An Investigation on Buprofezin Inhalation Toxicity and its Effects on Male Albino Mice

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A thesis submitted to the Department of Animal Sciences, Quaid-I-Azam University, Islamabad in the partial fulfillment of the requirements for the degree of

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بسم الله الرحمن الرحيم

CERTIFICATE

This dissertation "An investigation on Buprofezin inhalalation toxicity and its effects on male albino mice" submitted by **Ms. Momna Nazir**, is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Zoology (Physiology).

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DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is my original work. I have not previously presented this work elsewhere for any other degree.

Momna Nazir

Dedicated

To

My beloved Parents,

Sisters, Teachers, and Friends

whose love, encouragement, and

blessings helped me navigate life's

transitions.

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Momna Nazir

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LIST OF ABBRRVIATIONS

BSA	Bovine serum albumin\
CAT	Catalase
DEPPD	Diethyl para phenyldiamine
DTNB	5,5-Dithio-bis 2-nitrobenzoic acid
FeSo4	Ferrous sulfate
GSH	Reduced glutathione
HCL	Hydrochloric acid
H2O2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
H & E	Hematoxylins and eosin
MDA	Malondialdehyde
NBT	Nitro blue tetrazolium
PMSF	Phenyl-methyl-sulfonyl fluoride
PBS	Phosphate buffer saline
POD	Peroxide
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulfate
TBARS	Thiobarbituric acid reactive substances
TBA	Thiobarbutiric acid
TCA	Trichloroacetic acid

ABSTRACT

Buprofezin is a blocker of chitin synthesis belonging to the thiadiazine class that is effective at targeting insects during their molting process. Buprofezin is sprayed that is stable under acidic, and alkaline conditions and it is also thermostable and photostable, and its residues were insistent. Analyzing the potential toxicity of buprofezin, particularly its effects on the lungs, liver, and kidneys this experiment was performed. Mice were exposed to buprofezin mist using a nebulizer in a whole-body exposure. Group-I control was given distilled water mist whereas treated groups II, III, and IV were given 37mg/ml, 250 mg/ml, and 500 mg/ml doses of buprofezin respectively, for 60 min over 21 days. Statistics were applied to comparisons at a p- 0.05. Oxidative stress markers and antioxidant enzymes such as reactive oxygen species (ROS) and thiobarbituric acid reactive substance assay (TBARS) were also increased while tissue total protein, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) and non-enzymatic reduced glutathione (GSH) were decreased in lungs, liver, and kidney tissues. The results of histopathological examination of the lungs, liver, and kidney tissues indicate notable morphological deviations in the treated groups. Specifically, the lungs exhibited a decrease in the tunica media of pulmonary vessels, as well as hemorrhage and endothelial cell hypertrophy. In addition, bronchioles showed signs of catarrhal inflammation, and a decrease in the spaces of alveolar sacs and alveoli, along with the presence of inflammatory lesions. Regarding the liver, hepatic vacuolation, congestion, and vascular degeneration in blood vessels were observed, along with coagulative necrosis. Lastly, in the kidneys, there was evidence of vascular congestion, shrinkage of the glomerulus, an increase in the space of Bowman's capsule, and cellular infiltration. These results demonstrate the detrimental effects of exposure to buprofezin on the morphological integrity of the lungs, liver, and kidneys. Additionally, further research is still required to investigate other aspects like behavior and neurotoxicity.

INTRODUCTION

1 INTRODUCTION

1.1 Pesticides

Pesticide is defined as a product that contains chemical or biological substances or a mixture of them, which is designed to manage, control, repel, or eliminate any pests or regulate plant growth. Pesticides are composed of active molecules that belong to different chemical groups, and they can interfere with the normal biological processes of living organisms. As a result, they can disrupt various metabolic pathways, leading to the development of multiple illnesses. Pesticides are typically classified based on the grade of pest they are designed to control, such as herbicides, insecticides, or fungicides, or based on their chemical composition, such as organophosphates, organochlorines, or pyrethroids (Barros et al., 2016).

Pesticides are a commonly utilized tool in various areas of agricultural production, aimed at preventing or minimizing damage caused by pests, thereby enhancing both the return, efficiency, and refinement of crops. In addition, pesticides are often used to improve the aesthetic appeal of produce, which can be a crucial factor for many consumers (Cooper & Dobson, 2007; Oerke & Dehne, 2004).In some cases, the use of pesticides can also contribute to the enhancement of the nutritional quality of food, as well as its reliability (Boxall, 2001; Narayanasamy, 2006).

Although pesticides are widely used and well-liked, there are significant apprehensions in relation to the potential health liabilities that may arise from the vulnerability of farmers during the mixing and application of pesticides, as well as when working in fields that have been treated with these chemicals. Additionally, dregs of pesticides found in food stuff and water have raised concerns about the possible impact on the robustness of the general demography (Maroni et al., 2006; Pimentel, 2005; van der Werf, 1996; Viollet et al., 2009; Wilson & Tisdell, 2001).

1.1.1 Consequences on the natural world

The operations of pesticides in horticultural settings can have unintended consequences that affect the wider environment. For instance, when pesticides are splatter on crops, they can drift through the aeration and contaminate other modules of the environment, such as the soil or water. Furthermore, pesticides that are executed promptly to the soil can be swept away by surface runoff, or they can seep into the underneath of the topsoil and sink into the deeper soil regions and underground water. This can result in contamination of nearby surface water

bodies, which may have adverse effects on aquatic ecosystems and other living organisms. It is essential to use pesticides responsibly and to adopt sustainable farming practices to minimize the potential influences of pesticide usage on the natural world. (Zacharia, 2011).

Pesticides can have a variety of effects on the ecosystem, from minor disruptions in ecosystem functioning to the loss of species diversity. In some cases, pesticide use for a long time can cause damage, while in other cases, the effects can be acute and fatal. For instance, organochlorine pesticides, which persist in the environment for extended periods, can contaminate various environmental components, such as underground water, food items, the atmosphere, and soil. This can have severe consequences for both human well-being and natural surroundings. Therefore, it is crucial to consider the potential impacts of pesticide use and adopt sustainable and responsible practices to minimize these effects.

1.1.2 Consequences on the not intended organism.

The use of insecticides can have unintended consequences for non-intended organisms such as natural predators, earthworms, and pollinators(Ware, 1980). In addition to killing pests, insecticides can also harm beneficial insects and other animals that play important roles in ecosystem processes. For example, pollinators like bees, drosophila, a few beetles, and avian creatures can serve as a pointer of environmental health, and their activities can be adversely affected by environmental stress resulting from pesticide use and habitat modifications. Moreover, the direct loss of insect pollinators due to pesticide use can result in an indirect loss of crops due to inadequate pollination. It is crucial to take measures to minimize the impact of pesticides on non-intended organisms and to promote viable and responsible farming practices that are less damaging to the environment (Kevan, 1999).

1.1.3 Consequences on the human beings

The operations of pesticides have participated in the improvement of the human condition by limiting the burden of diseases that are transmitted to humans by vectors. However, the extended and indiscriminate application of these chemicals has led to severe health consequences. Toddlers and minors are particularly susceptible to the harmful impacts of pesticides owing to their non-specificity and inadequate use. With the rise in pesticide use in the recent time frame, the possibility of accessibility to these chemicals are more significantly (Mahmood et al., 2016).

Pesticides can come into the human body in several approaches, including breathing in toxic air, dust, and fumes containing pesticides, ingestion by eating and drinking from polluted

sources, and epidermal assimilation by coming into close skin interaction with pesticides. Fruits and vegetables, in particular, are sprayed with pesticides, which can lead to soil and groundwater contamination and ultimately finish with the water intended for human consumption. Additionally, pesticide sprays can drift and pollute the air (Sacramento, 2008).

1.1.3.1 Consequences of short-term exposure

The short-term exposure consequences can include immediate symptoms such as headaches, eye and skin itching and discomfort, nose and throat irritation, tingling, redness, and vesicles on the cutaneous, unsteadiness, diarrheal disease, abdominal distress and feeling queasy, as well as unclear vision, blindness, and in rare cases, death ("Pesticides and Human Health ", 2014).

1.1.3.2 Consequences of long-term exposure

Extended exposure to pesticides can result in severe and lasting effects that harm multiple body organs. Prolonged pesticide exposure has been linked to various neurological strength problems and recall impairment diminished visual capacity and decreased neuromuscular communication (Lah, 2011). Additionally, prolonged vulnerability can harm the immunoregulatory response, leading to sensitivity, respiratory disorders, and allergic reactions (Culliney et al., 1992). Pesticide deposits have been noticed in the bloodstream in patients of abnormal cell growth, and pesticides are linked with various cancers, including blood cell cancer, cerebral tumor, lymphatic system tumors, tumorous growth in the breast tissue, prostate neoplasm, and ovarian & testicular cancer. Furthermore, prolonged pesticide exposure can disrupt reproductive hormone levels, resulting in fetal death congenital anomalies, miscarriage, and failed conception. Lastly, prolonged vulnerability can also the destruction of the liver, lungs, and kidneys, and may result in hematological disorders ("Pesticides and Human Health ", 2014).

1.1.4 Pesticide stability in air

When pesticides are sprayed, a portion of the spray exists in the form of small droplets or particles known as aerosols, as well as in the gas phase as a pesticide. These aerosols are extremely tiny and are unable to reach the intended target area, making them difficult to collect using drift collectors. As a result, the amount of pesticide spray that exists in the form of aerosols and gas phases should be regarded as a loss that must be taken into check, in addition to any drift that may occur.

After applying pesticides, some of the residues can evaporate and transfer into the gas phase. This process is referred to as volatilization, which can cause the residues to disperse through the air. The speed and degree of emission depend on various factors, such as the pesticide's physical and chemical properties, the implementation conditions, the climate parameters during and after implementation, and the traits of the targeted area.

A commonly acknowledged fact is that the volatilization processes can be affected by several factors, including relative humidity, air temperature, air pressure, and wind flow (Grass et al., 1994; Guenzi & Beard, 1970; Harper et al., 1976). Moreover, the metabolism of substances can be impacted by irradiation through direct or indirect photolysis(Hock et al., 1995).

The intensity of volatilization is significantly concerned with the vapor pressure of a substance. Compounds that possess high vapor pressure tend to have a greater tendency for volatilization, irrespective of other external factors. Conversely, the volatility of compounds with standard vapor pressure is highly manipulated by various ecological and implementational-related attributes. In contrast, compounds with very low vapor pressures are not considered volatile.

When pesticides are used, they can move from the intended target area to other areas through three main activities:

(i) Emission into the air
(ii) Transportation and Transformation in the air
(iii) Settling onto surfaces from the air

Each of these activities is unique and requires a specific approach to modeling them.

1.1.4.1 Emission into the air

When a pesticide is being applied, it can release into the air by three paths:

a. The amount that settles onto the desired target area.
b. The amount that settles onto neighboring non-target areas.
c. The amount that is lost in the air during an implementation.

Depletion of pesticides to the air can occur via two mechanisms: firstly, the evaporation of small droplets while they travel from the spout to the designated region, and secondly, the conveyance of minute particles (less than 100μm) over longer distances, which cannot be detected by drift collectors. Volatilization of the deposited chemicals during treatment is the

primary act several intervals after implementation, with various operations having effectively ceased.

1.1.4.2 Transportation and Transformation in the air

Airborne transport of pesticides takes place via two mechanisms, namely dispersion, and convection. Wind-driven convection is typically the more dominant of the two, owing to its higher transport velocity, meaning that wind speed and direction exert a substantial impact on deciding the direction and extent of pesticide transport. Convection can transport pesticides in both gaseous and granular forms (i.e., tiny droplets absorbed onto tiny fragments). While the degeneracy of pesticides in the wind is of limited significance for nearby-distance delivery (up to 100m), it is a key parameter for estimating prolonged-distance delivery maximum and typical range of travel. Conversely, the upright diffusion layer is a more fluctuating parameter and thus has a greater impact on nearby-distance delivery compared to prolonged-distance delivery.

1.1.4.3 Deposition onto surfaces from the air

The deposition of pesticides is influenced by various aspects. Arid deposition is a highly significant process for nearby-distance delivery, as per Good Agricultural Practice, pesticide application is not conducted before rain occurrences. For, prolonged-distance delivery is the total of both arid and aqueous deposition, and it must be viewed as a reservoir for the matter present in the wind, which ultimately lessens its maximum travel (Kubiak et al., 2008).

1.2 Insecticides

Insecticides are chemical or biological substances that are designed to control and eliminate insects. They have been utilized for centuries across the globe, and people can be exposed to them in various settings, such as their homes, workplaces, and gardens, and through trace amounts in food. In agriculture, horticulture, and forestry, insecticides are used extensively, which makes farmers, their families, and farmhands most vulnerable to these chemicals. Additionally, insecticides are utilized to regulate vectors, like ticks and mosquitoes, that are responsible for spreading public health diseases like malaria, Lyme disease, and West Nile disease. However, insecticides can be harmful to humans and animals, and they are involved in malicious or accidental poisonings of pets, wildlife, and birds, as well as suicide attempts in developing countries. Insecticides comprise numerous chemicals belonging to different classes, and the impact on the different classes of insects, and it also impact on other animals, utilizing diverse modes of operation. As a consequence of their distinctive chemical nature,

insecticides act on various target sites, as well as on non-target sites like receptors and enzymes also with different types of molecules. Insecticides are mainly neurotoxic, meaning the primary focus is on the nervous system of insects, although on the additional bodily structures and physiological systems. The attachment sites and resulting chemical modifications can function as biomarkers for assessing exposure to and impact of insecticides. The insecticides undergo distinct cellular pathways, and the primary compounds or their derivatives are frequently employed as indicators of exposure through biomonitoring. Insecticides can cause detrimental health consequences that range from lesser to verse in non-target animal species, including human beings, as well as birds and wildlife. To summarize, insecticides are a vital tool in controlling and eliminating insect populations, but their usage must be balanced with their potential to cause harm to humans and animals (Gupta et al., 2019).

1.2.1 Insecticide types and their toxic effects

There are binary grades of insecticides: synthetic & natural insecticides. Synthetic insecticides are grouped into distinct categories sorted by their toxicological mechanism, like pyrethroids, organochlorines, organophosphates, carbamates, and organochlorines, insecticides. Secondly, natural insecticides include substances like azadirachtin, rotenone, spinosad, and abamectin. These natural insecticides are derived from plants, microbes, or other natural sources and are generally considered to be less harmful to humans and the environment compared to synthetic insecticides. However, it's worth noting that all insecticides have the potential to cause harm and must be used with caution.

The prolonged and extensive usage of synthetic insecticides has preceded to the buildup of their remnants in various environmental elements such as food, water, soil, and even milk. This accumulation has resulted in negative health impacts on both humans and ecosystems. Abundant investigations have demonstrated that insecticides that are synthetic like cyfluthrin, malathion, chlorpyrifos, pirimiphos-methyl, dimethoate, and methomyl induce oxidative damage to the liver and kidneys of experimental animals.(Akhgari et al., 2003; Farag et al., 2003; Ngoula et al., 2007; Patil et al., 2008; Soni et al., 2011)

Insecticides which are natural comprise chemical compounds, mineral elements, and biological substances, and certain of these manufactured goods are accessible in the market, such as pyrethrins, azadirachtin, spinetoram, cube resin, avermectin, Bacillus thuringiensis, allium sativum, cinnamomum verum, capsicum,and ethereal oil products. The principal sorts of such insecticides are plant, minerals, ethereal oils, microbial agents, and soaps. However, the safety

or selectivity of natural insecticides are not completely risk-free, as some of them have unintended consequences. For instance, historically, arsenic and nicotine were used as natural pesticides, but they are now deemed unsafe and not used in modern-day (Mossa et al., 2018).

