females), 6 weeks (males) ahead subsequent 5000 ppm lowered at 2000 ppm dosage. Liver weight increased at 2000 ppm dose and above at fifty-two weeks duration of treatment in females as well as males, nevertheless considerably improved in male mice

at 5000 ppm dosage at end of experiment. Liver shows hyperplastic changes and hepatocellular hypertrophy histologically, whereas liver cancer was also detected in female mice following buprofezin exposure

1.8 Antioxidant defense mechanism

Halliwell and Gutteridge, (1986) described that antioxidants are the substances that inhibits or delay the oxidation of any substrate containing oxygen nearby even more greater amounts apart from antioxidant itself. Among these, ROS are the natural metabolites of intracellular oxygen which could create either extrinsically or intrinsically. Cao et al. (2007) defined ROS generation by normal processes like cellular signaling or metabolism including oxidative phosphorylation. Klaunig et al. (1998) explained that mainly the reason of increased ROS levels comprise exposure to treatments, several hormones as well as overexpression of specific viral proteins.

It is not only environmental agents such as UV that promote oxidative stress. In fact, many intrinsic processes also drive the formation of ROS. Cellular mitochondria gradually produce ROS as a byproduct of normal aerobic metabolism, wherein the electron transport chain (ETC) forms superoxide. Mitochondrial nitric oxide (NO) synthase produces NO, itself a free radical, that associates with superoxide radical (O^{2-}) to form peroxynitrite ($ONOO^-$), a potent ROS. Although the mitochondria are oft-cited as the primary contributor of cellular ROS, many other cellular components also produce ROS. Other intrinsic sources of ROS include cytochrome p450, cyclooxygenases, NADPH oxidases, peroxisomal oxidases, and lipoxygenases (Finkel, 2011) (Figure 1.5).

ROS production can be further augmented in response to infection, malignancy, and other inflammatory states. Mice which are deficient in superoxide Cu-Zn dismutase (SOD1) and SOD2 were found to exhibit aging-related phenotypes, including skin atrophy, related to excess superoxide levels (Watanabe et al., 2014; Weyemi et al., 2012).

As such, ROS are associated with a multitude of pathologies whether within the skin or in other organ systems. Many diseases, such as cancer and neurodegenerative

disorders, have had oxidative stress implicated in their etiopathogenesis (Valko et al., 2007).

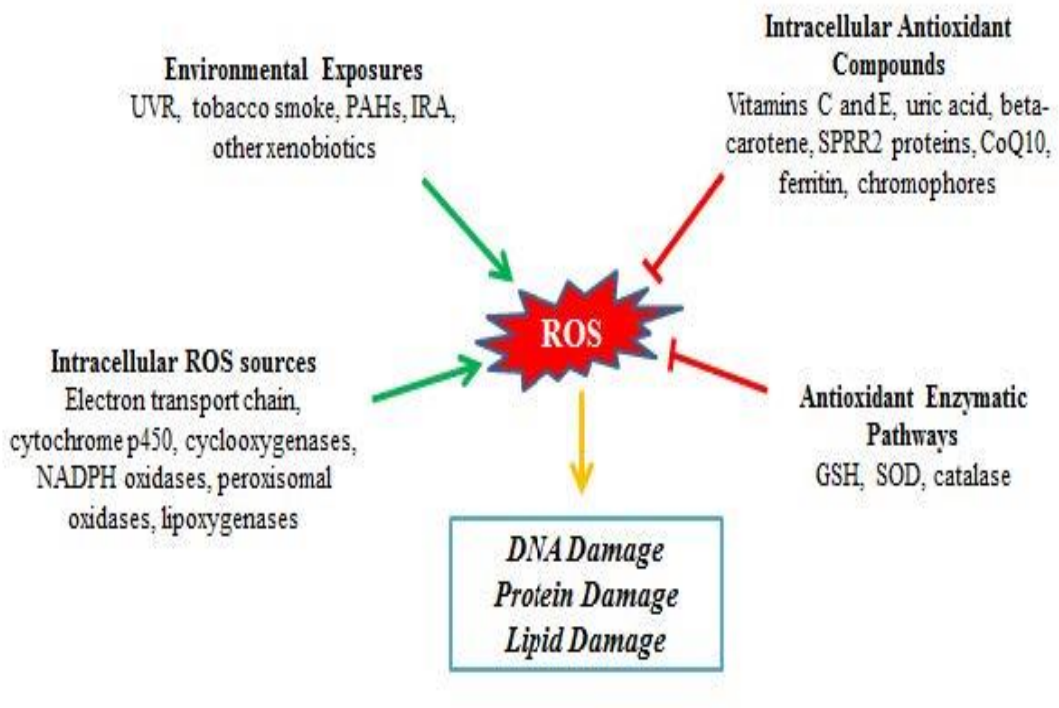


Fig. 1.5: Summary of antioxidant and Reactive Oxygen Species (ROS) sources within the cell (Ahsanuddin et al., 2016).

At low levels, it has been suggested that ROS and other free radicals are useful in cell signaling and may promote cell growth (Valko et al., 2007; Kohen and Nyska 2002). The role of ROS in potentiating oxidative stress has tilted more toward its detrimental effects (Ahsanuddin et al., 2016).

Arouma and Haliwell, (1987) reported that various enzymatic and non-enzymatic antioxidants are generally operated as defense mechanism by an extensive range of organisms against the toxic effects of these ROS. The enzymatic systems contain SOD, CAT, GPx whereas non-enzymatic systems comprise flavonoids, carotenoids, vitamins A, uric acid and suggested elements Se, Cu, Zn, Mn, Fe, numerous antioxidants present in the mitochondria, cytosol of the cell.

Approximately enzymes had multiple duplicate forms for example SOD possesses extracellular forms, cytosolic and membrane. GPx had been segregated for

example membrane, cytoplasm, hyaloplasm configurations. Mate´s and Sánchez-Jiménez, (1999) described furthermore, the activities and locations of such enzymes were firmly measured to sustain cell existence.

Principal antioxidant enzymes including SOD, GPX, CAT deactivates reactive oxygen species by overwhelming the production of free radicals. Thatha et al. (2015) explain although enzymes glutathione-s-transferase, glutathione reductase, ubiquinone and glucose-6-phosphate dehydrogenase are oxidative stress scavenging, intricate in detoxification of ROS the work by subduing chain commencement and interrupt chain proliferation reactions (Dempster et al., 1995).

Over the past few year, the use of buprofezin has extensively increased, but information regarding its adverse effects is limited, except for an extensive study done on liver. The present research work was planned to further examine buprofezin poisoning in male mice at biochemical, histological and DNA level. Buprofezin treatment clearly induced liver and kidney dysfunction. It determines that even short-term exposure to buprofezin is extremely toxic for mammalian tissues by generating ROS. This disruption and genotoxicity might lead to histopathologic changes in kidney and liver tissues. Our present study in laboratory mice and earlier studies in common carp necessitate the in-depth knowledge about cellular and molecular studies.

AIMS & OBJECTIVES

Aim

Core aim of study was to explore measures as the oxidative stress a precursor of molecular and histopathological complications following acute dosage of mice to buprofezin.

Objectives

The emphasis of the study was.

- To focus oxidative stress increase ROS and LPO generation due to buprofezin administration.
- Determine serum liver and kidney enzymes ALT, AST, Urea and Creatinine levels.
- Estimate tissue (liver and kidney) antioxidant enzymes SOD, POD, CAT & non antioxidant GSH activities.
- Determine the total protein concentrations in liver and kidney tissues.
- Detect histopathological and morphometric observations after buprofezin administration.
- Conclude DNA damage by Comet assay.
- Estimation of apoptosis by TUNEL assay.

MATERIALS AND METHODS

MATERIALS AND METHODS

All experiments were conducted in the laboratory of Animal and Human Physiology, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad. Adult Albino Balb/c mice of 8 weeks and 25 ± 3 g body weight (b.w.) were obtained from the National Institute of Health (NIH) Islamabad. Mice were acclimated for 15 days in the Animal House Facility of Department of Animal Science, Quaid-i-Azam University Islamabad.

They were provided standard rodent feed and water ad libitum. Photoperiod of 12:12 h, humidity 75-80 % and ambient temperature 27 ± 2 ºC was maintained. Handling of animals was accomplished according to the guidelines provided by the "Bio-Ethical Committee (BEC)" of Quaid-i-Azam University Islamabad, Pakistan". The study approval No. was BEC-FBS-QAU/2019-132. The mice were kept in separate hygienic rodent cages. No more than 7 mice were housed to avoid stress and overcrowding.

2.1 Reagents and Supplies

All chemicals were purchased from Sigma or Merck (Sigma, Merck Co., Germany). Buprofezin (25 % w/w) used in experiments was commercial formulation and obtained from Anpon Co. Ltd. (Jiangsu, China) marketed in Pakistan by Super pesticides, Bahawalpur.

2.2 Experimental Design

Mice were divided into groups; control and treatment groups (n = 7). Experiments were conducted in different sets to test more closely related parameters at one time. Three separate doses of toxicant buprofezin, low dose 4.0 µg/kg, medium 6.0 µg/kg & high 8 µg/kg (b.w.) were administered to mice via intraperitoneal injection (i.p).

Antioxidant and non-antioxidant enzyme assays were conducted at one time. Biochemical and enzymatic assays were carried out in the next set of experiments. Experiment for tissue and cell damage at light microscopic level was also carried out.

Lethal dosage LD_{50} of buprofezin has been reported, and the doses injected or otherwise given via other routes such as gavage, intraperitoneal, subcutaneous injections to evaluate the toxic effect are already available in literature (EFSA, 2010).

2.3 Dosage Preparation

Doses were prepared according to known LD_{50} for mice administered via i.p (intraperitoneal injection) based on (b.w.) of individual mice. LD_{50} is 10,000 mg/kg (b.w.) based on LD_{50} low, medium and high (4.0, 6.0 & 8.0 µg/kg b.w.) doses respectively were prepared in distilled water (EFSA, 2010).

2.4 Collection of Blood and Tissue Sample

Mice were sacrificed with cervical dislocation to avoid any possible effect of euthenization on biochemical parameters after short term (24 h) administration of buprofezin. Blood was drawn via cardiac puncture. It allowed to stand for 1 h at room temperature and later centrifuged at 960 *g* for 10 min (Eppendorf Centrifuge 5417C, USA) to obtain serum for enzyme assays and other biochemical tests it was store at 4 ºC until analyzed. Kidney and liver tissues were excised, washed with saline and fixed in 10 % formalin solution for histology.

2.5 Liver Function and Renal Function Tests

The liver function (LF) and renal function (RF) tests were carried out on serum samples, the enzyme levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) creatinine and urea by ELISA Reader (Platos R 496 Microplate reader, Austria). All enzyme activities were measured at 37 ºC and the results were obtained in µ/L. All enzymatic parameters were determined by using commercially available kits (AMP Diagnostics, Austria).

2.5.1. Alanine aminotransferase (ALT)

Reagent Composition

Regent (R1) contained Tris Buffer, pH 7.5 (150 mM/L), LDH (≥ 1.350 µ/L) and L-Alanine (750 mM/L), reagent (R2) contained NADH (1.3 mM/L) and 2-oxoglutarate (75 mM/L).

Procedure

To obtain working reagent

- Mixed four volumes of reagent 1 (R1) with one volume of reagent 2 (R2).
- Then serum sample of 20 µl and 200 µl of working reagent were mixed.
- After that incubation was carried out for 60 sec at 37 ºC in water bath (Jubalo, SW22 Germany).
- The change in absorbance per minute (A/min) was taken at 340 nm.
- Final value was calculated by the following formula:

Formula

ALT Activity (µ/L) = ΔA/min x 1746

2.5.2. Aspartate aminotransferase (AST)

Reagent Composition

Reagent (R1) contained Tris buffer, pH 7.8 (121 mM/L), L-Aspartate (362 mM/L), MDH (≥600 µl) and LDH (≥460 µl), reagent (R2) contained NADH (1.3 mM/L) and 2-oxoglutarate, (75 mM/L).

Procedure

The working reagent was obtained as

- Four volumes of reagent1 (R1) were mixed with one volume of reagent 2 (R2).
- Serum sample of 10 µl and 100 µl of working reagent was mixed.
- Incubation of 60 sec at 37 ºC in a water bath.
- Change in absorbance per minute (A/min) was measured at 340 nm.
- Final value was calculated by the following formula:

Formula

AST Activity (µ/L) = ΔA min x 1746

2.5.3. Creatinine

Reagent Composition

Reagent (A) contained alkaline buffer and reagent (B) contained picric acid surfactant (40 mM/L), while the standard contained 2 mg/dl creatinine.

Procedure

Working reagent was prepared

- Mixed five volumes of reagent (A) with one volume of reagent (B).
- Serum of 200 µl and standard were mixed with 2 ml of working reagent.
- Absorbance (A1) was read exactly after sample addition.
- Then again absorbance was read after 60 sec, at 490-510 nm (A2).
- For all samples and for the standard A2-A1 was calculated.
- Final value was calculated by the following formula:

Formula

Creatinine Activity (mg/dl) = ΔA sample/ ΔA standard x Concentration of standard

2.5.4. Urea

Reagent Composition

Reagent (R1) contained Tris buffer, pH 7.8 (80 mM/L), Urease (75 µ/ml), α-ketoglutarate (10 mM/L). Reagent (R2) contained NADH (80 mM/L), GLDH (60 µ/ml), and Urea 50 mg/dl (8.3 mM/L) as Standard.

Procedure

To obtain the working reagent

- Reagent 1 (R1), 4 ml was mixed with 1 ml of reagent 2 (R2).
- The initial absorbance (A1) reading was taken after 30 sec of sample addition and the next reading (A2) after 60 sec, at 340 nm.
- ΔA = (A2-A1) was calculated for all samples and for the standard value.
- Final value was calculated by the following formula:

Formula

Urea Activity (mg/dl) = ΔA sample/ΔA standard x Concentration of standard

2.6 Biochemical Study of Tissue

2.6.1 Tissue Analysis

Liver and kidney tissues were used for protein estimation, oxidative profile; ROS, lipid peroxidation (TBARS), and antioxidant enzymes, the superoxide dismutase (SOD), catalase activity (CAT), peroxidase (POD) and reduced glutathione (GSH)

2.6.2 Homogenate Preparation

Extract Buffer (Lysis buffer)

Lysis buffer was prepared by mixing **(**50 mM) HEPES, (150 mM) Sodium chloride (NaCl), (0.02%) Sodium azide (NaN_3), (0.1%) Sodium dodecyl sulphate (SDS) in (495 ml) distilled water and volume was raised to (500 ml) by adding 5 ml of (1%) Triton X-100. pH was adjusted to 7.0 (using 1 M NaOH and 1 M HCl).

Procedure

- Liver and kidney tissues (100 mg each) were taken.
- With the help of sterilized scalpel blades tissues were minced in ice cold petri plates.
- Homogenization of tissues was carried out for each sample after mincing.
- By adding 1000 µl of extract buffer (lysis buffer) containing 0.1 mg PMSF, the organs were homogenized in a hand-held glass homogenizer.
- Samples were taken in eppendorf tubes after homogenization. and centrifuged for 10 min at 10,286 *g* at 4 ºC.
- The supernatants were collected and stored at - 20 ºC.

2.6.3 Anti-oxidative Profile

Anti-oxidative profile included evaluation of the activity of enzymatic and non - enzymatic antioxidants. Three enzymatic antioxidants including peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) was estimated, while reduced glutathione (GSH) was determined from the non-enzymatic antioxidant group.

2.6.4. Superoxide Dismutase (SOD) Assay

Reagent Composition

Nitro blue tetrazolium (NBT. 2HCl) (1 ml), L-Methionine (1.5 ml), Triton X – 100 (0.75 ml), Phosphate buffer saline (PBS) (30 ml), Riboflavin (10 µl).

Procedure

To measure SOD activity

- Twenty microliter of tissue homogenate was mixed with 1 ml of this solution.
- For 8 min to fluorescent light this mixture was illuminated and incubated for 5 min at 37 ºC.
- Riboflavin 10 µl was added to each sample and incubated again for 8 min at 40 ºC.
- Absorbance was measured at 560 nm three times for each sample.
- NBT percentage was calculated by formula:

(Absorbance of Blank- Absorbance of Sample) / (Absorbance of Blank) × 100

- The amount of enzyme that cause 50 % inhibition in NBT reduction rate is defined as one unit of SOD activity.
- SOD activity = unit/min scale was measured.

2.6.5. Catalase Activity (CAT) Assay

Reagent Composition

Potassium phosphate buffer (50mM) 1990 µl, Hydrogen peroxide 1000 µl, Tissue homogenate sample 100 µl

Procedure

To measure CAT activity

- Fifty mM of Potassium phosphate buffer (1990 µl).
- Hydrogen peroxide (1000 µl).
- Homogenate sample (100 µl) was mixed in a cuvette.
- Three times for each sample at a time interval of 30 sec absorbance was measured at 240 nm.
- One-unit activity of catalase was unit per min an as absorbance change of 0.01.