1.2.2 Exposure to insecticides

Exposure to insecticides can occur through various routes, including:

1. Inhalation: Exposure to pesticides can occur when they are airborne, such as during pesticide application or from pesticide residues that have evaporated. Pesticides can enter the body through the respiratory system when inhaled.

2. Dermal Contact: Pesticides can be absorbed through the skin and enter the bloodstream. This can occur during pesticide application or from contact with surfaces or objects that have been contaminated with pesticide residues.

3. Oral Ingestion: Pesticide residues can be present in food and water, and when consumed, can enter the body through the digestive system.

4. Accidental exposure: Accidental exposure can occur when pesticides are not stored or handled correctly, or when protective equipment is not used during pesticide application.

These routes of exposure to pesticides can result in adverse health effects, including acute poisoning, chronic health problems, and increased risk of certain types of cancer.

1.2.3 Inhalation

Inhalation is a crucial method of being exposed to air-borne contaminants, like the pesticides (Emmendoerffer et al., 2000; Richards, 2008) .Two vital organs lungs and liver that are most vulnerable to environmental contaminants, particularly chemicals sprayed into the air like insecticides (Dixon et al., 2008).

Airborne contaminants, such as toxic gases, fumes of pesticide particulates, aerosols, and volatile organic solvents, can enter the body through the lungs. The absorption of such toxicants is contingent on their physical and chemical pr attributes and absorption can occur in the nasopharyngeal area, tracheobronchial region, or the surfaces of pulmonary exchanges in the lung. However, lung alveoli or terminal bronchioles are particularly efficient at absorbing these contaminants and cause the majority of the resultant toxicity during inhalation exposure. Long-term exposure to substances that are retained in the respiratory tract can result in area-specific toxicity, which might manifest as lung cancer. In addition, toxicants that are absorbed by the

lungs may disseminate through the bloodstream and impact other distant organs and tissues of the body, kidney, and particularly the liver, chemicals are taken up by the hepatocytes via the bloodstream (Richards, 2008).

Over the past few decades, cypermethrin and other pyrethroids have been widely used in agriculture, gardening, and households worldwide. Although these insecticides have their advantages, they can have adverse effects, particularly when used in excessive amounts. Prolonged exposure to these chemicals can lead to severe health problems, including mutations in the genetic material, which may cause cancer. Experiments on mice exposed to cypermethrin have revealed significant aberrant cellular changes in the liver and lungs. Inhalation of the cypermethrin resulted in time-dependent changes in the pathomorphological of these organs (Emmendoerffer et al., 2000; Richards, 2008)The lungs, in particular, showed signs of the alveolitis along with pulmonary edema, and destruction in the lung cells due to exposure to pyrethroids(Tian, 1993).

The liver and kidney are both vital organs in the body and are susceptible to damage from enduring to injurious substances and their metabolites. While the liver is responsible for metabolizing and detoxifying harmful compounds, the kidney is involved in the elimination of waste and maintenance of fluid balance in the body. In addition to these functions, the kidney also performs other crucial roles. However, it has been observed in experiments on animals that natural insecticides can lead to harmful effects on the kidney.

1.3 Buprofezin

Buprofezin chemically known as 2-*tert*-butylimino-3-isopropyl-5-phenyl-3,4,5,6-tetrahydro-2H-1,3,5-thiadiazine-4 -one is extensively used for management of insect worldwide.(Ponkarpagam et al., 2022) .Buprofezin has the following structural formula

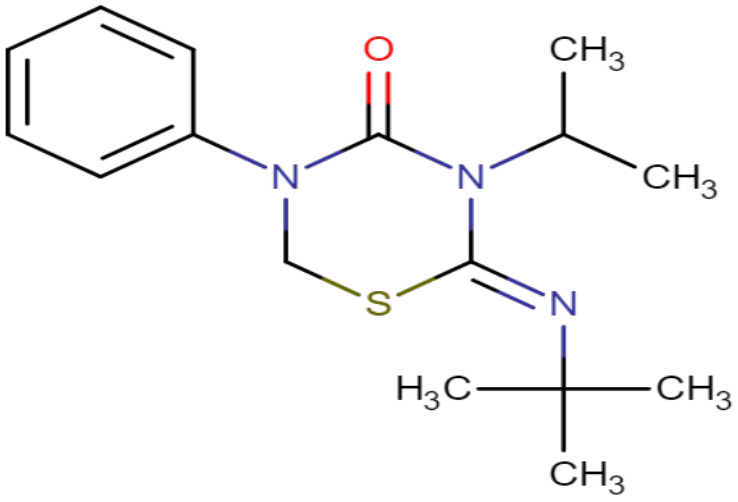

Figure 1.1 Structure of Buprofezin https://contaminantdb.ca/

Buprofezin is produced by Nihon Nohyaku in Japan.(Kanno et al., 1981)

Buprofezin is a unique insecticide that affects the growth and development of insects. It works by inhibiting the synthesis of chitin and shedding, which interferes with the insects' normal development process and leads to their death. Buprofezin is commonly applied to manage a variety of sucking pests, including leafhoppers, plant hoppers, jassids, thrips, whiteflies, aphids, and mealybugs. These pests belong to the orders Hemiptera and Thysanoptera and can cause significant damage to crops and other vegetation (Tan et al., 2014; Toscano et al., 2001)

Buprofezin is an insecticide that acts on insects through both inhalation and direct contact. It works by producing a vapor that insects inhale, which then disrupts their normal physiological processes and ultimately kills them. Additionally, the chemical can also adsorb onto the insect's cuticle or outer layer, leading to further toxicity through direct contact. This dual mode of action makes buprofezin an effective control option for a broad spectrum of pestilent insects in agricultural and horticultural settings (De Cock & Degheele, 1998; Konno, 1990).

Buprofezin is applied as a spray and is known for its stability under a wide range of conditions. This includes stability under both acidic and alkaline conditions, as well as its resistance to temperature changes and degradation by light. As a result, its residues can remain persistent in crops and soil, even with decreasing moisture content. It is vital to mention that the persistence of pesticide remnants, such as buprofezin, can lead to long-term environmental and health concerns. In some cases, it can contribute to the development of pesticide-resistant pests and affect the health of beneficial insects and non-target species. (MacBean, 2012)

Buprofezin has been usually used on crops such as fruit, tea, spud, rice, cotton, and vegetables, and it has been shown to have a long-lasting effect in killing the larvae of Nilaparvata lugens and Trialeurodes vaporariorum (Hegazy et al., 1990; Yang & Yang, 2007).

1.3.1 Mechanism of action

Buprofezin has the potential to execute as a chitin formation blocker. These inhibitors play a special role in the cuticle of insects, which is chiefly constituted of chitin, a basic polysaccharide made up of N acetyl glucosamine units. Chitin synthesis blockers have the potential to stop the biosynthesis and accumulation of chitin in the insect body, leading in the construction of new cuticles being prevented and the old cuticle becoming weakened, ultimately leading to the death of the insect (Gelbic et al., 2011). It decreases the number of Spodoptera litura (Fab.) young by blocking the biosynthesis of chitin during the molting procedure (Adel, 2012).

Buprofezin targets young, immature stages of homopteran pests, such as insects and mites, by preventing the formation of chitin in their exoskeletons. Chitin is a key component of the cuticle that provides structural support and protection to these pests. By preventing the incorporation of N-acetyl-[D-H3] glucosamine into chitin, Buprofezin causes the pests to die during the molting process when they shed their old exoskeleton and form a new one. This interferes with the pests' ability to grow and mature, leading to their eventual death. (Hatakoshi, 1992; Kanno et al., 1981) .

It has been noted that treatment with buprofezin leads to a reduction in fecundity and egg hatching in adult female insect (Ishaaya et al., 1988; Uchida et al., 1985). Buprofezin also has an impact on the group of Spodoptera litura (Fab.) by decreasing fertility, decreasing the hatch rate of eggs, making eggs sterile, and leading to the generation of abnormal larvae and pupae (Ragaei & Sabry, 2011).

Buprofezin is primarily used to control the growth and development of various insect pests, including the Nilaparvata lugens. One of the key advantages of using buprofezin is its long residual activity against N. lugens nymphs, which makes it an effective tool in controlling this pest. Unlike some other insecticides that have an immediate, acute insecticidal effect, buprofezin works gradually over time, making it a useful option for integrated pest-handling programs. Additionally, its unique way of operation, which targets the insect's growth and development, reduces the probability of insect populations developing resistance to the insecticide over time (Uchida et al., 1985).

The use of buprofezin has been shown to cause an increase in the death rate of S. furcifera by altering the manifestation of SfCHS1 and its two alternative exons as shown in figure (Wang et al., 2018).

1.3.2 Resistance to buprofezin

Buprofezin has the most extensively utilized insecticide for the management of brown planthopper. Unfortunately, this heavy usage has prompted in the rapid flourishing of resistance by the brown planthopper to buprofezin, extreme resistance seen within various territories of China ever since 2013 (Wang et al., 2008; Wu et al., 2018).

Research examining the processes of resistance to buprofezin in rice planthoppers, with a focus on the small brown planthopper, have revealed the expression of a cytochrome P450 monooxygenase gene (LsCYP6CW1) in a buprofezin-resistant strain of small brown planthopper. This overexpression was found to be 22.78-fold higher in the resistant strain, which showed a resistance level of 59.9-fold to buprofezin Studies using RNA interference techniques have confirmed that LsCYP6CW1 is responsible for the complex tolerance amongst the buprofezin and pymetrozine in the three wild population of small brown planthopper. These findings suggest that the tolerance to buprofezin in small brown planthoppers may be due to the involvement of cytochrome P450 enzymes (Zhang et al., 2017).

A new mutation, called the G932C non-synonymous amino acid switch, was found in the chs1 gene on chromosome 3. Interestingly, the occurrence and prevalence of the G932C mutation were found to be closely related to buprofezin resistance in brown planthopper. This same mutation, the G932C, is also present in buprofezin-resistant small brown planthoppers so it is confirmed that the G932C alteration is a significant factor in the buprofezin tolerance (Zeng et al., 2022).

1.3.3 Degradation of buprofezin

The widespread utilization of buprofezin has left behind residual traces in the environment, making it necessary to find effective solutions for its cleanup. Microbial degradation of buprofezin is seen as a viable option for the remediation of areas contaminated with this pesticide. The role of microbial degradation in getting rid of xenobiotics is significant (Cycon & Piotrowska-Seget, 2016). Up to now, several pure bacterial cultures that can break down buprofezin have been identified. Strain BF3 of Paracoccus sp. and strain DFS35-4 of Pseudomonas sp. were capacity to biotransformation of buprofezin, while strain YL-1 of Rhodococcus sp. and strain BF5 of Bacillus sp. were in a position of making in use of buprofezin just as the only supplier of the carbon and energy for their growth (Chen et al., 2011; Li et al., 2011; Li et al., 2012; Wang et al., 2016).

1.3.3.1 YL-1 and BF5 strain degradation pathway

The YL-1 and BF-5 strains were both derived from different sources. The YL-1 was obtained from a swampy field of rice contaminated with buprofezin, while BF-5 was obtained to the activated sludge of a buprofezin-supplying facility. Despite their different origins, both strains share a similar method of catabolizing buprofezin. This process involves the breakdown of the chemical into its constituent parts in the upstream catabolic pathway, resulting in the release of the heterocyclic ring 2-tert-butylimino-3-isopropyl-1,3,5- thiadiazinan-4-one (2-BI). The downstream catabolic pathway then further degrades the 2-BI. It's worth noting that the upstream catabolic pathway plays a key role in the degradation process of buprofezin.

The proposed catabolic pathway for buprofezin degradation by strain YL-1 is outlined in the diagram. Interim metabolites that have previously been identified are displayed in green, while indirect metabolites and enzymes that were found in this study are presented in blue. The key intermediates in the pathway include BF-DD (buprofezindihydrodiol), DHBF (dihydroxybuprofezin), RCDB (aromatic ring-cleaved dihydroxybuprofezin), 2-BI (2-tert-butylimino-3-isopropyl-1,3,5- thiadiazinan-4-one), N-BA (N-tert-butyl-thioformimidic acid formylaminomethyl ester), 2-IM (2- isothiocyanato-2-methyl-propane), and 2-IP (2-isothiocyanato-propane) (Li et al., 2012).

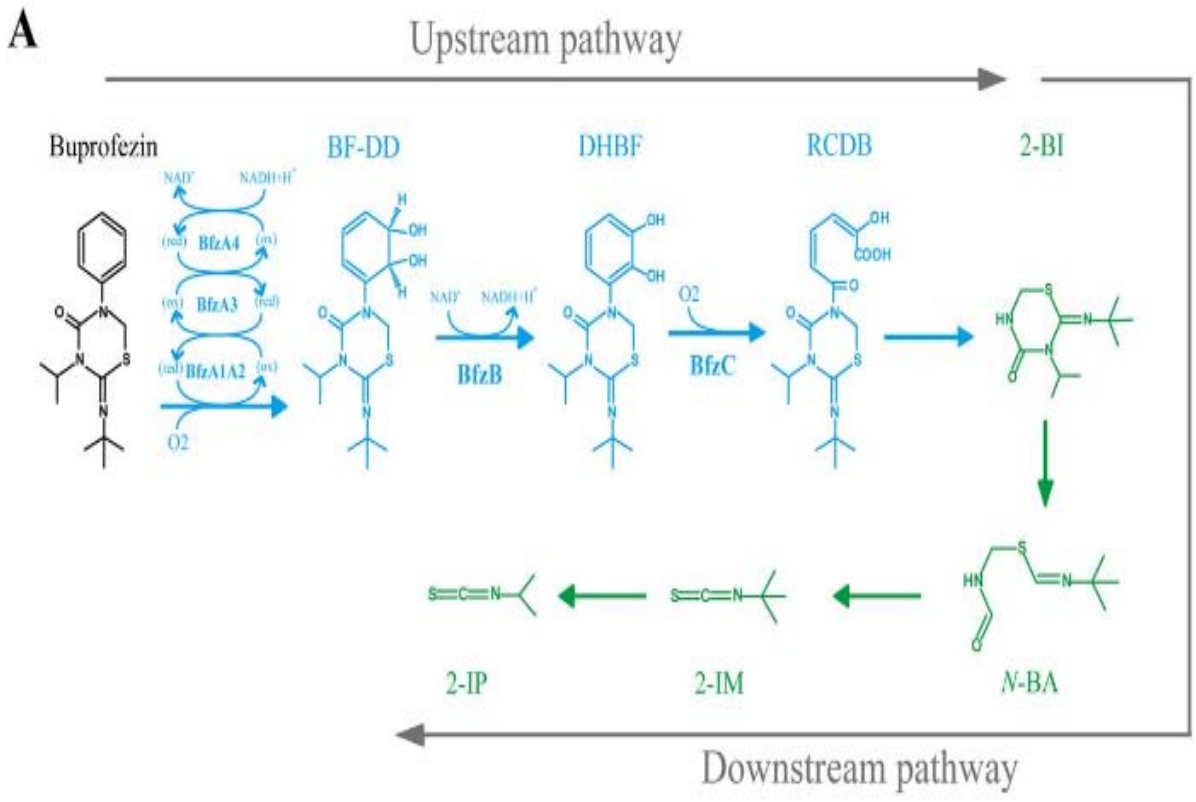

Figure 1.2 Buprofezin degradation pathway by strain YL-1 (Li et al., 2012).

1.3.3.2 Strain DFS35-4 degradation pathway

Pseudomonas sp. strain named DFS35-4, which is capable of breaking down buprofezin in the presence of additional carbon sources was discovered in soil that had been contaminated with the chemical over a long period of time. The strain was able to degrade more than 70% of the aggregate amount of buprofezin present dose of 50 mg/l within three days when grown in a culture medium. This strain also showed impressive degradation efficiency for buprofezin across a large range of pH amounts and temperatures. Results indicate that DFS35-4 has a significant capacity for use in biological treatment efforts to purify buprofezin-polluted areas.

Pseudomonas sp. DFS35-4 is capable of partially breaking down buprofezin through a transformation pathway illustrated in Fig. 5. This pathway involves successive removal of N-tert-butyl and Nisopropyl to form 2-imino-5-phenyl-3-(propan-2-yl)-1,3,5-thiadiazinan-4-one and 2-imino-5-phenyl1,3,5-thiadiazinan-4-one. The mechanism of N-dealkylation is thought to involve hydroxylating of the C-N carbon (α position), tracked by the decay of the unstable middle level into the Ndealkylated product and the alkyl snippet aldehyde or ketone. It was observed that acetone was not present and could not serve as a source of nutrition for the strain.

However, these two isopropanol and tert-butanol were found to be usable by the strain for growth, suggesting that the departing alkyls could be isopropanol and tert-butanol, despite the fact that these have not been discovered. Heterocyclic ring of the resulting outcome underwent hydrolyzed, leading to the formation of phenyl urea methyl (phenyl) carbamic acid from the assumed metabolite [(carbamimidoylsulfanyl) methyl] phenylcarbamic acid (Chen et al., 2011).

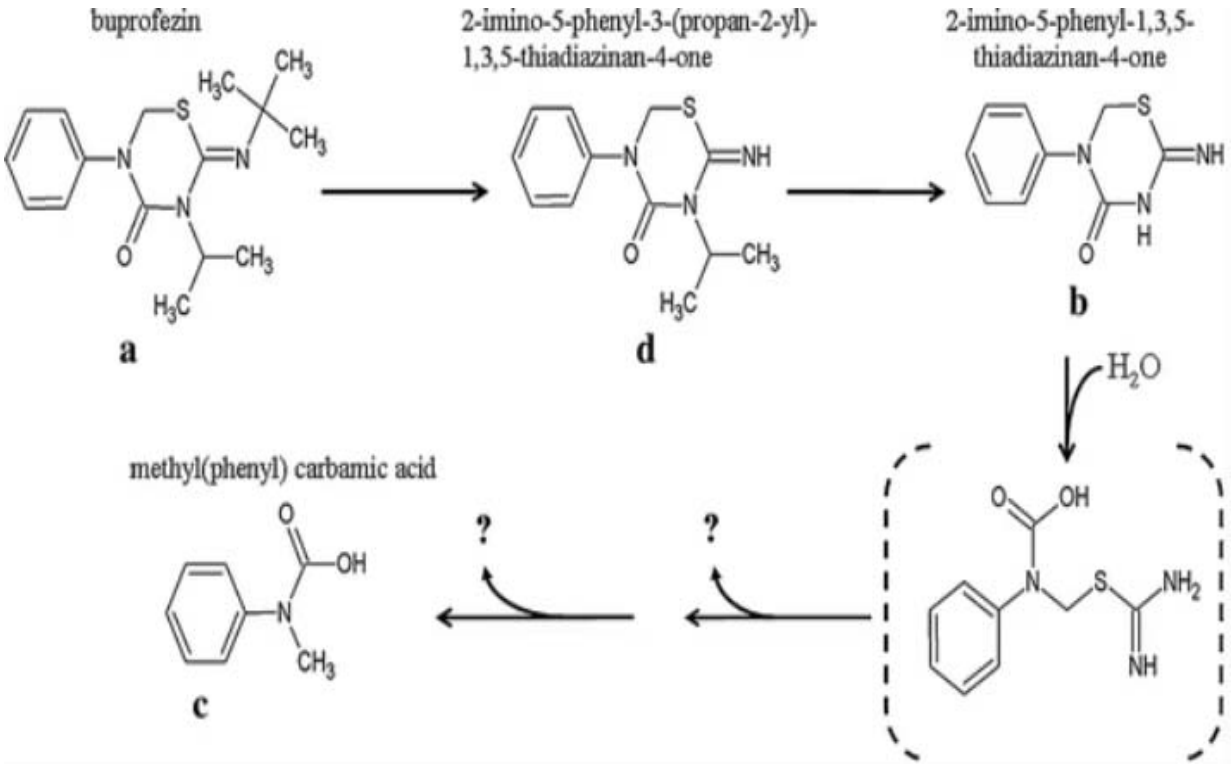

Figure 1.3 Buprofezin degradation path by DFS35-4 strain (Chen et al., 2011)

1.3.4 Toxicity

The presence of Buprofezin remnants can readily enter the human system through ingestion, skin contact, or inhalation, potentially causing harm to human health. (Authority, 2008). Due to its hydrophobic features, showing a logarithmic partition coefficient value of 4.31 buprofezin readily binds to soil debris, potentially leading to its prolonged presence in the soil (Toshiharu Funayama 1986) .

The half-life of Buprofezin in aerobic soils was found to range from 26 to 220 days and is dependent upon the microbial community within the soil. Underneath typical field circumstances, the half-life of Buprofezin in aerobic soil was estimated to be between 50 to 70

days, while in flooded fields, it was found to be between 36 to 104 days. The extensive application of Buprofezin has produced its residue being found at many locations. Studies have identified that Buprofezin has the dominant trace elements present in cotton tomatoes, and lettuce. The level of Buprofezin residue in citrus-type fruits, such as oranges, lemons, and mandarins, from various locations including Australia, New Zealand, Italy, Spain, and Portugal, was found to range between 0.05 and 0.69 milligrams per kilogram (Valverde-Garcia et al., 1993). When Buprofezin was suggested for use on grapes in 2006, frequent instances of its presence as a residue were noted during monitoring activities (Oulkar et al., 2009). Buprofezin residues have been found to range between 0.07 and 2.5 mg/kg in rice samples in Korea-based. Furthermore, the presence of Buprofezin residues is frequently detected in soil and water (Nguyen et al., 2008) .

African catfish's early embryonic and larval development is negatively impacted by even small amounts of buprofezin present in aquatic environments. Concentrations subdued as 16 µM have been shown to have detrimental effects (Marimuthu et al., 2013). Investigations have demonstrated that contact to buprofezin, at concentration between 12.5 to 100 µM, results in a significant escalate in the occurrence of micronuclei in Syrian hamster embryo cells that have been cultured (Herrera et al., 1993).

The research found that buprofezin poses a significant danger to the tissues of mammals such as the liver and kidney by examining the consequences of intraperitoneal administrated with doses of 4.0, 6.0, and 8.0 µg/kg according to bodyweight about 24 hours on Balb/c mice. The results indicate that buprofezin has a significant pessimistic impact on the liver and kidneys. (Bibi & Qureshi, 2019). The results of this study put forward concerns regarding the possible undesirable impacts of buprofezin on the human condition, even at non-toxic and sublethal concentrations.

As a primary focus of pesticides and the prime vulnerable body part of their impacts, the liver plays a crucial role in the detoxification (Mansour & Mossa, 2010) The liver, with its high-pitched metabolic rate and vigor demands, is central to retaining energy regulation. (Schneeweiss et al., 1990; Viollet et al., 2009)Disruptions in energy metabolism can contribute to liver injury and mitochondrial dysfunction, triggered by external stimuli, which has been linked to regulation of liver toxicity and the occurrence of liver-related diseases (Campillo et al., 1997; Krähenbühl, 2001).

When mice were treated with buprofezin, it resulted in an energy metabolism disorder. Cytochrome c oxidase activity is maintained by two key molecules SCO1 and COX17 and research showed that buprofezin interferes with this process. Buprofezin binds to the functional pockets of SCO1 and COX17, which inhibits the activity of cytochrome c oxidase and leads to an elevation in the intensity of reactive oxygen species (ROS). The reduced level of ATP and cytochrome c oxidase activity can be reversed by dealing with a ROS inhibitor (NAC), indicating the role of ROS in the modification of energy metabolism caused by buprofezin. This conversion from the aerobic process of the TCA cycle and oxidative phosphorylation to the anaerobic process of glycolysis triggers an oxidative stress response that follows after buprofezin is given via orally at sublethal concentrations. The result is a reduction in ATP production as shown in the figure. The alteration of energy metabolism caused by buprofezin reducing the activity of the enzyme of cytochrome c oxidase is at least partially due to the formation of chemically reactive oxygen species (ROS). Buprofezin directly affects the activity of several key enzymes in the tricarboxylic acid (TCA) cycle, stimulates glycolysis, and secondarily disrupts respiratory chain complex action by varying mitochondrial DNA (mtDNA). This highlights the role of ROS in the distraction of energy metabolism caused by buprofezin(Ji et al., 2016).

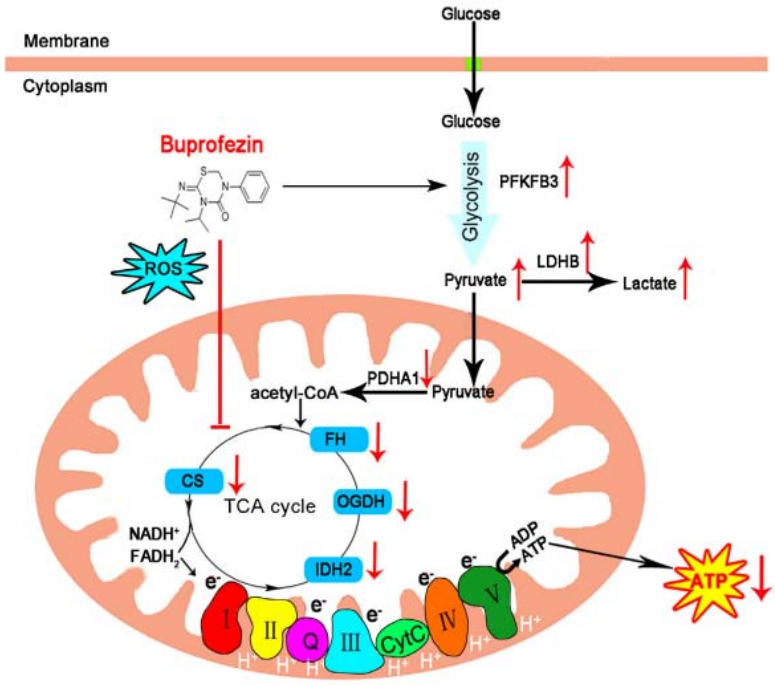

Figure 1.4 Disruption of energy metabolism by exposure of buprofezin (Ji et al., 2016)

1.3.5 Aim and Objectives

1.3.5.1 Aims

To investigate the toxic effects of Buprofezin inhalation on the lungs, liver, and kidney of male albino mice.

1.3.5.2 Objectives

The study objectives were to

- Investigate the level of oxidative stress markers reactive oxygen species (ROS) and lipid peroxidation (TBARS) in the tissues of the lungs, liver, and kidneys.
- Determine the level of anti-oxidative markers reduced glutathione (GSH), catalase activity (CAT), peroxidase (POD), and superoxide Dismutase (SOD) in the tissues of lungs, liver, and kidneys.
- Examine the histomorphological alterations in the lungs, liver, and kidneys.

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Animal housing and maintenance

Adult male albino mice were procured to the NIH (National Institute of Health) in Islamabad. Before conducting experiments, the mice were allowed to adjust to their new environment for 15 days in the Animal House Facility, which is under the purview of the Department of zoology at Quaid-i-Azam University Islamabad.

The animals were provided standard laboratory conditions with 12hr light/12hr dark, humidity 50-60%, and temperature of 25 ± 2 °C.

2.2 Bioethical approval

The experiments were carried out in the Animal and Human Physiology laboratory, which is located in the Zoology Department within the Biological Sciences Faculty at Quaid-i-Azam University Islamabad. Ethical approval was obtained from the "Bioethical committee of Biological sciences" regarding the utilization and well-being of animals.

2.3 Chemicals

A diverse array of chemicals was utilized in the course of this experimental work, which was obtained from the laboratory of Animal and Human Physiology. The majority of the chemicals were obtained from the Sigma Aldrich company. The following is a list of some of the chemicals that were employed in the experiment: Formaldehyde, xylazine, ketamine Triton-x100, NBT, Ferrous sulfate, Coomassie blue, Potassium chloride, Sodium chloride, BSA, Sodium azide, SDS, PMSF, DEPPD, Hydrogen peroxide, Sodium acetate, Tris-HCL, Ascorbic acid, TCA, TBA, Riboflavin, Sodium phosphate dibasic, Potassium phosphate monobasic, L-Methionine, Potassium phosphate dibasic, Sodium phosphate monobasic, Guaicol, Trisodium Citrate, DTNB, Eosin, Hematoxylin, Methanol, Phosphoric acid, among others.

2.4 Experimental Design

This experiment was performed on adult male albino mice with a body weight of 30 ± 5g. Healthy mice were randomly partitioned into four groups six in each group, approximately equal in weight. The animals were housed in plastic cages and subjected to 12h dark/light cycle, with a temperature of 25 ± 2 c° and humidity of 55 to 60%. Tap water with a standard laboratory diet was given before starting the experiment dose animals were given a week for acclimatization.

The male albino mice were acclimated to the whole-body exposure chamber for one week by keeping them inside for 30 minutes each day to reduce stress during the experiment. After the animals show the least resistance and normal behavior in the exposure chamber the experiment was started.

Group I: was control treated with distilled water mist.

Group II: was low dose group treated with 37 mg/ml.

Group III: was a medium-dose group treated with 250 mg/ml.

Group IV: was high dose group treated with 500 mg/ml.

2.5 Exposure chamber construction

A whole-body exposure chamber was designed by using fiberglass. The shape and structure of the exposure chamber is a rectangular idea taken from of the previous study (Tully et al., 2014). The exposure chamber has 46 cm in length and 46 cm in width and 25 cm in height. On the side of the chamber, a hole with a radius of 1.18 inches from both inside and outside, that act as an inlet of air. On the other side another hole on the top side with a radius of 0.78 inches from the inside and a 1.18 radius from the outside that is an outlet of air. The difference in the radius from inside and outside helps in maintaining the maximum mist of buprofezin remaining in the chamber because the inside radius is small in diameter while the outside diameter is bigger in size, so the maximum amount of oxygen enters the chamber.