2.6.6. Peroxidase (POD) Assay

Reagent Composition

Phosphate buffer (50mM) pH 5.0 2.5 ml, Guaiacol 20 mM 0.3 ml, Hydrogen peroxide 40 mM, Tissue homogenate sample (enzyme extract) 0.1 ml

Procedure

To measure POD activity

- Assay reaction mixture was prepared.
- By adding 50 mM phosphate buffer (2.5 ml) of pH 5.0.
- Twenty millimole Guaiacol (0.3 ml) and enzyme extract (0.1 ml).
- The contents were mixed thoroughly till solution became homogenous and then was poured into a cuvette.
- In the last step, 40 mM hydrogen peroxide was added to the reaction mixture.
- Change in the absorbance of reaction mixture was determined after 1 min at 470 nm.
- Three readings were taken for each sample at every 30 sec intervals.
- One unit of peroxidase activity; unit per min as an absorbance change of 0.01.

2.6.7 Reduced Glutathione (GSH) Assay

Reagent Composition

Disodium hydrogen phosphate buffer 0.4 M 1 ml, DTNB reagent 0.5 ml, Tissue homogenate sample (enzyme extract) 0.1 ml.

Procedure

To measure GSH activity

- By mixing homogenate sample (0.1 ml), 0.4 M disodium hydrogen phosphate buffer (1 ml) reaction mixture was prepared.
- DTNB reagent (0.5 ml) was prepared by mixing 40 mg of DTNB in 100 ml of 1 % Trisodium citrate.
- The absorbance of the yellow color developed was measured at 412 nm.
- Average was calculated after three readings for each sample were taken.

2.7. Parameters for oxidative profile

2.7.1. Reactive Oxygen Species (ROS) Assay

Reagent Composition

Reagent 1 (R1): N.N-Diethyl para phenyl diamine (DEPPD) sulfate 10 mg, Reagent 2 (R2): Ferrous sulfate ($FeSO_4$) 50 µL, Sodium acetate buffer (pH 4.8) 10 ml, Tissue homogenate 60 µl.

Procedure

- By mixing 10 mg of N.N-Diethyl para phenyl diamine (DEPPD) sulfate in 100 ml of distilled water reagent 1 (R1) was prepared.
- By adding 50 µl of stock solution of ferrous sulfate ($FeSO_4$) in 100 ml of buffer reagent 2 (R2) was prepared.
- Ferrous sulfate ($FeSO_4$) stock solution was prepared by dissolving 50 mg of ferrous sulfate in 10 ml of sodium acetate buffer (pH 4.8).
- R1 and R2 were mixed in a ratio of 1:25 and kept in dark for 2 min.
- Reagents mix (1680 µl), buffer (1200 µl) and homogenate sample (60 µl) were mixed in a cuvette.
- Absorbance was measured at 505 nm.
- Average was calculated after readings were taken in triplicate with an interval of 15 sec for each sample.

2.7.2. Lipid Peroxidation (TBARS) Assay

Reagent Composition

Tris – HCl 150 mM 0.1 ml, Ascorbic Acid 1.5 mM 0.1 ml, Trichloroacetic Acid TCA 10 % 1ml, Thiobarbituric Acid (TBA) 0.375 % 1 ml Distilled water 0.6 ml, Tissue homogenate 0.1 ml

Procedure

- Thiobarbituric acid reactive substances (TBARS) reaction mixture was prepared.
- Adding 150 mM Tris – HCl (0.1 ml).
- 1.5 mM ascorbic Acid (0.1 ml)

- Distilled water (0.6 ml),
- Homogenate sample (0.1 ml) in a test tube at same time.
- The contents were mixed thoroughly and incubated at 37 ºC for 15 min.
- Then into the reaction mixture 10 % Trichloroacetic Acid TCA (1ml) and 0.375 % Thiobarbituric Acid (TBA) (1 ml) were also added.
- Contents were mixed and boiled in water bath for 15 min.
- The reaction mixture was centrifuged after 15 min at 926 *g* for 10 min and after centrifugation supernatant was collected.
- Absorbance was measured three times for each sample at 532 nm.

2.8 Total Tissue Protein

Procedure

Protein concentrations were measured through Bradford method using bovine serum albumin as the standard (Bradford, 1976).

2.9 Estimation of DNA damage

DNA damage was predictable by using the standard procedure of single cell gel electrophoresis (SCGE) or comet assay according to the process of Dhawan and Anderson (2009) with minor modifications. Comet assay is a simple method for identifying single and double strand DNA break and alkali-labile sites in DNA.

2.9.1. Comet assay or Single cell gel electrophoresis (SCGE)

This method includes, embedding of single cell in agarose, lysing the cell and liberating out DNA by electrophoresis cleaving the head of intact DNA to form comet tail. Intact and damaged DNA is then visualized by fluorescent microscopy and calculated through image study.

2.9.2 Comet Assay process

A: Reagents preparation

Lysing solution

Reagent Composition

Disodium EDTA 3.72 g, Tris-Base 0.121 g, Sodium chloride (NaCl) 14.61 g, Distilled water 90 ml, Triton X-100 1 % 1 ml, Di-methyl sulfoxide 10 % 10 ml, Triton X-100 100 ml.

Procedure

Lysing solution was prepared.

- Mixing 3.72 g of disodium EDTA in 90 ml of distilled water and stirred on a magnetic stirrer.
- After thorough mixing 14.61 g of NaCl and 0.121 g of Tris-Base were added and stirred till completely mixed.
- 1 ml of 1 % Triton X-100 and 10 ml of 10 % di-methyl sulfoxide were added and then volume 100 ml was raised.
- Just 30 min before use Triton X-100 was added.
- With 1 M HCl and 1 M NaOH pH was adjusted to 10.0.

PBS buffer

Reagent Composition

PBS buffer 10 ml, Distilled water 990 ml.

Procedure

PBS buffer was prepared in 990 ml of dH_2O.

- Then volume up to 1000 ml was raised.
- pH was adjusted to 7.4 at room temperature.

Electrophoresis buffer

Reagent Composition

EDTA 200 mM 0.37 g, NaOH 300 mM 12.0 g.

Procedure

The 1x buffer preparation, in dH_2O as

- 0.37 g of 200 mM EDTA.
- And 12.0 g of 300 mM NaOH were mixed and volume 1000 ml was adjusted.
- With 1 M NaOH and 1 M HCl pH was adjusted to >13.0.

Neutralization buffer

Reagent Composition

0.4 M Tris 48.5 g, double distilled water 1000 ml,

Procedure

- Mixed double distilled water 1000 ml and 48.5 g of 0.4 M Tris.
- With concentrated HCl pH was adjusted to 7.5
- And buffer was left at room temperature.

Staining solution

Reagent Composition

Ethidium bromide 10 mg, distilled water 50 ml

Procedure

A 10x stock ethidium bromide 30 µg/ml

- By adding 10 mg ethidium bromide to 50 ml dH_2O stock solution was prepared.
- Mixing 1 ml of stock solution in 9 ml dH_2O 1 x ethidium bromide from the stock was prepared.

0.5 % LMPA

Reagent Composition

LMPA 650 mg, PBS solution 1 % 100 mL

Procedure

Prepare Low melting point agarose

- Adding 650 mg LMPA in 100 mL of 1 % PBS solution.
- Solution was kept in the refrigerator.

- And prior to the use temperature was stabilized.
- By keeping it in a water bath at 37 ˚C.

1 % NMA

Reagent Composition

NMA 750 mg, distilled water 100 ml

Procedure

Normal melting agarose get prepared by

- Adding 750 mg NMA in 100 ml distilled water.
- Till dissolution of the gel heating was finished.

B: Preparation of slides

The slides were prepared by dipping in methanol and warmed to eradicate dust particles over a flame. Slides were then labelled at one end. About one third area of slide was rinsed in hot NMA. Lower surface of slides was cleaned and kept on tray for solidification. The slides were then dried and kept at room temperature. For isolation purpose small fraction of tissue was placed in 1 ml cold HBSS solution containing 20 mM EDTA.

Tissue was sliced into small pieces and then 75 µl of LMPA was mixed in it. This mixture was then coated on slides which were then cover slipped. These slides were kept in icepacks. After 5 - 10 min cover slips were removed gently and third layer of 80 µl of LMPA was poured on same slide and let it dry again. Cover slips were removed, and slides were placed in lysing solution. Later the slides were refrigerated for 2 h and were kept away from light.

C: Electrophoresis

After 2 h slides were taken out from lysing solution and adjusted in a horizontal gel apparatus. Freshly prepared buffer was poured, and all the slides were dipped in buffer in the gel tank. Slides were left for 20 min until unwinding of DNA. Power supply was turned on for 30 min at 24 volts. Slides were taken out gently after 30 min and then added neutralizing buffer. Similar steps were repeated twice. Staining was done with 80 µl of 1 x ethidium bromide and then cover slips were placed on the slides.

D: Slides visualization

The detection of DNA damage, fluorescent microscope (Nikon AFX-1 Optiphot, Japan) was utilized at 40 x. Image analysis software (CASP 1.2.3.b, UK) was used to evaluate the extent of DNA damage by estimating the percentage of length of migration and DNA migrated. Generally, in each sample 50 - 100 cells were detected. DNA comet parameters including Comet length (CL, µm), Head length (HD, µm), % DNA in head (% H), Tail length (TL, µm), Tail moment (TM, µm), Tail DNA (TDNA, %) and olive moment (OTM, µm) were recorded. Evaluation was also made between the amount of migration per cell, number of cells with increased migration, viability and ability of migration between damaged cells.

2.10 Histology

2.10.1 Hematoxylin and Eosin (H & E) Staining

Standard method was followed for fixation and staining purpose. Liver and kidney tissues of control and treated groups of mice were fixed in 10 % formalin and then dehydrated, taken to two changes of xylene and finally embedded in molten paraffin wax. Tissues were cut with a rotary microtome (Shandon, Finesse 325, UK) at 5 µm thickness and stained with conventional Hematoxylin and Eosin.

For hematoxylin preparation,

Reagent Composition

Procedure

- For preparation of hematoxylin stain; Harris's hematoxylin crystals (0.5 g), mercuric oxide (0.25 g) and potash alum (10 g) were dissolved in 95% ethanol (5 ml) and glacial acetic acid (4 ml), final volume was raised with distilled water to 100 ml.

For Eosin preparation

Reagent Composition

Procedure

Eosin solution was prepared by

- Adding 1g of eosin powder and glacial acetic acid 0.5 mL to 99.5 ml distilled water Eosin solution was ready.

- Sections were stained in Harris's Hematoxylin for 2 - 3 min and eosin for 1 min. Tissue sections were dehydrated and mounted in DPX mountant medium (BDH, Germany). Tissue sections were photographed on compound microscope (Leica, Germany) with digital camera attachment. Histopathologic parameters were calculated by Image J software (Project was developed at NIH, US Govt. Org.).

2.10.2 Periodic Acid & Schiff (PAS) Staining

To differentially stain the liver tissue sections of mice, PAS staining reagent were prepared. PAS was prepared as 1 % solution, whereas for preparation of Schiff reagent, 1 g basic fuchsin was dissolved in boiling distilled water, allowed to cool to temperature of 50 ºC and by mixing potassium metabisulphate was added. The solution was cooled to come near to room temperature, 2 ml of concentrated HCl was then added, and allowed to stand in dark for overnight. 0.2 g of activated charcoal was added and mixed it for 1 - 2 mins, solution was filtered and stored in brown bottle till use at 4 ºC for use.

The tissue sections were dewaxed, dipped in descending grades of alcohol and then in distilled water. Tissue sections were oxidized in 1 % Periodic acid for 5 min, washed with tap water for 1 min and in distilled water again rinsed. The sections were then treated with Schiff's reagents for 15 min and then dipped in first with distilled water and then tap water for 10 min. These sections were at that time stained with Harris's Hematoxylin for 1 min, dipped in tap water for 5 min, dehydrated through ascending grades of alcohol and mounted with DPX mountant medium. Tissue sections were photographed on compound microscope (Leica, Germany) with digital camera attachment. Histopathologic parameters were determined by Image J software (Project was developed at NIH, US Govt. Org.).

2.10.3 Iron/Prussian blue Staining

For Iron staining, Perl's reaction or Prussian blue stain was used for the staining of liver tissue sections. Potassium Ferrocyanide solution 2 % as reagent 1 (R1) and HCl solution 1 % as reagent 2 (R2) were used as two reagents. One part of reagent 1 (R1)

and three parts of reagent 2 (R2) were mixed and cover the slides for 10 min or more, then air dry the tissue sections and contrast with H & E staining. Sections were mounted with DPX mountant medium. Tissue sections were photographed on compound microscope (Leica, Germany) with digital camera attachment. Histopathologic parameters were calculated by Image J software (Project was developed at NIH, US Govt. Org.).

2.11 TUNEL Assay

Principle

Paraffin-embedded liver and kidney sections were used in DNA fragmentation indicative of cell death using ApopTag® Fluorescein *in Situ* Apoptosis Detection Kit S7110. (MILLIPORE International, Inc. USA). The chemicals provided in all ApopTag Kit were designed to label the free 3'OH DNA termini *in situ* with chemically labelled and unlabeled nucleotides. The nucleotides confined in the Reaction Buffer are enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). DNA fragments which have been labelled with the digoxigenin-nucleotide are then permissible to bind an anti-digoxigenin antibody that was conjugated to fluorescein. The ratio of labeled to unlabeled nucleotide in ApopTag® Kits is optimized to minimize fluorescein self-quenching or to promote anti-digoxigenin antibody binding.

DNase1 as a positive control was applied before the TUNEL reaction. The negative control (control tissue sections) consisted of omission of the TUNEL reaction enzyme

Reagent Composition

Sr. No.	Chemicals	Volume
1.	DNase 1	20 µl
2.	proteinase K	20 µg/ml
3.	Equilibration buffer	75 µl
4,	TdT enzyme	55 µl
5.	Anti-digoxigenin	65 µl
6.	DAPI	0.5 - 1 µg/ml

Procedure

- Tissue sections (liver and kidney) of 6 µm thickness were deparaffinized on glass slides, and in 3 changes of xylene were washed in coplin jars for each wash of 5 min, two changes of absolute alcohol for 5 min, then in 95 % and 70 % ethanol for 3 min and 1 change of PBS for 5 min.
- For positive control, sections were prepared by treating with DNase 1, pretreated sections with DN buffer, DNase solution (containing DNase 1 and DN buffer) 20 µl was applied to each tissue section and incubated for 10 min at room temperature, then wash with 5 changes for 3 min each wash of distilled water.
- Tissue sections were pretreated by applying proteinase K (20 µg/ml) in the specimen for 15 min at room temperature in coplin jars or directly on the slide, then washed in 2 changes for 2 min each wash of PBS.
- Equilibration buffer 75 µl/5cm^2 was immediately applied directly on the sections and slides containing sections were incubated for 10 sec at room temperature, then immediately apply 55 µl/5 cm^2 of working strength TdT enzyme on the sections, gently tap off excess liquid around the sections, the section on slides were then incubated in humidified chamber at 37 ºC for 60 min.

- Place the slides in coplin jars comprising working strength/ stop wash buffer, agitated for 15 sec and incubated them for 10 min at room temperature. Excess liquid was tapped off after washing in 3 changes for 1 min each wash of PBS.
- Anti-digoxigenin 65 µl/5cm^2 was applied then covers the slides with cover slip (plastic) in the coplin jars at room temperature, incubated them in humidified chamber for 30 min at room temperature and also avoids light exposure. The slides were washed in coplin jars in 4 changes for 2 min each wash of PBS at room temperature.
- Counterstained and mounted the sections after fluorescein staining, 0.5 - 1 µg/ml of DAPI mount was smeared under glass coverslip, if storage is required stored at - 20 ºC in dark.
- View by fluorescence/confocal laser microscope using appropriate emission filters. Tissue sections were viewed and photographed by using confocal laser microscope ((LEICA DMIRE2, Germany).

2.12 Statistical Analysis

Statistics are accessible as mean ± SE (Standard error of mean). One way analysis of variance (ANOVA) was applied using the Graph pad prism software Inc. (version 5.1, California, USA) to relate the concentrations of antioxidant enzymes, ROS and' TBARS activities in control and buprofezin administered groups followed by Post Hoc Tukey's test for comparison among dissimilar treatment groups. A ($P < 0.05$) was considered as statistically significant difference. DNA impairment was evaluated using CASP lab image analysis software (version 1.2.3 beta1, USA).

RESULTS

RESULTS

3.1 Body and Organs Weight

Body weight and weight of organs (liver and kidney) showed no considerable difference ($P > 0.05$) between 4.0 µg, 6.0 µg and 8.0 µg buprofezin treated groups as compared to those of healthy controls (Table 1).

Table. 1 Body weight and organs liver and kidney weight (g) after 24 h exposure of buprofezin to mice

	Body Weight	Liver Weight	Kidney Weight
Control	28.57 ± 0.18	1.23 ± 0.06	1.24 ± 0.03
4.0 µg	28.43 ± 0.20	1.33 ± 0.03	1.27 ± 0.02
6.0 µg	28.27 ± 0.15	1.66 ± 0.05	1.31 ± 0.04
8.0 µg	28.23 ± 0.17	2.03 ± 0.06	1.34 ± 0.03

Values are mean ± S.E, (n=7 mice per group) show no significant change ($P > 0.05$) of control and those of treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w)

3.2 Liver and Kidney Function Tests

3.2.1 Alanine Aminotransferase

Serum ALT levels were found significantly higher at all doses (2010.64 ± 108.79, 2380.29 ± 140.11, 2568.62 ± 422.18) when compared to the healthy controls (1111.45 ± 32.30) (Table 2). Increase ALT levels were dose dependent (Fig.3.1).