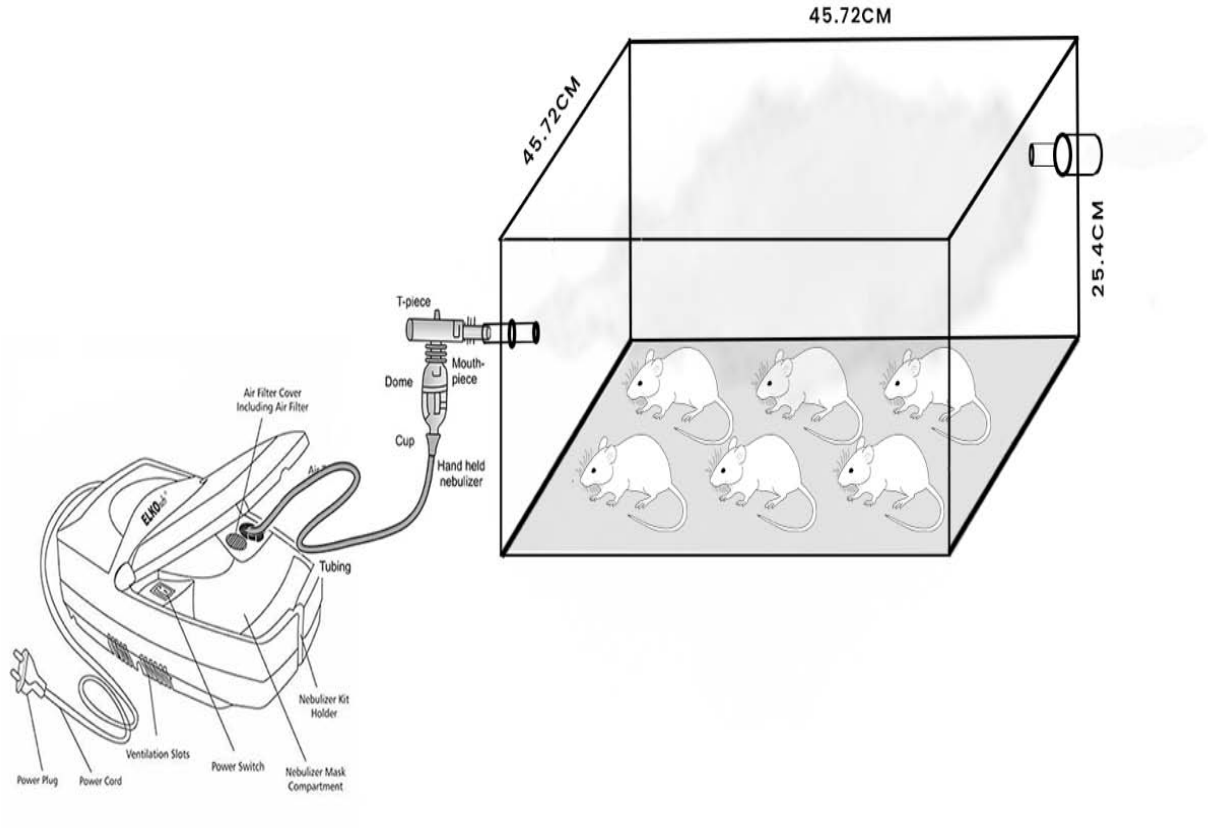

Figure 2.1 Experimental Arrangement

2.6 Dose preparation

Buprofezin solution was prepared by dissolving 35, 250, and 500 mg of powder buprofezin by dissolving in 5 ml distal water.

2.7 Experimental procedure

Buprofezin mist was administered to the mice for 1 hour per day over a period of 21 days using a nebulizer in the whole-body exposure chamber. The nebulizer was filled with a solution of buprofezin, and its pipe was attached to the inlet hole of the inhalation chamber. This enabled the male albino mice to be exposed to the buprofezin mist.

2.8 Dissections of animals and procuring biological samples

After concluding the experiment, the subsequent step involved dissecting the animals and procuring biological samples for analysis. It's important to note that all animals in each group survived until the day of dissection. To begin the dissection process, each animal was administered a xylazine and ketamine cocktail for anesthesia. The thoracic and abdominal cavities were opened, and organs such as the lungs, liver, and kidneys were extracted. To ensure that the organs were clean, they were soaked in saline solution.

2.9 Investigation of the biochemical properties of tissues

The analysis of the biochemical components in the lung, liver, and kidney tissues was executed to the levels of ROS, lipid peroxidation (TBARS), and antioxidant enzymes such as superoxide dismutase (SOD), catalase activity (CAT), peroxidase (POD), and reduced glutathione (GSH), as well as to estimate the total protein content.

2.9.1 Tissue homogenate preparation

2.9.1.1 Extract buffer

To make the lysis buffer, combine 5.95 g HEPES,4.38g sodium chloride (NaCl), 0.1g sodium azide (NaN3), and 0.5 g sodium dodecyl sulfate (SDS) in 495 mL of distilled water. Then, add 5 mL of 1% Triton X-100 to bring the volume up to 500 ml. Adjust the 7 pH by using the one molar solution of NaOH or HCl as needed.

2.9.1.2 Procedure

To prepare tissue homogenate, 100mg of tissue was weighed using a microbalance for each sample. The tissue was minced on a petri dish that was kept on ice. Each sample was homogenized manually by using the hand-driven homogenizer and add the 1000µl of the

extract buffer, which contained 0.1mg of PMSF. Subsequently, the material was conveyed in the 1.5ml eppendorf tubes and centrifuged for 10,000 rpm about ten minutes. The supernatants were collected in 1.5ml eppendorf tubes and kept at -20°C until analyzed.

2.9.2 Markers of oxidative status

Assessment of the oxidative profile was carried out by measuring the levels of the reactive oxygen species (ROS) and conducting the lipid peroxidation (TBARS) assays.

2.9.2.1 Reactive oxygen species (ROS)

The procedure for analyzing ROS (reactive oxygen species) levels in tissue homogenate, was conducted based on the (Hayashi et al., 2007) protocol. First, a 0.1M sodium acetate buffer with a pH of 4.8 was created by fusing 4.1g of sodium acetate in 500 ml of distilled water. The chemical Reagent 1 was subsequently made by dissolving 10mg of the N.N-Diethylpara phenyldiamine sulfate (DEPPD) in 100 ml of the buffer. The FeSo4 stock solution was subsequently made by mixing the 50mg of FeSo4in the 10ml of the 0.1M sodium acetate buffer, and then 50µl of FeSo4 from the stock solution was dissolved in 100ml of sodium acetate buffer to create Reagent 2. Reagent 1 and Reagent 2 were combined in a ratio of 1:25 and left in the blackness for two minutes. Next, 1680µl of the reagent mixture, 1200µl of sodium acetate buffer, and 60µl of tissue homogenate sample were added in a cuvette, and the absorbance was measured at 505nm utilizing an Ultraviolet-Visible spectrophotometer of (Agilent 8453, USA). Three readings were noted for each sample, with a 15-second interval between each reading.

2.9.2.2 Lipid Peroxidation (TBARS) Assay

For measuring amounts of malondialdehyde (MDA), that is indicated as a sign of oxidative stress caused by lipid peroxidation. The method used is based on the reaction of MDA with thiobarbituric acid (TBA). To perform the assay, a reaction mixture is prepared by combining 100µl of ascorbic acid (1.5mM), 100µl of FeSO4 (1mM), 100µl of 50mM Tris-HCl, 600µl of distilled water, and 100µl of sample in a 15ml Falcon tube. The mixture is then vigorously mixed, and the mixture is incubated for about 15 minutes at 37°C in a paraffin oven. After incubation, 1 ml of thiobarbituric acid (0.375%) and 1 ml of trichloroacetic acid (10%) are added to the reaction mixture. Then the mixture is boiled in the water bath at 90°C for 15 minutes. Following this, the mixture is centrifuged at 3000 rpm for about 10 minutes, and the supernatant is collected in a cuvette. The absorbance of the supernatant is measured at 532nm, which provides an indirect measurement of the level of oxidative stress produced by lipid peroxidation. It is worth noting that this method was first described by(Iqbal et al., 1996)

2.9.3 Markers of anti-oxidative status

The assessment of the anti-oxidative profile involved the measurement of both enzymatic as well as non-enzymatic antioxidants. Within the group of enzymatic antioxidants, the activity levels of three different antioxidants were estimated: peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT). Additionally, the non-enzymatic antioxidant group was estimated by determining the levels of reduced glutathione (GSH)

2.9.3.1 Reduced glutathione (GSH)

To evaluate the level of reduced glutathione, a method involves the preparation of a reagent mixture by combining 0.1 ml of disodium phosphate buffer (0.4M), 0.5 ml of DTNB, and 0.1 ml of tissue homogenate.

Chemical reagent DTNB, also referred a Ellman's reagent, was prepared by dissolving 40mg of the DTNB in 100 ml of one percent trisodium citrate. After mixing of reagent mixture, a yellow-colored product established and measured its absorbance at 412nm. This provides an indirect measurement of the level of reduced glutathione present in the tissue homogenate.

It should be noted that the method used for measuring reduced glutathione in this study was first described by (Jollow et al., 1974)

2.9.3.2 Catalase Activity (CAT)

The activity of catalase (CAT) with determined by slight modifications to the protocol (Chance & Maehly, 1995). A reagent solution was prepared by combining 1.99ml of 50mM potassium phosphate buffer (7 pH), 1000µl of H2O2 (5.mM), and 100µl of sample in a cuvette. The absorbance of the resulting mixture was then measured at 240nm, with three readings taken for each sample at 30-second intervals.

2.9.3.3 Peroxidase (POD) Assay

The concentration of peroxidase (POD) in tissue homogenate was identified by the protocol of (Chance & Maehly, 1995). To prepare the reaction mixture for the POD assay, 2.5ml of 50mM phosphate buffer, 0.1ml of 20mM guaiacol, and 0.1ml of enzyme extract were mixed vigorously until a homogeneous solution was obtained. Then, 0.3ml of 40mM hydrogen peroxide was added in the homogeneous solution, and after one minute, the absorbance of the homogeneous solution was noted at 470nm. Three readings of absorbance were noted for each sample, with a gap of 30 seconds.

2.9.3.4 Superoxide Dismutase (SOD) Assay

The evaluation of superoxide dismutase (SOD) level in the tissue homogenate was conducted by the following steps narrated by (Kakkar et al., 1984) with some enhancements. The reagent mixture was prepared by adding1.5 ml solution of L-methionine, which is 9.9 millimolar, 1ml of NBT (Nitroblue Tetrazolium) which is 57 micromoles, and 0.75 ml solution of Triton X-100 (0.025%) to 50 mM Phosphate Buffer Saline (PBS) pH: 7.8, and the final volume was adjusted to 90 ml. One milliliter of the mixture was then transferred to a cuvette, followed by the addition of 20 µL of the sample. The cuvettes were exposed with the light of fluorescent lamp for about 7 minutes and after that, it incubates in a paraffin oven at 37°C for 5 minutes. To commence the reaction, 10 µL of the 0.9 µM chilled riboflavin was melded in the beyond mixture, after that it incubate again for 37°C about 8 minutes. The absorbance was metered at 560 nm, and three readings were taken within one minute.

2.9.4 Total Tissue Protein

To quantify the protein proportion in the lung, liver, and kidney tissues, the Bradford assay was used. The protocol followed was as described below.

Reagents:

1. 25 mL of methanol
2. 50 mg of Coomassie blue
3. 50 mL of phosphoric acid (H3PO4)
4. 100 mL of distilled water
5. Bovine serum albumin (BSA) serial dilution

Protocol

A stock solution was prepared by mixing 25 mL of methanol, 50 mg of Coomassie blue, 50 mL of H3PO4, and 100 mL of distilled water. The stock solution was kept in the dark at 4°C until use. To make the working solution, the stock solution was mixed with distilled water in a 1:4 ratio, respectively. Similarly, BSA stock solution ready by dissolving 10 mg BSA in the 10 mL of phosphate-buffered saline (PBS). Serial dilutions of BSA were made from the stock solution at concentrations of 100x, 50x, 25x, 12.5x, and 6.25x. To generate a standardization curve, 2900 µL working solution was added in 100 µL of the BSA serial dilutions in a cuvette, and the change in absorbance was noted at 595 nm. For protein estimation in tissue homogenates, 2900 absorbance was noted at 595 nm. Three readings were noted within one minute.

2.10 Histopathology

Tissue histology was performed using the Hematoxylin and Eosin (H&E) staining method. The organs, including the lungs, liver, and kidney which were fixed in 10% buffered formalin, underwent a histopathological assessment to determine any pathological alterations.

Reagent preparation

2.10.1 Harris Hematoxylin solution

To prepare the Harris hematoxylin stain, 1 gram of hematoxylin was dissolved in 10 ml of absolute alcohol. Separately, 20 grams of potassium alum were dissolved in 200ml of distilled water. Then two solutions of Hematoxylin and alum were combined and rapidly boiled, then 0.5 grams of mercuric oxide was added. Once cooled, 8 ml of glacial acetic acid was added to the stain solution. The resulting stain was filtered through a 0.45µm filter paper and stored at room temperature.

2.10.2 Eosin solution

To prepare the eosin solution, 1 gram of eosin was dissolved in 0.05ml of acetic acid, followed by the addition of distilled water to bring the final volume up to 100ml

2.10.3 Acid alcohol

To prepare acid alcohol, 1 ml of concentrated hydrochloric acid was mixed with 99 ml of 70% ethyl alcohol.

2.10.4 Procedure

Small pieces of tissue were extracted from organs such as the brain, liver, kidneys, testes, and epididymis, which were then preserved in 10% buffered formalin. These tissue samples were appropriately sized, and dehydrated in 30 percent of 2-propanol, 50 percent, 70 percent, 90 percent, and finally in 100% ethanol for a duration of 1-2 hours. The samples were cleared in xylene1 and xylene2, followed by embedding in paraffin wax preheated near 59°C by using a dispenser of wax from the Triangle Biomedical Sciences in Durham, NC, USA. Subsequently, 5µm tissue blocks have been taken from the wax-embedded samples using a rotary microtome from Shandon, Finesse in Italy. These tissue-containing sections were placed in a water bath from Boekel Scientific and then transferred to clean glass slides, which were placed on a glass slide warmer overnight. The deparaffinization of tissues was performed in xylene, with two changes, and the slides were submerged for 5 minutes in each change. The slides were then hydrated in a falling sequence of alcohol solution concentrations of 100 percent, 90 percent, 80

percent, 70 percent, and 50 percent, with 3 minutes in each grade. Finally, the slides underwent a gentle washing process in tap water that was running.

2.10.5 Hematoxylin and Eosin Staining

The processed tissue samples were stained using the standard procedure of Hematoxylin and Eosin staining. The tissues were initially stained in Harris' Hematoxylin working solution for about five minutes, cleaned gently in flowing water for two minutes, and dipped into alcohol for about five seconds. They have then washed again gently in flowing water for 45 seconds. The slides were subsequently stained in a 1% working solution of Eosin for two minutes, washed for 45 seconds in running tap water, and dehydrated in increasing order of the concentrations of alcoholic solution like 50percent, 70 percent, 80 percent, 90 percent, and 100 percent about 3 minutes in each concentration. The tissues were cleared in xylene using two changes, with each change taking approximately 3 minutes. Finally, the slides were air dry at ambient temperature by using the DPX as a mountant for the slides medium. The slides have been observed examined under a light microscope at 10x and 40x magnification and images were taken using an Olympus camera microscope.

RESULTS

3 RESULTS

3.1 Markers of anti-oxidative status

3.1.1 Activity of Reduced glutathione (GSH)

3.1.1.1 Lungs

In the lungs, the level of GSH has significantly decreased in group IV (p=.001) and group III (p=.004) but non-significantly in group I as similitude to the control one as shown in fig 3.1

3.1.1.2 Liver

In the liver, the level of GSH has significantly decreased in group IV (p<.001), group III (p<.001), and group I (p<.001) as similitude to the control one as shown in fig 3.2.

3.1.1.3 Kidney

In the kidney, the level of GSH has significantly decreased in group IV (p= .017) and group III (p= .023) but non-significantly in the group I as similitude to the control one as shown in fig 3.3.

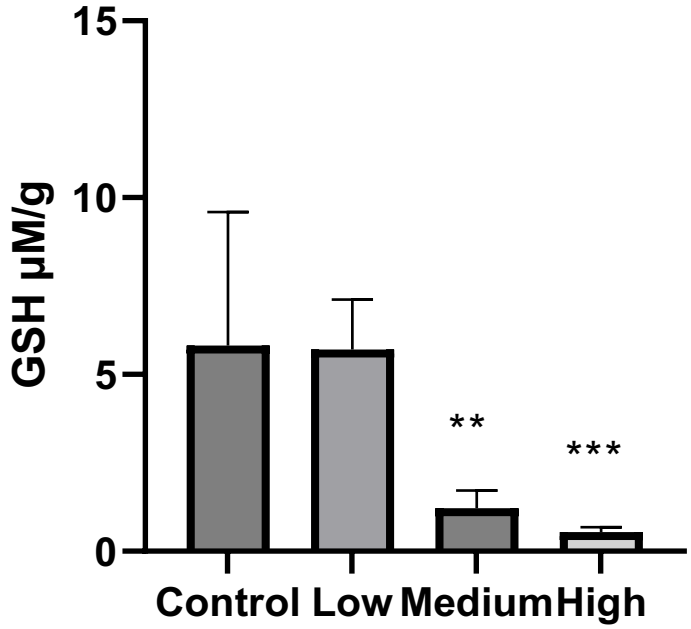

Figure 3.1 The level of GSH in the lungs of the mice demonstrated a significant decrease in group IV administered with a high dose of buprofezin (p=.001) and group III administered with a medium dose of buprofezin (p=.004), whereas there was no significant difference in group I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (*** = p=0.001 and **= p=0.004), n = 6

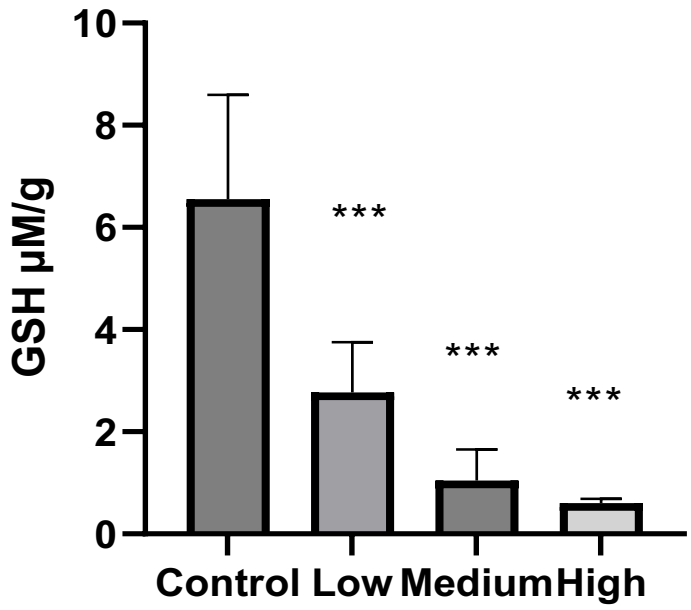

Figure 3.2 The level of GSH in the liver of mice was reduced significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and group I administered with a low dose of buprofezin (p<.001) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001), n = 6

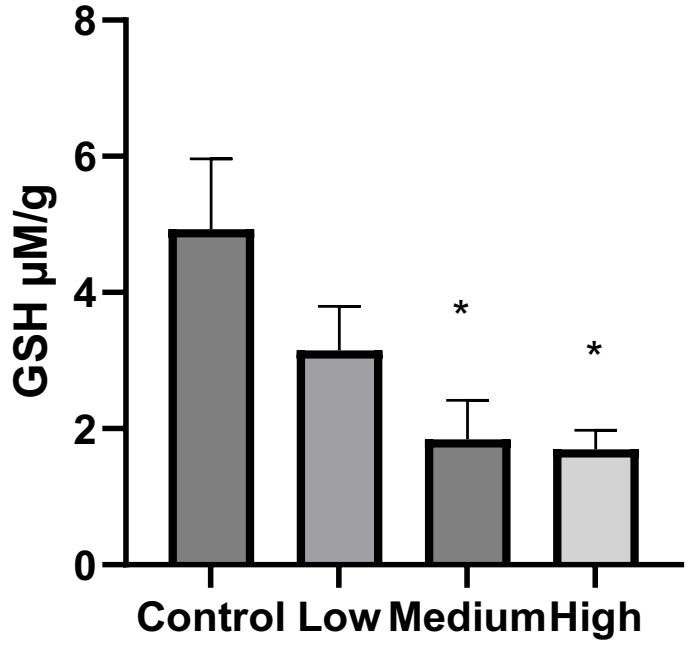

Figure 3.3 The level of GSH in the kidney of mice was reduced significantly in group IV administered with a high dose of buprofezin (p= .017) and group III administered with a high dose of buprofezin (p= .023) but non-significantly in the group, I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (* = p=.017 & p=.023), n = 6

3.1.2 Activity of Superoxide Dismutase (SOD)

3.1.2.1 Lungs

In the lungs, the level of SOD was decreased significantly in group IV (p= .010) and group III (p= .038) but non-significantly in group I as similitude to the control one as shown in fig 3.4.

3.1.2.2 Liver

In the liver, the level of SOD was decreased significantly in group IV (p<.001), group III (p<.001), and the group I (p=.001) as similitude to the control one as shown in fig 3.5.

3.1.2.3 Kidney

In the kidney, the level of SOD decreased significantly in group IV (p= .020) and group III (p= .040) but non-significantly in the group I as similitude to the control one as shown in fig 3.6.

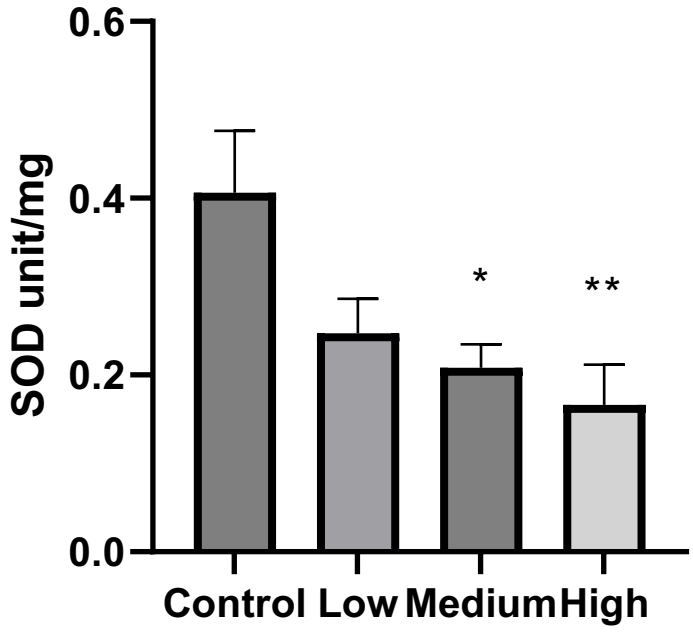

Figure 3.4 The level of SOD in the lungs of mice was decreased significantly in group IV administered with a high dose of buprofezin (p=.010) and group III administered with a medium dose of buprofezin (p= .038) but non-significantly in the group I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (* = p=.038 & ** =p=.010), n = 6

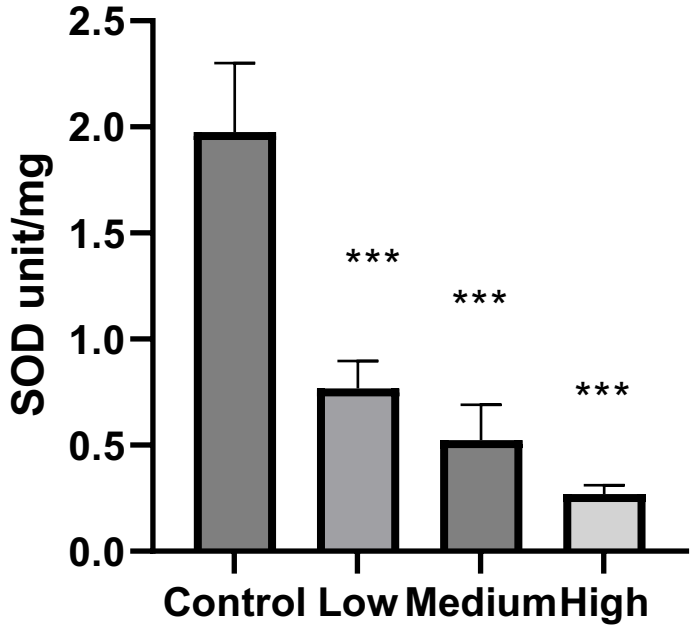

Figure 3.5 The level of SOD in the liver of mice was decreased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and group I administered with a low dose of buprofezin (p=.001) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001&p=.001), n = 6

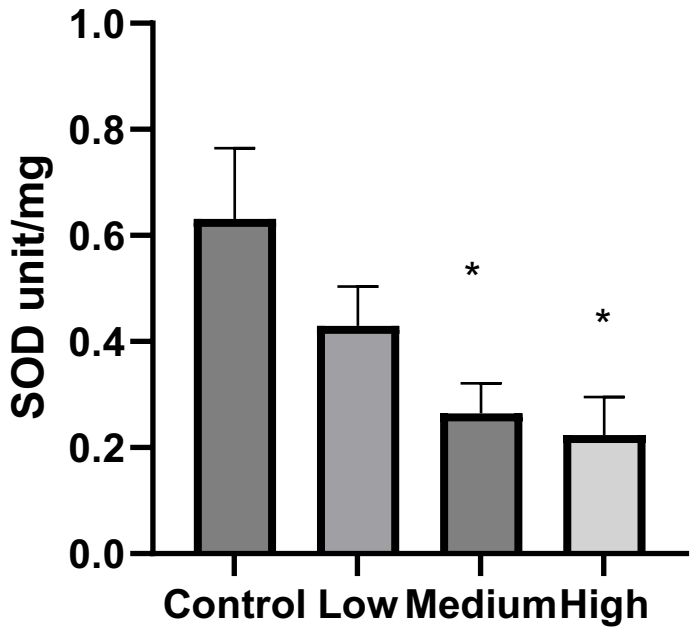

Figure 3.6 The level of SOD in the kidney of mice was decreased significantly in group IV administered with a high dose of buprofezin (p= .020) and group III administered with a medium dose of buprofezin (p= .0.40) but non-significantly in the group, I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (* = p=.020 & .040), n=6

3.1.3 Activity of Peroxidase (POD)

3.1.3.1 Lungs

In the lungs, the level of POD was decreased significantly in group IV ($p<.001$), group III ($p<.001$), and the group I ($p<.001$) as similitude to the control one as shown in fig 3.7.

3.1.3.2 Liver

In the liver, the level of POD was decreased significantly in group IV ($p<.001$), group III ($p<.001$), and the group I ($p= .002$) as similitude to the control one as shown in fig 3.8.

3.1.3.3 Kidney

In the kidney, the level of POD was decreased significantly in group IV ($p<.001$), group III ($p<.001$), and the group I ($p= .028$) as similitude to the control one as shown in fig 3.9.

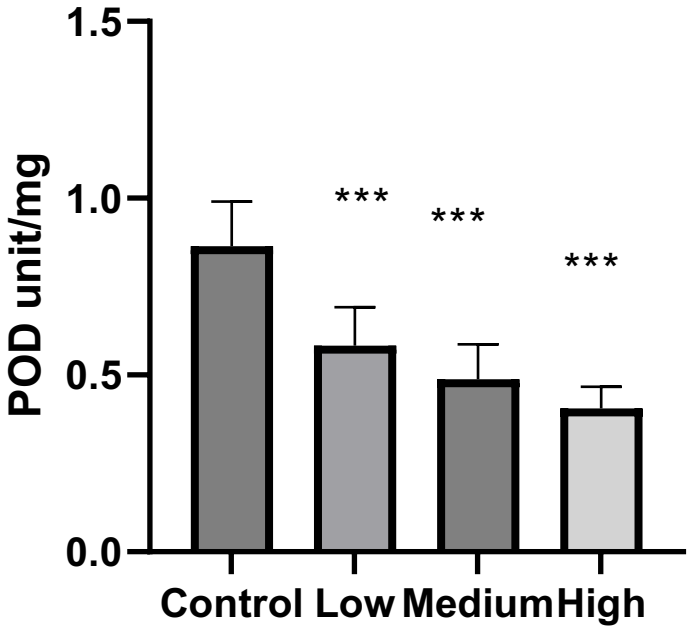

Figure 3.7 The level of POD in the lungs of mice was decreased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and the group I administered with a low dose of buprofezin (p<.001) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001), n = 6

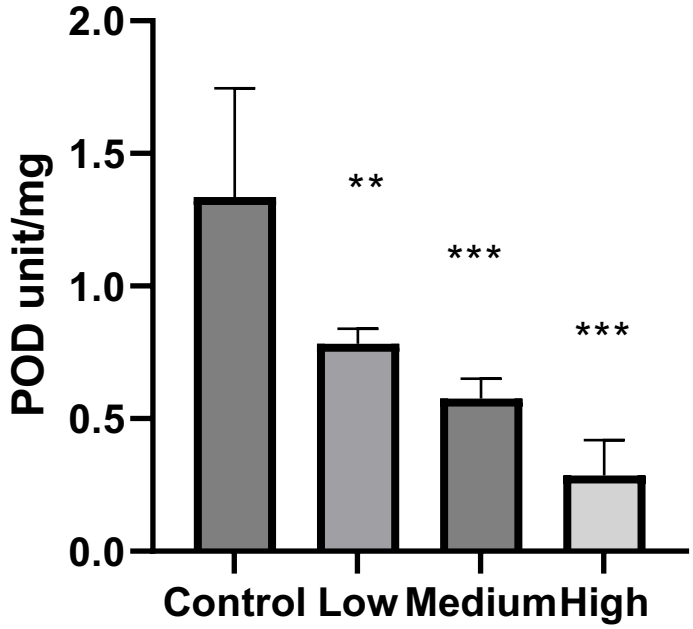

Figure 3.8 The level of POD in the liver of mice was decreased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and the group I administered with a low dose of buprofezin (p=.002) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001& ** =p=.002), n = 6

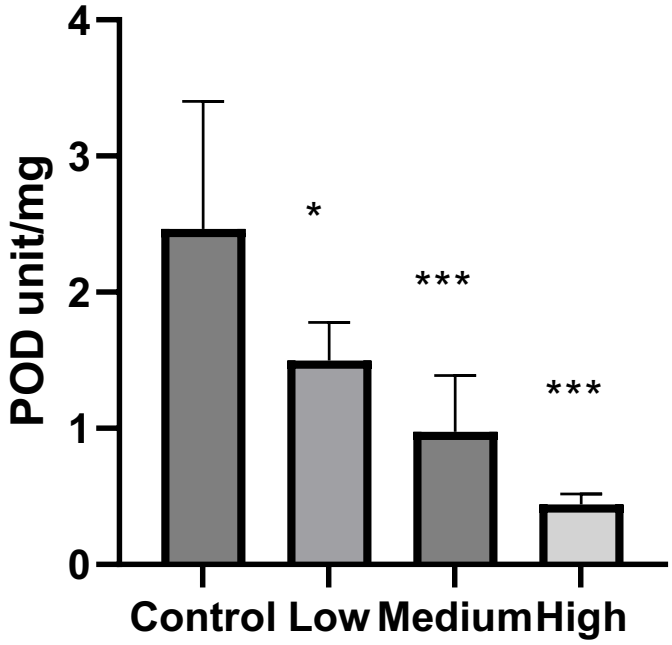

Figure 3.9 The level of POD in the kidney of mice was decreased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and the group I administered with a low dose of buprofezin (p=.002) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001& * =p=.024), n = 6