3.2.2 Aspartate Aminotransferase

Serum AST levels were increased significantly ($P < 0.05$) in the treated groups when compared to control animals (Table 2). Inter group comparison showed that the AST level of mice treated with the 8.0 µg/kg (39.94 ± 4.98) was significantly greater ($P < 0.05$) than those treated with the 4.0 µg/kg (21.41 ± 0.38) treated group (Fig. 3.2).

Table. 2 Serum ALT & AST levels variation following 24 h BPFN exposure of Albino Balb/c mice

Liver Function Tests	Control	4.0 µg	6.0 µg	8.0 µg
ALT (µ/L)	1111.45 ± 32.30 [a]	2010.64 ± 108.79 [ab]	2380.29 ± 140.11 [ac]	2568.62 ± 422.18 [a]
AST (µ/L)	12.23 ± 0.55 [a]	17.88 ± 1.57 [ab]	30.99 ± 4.68 [ac]	39.94 ± 4.98 [a]

Values are mean ± SE, (n=7 mice per group) Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

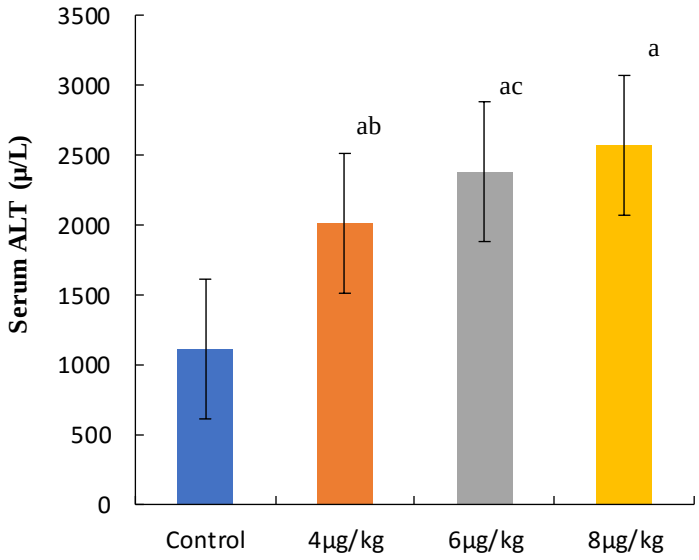


Fig. 3.1 Serum ALT levels in buprofezin treated groups. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

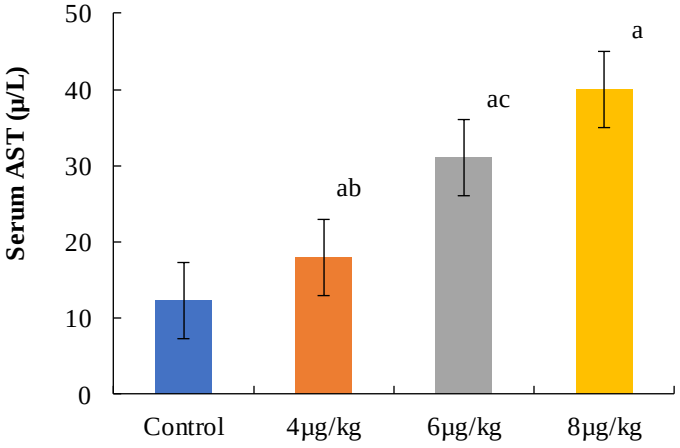


Fig. 3.2 Levels of serum AST. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

3.2.3 Serum Creatinine

Serum Creatinine levels significantly increased ($P < 0.05$) in the buprofezin administered groups as compared to control groups of mice (Table 3). Elevation in serum creatinine was also dose dependent (Fig. 3.3).

3.2.4 Urea

Serum urea levels were significantly higher ($P < 0.05$) in the buprofezin treated groups as compared to the control animals (Table 3). The urea levels were significantly higher ($P < 0.05$) in the 8.0 µg/kg (54.11 ± 0.64) treated group than those of 4.0 µg/kg (17.88 ± 1.57) treated group (Fig. 3.4)

Table. 3 Serum Creatinine & Urea levels variation followed by 24 h buprofezin exposure on the Albino mice

Renal function tests	Control	4.0 µg	6.0 µg	8.0 µg
Creatinine (mM)	132.6 ± 0.37 [a]	151.54 ± 8.85 [ab]	176.8 ± 1.14 [ac]	239.94 ± 8.85 [a]
Urea (mM)	11.29 ± 0.42 [a]	21.41 ± 0.38 [ab]	31.81 ± 1.17 [ac]	54.11 ± 0.64 [a]

Values are mean ± SE, (n=7 mice per group) Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

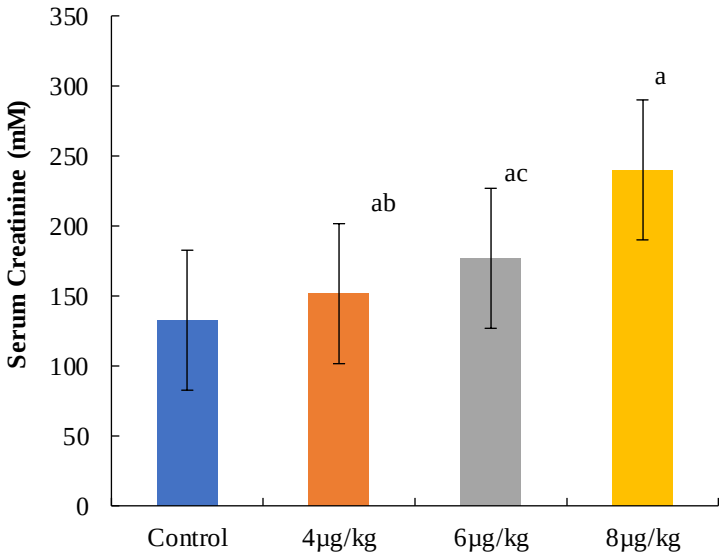


Fig. 3.3 Levels of serum creatinine. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

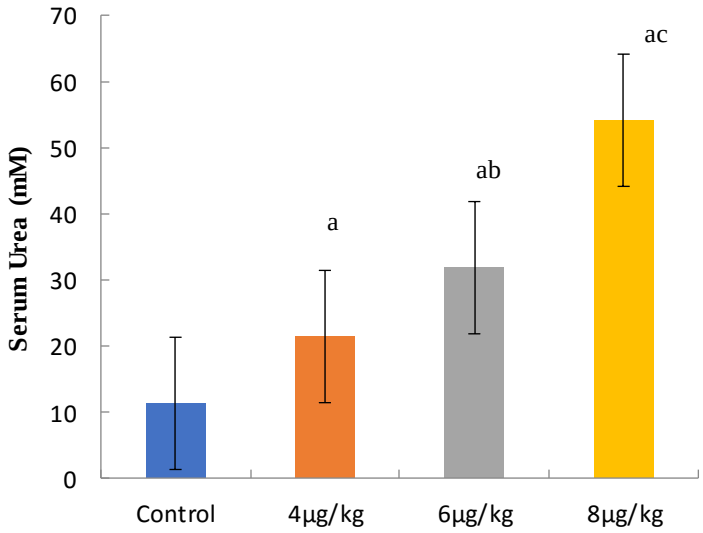


Fig. 3.4 Serum urea levels. Common superscript letter (a) shows difference of the dosed groups from control groups and different superscript letters (b, c) on the bars show significant ($P < 0.05$) difference among dosed groups. Values are expressed as mean ± S.E in the groups.

3.3 LIVER

3.3.1. Antioxidant Enzymes

3.3.1.1 Super Oxide Dismutase (SOD)

SOD activity decreased significantly ($P<0.05$) in buprofezin treated groups as compared to healthy control mice (Table 4). SOD activity decreased among 4.0 µg, 6.0 µg and 8.0 µg treated groups (23.48 ± 8.89, 22.58 ± 8.55, 20.58 ± 7.79) (Fig.3.5).

3.3.1.2 Catalase (CAT)

Significantly decreased ($P<0.05$) CAT activity was found in buprofezin administered animals in comparison to untreated groups (Table 4). Significant decrease in CAT activity (0.07 ± 0.03, 0.05 ± 0.02, 0.02 ± 0.01) was observed between treated groups (Fig.3.6).

3.3.1.3 Peroxidase (POD)

POD activity reduction was significantly ($P < 0.05$) observed in liver tissue on BPFN administered animals in comparison to control animals (Table 4). Significant decline in POD activity was detected among dose administered animals (Fig.3.7).

Table. 4 Changes in the antioxidant enzymes SOD, CAT & POD activities in Liver tissue of Albino mice following 24 h buprofezin treatment

Liver biochemical assays	Control	4.0 µg	6.0 µg	8.0 µg
SOD (unit/min)	26.06 ± 9.87^{a}	23.48 ± 8.89^{ab}	22.58 ± 8.55^{ac}	20.58 ± 7.79^{a}
CAT (unit/min)	0.12 ± 0.05^{a}	0.07 ± 0.03^{ab}	0.05 ± 0.02^{ac}	0.02 ± 0.01^{a}
POD (unit/min)	0.11 ± 0.04^{a}	0.08 ± 0.03^{ab}	0.06 ± 0.02^{ac}	0.04 ± 0.01^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

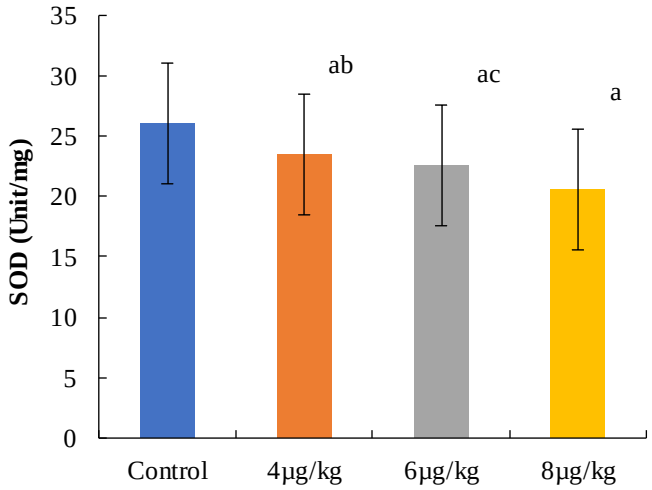


Fig. 3.5 SOD activity in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

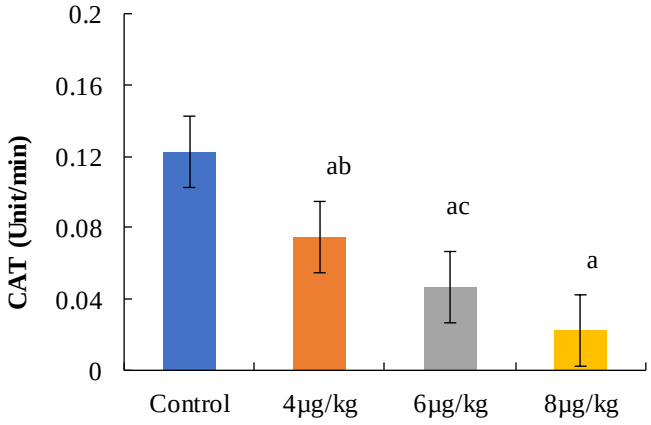


Fig. 3.6 CAT activity in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

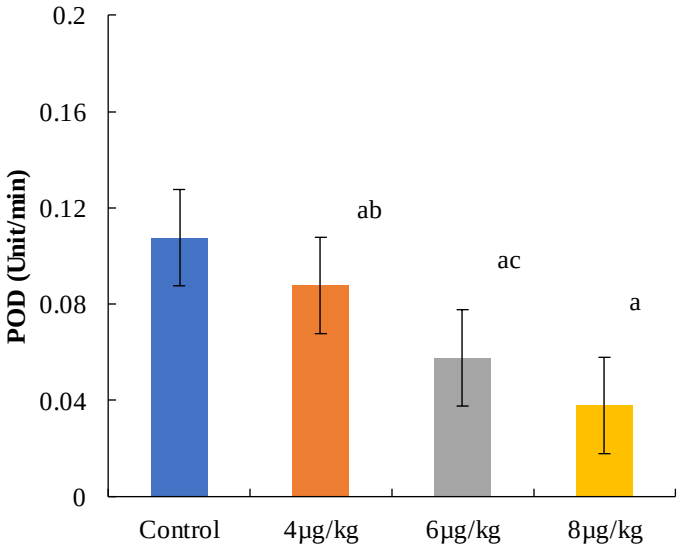


Fig. 3.7 POD activity in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

3.3.2. Reduced Glutathione (GSH)

Liver GSH activity lowered significantly ($P < 0.05$) among all the treated groups as compared to control animals (Table 5). The GSH activity significantly reduced between treated groups 4.0 µg, 6.0 µg and 8.0 µg (1.05 ± 0.39, 0.95 ± 0.36, and 0.66 ±0.25) (Fig. 3.8)

Table. 5 GSH (Reduced Glutathione) activity variations following 24 h BPFN treatment in Liver tissue of Albino mice

Liver biochemical assays	Control	4.0 µg	6.0 µg	8.0 µg
GSH (µM/g of tissue)	2.04 ± 0.77 [a]	1.05 ± 0.39 [ab]	0.95 ± 0.36 [ac]	0.66 ±0.25 [a]

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

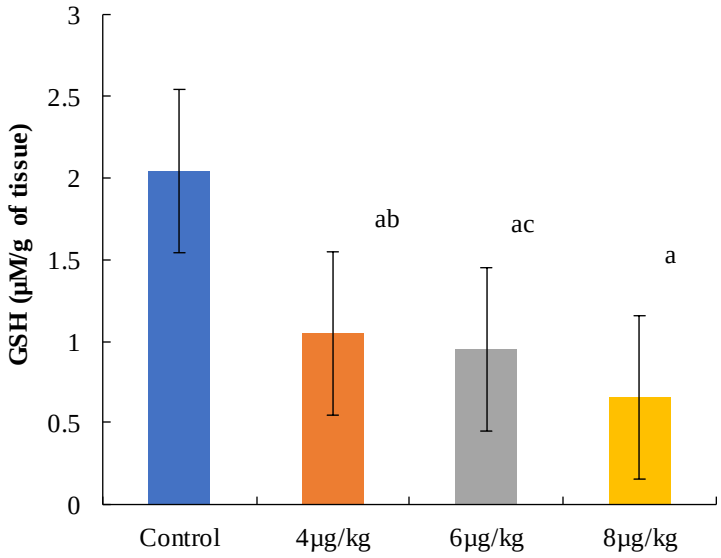


Fig. 3.8 GSH activity in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

3.3.3. Oxidative Stress Markers

3.3.3.1. Reactive Oxygen Species (ROS)

The ROS production increased significantly ($P<0.05$) in the buprofezin treated groups as compared to control animals (Table 6). Significantly increased ROS level ($P<0.05$) among treated groups (1.55 ± 0.59, 1.65 ± 0.62, 2.07 ± 0.79) was observed (Fig.3.9).

3.3.3.2. Lipid Peroxidation (TBARS)

TBARS level increased significantly ($P < 0.05$) at 4.0 µg, 6.0 µg and 8.0 µg dosages of buprofezin as compared to healthy control groups (Table 6). Increase in the levels of TBARS among treated groups was observed (Fig.3.10).