3.1.4 Activity of Catalase Activity (CAT)

3.1.4.1 Lungs

In the lungs, the level of CAT was decreased significantly in group IV (p=.001), group III (p=.005), and the group I (p=.034) as similitude to the control one as shown in fig 3.1o

3.1.4.2 Liver

In the liver, the level of CAT was decreased significantly in group IV (p= .007) and group III (p= .028) but non-significantly in the group, I as similitude to the control one as shown in fig 3.11

3.1.4.3 Kidney

In the kidney, the level of CAT was decreased significantly in group IV (p<.001), group III (p<.001), and group I (p=.020) as similitude to the control one as shown in fig 3.12

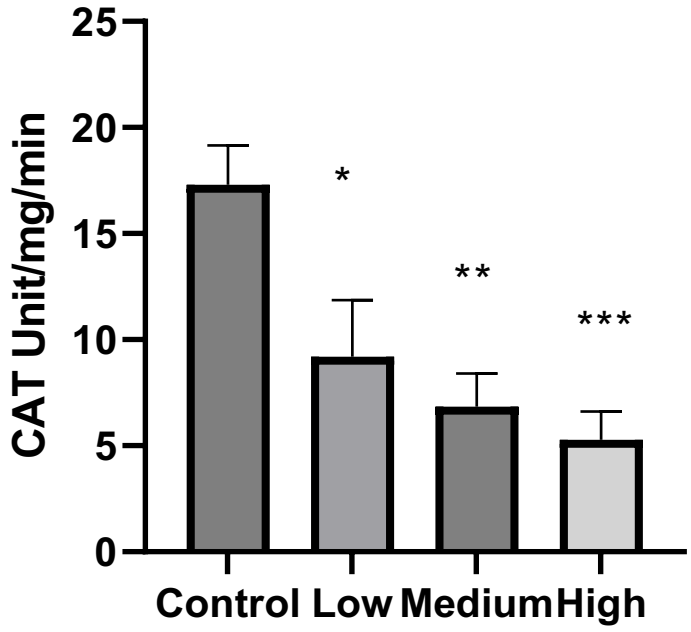

Figure 3.10 The level of CAT in the lungs of mice was lessened significantly in group IV administered with a high dose of buprofezin (p=.001), group III administered with a medium dose of buprofezin (p=.005), and the group I administered with a low dose of buprofezin (p=.034) as similitude with the control one. Values are stated as mean ±S. E (*** = p=0.001& ** =p=.005and *=p=.034), n = 6

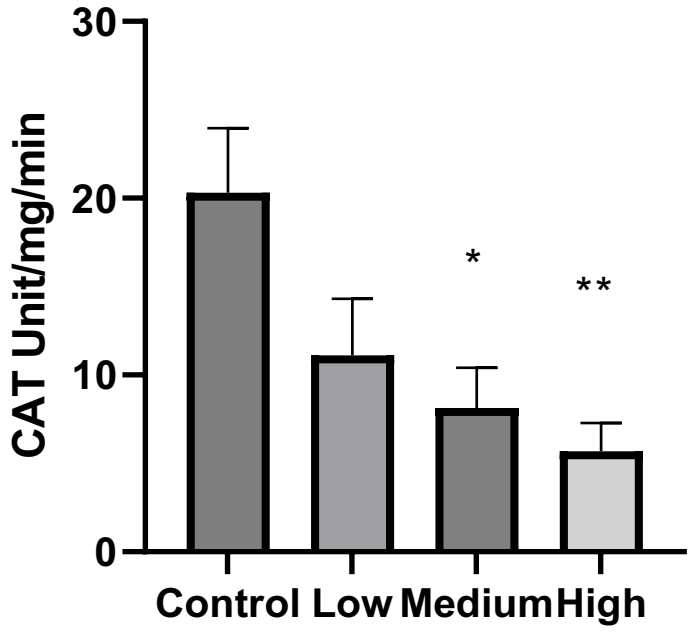

Figure 3.11 The level of CAT in the liver of mice was lessened significantly in group IV administered with a high dose of buprofezin (p=.007) and group III administered with a medium dose of buprofezin (p= .028) but non-significantly in the group, I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (* = p=.028 & ** =p=.007), n = 6

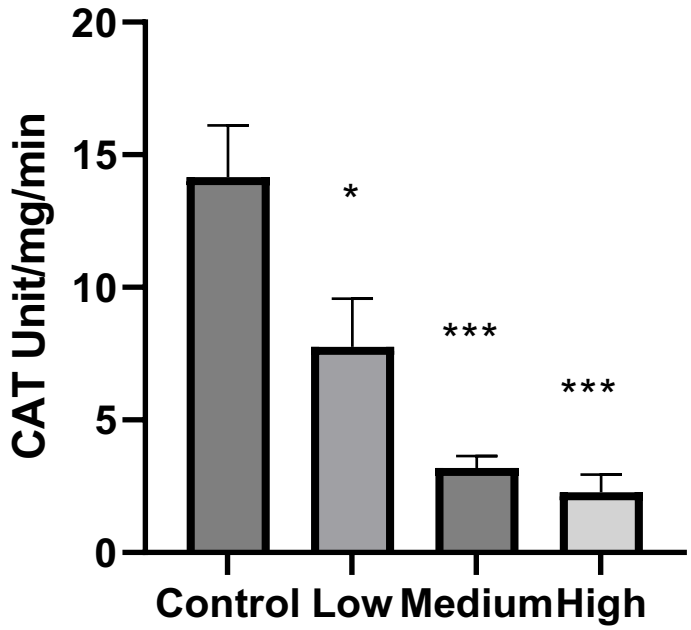

Figure 3.12 The level of CAT in the kidney of mice was lessened significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and the group I administered with a low dose of buprofezin (p=.020) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001& * =p=.020), n = 6

3.1.5 Analysis of total tissue protein

3.1.5.1 Lungs

In the lungs, the level of total tissue protein of was decreased significantly in group IV ($p<.001$) and group III ($p<.001$) but non-significantly in group I as similitude to the control one as shown in fig 3.13

3.1.5.2 Liver

In the liver, the level of total tissue protein was decreased significantly in group IV ($p<.001$), group III ($p<.001$), and the group I ($p<.001$) as similitude to the control one as shown in fig 3.14.

3.1.5.3 Kidney

In the kidney, the level of total tissue protein was decreased significantly in group IV ($p<.001$), group III ($p<.001$), and the group I ($p<.001$) as similitude to the control one as shown in fig 3.15.

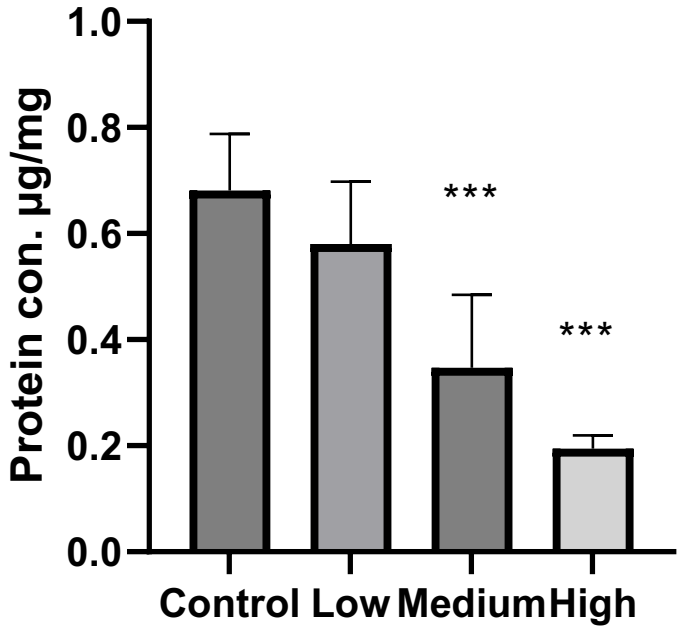

Figure 3.13 The level of total tissue protein in the lungs of mice was reduced significantly in group IV administered with a high dose of buprofezin (p <.001) and group III administered with a medium dose of buprofezin (p<.001) but non- significantly in group I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (*** =p <.001), n = 6

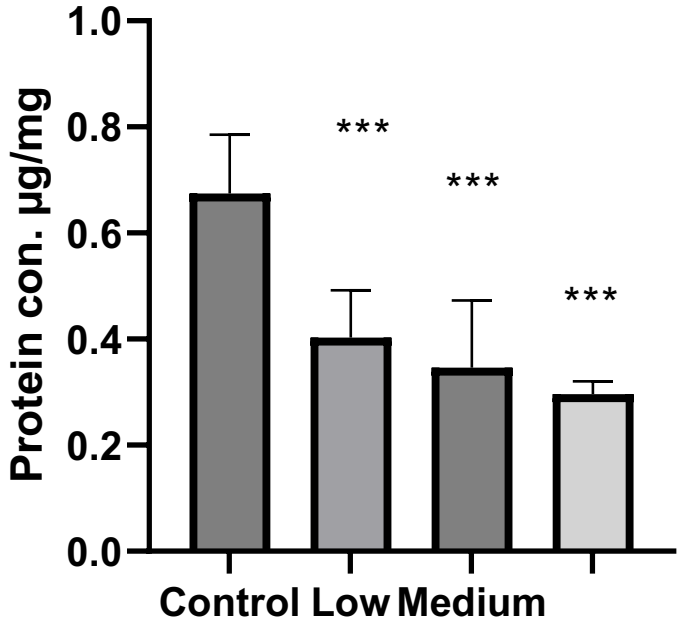

Figure 3.14 The level of total protein in the live tissues of mice was decreased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001) and the group I administered with a low dose of buprofezin (p<.001) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001), n = 6

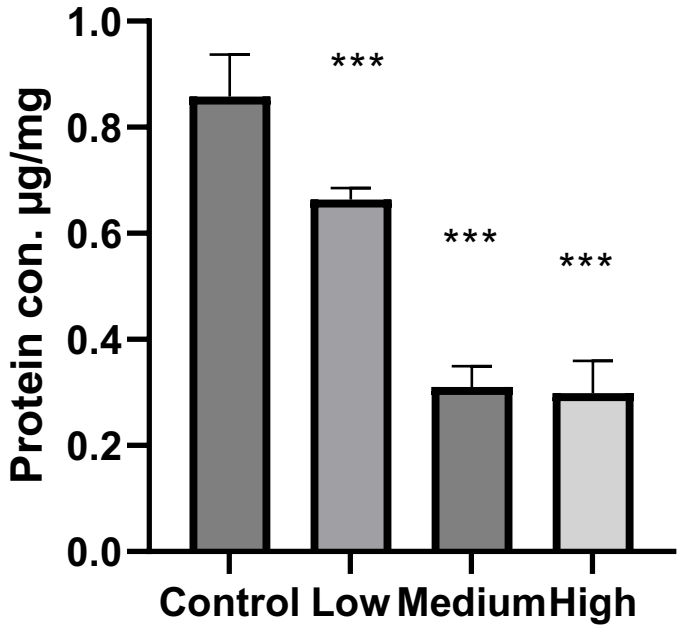

Figure 3.15 The level of total protein in the kidney tissues of mice was decreased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001) and group I administered with a low dose of buprofezin (p<.001) as similitude with the control on. Values are stated as mean ±S. E (*** = p<0.001), n = 6

3.2 Markers of oxidative status

3.2.1 Reactive oxygen species (ROS)

3.2.1.1 Lungs

In the lungs, the level of ROS was increased significantly in group IV ($p<.001$), group III ($p=.001$), and group I ($p=.047$) as similitude to the control one as shown in fig 3.16

3.2.1.2 Liver

In the liver, the level of ROS was increased significantly in group IV ($p<.001$), group III ($p<.001$), and the group I ($p=.027$) as similitude to the control one as shown in fig 3.17

3.2.1.3 Kidney

In the kidney, the level of ROS was increased significantly in group IV ($p<.001$), and group III ($p<.001$) but non-significantly in group I as similitude to the control one as shown in fig 3.18.

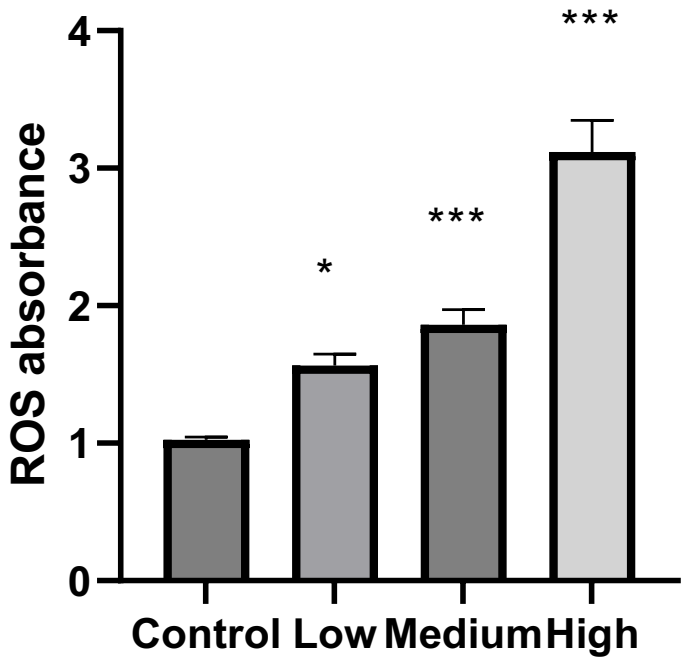

Figure 3.16 The level of ROS in the lungs of mice was increased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p=.001), and the group I administered with a low dose of buprofezin (p=.047) as similitude with the control on . Values are stated as mean ±S. E (*** = p<0.001 & p=.001 and *=p=0.47), n = 6

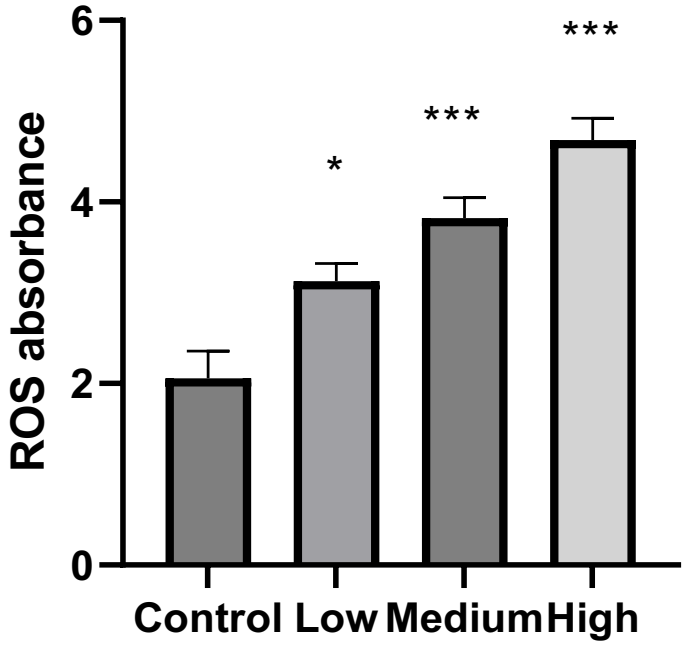

Figure 3.17 The level of ROS in the liver of mice was increased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and the group I administered with a low dose of buprofezin (p=.027) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001 and *=p=0.27), n = 6

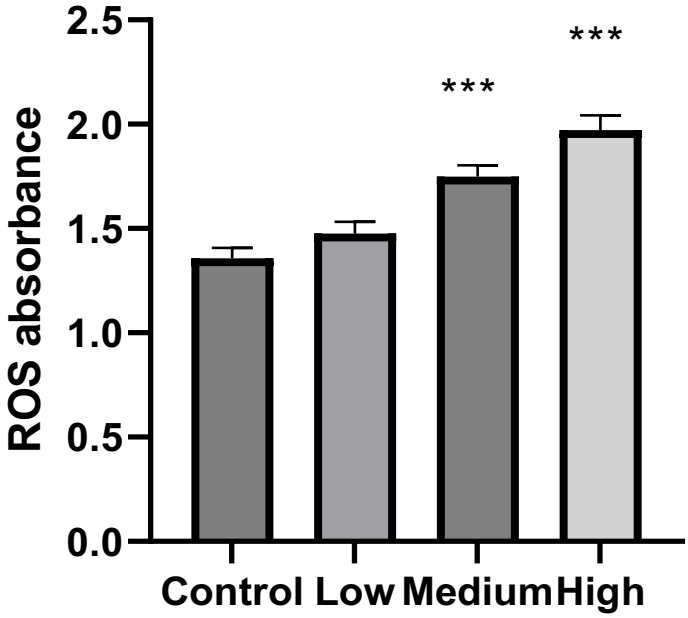

Figure 3.18 The level of ROS in the kidney of mice increased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001) but non significantly in group I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001), n = 6

3.2.2 Lipid Peroxidation (TBARS) Assay

3.2.2.1 Lungs

In the lungs, the level of TEBARS was increased significantly in group IV ($p<.001$), and group III ($p=.003$) but non-significantly in group I as similitude to the control one as shown in fig 3.19.