Table. 6 Oxidative stress profile, reactive oxygen species (ROS) and lipid peroxidation (TBARS) levels in Liver tissue of Albino mice following 24 h BPFN exposure

Liver biochemical assays	Control	4.0 µg	6.0 µg	8.0 µg
ROS (absorbance)	1.29 ± 0.49^{a}	1.55 ± 0.59^{ab}	1.65 ± 0.62^{ac}	2.07 ± 0.79^{a}
TBARS (nM/min/mg of protein)	0.07 ± 0.03^{a}	0.09 ± 0.03^{ab}	0.11 ± 0.04^{ac}	0.20 ± 0.08^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

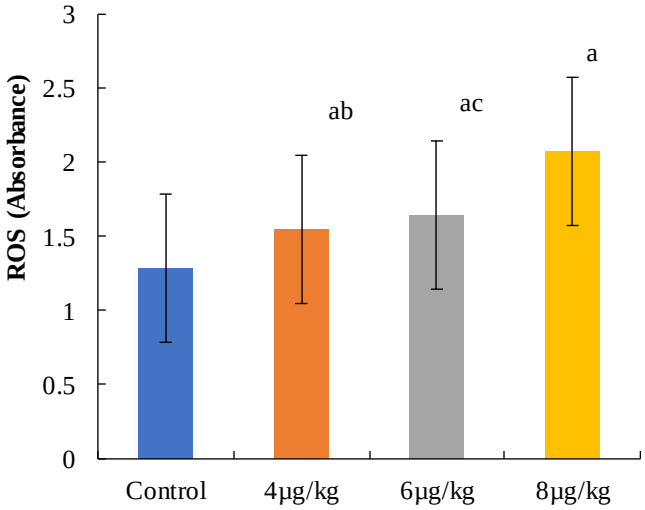


Fig.3.9 ROS level in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

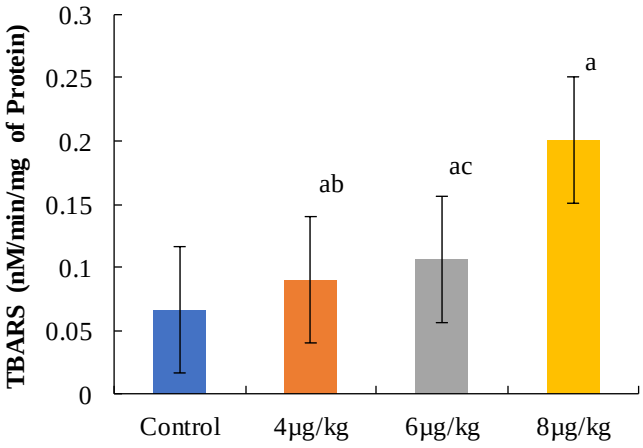


Fig.3.10 TBARS level in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

3.3.4. Tissue Total Protein

The total tissue protein concentration of mice treated with all increasing doses (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) of buprofezin decreased significantly ($P < 0.05$) as compared to control mice (Table 7). Decrease in protein concentration among treated groups was observed (Fig.3.11).

Table. 7 Total Protein concentration variations in Liver tissue of Albino mice following 24 h BPFN treatment

Liver biochemical assays	Control	4.0 µg	6.0 µg	8.0 µg
Protein (µg/mg of tissue)	0.16 ± 0.06^{a}	0.12 ± 0.04^{ab}	0.09 ± 0.03^{ac}	0.08 ± 0.03^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA)

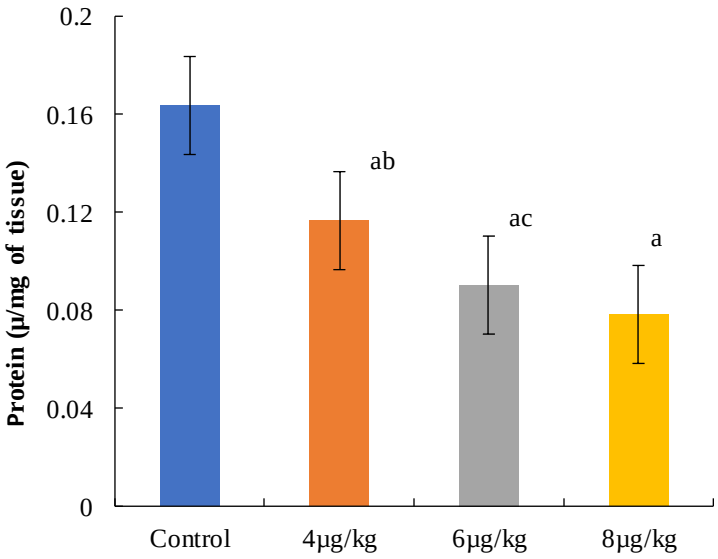


Fig. 3.11 Total tissue protein concentration in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

3.3.5 Histopathological Observations

The congestion and degeneration in the liver tissue of mice treated with all increasing doses (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) of buprofezin increased significantly ($P < 0.05$) as compared to those of healthy control groups. No significant changes in hemorrhage of tissue were observed (Table 8).

Table. 8 Histopathological observations in liver tissue after 24 h BPFN exposure on Albino mice

Histopathological changes	Control	4.0 µg	6.0 µg	8.0 µg
Congestion	0.00 ± 0.00^{a}	1.89 ± 0.31^{ab}	3.0 ± 0.2^{ac}	3.2 ± 0.1^{a}
Degeneration	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.1 ± 0.1^{ac}	1.3 ± 0.2^{a}
Hemorrhage	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

3.3.5.1. Morphometric Observations

According to morphometric calculations, significant difference ($P < 0.05$) in the hepatocyte area and nuclear area of hepatocyte in all increasing doses (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) of buprofezin were observed as compared to control animals. Decrease in hepatocyte and nuclear area among treated groups were also observed (Table 9).

Table. 9 Morphometric observations in the hepatocyte area and nucleus area of hepatocyte of liver tissue after 24 h BPFN exposure on Albino mice

Parameters	Control	4.0 µg	6.0 µg	8.0 µg
Hepatocyte area (μm^2)	517.16 ± 58.38^{a}	391.91 ± 91.45^{ab}	382.35 ± 55.85^{ac}	351.24 ± 48.85^{a}
Nucleus area of hepatocytes (μm^2)	93.63 ± 23.54^{a}	86.42 ± 19.17^{ab}	78.35 ± 17.41^{ac}	67.89 ± 8.35^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA)

3.3.5.2. H & E, PAS and Iron Staining

Control liver sections showed normal tissue architecture, normal hepatocytes, sinusoids, radiating canals and central vein. In the treatment groups (4.0 µg/kg, 6.0 µg/kg and 8.0 µg/kg b.w.) showed altered morphology of hepatocytes, liver tissue congestion, prominent radiating canals but deshaped nuclei blood accumulation in central canal, sinusoidal spaces which widened in some regions but become narrowed in other regions and nuclear atypia. Sinusoidal architecture showed severe alterations, pyknosis and nuclear shrinkage evident. Signs of necrosis were depicted by dark chromatin, loss of chromatin excess vacuolization, breakage of nuclear membrane and gross tissue disruption and accumulation of fatty globules; nuclei were pyknosed and look as if apoptotic (Fig.3.12, 3.13 & 3.14)

Fig. 3.12 Photomicrographs showing assessment of buprofezin treated groups with controls. In control H & E stained liver histology showed normal and large central vein, bi nucleated nuclei, normal sinusoids, radiating canals, nuclear connective tissue, prominent nuclei with chromatin (A, B). In 4.0 µg treated sections showed parenchyma tissue congestion, radiating canals, sinusoids, sinusoidal spaces widen some places and narrowed, blood accumulation in the central vein, hepatocytes with deshaped nuclei, nuclear atypia, , 6.0 µg treated group tissue sections indicated sinusoidal architecture with severe alteration, central vein show deformity, connective tissue breakage signs of necrosis appear as depicted by dark chromatin, nuclear shrinkage and pyknosis evident, breakage of nuclear membrane also evident and 8.0 µg treated group sections showed extremely wide sinusoidal spaces, radiating canals and sinusoids visible but show severe alterations in morphology, increased veins, blood accumulation/hemoglobin in central canal increased number of cells apparent, clear tissue/cell necrosis, loss of chromatin, membrane breakage and fatty globules, liver parenchyma appear completely disrupted (C, D, E, F, G, H) respectively. Magnification = 100X, 1000X, Scale bar = 30µm, 160µm.

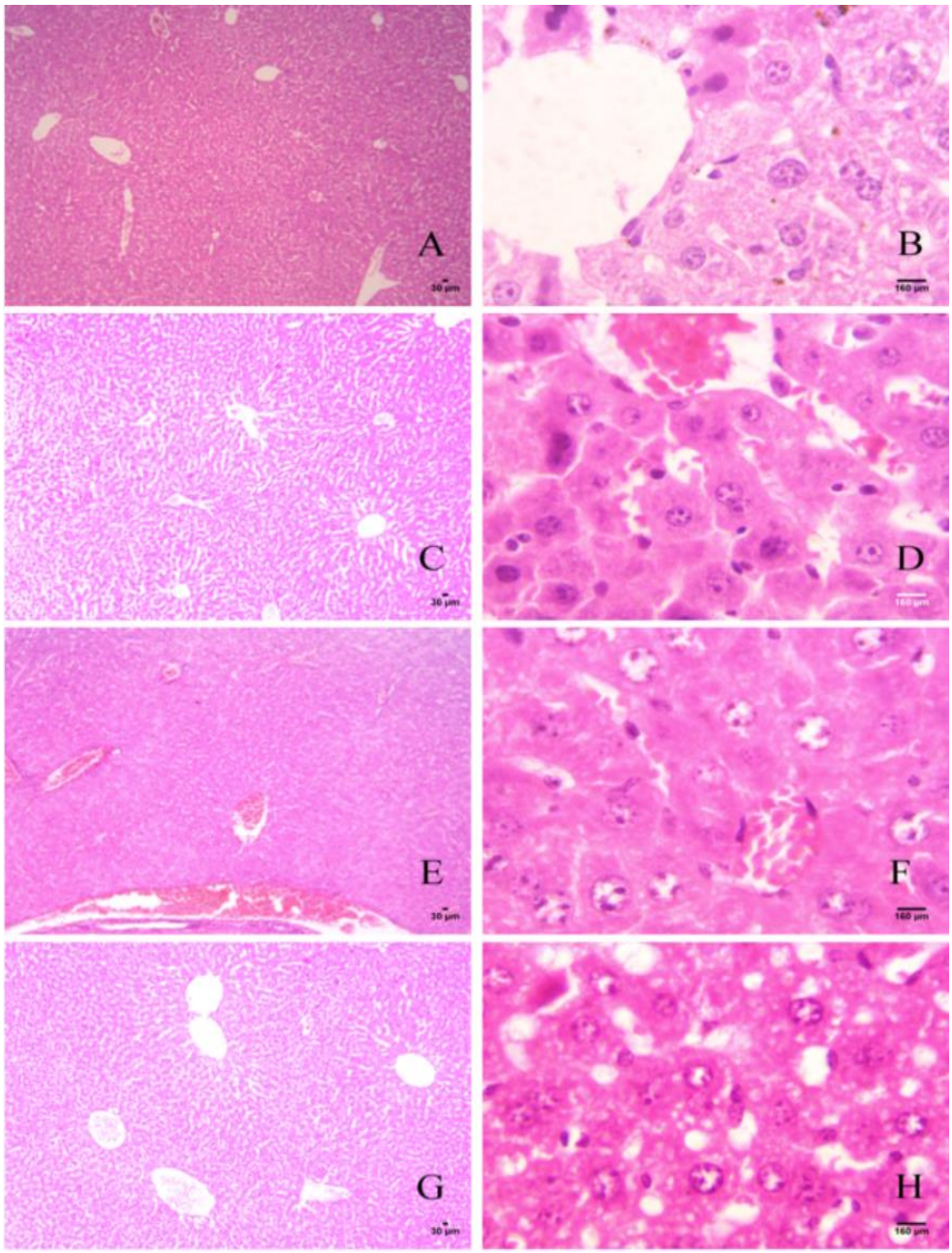

A
B
C
D
E
F
G
H

Fig. 3.13 Photomicrographs showing buprofezin treated groups with control group comparison. In control tissue sections stained with PAS showed, normal liver macrophages, sinusoidal hepatocytes (A, B). In 4.0 µg treated group tissue sections showed breakage of sinusoidal architecture, abundant macrophages, tissue necrosis, severe tissue damage altered central vein, pyknotic, 6.0 µg treated group showed loss of sinusoids and radiating canal, loss of chromatin excess vacuolization and gross tissue disruption excessive and tissue damage with pyknosis and necrosis and 8.0 µg showed widened sinusoidal spaces, excessive venous capillaries, lobulated appearance, excessive accumulation of fatty globules, loss of parenchyma, nuclei pyknosed and apoptosed hepatocyte architecture loss (C, D, E, F, G, H) respectively. Magnification = 100X, 1000X, Scale bar = 30µm, 160µm.

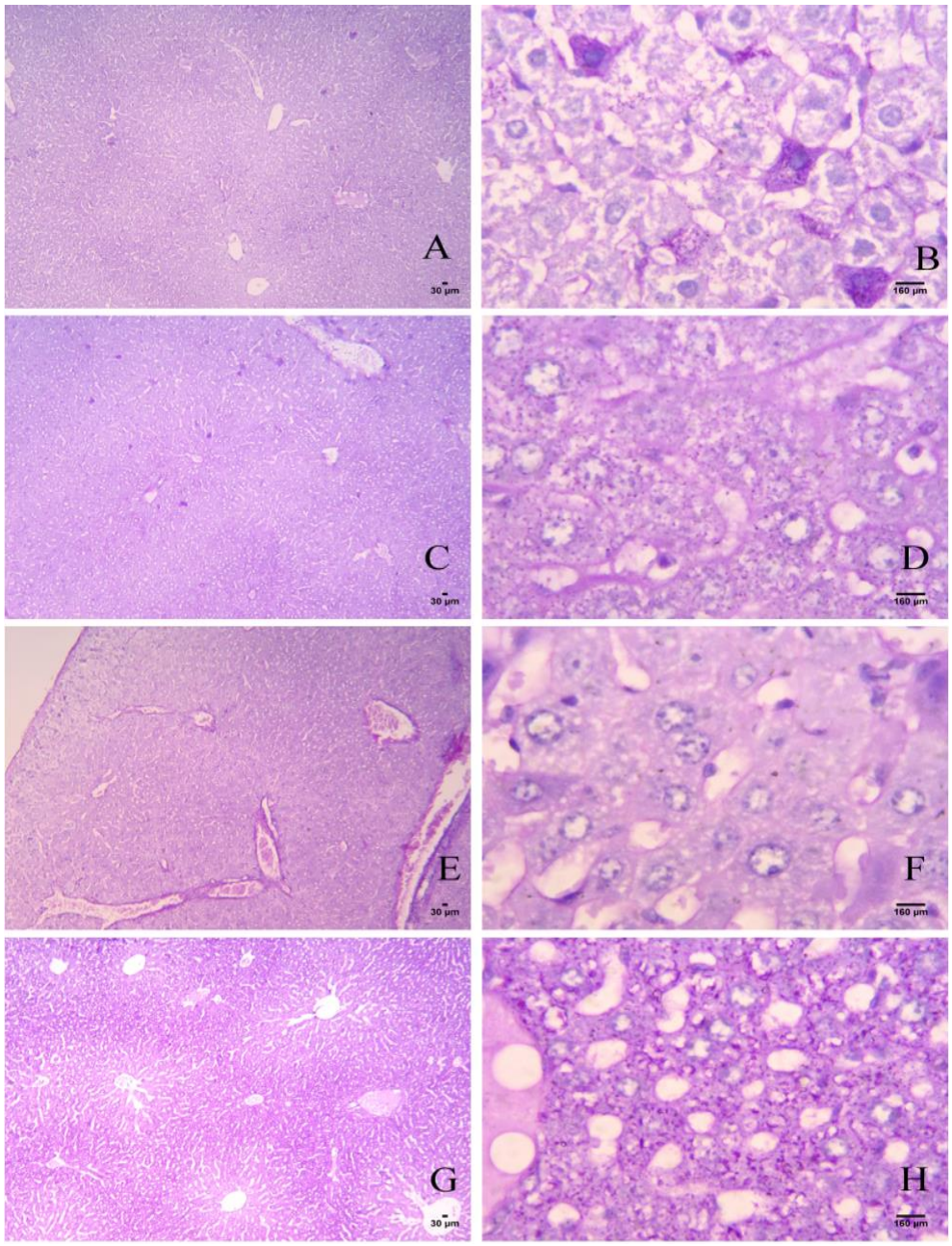
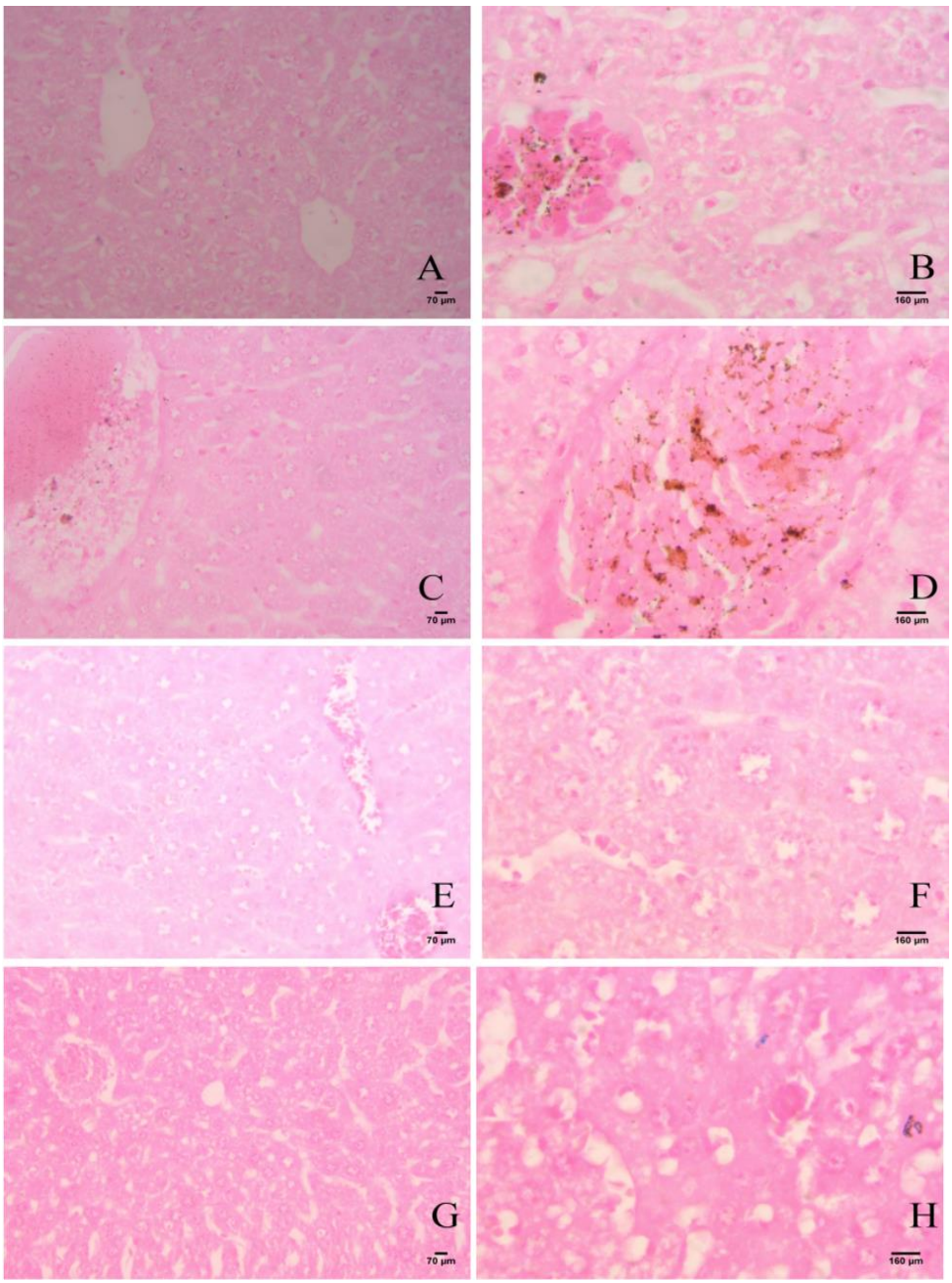