3.2.2.2 Liver

In the liver, the level of TBARS was increased significantly in group IV ($p<.001$), and group III ($p =.012$) but non-significantly in group I as similitude to the control one as shown in fig 3.20.

3.2.2.3 Kidney

In the kidney, the level of TBARS was increased significantly in group IV ($p<.001$), group III ($p<.001$), and the group I ($p =.023$) as similitude to the control one as shown in fig 3.21.

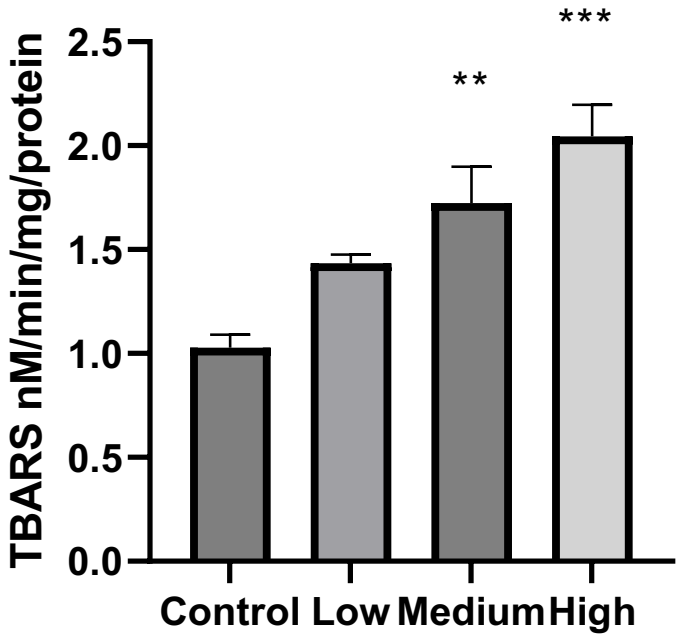

Figure 3.19 The level of TBARS in the lungs of mice increased significantly in group IV administered with a high dose of buprofezin (p<.001), and group III administered with a medium dose of buprofezin (p=.003) but non significantly in group I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001 &**=p= p=.003), n = 6

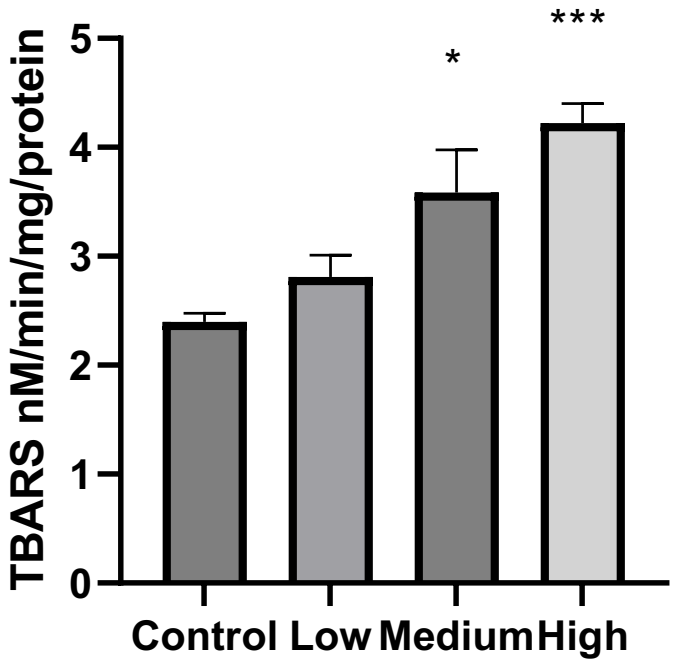

Figure 3.20 The level of TBARS in the liver of mice increased significantly in group IV administered with a high dose of buprofezin (p<.001), and group III administered with a medium dose of buprofezin (p=.012) but non significantly in group I administered with a low dose of buprofezin as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001 &*=p= p=.012), n=6

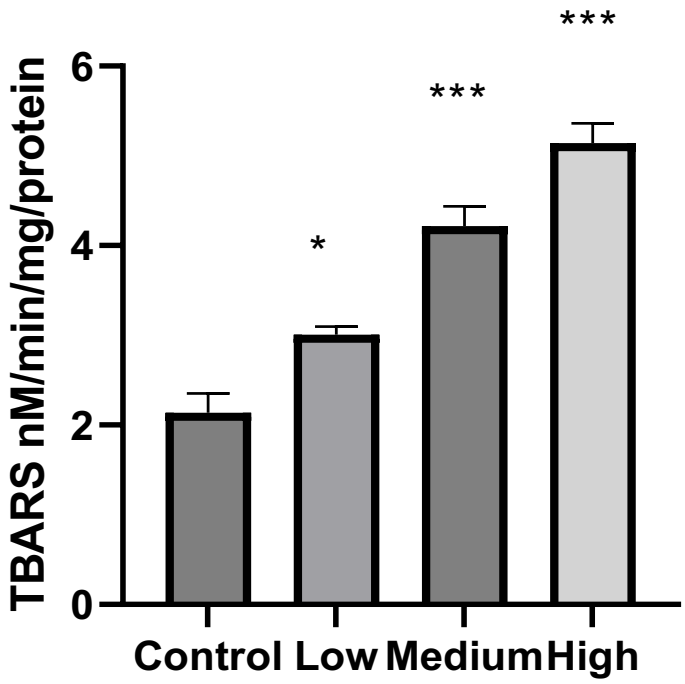

Figure 3.21 The level of TBARS in the kidney of mice increased significantly in group IV administered with a high dose of buprofezin (p<.001), group III administered with a medium dose of buprofezin (p<.001), and group I administered with a low dose of buprofezin (p=.023) as similitude with the control one. Values are stated as mean ±S. E (*** = p<0.001 and *=p=0.23), n = 6

3.3 Histopathology

3.3.1 Lungs

The control group's lungs exhibited a normal histomorphology as compared to the treated groups The lungs of the control group show normal pulmonary vessels, alveolar sacs, alveolar ducts, and alveoli. However, the treated groups showed various abnormalities in their lungs like pulmonary vessels show a decrease in the tunica media along with hemorrhage and endothelial cell hypertrophy, bronchioles show a catarrhal inflammation along with damaged clara cells, a decrease in the spaces of alveolar sacs, and alveoli also the inflammatory lesions form in especially in the medium and high dose group as shown in fig 3.22

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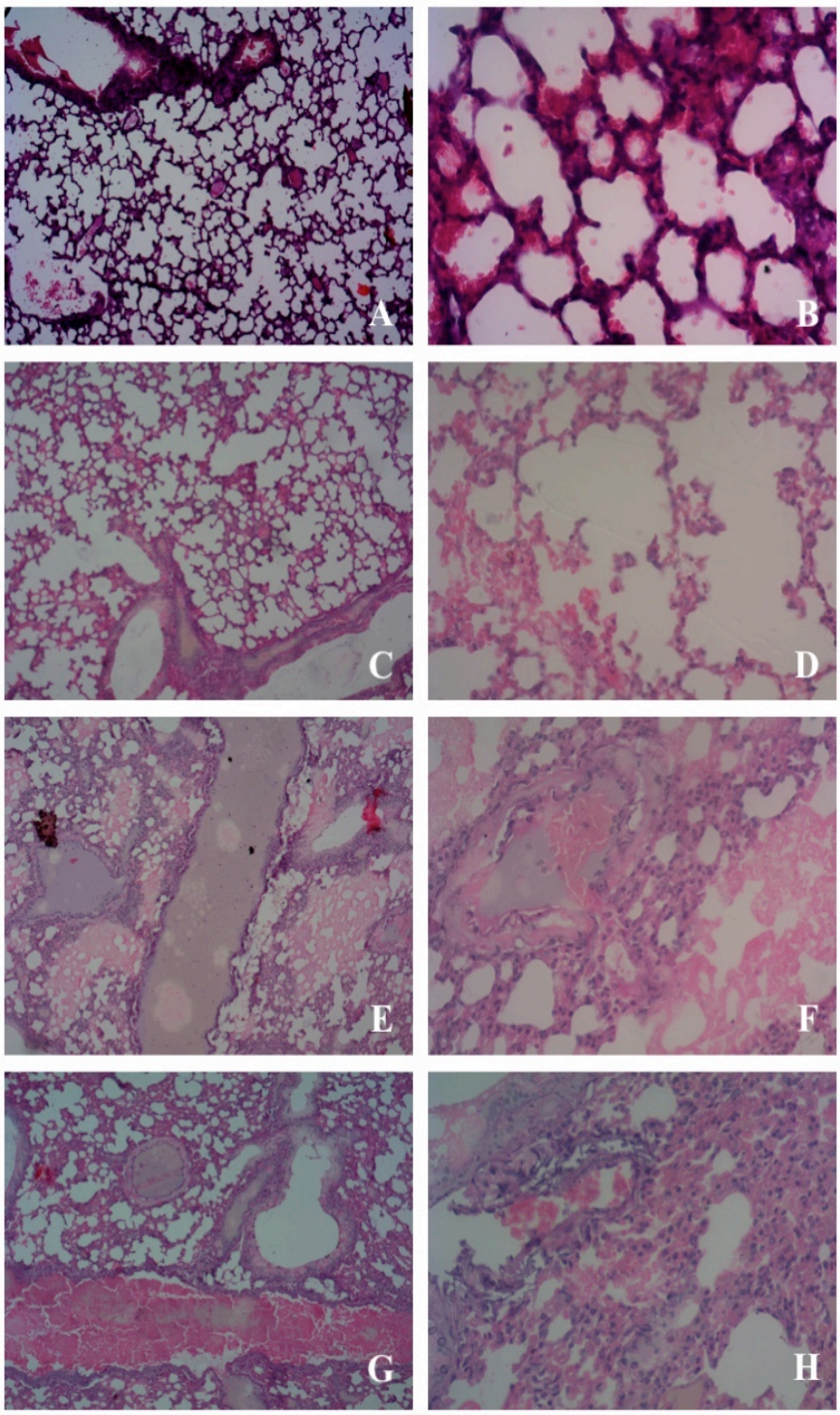

Figure 3.22 A and B are control lung photomicrographs of mice showing normal pulmonary vessels, alveolar sacs, alveolar ducts, and alveoli taken at 10 X and 40 X, respectively. C and D are low dose lung photomicrographs that show damage in alveolar sacs, alveolar ducts, and alveoli 10 X and 40 X, respectively. in the medium and high dose groups decrease in the tunica media of pulmonary vessels along with hemorrhage and endothelial cell hypertrophy, bronchioles show a catarrhal inflammation decrease in the spaces of alveolar sacs and alveoli also the inflammatory lesions form shown in the E, F, G, and H photomicrographs taken at 10X, 40X, 10X, and 40 X respectively.

3.3.2 Liver

The control group liver shows normal hepatocytes with normal blood vessels, normal central vein, bile duct, prominent nuclei, binucleated hepatocyte, and normal hepatic sinus some damage occurs in the low dose group. Medium and high-dose groups show hepatic vacuolation, congestion, vascular degeneration in blood vessels, and coagulative necrosis as shown in fig 3.23

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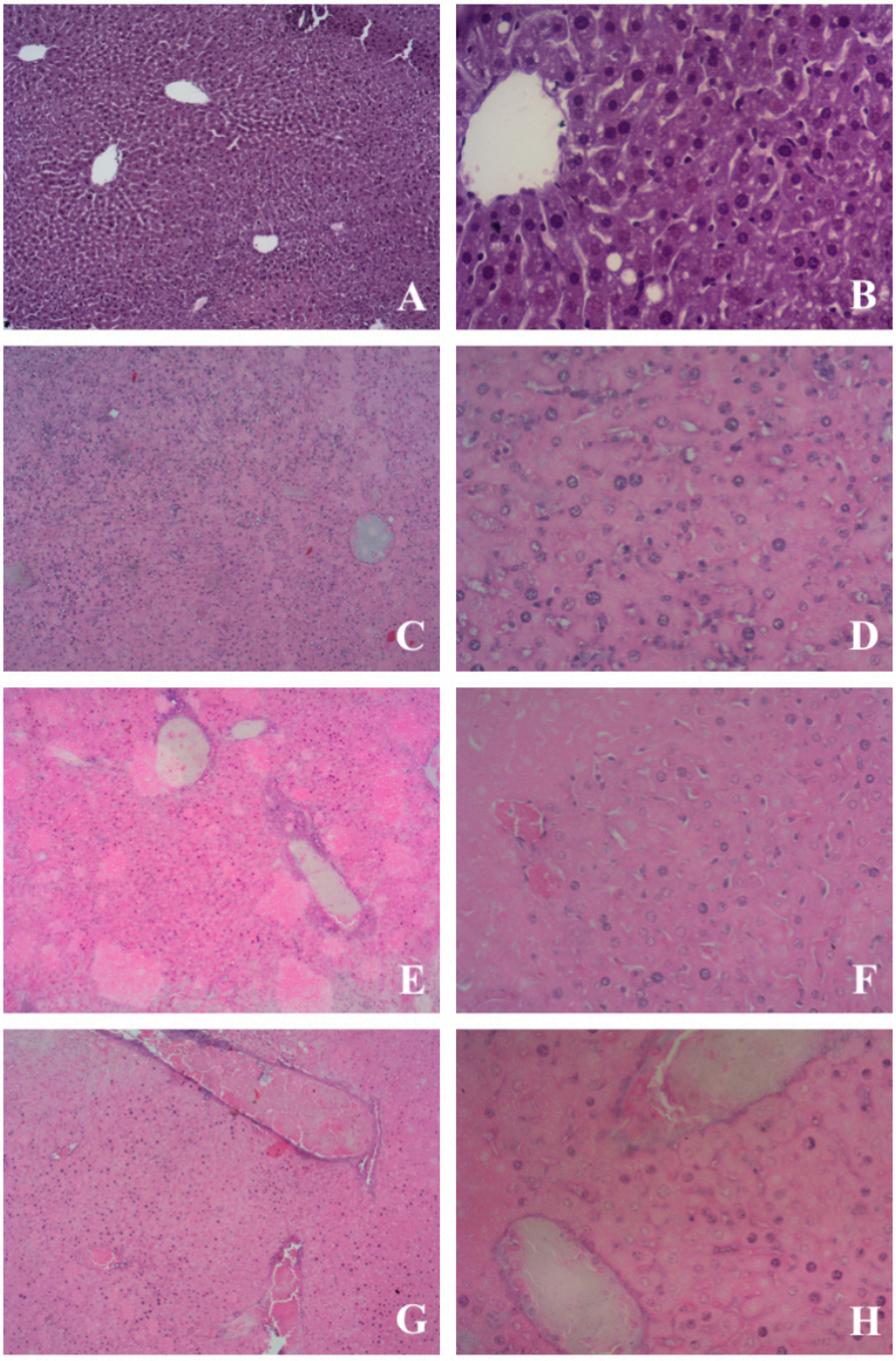