A
30 µm
B
160 µm
C
30 µm
D
160 µm
E
30 µm
F
160 µm
G
30 µm
H
160 µm

Fig. 3.14 Photomicrographs showing comparison of buprofezin exposed groups with controls. In control tissue section stained with iron showed, normal sinusoids, nuclei become bi nucleated, radiating canals (A, B). In 4.0 µg treated group showed tissue congestion prominent, sinusoidal spaces widen some places and narrowed, radiating canals and sinusoids, parenchyma tissue congestion, tissue show altered morphology, hepatocytes with deshaped nuclei, nuclear atypia, and, 6.0 µg treated tissue sections showed loss of sinusoids and radiating canal, loss of chromatin excess vacuolization and tissue disruption, excessive tissue damage with pyknosis and necrosis, and 8.0 µg buprofezin treated tissue sections showed widen sinusoidal spaces, excessive venous capillaries, lobulated appearance, excessive accumulation of fatty globules, loss of parenchyma, nuclei pyknosed, hepatocyte architecture loss, (C, D, E, F, G, H) respectively. Magnification = 400X, 1000X, Scale bar = 70µm, 160µm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B
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C
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D
160 µm
E
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F
160 µm
G
70 µm
H
160 µm

3.3.6. DNA damage

3.3.6.1. Genotoxic effect in Liver

The mean TL of comet was increased significantly ($P < 0.05$) in BPFN administered groups. The % DNA, comet tail enlarged significantly in treated groups as compared to control and this increase also observed among treated groups (6.35 ± 0.03, 9.06 ± 0.06, 11.37 ± 0.02). A significant increase occurred in the head length among dose administered groups when compared to untreated group. Similarly, in other parameters like the length of comet, head DNA, tail DNA, tail moment (TM) and the olive tail moment (OTM) significant increase ($P < 0.05$) arose in buprofezin treated groups in comparison to untreated animals (Fig. 3.15). Substantial proliferation was detected among treated groups (4.0 µg/kg, 6.0 µg/kg and 8.0 µg/kg b.w.) (Table 10). Photomicrograph showed intact DNA in liver cells of control group, while in buprofezin treated groups damage were observed in the form of comets viewing damaged and degraded DNA in liver cells (Fig.3.16).

Table. 10 Genotoxicity in liver tissue after buprofezin exposure for 24 h on Albino mice

Liver comet assay	Control	4.0 µg	6.0 µg	8.0 µg
L Head (mean value)	9.29 ± 0.06 [a]	12.67 ± 0.56 [ab]	29.86 ± 0.05 [ac]	43 ± 0.05 [a]
L Tail (mean value)	3.57 ± 0.29 [a]	6 ± 0.34 [ab]	9.71 ± 0.04 [ac]	12.29 ± 0.03 [a]
L Comet (mean value)	10.8 ± 0.01 [a]	15.67 ± 0.51 [ab]	26.57 ± 0.04 [ac]	34.29 ± 0.08 [a]
Head DNA (mean value)	70.67 ± 0.05 [a]	81.± 0.23 [ab]	89.03 ± 0.02 [ac]	98 ± 0.06 [a]
Tail DNA (mean value)	4.57 ± 0.03 [a]	6.35 ± 0.03 [ab]	9.06 ± 0.06 [ac]	11.37 ± 0.02 [a]
TM (mean value)	0.36 ± 0.01 [a]	0.53 ± 0.06 [ab]	0.89 ± 0.03 [ac]	1.17 ± 0.03 [a]
OTM (mean value)	0.28 ± 0.04 [a]	0.55 ± 0.03 [ab]	0.76 ± 0.04 [ac]	1.39 ± 0.04 [a]

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA)

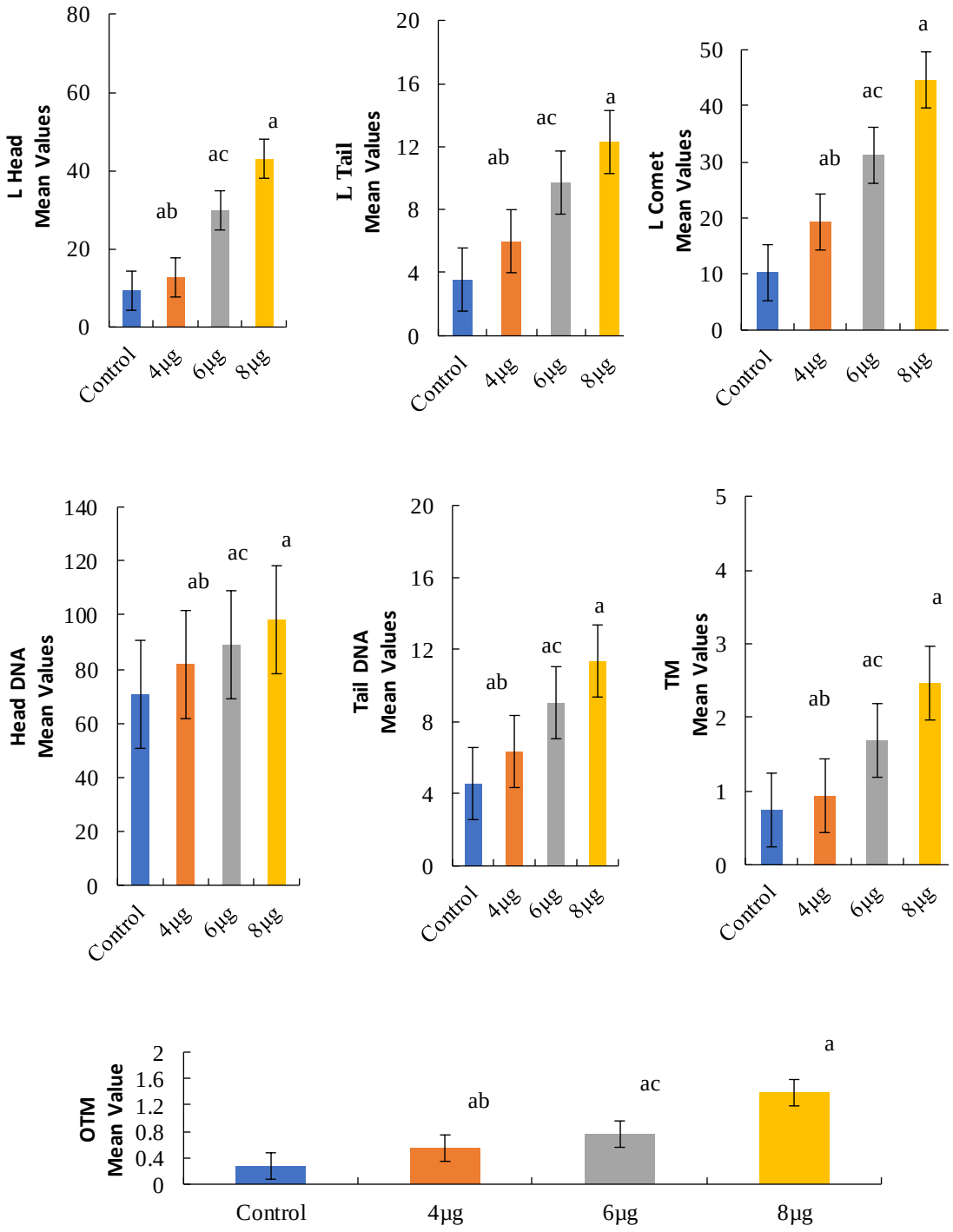


Fig. 3.15 Genotoxicity in liver tissue of mice. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups. L Head= head length; L Tail= tail length; L Comet= comet length; TM= tail moment; and OTM= oil tail moment. Values are expressed as mean ± S.E and bars showing.

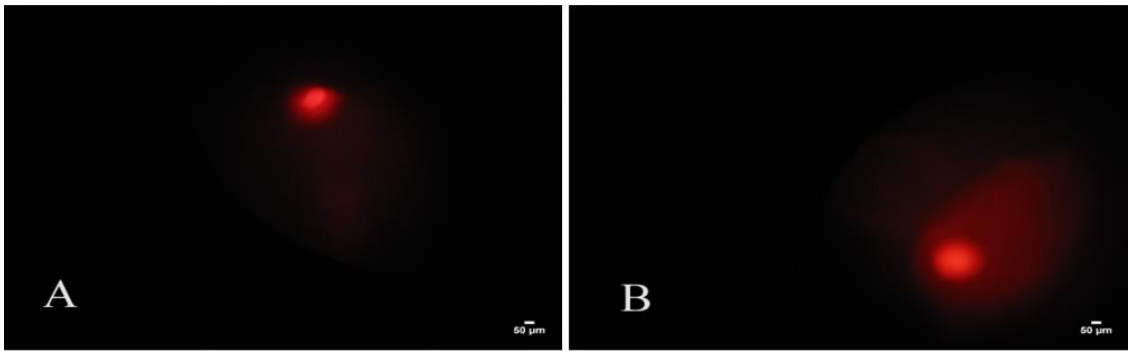
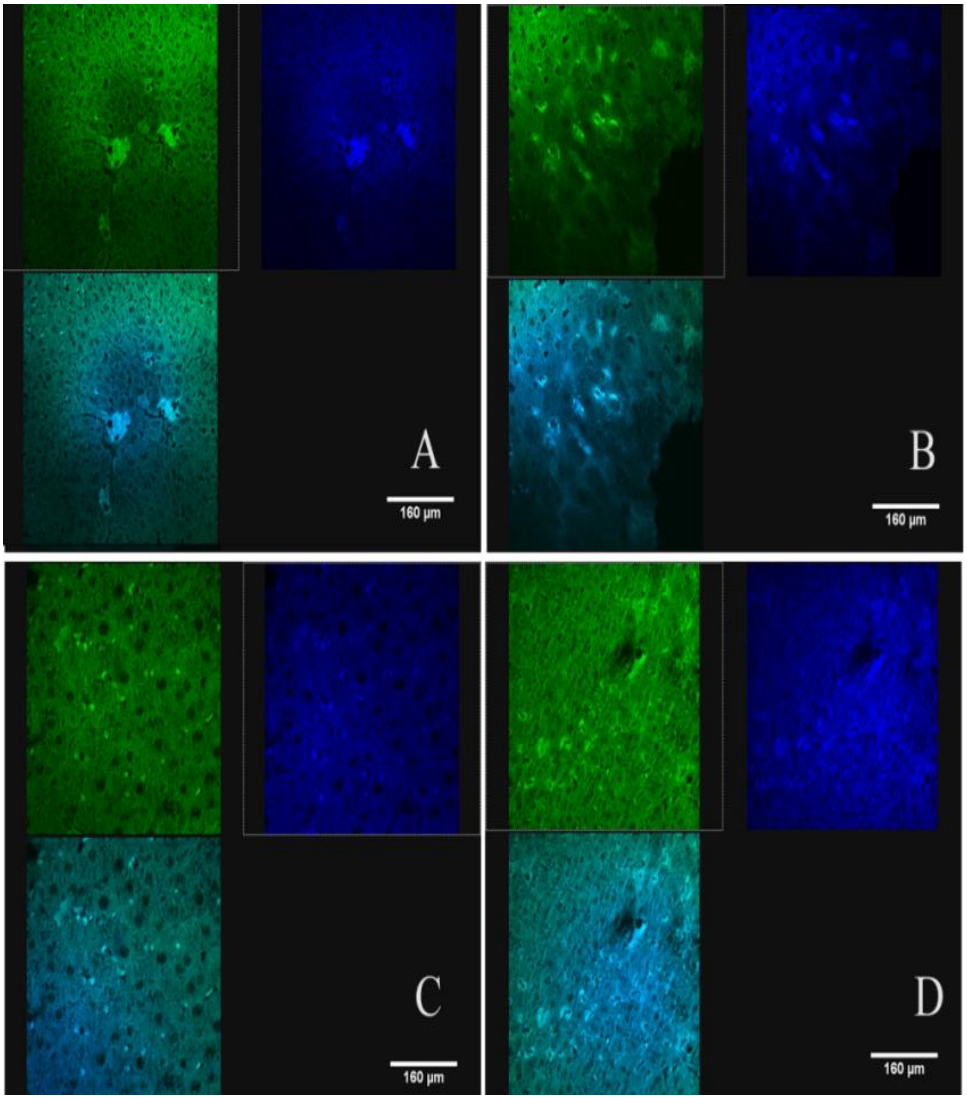


Fig. 3.16 Photomicrographs of DNA damage in liver cells of buprofezin treated mice (A) Untreated Control, (B) Treated group. Magnification = 400X, Scale bar = 50µm.

3.3.7. TUNEL Assay

TUNEL assay revealed apoptosis in the paraffin fixed embedded tissue sections of liver. Two folds increase occurred in apoptosis in cells with 2 µg increase in buprofezin dose (4.0 µg/kg, 6.0 µg/kg and 8.0 µg/kg b.w.) in comparison to control groups (Fig.3.17).

Fig. 3.17 Photomicrographs showed apoptosis in liver tissue sections of mice treated with buprofezin (A) Positive control (B) 4.0 µg, (C) 6.0 µg (D) 8.0 µg dosed groups. Magnification = 1000X, Scale bar=160µm.


A
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B
160 µm
C
160 µm
D
160 µm

3.4. KIDNEY

3.4.1. Antioxidant Enzymes

3.4.1.1.Super Oxide Dismutase (SOD)

A significantly decreased ($P < 0.05$) SOD activity was found in the kidney tissues of buprofezin treated mice compared to control animals was observed (Table 11). SOD activity decreased significantly ($P < 0.05$) among the treated groups (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) (Fig.3.18).

3.4.1.2.Catalase (CAT)

The kidney tissue of mice treated with buprofezin showed significant decreases ($P < 0.05$) in CAT activity compared to control (Table 11). CAT activity decreased significantly ($P < 0.05$) among buprofezin treated groups (Fig.3.19).

3.4.1.3.Peroxidase (POD)

POD activity was significantly lowered ($P < 0.05$) in kidney tissue treated with buprofezin compared to control group. (Table 11) Significant reduction in the POD activity also observed among the toxicant given groups (Fig.3.20).

Table. 11 Changes in the antioxidant enzymes SOD, CAT & POD activities in kidney tissue after 24 h buprofezin treatment

Kidney biochemic assays	Control	4.0 µg	6.0 µg	8.0 µg
SOD (unit/min)	52.46 ± 19.87 [a]	26.19 ± 9.92 [ab]	24.20 ± 9.17 [ac]	16.02 ± 6.07 [a]
CAT (unit/min)	0.14 ± 0.05 [a]	0.09 ± 0.04 [ab]	0.063 ± 0.024 [ac]	0.030 ± 0.011 [a]
POD (unit/min)	0.11 ± 0.04 [a]	0.09 ± 0.03 [ab]	0.06 ± 0.02 [ac]	0.03 ± 0.01 [a]

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

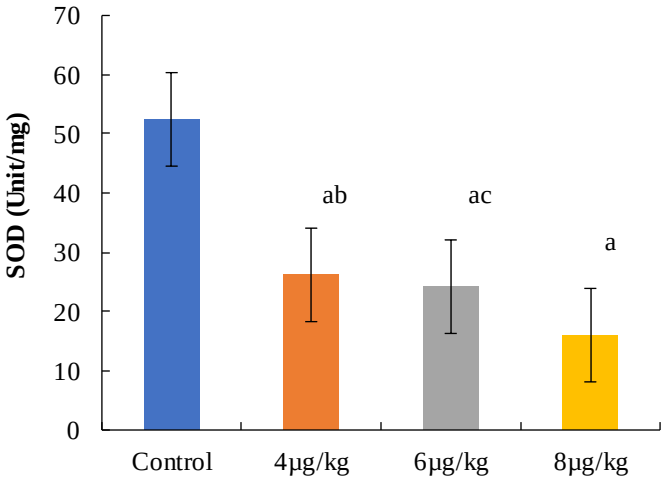


Fig.3.18 SOD activity in kidney tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

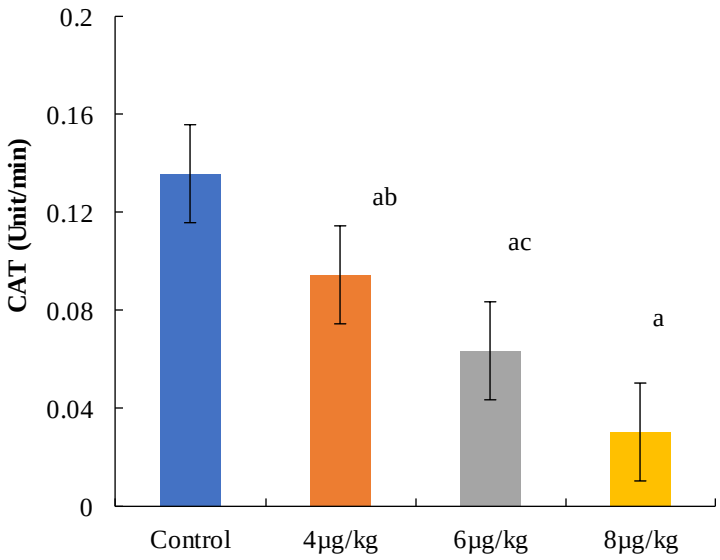


Fig. 3.19 CAT activity in kidney tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

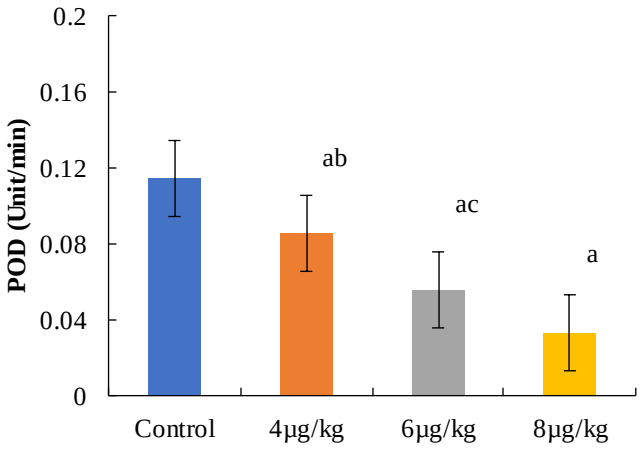


Fig. 3.20 POD activity in kidney tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

3.4.2. Reduced Glutathione (GSH)

GSH activity in kidney tissue of mice was considerably lowered ($P < 0.05$) in all treated groups compared to control animals (Table 12). GSH activity reduced significantly ($P < 0.05$) between dose administered groups was observed (Fig.3.21).

Table. 12 GSH (Reduced Glutathione) activity alteration following 24 h exposure of BPFN treatment in Kidney tissue of Albino mice

Kidney biochemical assays	Control	4.0 µg	6.0 µg	8.0 µg
GSH (µM/g of tissue)	1.43 ± 0.54^{a}	0.85 ± 0.32^{ab}	0.64 ± 0.24^{ac}	0.32 ± 0.12^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

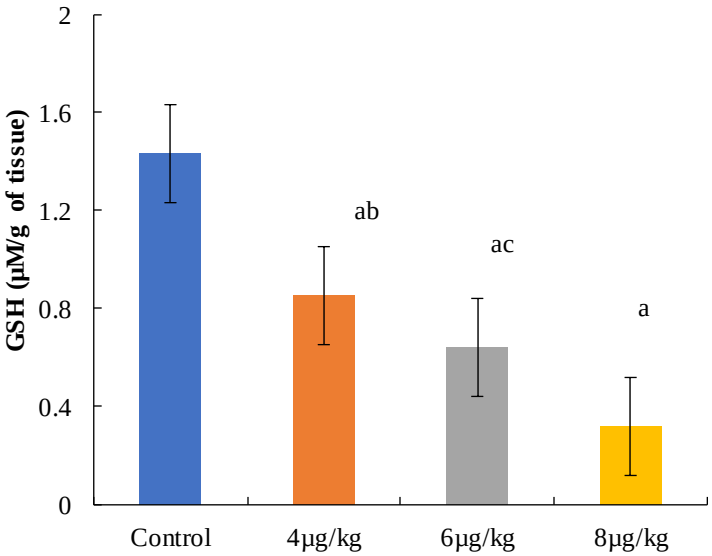


Fig. 3.21 GSH activity in kidney tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups

3.4.3. Oxidative Stress Profile

3.4.3.1. Reactive Oxygen Species (ROS)

ROS levels in all buprofezin administered groups (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) increased significantly ($P < 0.05$) as compared to the control groups (Table 13). Significant increased ($P < 0.05$) ROS levels among treated groups were also observed (Fig.3.22).

3.4.3.2. Lipid Peroxidation (TBARS)

TBARS level increased significantly ($P < 0.05$) in the kidney tissue of buprofezin treated mice compared to the healthy controls (Table 13). TBARS level (0.09 ± 0.03, 0.19 ± 0.07 and 0.21 ± 0.08) also increased among buprofezin treated groups (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) (Fig.3.23).

Table. 12 Oxidative stress profile, reactive oxygen species (ROS) and lipid peroxidation (TBARS) levels alteration in Kidney tissue of Albino mice following 24 h BPFN exposure

Kidney biochemical assays	Control	4.0 µg	6.0 µg	8.0 µg
ROS (absorbance)	1.34 ± 0.51^{a}	1.64 ± 0.62^{ab}	1.81 ± 0.69^{ac}	2.08 ± 0.79^{a}
TBARS (nM/min/mg of protein)	0.05 ± 0.02^{a}	0.09 ± 0.03^{ab}	0.19 ± 0.07^{ac}	0.21 ± 0.08^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

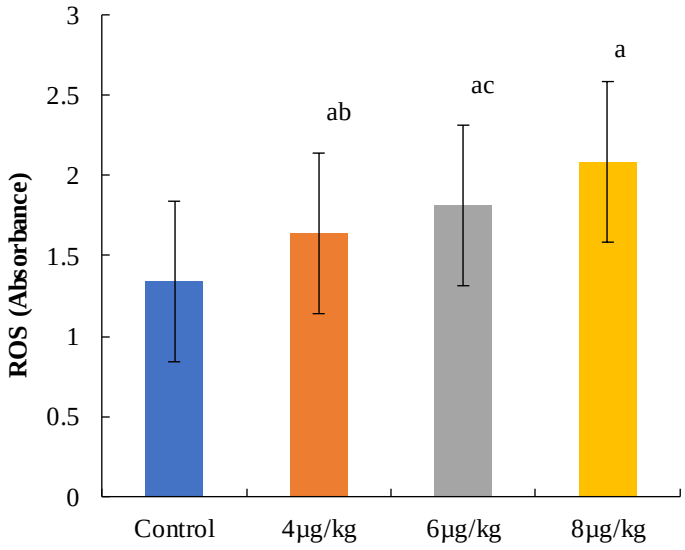


Fig. 3.22 ROS levels in liver tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

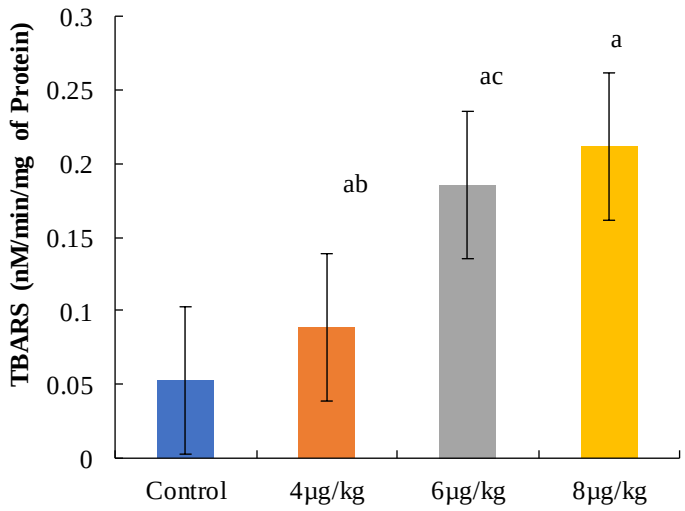


Fig. 3.23 TBARS level in kidney tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups

3.4.4. Tissue Total Protein

In the kidney tissue total protein concentrations were lowered significantly ($P < 0.05$) in BPFN administered groups compared to control animals (Table 14). Tissue total protein concentrations were considerably decreased ($P < 0.05$) among buprofezin treated groups (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) (Fig.3.24)

Table. 14 Total Protein concentrations alteration in Kidney tissue of Albino mice following 24 h BPFN treatment

Kidney biochemical assays	Control	4.0 µg	6.0 µg	8.0 µg
Protein (µg/mg of tissue)	0.12 ± 0.05^{a}	0.03 ± 0.01^{ab}	0.09 ± 0.04^{ac}	0.06 ± 0.02^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

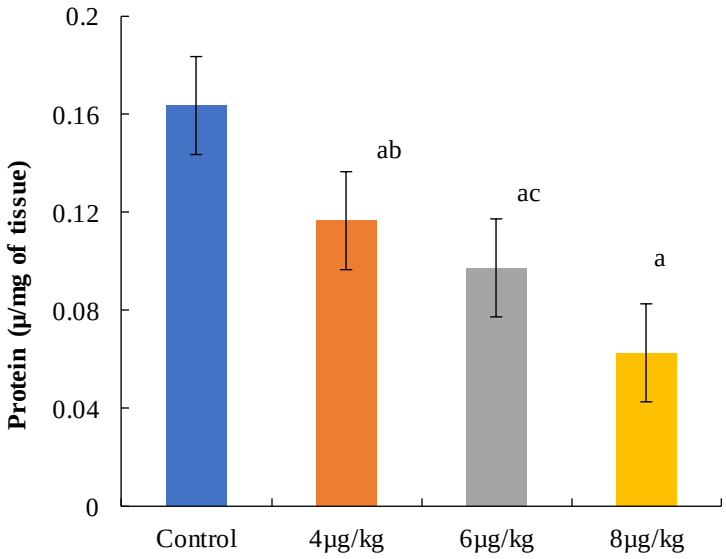


Fig. 3.24 Total tissue protein concentrations in kidney tissue. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

3.4.5. Histopathological Observations

Tissue congestion and degeneration were found in the kidney tissue of mice treated with all buprofezin doses (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) as compared to control groups. Not any significant change in hemorrhage were detected (Table 15)

Table. 15 Histopathological observations in kidney tissue after 24 h BPFN exposure on Albino mice

Histopathological changes	Control	4.0 µg	6.0 µg	8.0 µg
Congestion	0.00 ± 0.00^{a}	1.3 ± 0.2^{ab}	1.7 ± 0.3^{ac}	2.6 ± 0.1^{a}
Degeneration	0.00 ± 0.00^{a}	1.6 ± 0.2^{ab}	1.6 ± 0.2^{ac}	2.6 ± 0.2^{a}
Hemorrhage	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

3.4.5.1. Morphometric observations

A significant alteration ($P < 0.05$) was found in the glomerular area and renal corpuscular area in the buprofezin treated groups (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) as compared to control animals. Shrinkage in the glomerular area and renal corpuscular area among treated groups were also observed. (Table 16)

Table. 16 Morphometric observations in the glomerular area and renal corpuscular area of kidney tissue after 24 h BPFN exposure on Albino mice

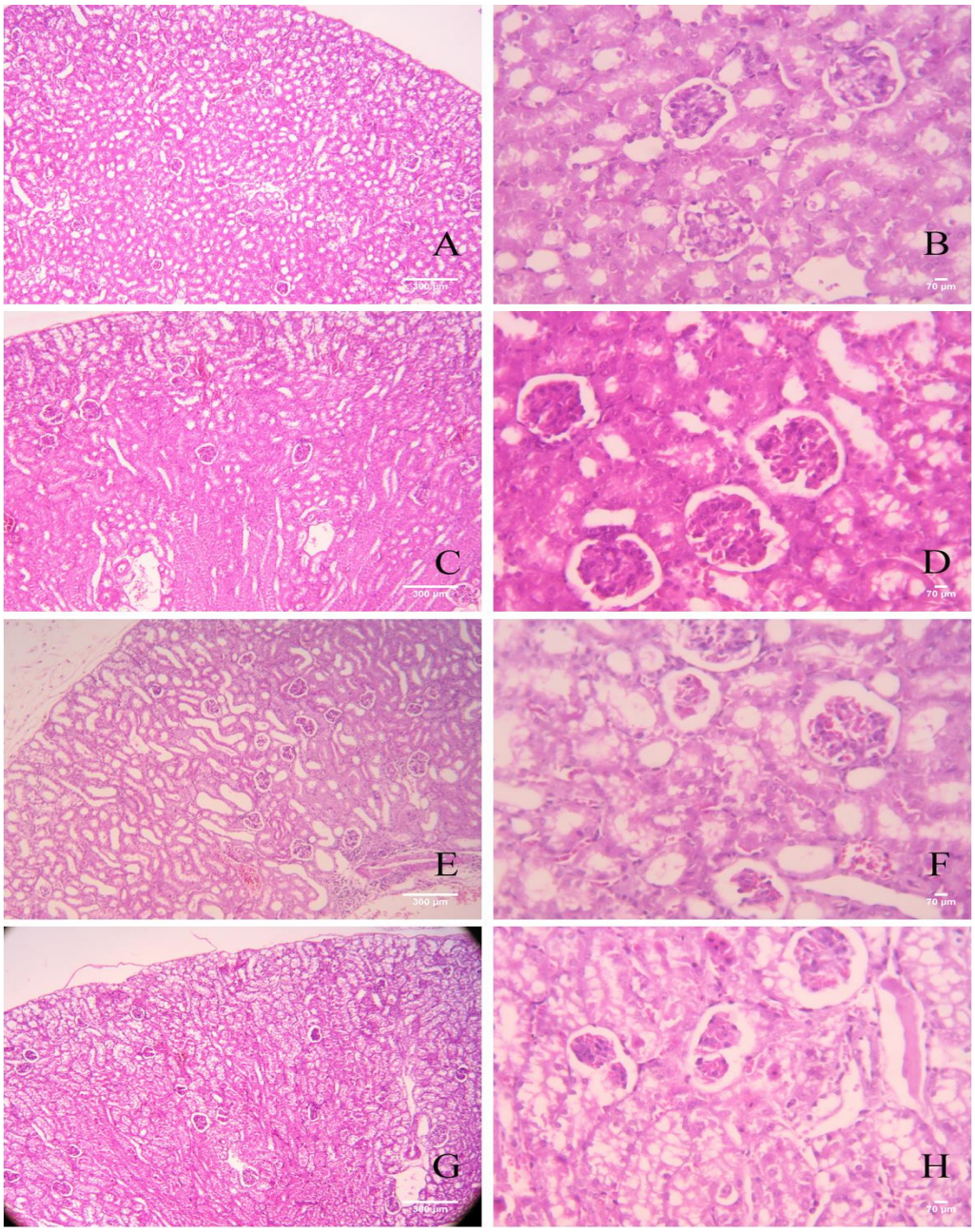
Parameters	Control	4.0 µg	6.0 µg	8.0 µg
Glomerular Area (µm^2)	3640.98 ± 472.74 ^{a}	2583.88 ± 700.28^{ab}	1992.06 ± 624.33 ^{ac}	1449.68 ± 307.78 ^{a}
Renal Corpuscular Area (µm^2)	4316.52 ± 690.0 ^{a}	3437.59 ± 678.94 ^{ab}	2864.41 ± 770.62 ^{ac}	2733.71 ± 744.61 ^{a}

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA)

3.4.5.2. H & E Staining

The control group kidney tissue sections, stained with H & E showed normal kidney cells with glomeruli, Bowmann's space, tubular lumen as compared to the buprofezin treated groups (4.0 µg, 6.0 µg and 8.0 µg/kg b.w.) noted alterations in the kidney tissue architecture, glomeruli showed shrinkage, enlarged Bowmann's space along with obliteration of tubular lumen, enlarged tubules, parenchyma showed congestion and gave vacuolated appearance, nuclear dislodge from epithelia, mononuclear infiltration, while tubular epithelia show severely altered morphology, necrosis at some places tissue revealed fibrotic appearance (Fig. 3.25).