Figure 3.23 A and B control liver photomicrographs of mice showing a normal blood vessel, normal central vein, bile duct, prominent nuclei, binucleated hepatocyte, and normal hepatic sinus at 10 X and 40 X, respectively. C and D are low-dose liver photomicrographs that show damage in hepatocytes and blood vessels at 10 X and 40 X, respectively. In the medium and high dose groups hepatic vacuolation, congestion, vascular degeneration in blood vessels, and coagulative necrosis occur shown in the E, F, G, and H photomicrographs were taken at 10X, 40X, 10X, and 40 X respectively

3.3.3 Kidney

The control group kidney exhibited a normal histomorphology as compared to the treated groups. The control group kidneys have the normal glomeruli, bowman's capsule and space of bowman's capsule, normal vessels, normal proximal and distal convoluted tubules, and normal and medullary rays. While in the treated groups vascular congestion occurs, the glomerulus shrinks, and space of the bowman's capsule increase, and cellular infiltration as shown in fig 3.24

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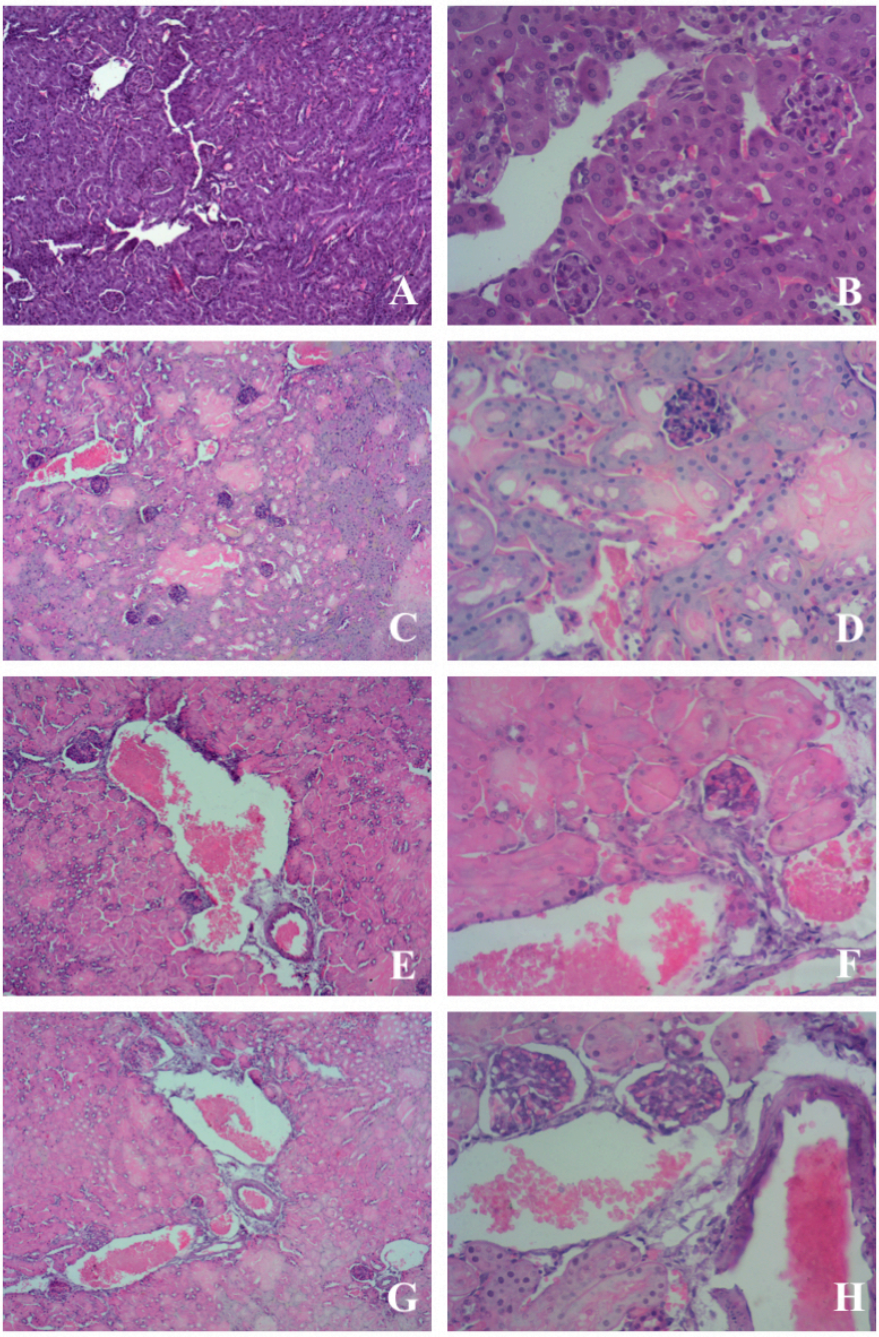

Figure 3.24 A and B control kidney photomicrographs of mice that show normal glomeruli, bowman's capsule and space of bowman's capsule, normal vessels, normal proximal and distal convoluted tubules, normal and medullary rays at 10 X and 40 X, respectively. C and D are low-dose kidney photomicrographs that show damage in the glomeruli, bowman's capsule, and space of bowman's capsule, and vascular congestion and cellular infiltration occur at 10 X and 40 X, respectively. In the medium and high dose groups vascular congestion occurs, glomerulus shrinks, and space of bowman's capsule increase, and cellular infiltration form shown in the E, F, G, and H photomicrographs taken at 10X, 40X, 10X, and 40 X respectively.

DISCUSSION

4 DISCUSSION

The key intent of the study is to scrutinize the toxic consequences of buprofezin on adult male albino mice by inhalation. Inhalation is the critical means of infusion into the body system and the organs that are most susceptible are the lungs, liver, and kidney. In this study, the main focus was also on the lung, liver, and kidneys. Buprofezin is an insecticide that falls under the thiadiazol class and is known to hinder molting. Residues of this chemical can persist in both crops and soil for extended durations of phase, and the concentration of buprofezin residues is known to increase as the moisture content in the environment decreases (Noh et al., 2021).

Pesticides and insecticides can incite oxidative stress, which can result in the creation of free radicals as well as alterations in the radical and antioxidant enzymes of the body. when the radicals that are free and antioxidant enzymes are not produced in the balance it produces oxidative. Radicals that are free are unstable chemical compounds that contain ions that are negatively charged. These molecules, such as H2O2 (hydrogen peroxide), OH (hydroxyl ions), O2– (superoxide radicals), and LOO (lipid peroxyl), can cause oxidative degradation of lipids layer of the cells, damage to the structure or function of cells. The activation of reactive oxygen species (ROS) results in the process of oxidation. ROS are free radicals that are highly reactive and unbalanced because it has unpaired electrons. These free radicals are able to damage the other molecules and get more charged negatively leading to structural plus functional destruction to the molecule. ROS are responsible for most of the potentially harmful effects associated with oxidative stress. To summarize, oxidative damage occurs when free ROS, overwhelms the body's antioxidant enzymes and damages to the cellular structures (Sharma et al., 2012).

The body has multiple systems to covert the damage engendered by radicals that are free, including enzymatic systems. The first defense against radicals are provided through antioxidant enzymes of the body like superoxide dismutase (SOD) and catalase (CAT). SOD converts superoxide radicals into peroxides, while CAT converts peroxides into water (Abdollahi et al., 2004). When the level of oxidative damage overtakes the ability of the first defense low then the second line of defense, which includes glutathione (GSH), comes into play. Then the electrons from the Radicals are taken and deactivated the radicals during this process (Al-Dalaen & Al-Qtaitat, 2014). To assess the oxidative damage levels, measure the amount of enzymes of the body like CAT, POD, and SOD, and the degradation of the lipids layer can be analyzed by TBARS.

We found evidence suggesting that buprofezin tends to accumulate in the liver and that such accumulation may lead to oxidative stress as a result of oral exposure to this chemical. Buprofezin hindered ATP making by disrupting the normal tricarboxylic acid cycle and oxidative phosphorylation system, leading to an increased reliance on anaerobic glycolysis. Our study also identified that the creation of reactive oxygen species (ROS) played a role in the changes to energy metabolism induced by buprofezin. This was caused by the direct suppression of cytochrome c oxidase function by the chemical, as well as by indirect disruptions to respiratory chain complex activity through alterations to mitochondrial DNA. Additionally, key enzymes involved in the TCA cycle were also disturbed, which further promoted the glycolysis (Ji et al., 2016).

By the inhalation of buprofezin, the level of ROS and TEBARS increased and the level of total protein and antioxidant enzymes like SOD, POD, CAT, and GSH was decreased in the lungs. This is supported by the study of adenine causes lung injury, to measure oxidative damage by LPO, ROS, and catalase activity. Findings presented that adenine led to a major boost in LPO and ROS levels, while the activity of catalase decreased. This suggests that oxidative stress consumed the antioxidant catalase, leading to its decreased activity (Nemmar et al., 2017).

Inhalation of pesticides affects the lungs tissue, lipid peroxidation, and antioxidant enzymes demonstrated by the encountering to a combination of chlorpyrifos and cypermethrin pesticides via inhalation for 28 days consecutively was found to cause moderate effects on mutagenicity and neurodegeneration, but severe histological changes were observed in lung tissue. The study found that oxidative damage was generated in the tissues only at high inhalation doses by an intensify in TBARS amount and a deplete in the amounts of antioxidant enzymes like CAT and GSH in the tissues rat. (Noaishi et al., 2013).

By the inhalation of buprofezin damage, the amount of ROS and TEBARS intensify and the amount of total protein and antioxidant enzymes like SOD, POD, CAT, and GSH was decreased in the liver and kidney. Supported by the study examined the impact of administering Fipronil to male rats through their drinking water at concentrations in that is .1 mg/L, 1 mg/L, and 10 mg/L for 45 days. The findings revealed that exposure to Fipronil caused a concentration-dependent to deplete in the amount of superoxide dismutase (SOD), catalase (CAT), and glutathione reduced (GSH). Furthermore, Fipronil exposure caused a major increase in the destruction of lipids in the rats. These observations suggest that sub-chronic exposure to Fipronil has the potential to induce liver and kidney damage in male rats.

Additionally, oxidative stress biomarkers could be useful indicators for such damage. (Mossa et al., 2015). The experimental rats were administered a cypermethrin concentration of 14.5 mg/kg given. This led to a reduction in their overall protein levels(Grewal et al., 2009).

The boost in TBARS amount in the liver, kidney, and lung by the inhalation of buprofezin may also be supported by the impact of malathion exposure caused damage by reactive oxygen species to organs or body tissues of rats. Malathion was delivered into the peritoneal cavity doses of the body were 25, 50, 100, and 150 mg/kg of body. The results revealed that exposure to higher doses of malathion caused an intensify in thiobarbituric acid reactive substances (TBARS) levels in the hepatic, renal, and pulmonary tissues. Additionally, malathion exposure prompted oxidative stress and altered the actions of catalase (CAT) and superoxide dismutase (SOD) in different organs of rats.(Possamai et al., 2007).

Inhalation of buprofezin causes damage in the tissues of the lung, liver, and kidney tissues. The histopathological of the lungs revealed that group 1 which was given no dose had a normal histomorphology in similitude to the treated one. The lungs of the control one displayed normal pulmonary vessels, alveolar sacs, alveolar ducts, and alveoli. Conversely, the lungs of the treated groups exhibited various abnormalities, such as a decrease in the middle layer of the vessels, hemorrhaging, hypertrophy of endothelial cells in the pulmonary vessels, catarrhal inflammation, and damage to Clara cells in bronchioles, a decrease in the spaces of alveolar sacs and alveoli, and the formation of inflammatory lesions primarily in the medium and high dose group.

This is backed by the investigation into whether thymoquinone administration can protect against malathion-induced pulmonary toxicity at a concentration of 100 mg/ml/m3 through this study. The examination of lung tissue from the control group showed normal bronchi and alveoli, while given thymoquinone displayed normal lung tissues. However, breathing in malathion caused severe inflammation in the lung tissues, specifically between the tissues. The blood vessels also showed signs of enlargement, swelling, and bleeding, with a decrease in the middle layer of the vessels. The air sacs in the lungs were notably reduced in size, with an increase in fibrous tissue between cells, and enlargement and growth of specific lung cells (Abdo et al., 2021).

The histologic analysis of the liver manifested that the control group revealed normal hepatocytes with normal blood vessels, a normal central vein, a bile duct, prominent nuclei, binucleated hepatocytes, and normal hepatic sinusoids. However, in the low-dose group, some

damage was observed. The medium and high-dose groups showed hepatic vacuolation, congestion, vascular degeneration in blood vessels, and coagulative necrosis. This is also backed by the same study investigation into whether thymoquinone administration can provide protection against malathion-induced pulmonary toxicity at a quantity of 100 mg/ml and the rats given to malathion told a dose-dependent decline in degenerative and necrotic changes (Abdo et al., 2021).

The histologic analysis of the liver manifested that the kidney of the control one revealed normal renal corpuscles, renal capsule, renal vessels, proximal tubules, and distal tubules, and ray of the renal medulla showed no abnormalities. Additionally, the space of Bowman's capsule is normal. In contrast, the treated groups showed various abnormalities, including vascular congestion, shrinking of glomeruli, enlargement of the space of Bowman's capsule, and cellular infiltration. This is backed by the study of the toxic effects of administering an ethanolic leaf extract of Clinacanthus nutans orally to the Institute of Cancer Research mice for a sub-chronic duration. The kidney's histomorphology analysis showed vacuolation in renal tubules and renal tubular cells, as well as cellular infiltration. Synonyms could include: The kidney's microscopic examination displayed renal tubular cells with vacuolation, renal tubules with vacuolation, and cellular infiltration (Aliyu et al., 2021).

As demonstrated by the outcomes of the recent investigation, the inhalation of buprofezin leads to detrimental effects on the lungs, liver, and kidneys of mice. The harmful effects of exposure to buprofezin are manifested by the induction of oxidative stress, which is evidenced by a reduction in the level of antioxidant enzymes, tissue total protein and a boost in the level of reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS). Moreover, damage to the lung, liver, and kidney tissues is also observed as a result of this exposure. Thus, it can be concluded that buprofezin exposure has a deleterious impact on the oxidative status and tissue integrity of the lungs, liver, and kidneys. Therefore, in verdict, the current research supports testimony that exposure to buprofezin through inhalation has negative consequences on these vital organs of the mice.

Moreover, it is important to note that additional research is still necessary to thoroughly explore and analyze other potential effects of buprofezin exposure, such as its impact on behavior and neurotoxicity. This would provide a broader understanding of the potential risks and health consequences related with exposure to this chemical, which could inform the development of effective prevention and mitigation strategies.

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