Fig. 3.25 Photomicrographs of kidney sections stained with H & E revealed comparison of buprofezin treated groups with healthy controls. Control group kidney tissue sections showed normal with prominent glomeruli and nephric tubules, proximal and distal tubule show normal epithelia, normal Bowmann's space, (A, B). In 4.0 µg tissue sections showed glomeruli shrinkage, enlarged Bowmann's space, distended tubular tubules, tissue congestion and vacuolated appearance, mononuclear infiltration, excessively widened lumen of tubules and collecting ducts, nuclear dislodge from epithelia observed at some places tissue necrotic and fibrotic appearance in 6.0 µg buprofezin treated group and 8.0 µg showed abnormal architecture of medullary rays and collecting duct, excessive shrinkage of glomeruli, enlarged Bowmann's spaces along with obliteration of tubular lumen, mononuclear infiltration, , tubular epithelia show severely altered morphology, necrosis (C, D, E, F, G, H) respectively. Magnification = 100X and 400X). Scale bar = 300µm, 70µm.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3.4.6. DNA damage

3.4.6.1. Genotoxic effect in Kidney

To detect DNA damage the Comet assay techniques was used in the kidney of albino mice after 24 h exposures to three doses of buprofezin (4.0 µg/kg, 6.0 µg/kg and 8.0 µg/kg b.w.). Significant increase ($P < 0.05$) was found in the mean tail length (6.14 ± 0.06, 8.2 ± 0.03, 13.53 ± 0.04) in buprofezin treated groups. The percent DNA in comet tail enlarged significantly in treated groups as compared to control and among treated groups. A significant increase occurred in the head length among treated groups in comparison to control group. Also, other parameters like the length of comet, tail DNA, head DNA, the olive tail moment (OTM) and tail moment (TM) also indicated significant increase ($P < 0.05$) in buprofezin treated mice as compared to control mice (Fig. 3.26). Significant increase in the mean tail length, percent DNA, length of comet, head DNA, tail DNA, tail moment (TM) and the olive tail moment (OTM) observed among treated groups (Table 17). Photomicrograph revealed undamaged DNA in control group, damage was observed in the form of comets showing damaged and degraded DNA in buprofezin treated groups (Fig.3.27).

Table. 17 Genotoxicity in kidney tissue after 24 h buprofezin exposure on Albino mice

Comet Assay Kidney	Control	4.0 µg	6.0 µg	8.0 µg
L Head (mean value)	14.16 ± 0.07 [a]	19.14 ± 0.04 [ab]	24.43 ± 0.20 [ac]	28.6 ± 0.16 [a]
L Tail (mean value)	3.13 ± 0.02 [a]	6 ± 0.02 [ab]	8.2 ± 0.03 [ac]	13.53 ± 0.04 [a]
L Comet (mean value)	10.24 ± 0.04 [a]	19.29 ± 0.03 [ab]	31.19 ± 0.03 [ac]	44.63 ± 0.02 [a]
Head DNA (mean value)	69.26 ± 0.03 [a]	77.19 ± 0.03 [ab]	88.21 ± 0.03 [ac]	95.59 ± 0.03 [a]
Tail DNA (mean value)	5.84 ± 0.02 [a]	9.69 ± 0.03 [ab]	13.81 ± 0.03 [ac]	19.11 ± 0.01 [a]
TM (mean value)	0.74 ± 0.02 [a]	0.94 ± 0.04 [ab]	1.69 ± 0.02 [ac]	2.46 ± 0.01 [a]
OTM (mean value)	0.62 ± 0.03 [a]	0.95 ± 0.02 [ab]	1.43 ± 0.03 [ac]	2.26 ± 0.01 [a]

Values are mean ± SE, (n=7 mice per group). Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups (4.0 µg, 6.0 µg & 8.0 µg/kg b.w.) followed by One Way Analysis of Variance (ANOVA).

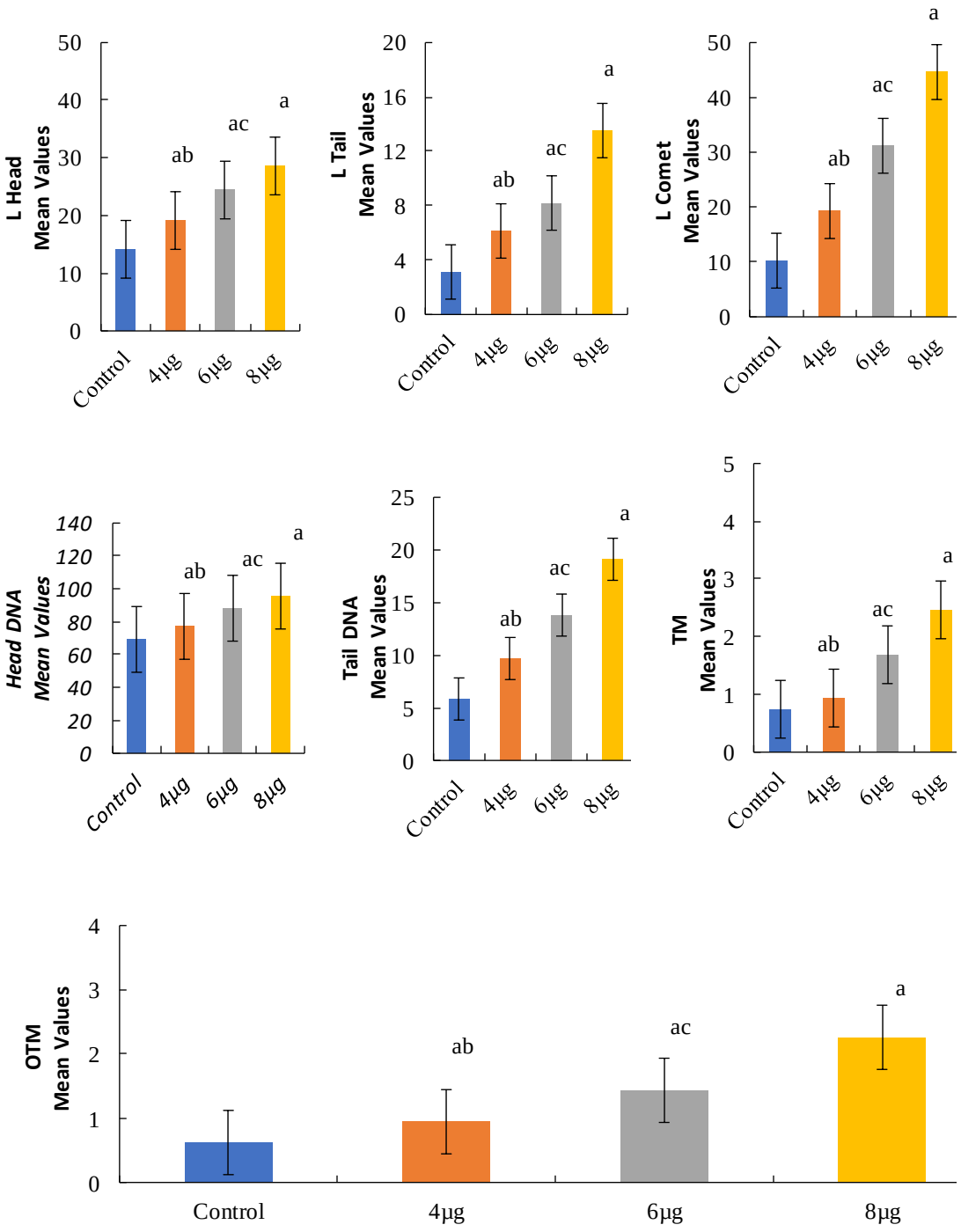


Fig. 3.26 Genotoxicity on kidney tissue of mice. Common superscript letter (a) shows difference of treated groups with the control, while different letters (b, c) show significant ($P < 0.05$) difference among treated groups.

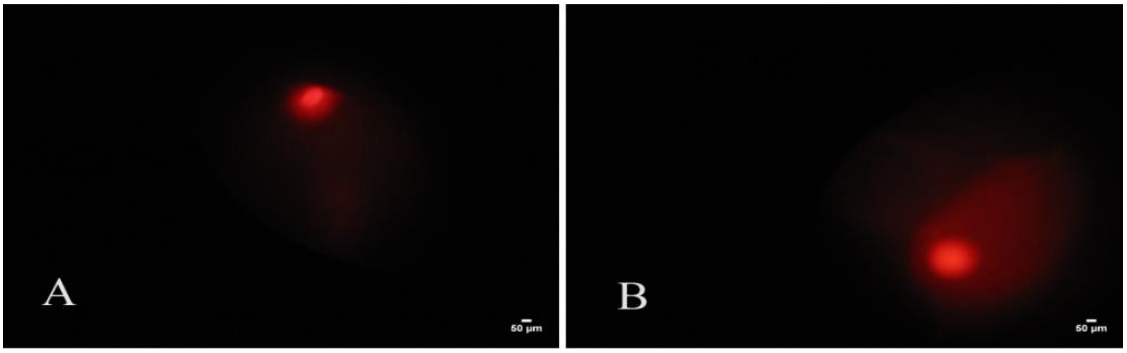
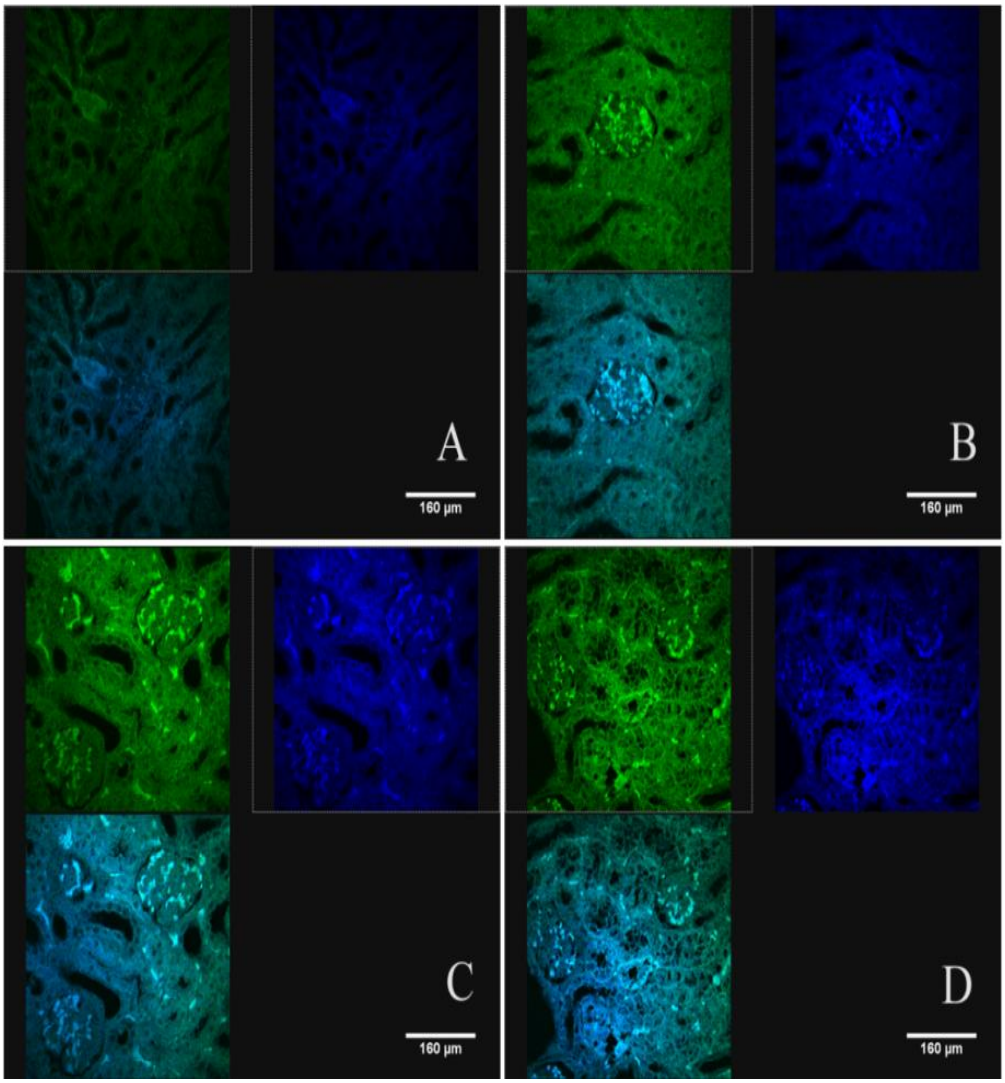


Fig. 3.27 Photomicrographs of DNA damage in kidney cells of buprofezin treated mice (A) Untreated Control, (B) Treated group. Magnification = 400X, Scale bar = 50µm.

3.4.7. TUNEL Assay

TUNEL assay revealed apoptosis in the paraffin fixed embedded tissue sections of kidney A two-fold increase in apoptosis in cells with 2 µg increase occurred in buprofezin doses (4.0 µg/kg, 6.0 µg/kg and 8.0 µg/kg b.w.) compared to controls (Fig.3.28).

Fig. 3.28 Photomicrographs showed apoptosis in kidney tissue sections of mice treated with buprofezin (A) Positive control (B) 4.0 µg, (C) 6.0 µg (D) 8.0 µg dosed groups. Magnification = 1000X, Scale bar=160µm.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DISCUSSION

DISCUSSION

The current study investigated the potential toxic effects, a moulting inhibitor insecticide buprofezin (BPFN), a thiadiazine compound on albino mice Balb/c following exposure to short term (24 h) increasing doses. The study demonstrated no detectable changes in the body weight or organs (liver & kidneys) weight. All biochemical, genotoxic and morphological alterations were found dose dependent. Significant increases occurred in liver enzymes, the ALT and AST. Increases occurred in serum urea and creatinine, while decrease occurred in tissue total proteins. Oxidative stress markers, ROS and TBARS were elevated, while noticeable decreases were found in the antioxidant enzymes, SOD, CAT and POD. Tissue histology demonstrated alterations in cellular morphology, which was indicative of tissue necrosis and DNA damage. TUNEL and Comet assays further confirmed excessively cellular and genotoxic damage caused by buprofezin to mammalian tissues.

Although no alteration was detected in the body weight or organs weight upon buprofezin treatment, past studies with pesticides have shown reduction in body weight and proliferation in organs weight. After other finding decrease in body weight of rats were determined once administered orally with 0.2, 2.0 and 20 µg/kg body weight doses of bisphenol A (BPA) for 30 days (Bindhumol et al., 2003). Thirty days exposure upon 2.0 and 20 g/kg BPA doses via s.c (subcutaneous) injections to male and female rats revealed no effect on body weight (Honma et al., 2002).

In current study no alteration was identified in body weight of buprofezin administered mice. Yasir et al. (2014) demonstrated significant reduction in the body weight of mice exposed for 15 days to high dose 15 mg/kg imidacloprid. Oral toxicity of imidacloprid conducted for 90 days with doses of 0, 5, 10 and 20 mg/kg in female rats indicated reduction in body weight and increase in liver, adrenal and kidney weights at this dosage levels (Bhardwaj et al., 2010).

The body weight increases of all malathion concentrations 0.1, 0.5, 0.10, 0.20 mg/kg/day through diet compared to untreated mice up to 90 days, and increase in liver weight occurred of all malathion concentrations 0.1, 0.5, 0.10, 0.20 mg/kg/day via diet

to mice (Karware 2013). Slimen et al., (2018) demonstrated sub-acute exposure of prepubertal male mice to malathion in corn oil at the dosage of 200 mg/kg (b.w.) through gavage indicated decrease in body weight, in disparity by increase in weights of both kidney and liver.

In contrast no significant consequence on body weights occurred nevertheless an increase in liver weight, after the effects of oral dosage of endosulfan 0.24 mg/ 100 g body weight to *Mus musculus* daily for 90 and 180 days (Ergul et al., 2006). This increase and decrease in body or organ weight were detected due to continuous exposure to pesticides for 15, 28, 30, 90 and 180 days. Reason was seeming to be that experiment was conducted for 24 h short exposure, obviously it was not expected that change. Trial experiments showed that high dose animals died, thus it was change in body weight.

Liver damage is indicative of liver enzymes. The present study showed elevation of the ALT, AST and creatinine levels with buprofezin. Liver enzymes ALP, ALT and AST levels elevated when male mice administered the dose of chloropyriphos and cypermetherin in combination on alternate day for 15 days resulted in subacute toxicity (Khan, 2006). Other study, significantly increased levels of serum enzymes ALP, ALT, AST, and LDH subsequently 45 days exposure to 1 and 10 mg/l of FPN (Abdel-Tawab et al., 2015). The present finding of buprofezin treatment to mice demonstrated elevated levels of AST, ALT. ALT is an enzyme found chiefly in the liver. Cellular damage to liver tissue produced a substantial rise in the blood level of this enzyme, a fact interpreting it an exceptional marker of cellular necrosis. Serum ALT activity is commonly related with hepatotoxic effect but later is not always associated with the histological conclusions (Ozer et al., 2010).

The presence of tissue degeneration was revealed by histopathological analysis, such as cytoplasmic vacuolization, cell degeneration-necrosis. In addition to cytoplasmic degenerative characteristics, hepatocytes also presented nuclear pyknosis symptomatic of apoptosis. Yano et al. (2002) reported that rats which were administered 0.1 to 0.2% Spinosad (SPD) showed minor increase in ALT, AST, nitrogen, urea, cholesterol, and triglyceride level. Further study, (Stebbins et al., 2002)

indicated mice administered to 0.12% spinosad had significant rise in AST and ALT enzymatic levels.

The common enzymes for the analysis of liver cell destruction are enzymes which comprise ALT and AST (Nnodim, 2010; Tousson, 2011; Alm-Eldeen, 2015). The rise in levels are unevenly proportional to tissue destruction (Ekam, 2007; Ogbonnia, 2008; Nwaehujor, 2011; El-Naggar, 2015).

Proteins are the functional and essential sources of the cells; thus, proteins play extensive part in cellular biological functions in fish and other vertebrates (David et al., 2004). It was revealed that prolonged exposure to carbosulfan, which is an extensive systemic carbamate insecticide strictly related to its core metabolite carbofuran, carbosulfan delivers control of a wide variety of foliar insect pests and soil-dwelling caused decline in the level of protein in the liver of male and female mouse. Harper et al., (1977) demonstrated significant reduction of overall protein level was owing to catabolism of protein and/ or malfunction of liver. Swamy et al., (1992) have described that the decrease in soluble proteins and total proteins show their metabolic consumption.

There was also claim that the cypermethrin showed no substantial impact on the liver tissue of rat total protein content (Altug et al., 2006) while decrease in the protein level of mice kidney and liver tissue due to buprofezin toxicity was revealed in the current study. As protein degradation was caused by elevated levels of urea and creatinine detected in serum examination.

Apart from other roles most important tasks of kidneys as blood filtering and purifying organ from waste materials ultimately expelled from the body as urine. The kidneys also support thermoregulation of the body. The activity of serum enzymes; creatinine and urea were significantly increased after forty-five days treatment of 1.0 and 10.0 mg/L of fipronil in rats (Abdel-Tawab et al., 2015). This relates the present finding of buprofezin exposure to mice showed increase levels of creatinine and urea in the kidneys when compared to control group and among the buprofezin administered groups.

ROS generation was identified in the present study significantly high in toxicant exposed mice associated to control mice. Nordberg and Arnér, (2001) explained, ROS obviously produced in animal cells during following respiration. Le Bras et al. (2005) demonstrated chief ROS generating radical like superoxide anion, hydroxyl, hydrogen peroxide. However, Small et al., (2012) stated that ROS are cellular toxins for cell survival, they are naturally counteracted by the internal antioxidant defense system primarily GSH, GPx, CAT, and SOD. Once an imbalance between ROS production and antioxidants was noted, the cell becomes susceptible to severe oxidative stress-induced damage. Abdel-Daim et al. (2015) and others explained that ROS can attack cell membranes and other cellular molecules, producing lipid peroxidation, protein oxidation, and DNA damage, which results in loss of function and cell disruption and can lead to diseases such as cancers, renal failure, diabetes, and atherosclerosis (Klaunig et al., 2010)

It was described earlier as antioxidant summary as well as LPO offered strong depiction of oxidative stress beside insecticide noxiousness. The basic origin of ROS generation within a cell was H_2O_2 production, by various pathological methods and physiological circumstances (Krohn et al., 2007). Reactive oxygen species (ROS) are mostly produced as free oxygen radicals, hydroxyl radicals by H_2O_2 through several mechanisms (Halliwell et al., 1992). LPO was started with ROS generation, (Patil and David, 2013) furthermore explained effects on DNA impairment. Earlier Yang et al., (1999) described LPO main method for H_2O_2 initiated cellular impairment.

Aboul-Enein et al. (2012) stated that the toxic results of different doses of spinosad (SPD) 35 and 350 mg/kg (b.w.) on liver tissue architecture was facilitated mainly through ROS production. ROS production could alter function and activity including nuclear pyknosis and DNA impairment. These evidences related to the present results in mice when ROS level increased significantly also TBARS in verified tissues (liver, kidney) of mice Balb/c exposed to buprofezin.

It was described earlier the physiological pressure in different organs like brain, stomach, liver and kidney of mice via ROS production brought about by harmful buprofezin treatment. This discovery is in favor of the present study as ROS and TBARS level elevated upon short term dose administration of buprofezin as related to the control group Ji et al., (2016). Currently, however activities of antioxidant enzyme

(SOD, CAT) showed reduction in its affects in kidney and liver tissues in response to buprofezin treatment. This shows pressures and letdown antioxidant defense mechanism owing buprofezin-produced physiological pressure on body. Increase LPO, ROS levels described, when rats orally administered different concentrations of 62.5 mg/kg/day, 125 mg/kg/day, and 250 mg/kg/day pendimethalin, member of dinitroaniline herbicide used to control crop and non-crop areas to for landscape conservation and lawn maintenance (Irshad et al., 2018).

Al Harib et al. (2014) explored insecticide originated physiological pressures stimulated oxidative degradation of lipids that carried antioxidant enzymes utilization to prevent peroxides. Generally, stability sustained between oxidative stress production whereas removal by antioxidant enzymes.

Earlier describes the disproportionate ROS production effect in damaged antioxidant resistance mechanism one after the other, reasons physiological pressures (Kavitha and Rao, 2008). Presently, CAT, SOD, POD activities decreased significantly following buprofezin administered groups. GSH activity reduced significantly between the buprofezin administered groups. Comparable decrease in GSH activity was obtained when rats administered orally 250 mg/kg/day pendimethalin (Irshad et al., 2018).

SOD and CAT are defensive Monteiro et al., (2006) describes beside defense impairment initiated via oxidative degradation of lipid by their capability to search superoxide ions and OH-. SOD and CAT illustrate mutual reaction, Trenzado et al., 2006 explained that like SOD brings about disproportionation of O_2- anion into H_2O_2 and H_2O brought about by SOD, while CAT activity detoxifies this H_2O_2.

Oxidative stress caused by methomyl, a carbamate insecticide used for the control of insects and spiders mites concluded direct interaction as well as absorption, exposure to 1 mg/kg (b.w.) for 10, 20 and 30 days in CD-1 mice not only restricted elevation in oxidative degradation of lipid, reduction in glutathione, similarly obstructed oxidation resistance mechanism (Fatma et al., 2013). It was reported that sustaining ROS levels currently reflected resistance mechanisms were operational below appropriate levels.

Amongst SOD, CAT and GST operated in assistance non-enzymatic defense mechanisms such as GSH to deactivate free radicals generation respond oxidative stress, and little variations in the biochemical mediations of enzymes might show affected consequences cell proteins fighting, DNA and lipids to physiological impairment (Auten and Davis, 2009).

Droge, (2002) describes that SOD played an important part in the purification of ROS by bringing out disproportionation of O_2- into H_2O_2. Reduced SOD and CAT activities have been found in rats administered orally with different meditations 62.5, 125, and 250 mg/kg/day of pendimethalin (Irshad et al., 2018).

Reduction in CAT action was established during present research work. Klaassen, (1990) explained that this might be owing to normal H_2O_2 alteration through Fenton reaction to the OH^1-radicals that was Fenton reaction's last toxic agents. Cheng et al., (1981) described catalase as universal enzymes, principal antioxidant defense component catalyze decomposition of H_2O_2 to H_2O.

Ozden et al. (2009) demonstrated the consequence of methomyl on SOD and CAT activities were agreement with several studies. Kamboj et al. (2006) those described significant changes in SOD and CAT activities in diverse insecticides exposure to rat organs. The substantial decline of GST activity in kidney of male mice, when administered methomyl which might show inadequate detoxification and undoubtedly manifested to reduce GSH levels (Fatma, 2013).

Histological observations of mice administered to increasing doses of buprofezin marked alterations, abnormal hepatocyte architecture, tissue necrosis, vacuolization, and damaged glomeruli, vacuolation, pyknosis in nuclei. Histologically, low dose of cypermethrin, induce neurotoxicity in insects produced slight disorganization of hepatic laminae and high dose produced necrosis of hepatic cells, with pyknotic nuclei and dilation of sinusoids with highly disordered hepatic laminae in both male and female rats for 30 days (Grewal et al., 2010). Biernacki et al. (1995) described hepatotoxic effect of cypermethrin in rabbits. It was detected following exposure of animals to several hepatotoxicants that, necrosis of hepatocytes and fatty degeneration (Thomas, 1984).

Mature male rats treated via gavage to 0.0, 2.0, 4.0, or 8.0 mg/kg of diflubenzuron doses for twenty-eight days, no substantial histological variations could

be observed in the kidney and liver tissues (de Barros, 2014). Other study, hepatocytes of rats exposed to different concentrations 62.5, 125, 250 mg/kg/day of pendimethalin for 14 days showed appearance of hyperplasia and swelling, pyknotic nuclei, vacuolization and dilation in sinusoids. In kidney, increased Bowmans space, dilation of renal tubules, dilation of blood vessels and severe necrosis was histologically observed (Irshad et al., 2018).

Kerem et al. (2007) demonstrated at high dose fenthion groups 75.0 or 100.0 mg/kg, and insignificant damage, like sometimes moderate central lobular injury and hepatocyte swelling and vacuolization was described. Further study validated, rats were daily injected for two to four weeks to 80 and 100 mg/kg FNT separately. Histopathological alterations in liver of treated animals indicated, tissue congestion, hepatocyte swelling, necrosis and fatty degeneration (Al-Jahdali et al., 2007). Histopathological variations in the liver of male Wister rat in other result exposed to different doses of malathion; the oral single dose administration to malathion (1/50 LD_{50}) initiated degenerative changes in the liver in the form of cell parenchyma degeneration in 80 % of animals, whereas in 10 % of rats following the dermal application, changes in cell parenchyma degeneration were detected (Tos-Luty et al., 2003).

Luty et al., (1998) demonstrated changes in the liver histo-architecture of rats after dermal exposure to organophosphate dichlorvos initiated in appearance of mononuclear cell infiltration in the liver. The albino mice following 10, 20 and 30-days administration of carbosulfan indicated histopathological changes liver for like dilation in central vein and sinusoidal spaces among overgrown liver cells through impaired nuclei, hyalinization, vacuoles (Ksheerasagar and Kaliwal, 2006).

It was described earlier that male rats treated with dimethoate, carbaryl and endosulfan showed histopathological variations in liver comprising cells having single nucleus infiltration, overcrowding, and hepatocellular damage, hydropic degeneration (Selmanoglu and Akay, 2000). Sharma et al. (2005b) recognized in earlier times when of male rats were exposed to dimethoate at doses of 6.0 and 30.0 mg/kg for 30-day initiated infected veins, focal hepatocyte necrosis, centrizonal congestion. The effects

of four weeks management of imidacloprid on male Sprague Dawley rats exhibited histological alterations in liver noticeable vasodilation, blockage of central vein, sinusoidal spaces, degenerated hepatocytes and vacuolation/fatty change (Soujanya et al., 2013).

The eight-week study of four groups of Wister albino rat exposed to three treated groups sequentially 25.0, 50.0, 100.0 mg/kg (b.w.) of fentrothion for continual 28 days in comparison to control groups, showed histopathological variations in liver and kidney. In low treated group 25.0 mg/kg (b.w.), noticeable inflammation of liver cells, necrosis, adequate overcrowding of the blood vessel and inflammatory leucocyte infiltration were found, in modest and high treated groups 50.0, 100.0 mg/kg (b.w.), hepatocytes disintegration by necrosis, severe congestion hepatocyte swellings and hemorrhage hypertrophy of hepatocytes. In kidney histological explanations, mild degeneration, mild congestion and hemorrhage was observed in rats treated with low dose 25.0 mg/kg (b.w.) FNT, at doses 50.0, 100.0 mg/kg (b.w.) more disintegration of cells of renal tubule, tubular enlargement, adequate overcrowding, reasonable loss was noted. In the current study, when 24 h treatment of BPFN followed by mice presented damaged liver and kidney tissues. The histopathological observation of liver and kidney exposed to high dose 100 mg/kg FNT showed significant damage in rats (Afshar 2008).

The histological consequences of the study showed that 30 days sub lethal diazinon, is an organophosphate insecticide which is the most frequently used to control cockroaches, fleas, silverfish and ant mechanism of action is to influence the enzyme acetylcholinesterase, exposure to mice at dose rates of 30, 60 and 120 mg/kg (b.w.) caused deteriorating variations, comprising vacuolization, hepatocytes increased in the number of kupffer cells, enlargement of vein, congestion and mononuclear cells infiltration. Kidney tissue showed many histopathological variations like cell containing single nucleus penetration, overcrowding, glomerular loss, glomerular absorption by hypertrophic cells of Bowman's capsule (Sabry, 2017).

Examination of liver sections of mice received low (35.0 mg/kg (b.w.) and high 350.0 mg/kg (b.w.) doses of spinosad, exhibited mild histopathological changes like vanishing frequently as well as outwardly organized liver cells, recognition of fainter stained hepatocytes widespread disintegration and localized liver cell death and aggregation into small patches of collagen fibers.

Likewise, the above said doses of SPD in kidney sections of mice presented inadequate histological variations, widening of renal tubules, damaged glomeruli, localized cells and nuclear death, cytoplasmic vacuolation, vacuolation, severe lymphatic infiltration were also detected.

Sabry and El-Naggar (2017) established histopathological deformities in kidney tissue of methomyl treated mice. Disintegrated glomeruli as well as proximal tubules were identified in the kidney after 20 and 30 days methomyl action. Manna et al. (2004) described comparable histological observations in kidney of rats treated with cypermethrin for twenty-eight days where localized cell death turgid as well as shedding of outer layered cells. Also, Ksheerasagar and Kaliwal (2006) recommended that carbosulfan, showed deteriorating alterations due to the lipoperoxidative damage brought by methomyl treated mice kidneys as well as subsequent accumulation of free radicals. Fipronil-induced histopathological changes in kidney tissue with variations in renal function tests, comprising creatinine and urea, intervened by oxidative impairment (Mohamed et al., 2018).

Male Balb/c mice administered doses 0, 0.25, 0.75, 2.25 or 6.25 mg/kg (b.w.) of fumonisin B_1 via subcutaneous injections for 5 days. TUNEL assay established the presence of dose-related apoptotic cells at all dose levels in kidney and liver (Sharma, 1997). Ji et al., (2016) confirmed the study of living properties of sub lethal mediations 3, 10 and 30 μM of buprofezin on vitality mechanisms of liver to regulate its hypothetically hepatotoxic properties. The 30 μM concentration buprofezin following cell sustainability, TUNEL, cleaved casepase-3 cause nonsignificant cellular damage. It was described earlier LPO was introduced because of ROS production, approached DNA impairment (Patil and David, 2013).

Krohn et al., (2007) defined H_2O_2, an effective deadly chemical, and affect important constituents during ROS production as mediator in cell activities like cancer. Halliwell and Gutteridge, (1985) explained cell damage beneath definite toxicological conditions explained that ROS basically obtained from hydrogen peroxide. Ali et al., (2015) and others described that ROS, H_2O_2 strictly impair the macromolecules and other biological entities (Ahsanuddin et al., 2016). Amongst all the macromolecules, DNA furthermost susceptible to noxious constituents, deadly chemicals, contaminants thus exposed to genetic impairment (Ullah et al., 2016).

Collin, (2004) explained comet assay technique to evaluate DNA damage. Comet assay was consequently cast-off to conclude the genetic effects brought by buprofezin because of physiological stress. McKelvey-Martin et al. (1993) demonstrated comet development was attained due to DNA ends and allowed to move to anode in electrophoretic areas. Chlorpyrifos (CPF), methyl parathion (MPT), and malathion (MLT) when given singly or in combination to rats showed DNA damage in liver, kidney, and other tissues (Ojha et al., 2013). Irshad et al. (2018) demonstrated significant levels of DNA damage both in rat liver and kidney tissues at orally administered varying doses of 62.5, 125, 250 mg/kg body weight/day for 14 days of pendimethalin (PND).

Kidney is involved in elimination of noxious elements, homeostasis, and erythropoiesis kidney is exposed towards lethal effects openly. The noticeable genotoxic effect of fipronil on all used doses 4.75, 9.50, 19.00, and 31.70 mg/kg (b.w.) consumed at single exposure to lungs and spleen and other organs of rat was ascertained through the Comet assay. Organ specificity of genotoxic effects of the pesticide was revealed. The liver was most sensitive to fipronil (Lovinskaya et al., 2016) whereas in the current study, the genotoxic findings were more pronounced to buprofezin toxicity on kidney and liver tissues both.

The present study indicated that the DNA damage in liver and kidney tissue of mice shows significant alterations. DNA impairment was resolved in relations of parameters % tail DNA as well as mean tail length of comet. Noticeable significant decrease was observed in head length, comet length, tail length, tail moment and oil tail moment of liver and kidney tissues of mice on buprofezin exposure.

Future Perspective

In future, study should be directed

- Protective effects of vitamin E and C treatment on buprofezin induce Biological, Physiological, and Histopathological alterations in mice.
- Advance studies are needed to enhance the understanding of effects of buprofezin, including the chronic effect.
- Records should also be collected from human population, chiefly farmers, as regards determination of buprofezin levels in humans to create and adjust preventive measures as to date no specific antidote against buprofezin poisoning is identified.
- To collect facts from other invertebrates and vertebrate's species as inadequate work has been reported in regard to this insecticide.

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