Protective effect of folic acid against imidacloprid-induced toxicity in liver and kidney of adult female rats

A dissertation submitted in the partial fulfillment of the requirement for the degree of Master of Philosophy in Zoology, In laboratory of Animal and Human Physiology

By

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CERTIFICATE

This dissertation “Protective effect of folic acid against imidacloprid induced toxicity in liver and kidney of female albino rats” submitted by Abdul Qadeer Khan, 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 with specialization in Human and Animal Physiology.

Supervisor: _____

External Examiner: _____

(O Prophet), Those who have knowledge see clearly that what has been revealed to you from your Lord is the Truth and directs to the Way of the Most Mighty, the Immensely Praiseworthy Lord (Surah Saba Ayat 6)

Now let man observe from what he was created! He was created from a fluid, ejected from between the backbone and the ribs (Surah At-Tariq, 86:5-7)

Then We made the sperm-drop into a clinging clot, and We made the clot into a lump [of flesh], and We made [from] the lump, bones, and We covered the bones with flesh; then We developed him into another creation. So blessed is Allāh, the best of creators (Surah Al-Muminun)

Dedicated to my Beloved Parents

Their unwavering commitment to my education and their relentless efforts in shaping me into a better human being are truly unparalleled.

Your unwavering love continues to serve as an inspiration for me to strive towards becoming a better individual

"We used to think our fate was in stars. Now we know, in large part, our fate is in our genes" **James Watson** (Molecular Biologist)

I can never fully repay my parents' kindness, but I'll strive to make positive contributions to society, guided by their values.

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Abdul Qadeer Khan

Abstract

The widespread use of pesticides in agriculture has raised concerns about their potential impact on human health and the environment. Imidacloprid, a widely used neonicotinoid insecticide, has been associated with oxidative stress and cellular damage. This study investigated the potential protective role of folic acid (FA) against imidacloprid-induced toxicity. Previous studies have shown that imidacloprid can cause oxidative stress and toxicity in rat kidney and liver. However, the potential protective role of FA against imidacloprid-induced toxicity has not been fully investigated. This study aimed to fill this research gap by investigating the effects of FA on oxidative stress markers, serum ALT and AST levels, and histopathological damage in rats exposed to imidacloprid. Adult female Sprague Dawley rats were divided into four groups: control(G1), imidacloprid (IMI) treatment (G2), FA supplementation(G3), and combined IMI and FA treatment (G4). IMI treatment resulted in a significant increase in oxidative stress markers in the liver and kidney, including ROS (**$p<0.01$), TBARS (***$p<0.001$), and decreased SOD (**$p<0.01$), POD (*$p<0.05$), CAT (**$p<0.01$), and GSH (****$p<0.0001$) levels. FA treatment significantly reversed the effects of IMI on oxidative stress markers in the liver and kidney. This was evident by the significant decrease in ROS (**$p<0.01$), TBARS (**$p<0.001$), and increased SOD (**$p<0.01$), POD (*$p<0.05$), CAT (**$p<0.01$), and GSH (****$p<0.0001$) levels in the FA-treated groups compared to the IMI-treated groups. Folic acid treatment also significantly reduced serum ALT (****$p<0.0001$) and AST (****$p<0.0001$) levels. Histopathological analysis of the liver and kidney tissues showed that IMI treatment caused significant damage, including the presence of lipofuscin, dilation and congestion of hepatic sinusoids, leucocytic infiltration, Kupffer cells hyperplasia, granuloma formation, and eosinophilic infiltration. FA treatment partially protected against this damage, as evidenced by the decreased severity of these histopathological findings in the FA-treated groups compared to the IMI-treated groups. The findings of this study suggest that folic acid has a protective effect against imidacloprid-induced oxidative stress and toxicity in rat kidney and liver. This is supported by the significant decrease in oxidative stress markers and histopathological damage in the FA-treated groups. These findings highlight the potential benefits of FA in reducing imidacloprid-induced toxicity and contribute to the broader conversation about safeguarding human health and the environment from pesticide-related challenges. The study has few limitations, including the small sample size and the short duration of the study. Further studies are needed to confirm the findings of this study and to investigate the mechanisms by which FA

exerts its protective effects against imidacloprid-induced toxicity. Overall, the findings of this study suggest that FA is a promising candidate for the prevention and treatment of imidacloprid-induced toxicity.

INTRODUCTION

Pesticides and insecticides are critical to the productivity and security of food crops. Nonetheless, the use of these chemical agents has raised concerns because of the possible effects on non-target creatures and human health. Balancing crop protection with ecosystem preservation remains crucial, requiring extensive research and precise risk assessment. Understanding and managing these issues is critical for long-term agricultural practices and the health of both ecosystems and human populations (Nicolopoulou-Stamati et al., 2016a). In modern times, environmental contamination is a widespread issue and concerns regarding potential negative impacts on the environment, human health, and non-target species have been greatly increased because of the overuse of pesticides to increase the crop yield. Exposure to insecticides has reportedly caused issues such as cancer, reproductive problems, endocrine disruption, kidney, and liver damage in both humans and animals (El-Nemr, 2010). Understanding the mechanisms of chemical toxicity and identifying strategies to reduce harmful effects is essential for safeguarding humans and environmental health (Nicolopoulou-Stamati et al., 2016a).

1.1 Pesticides Use in Agriculture and Its Adverse Effects on Public

Pesticides can have negative impacts on ecosystems, human health, and the environment despite being created to protect crops and increase agricultural productivity. Pesticides can harm beneficial insects, birds, and aquatic organisms, disrupting ecosystems and reducing biodiversity. Excessive pesticide use can result in runoff, carrying these chemicals into water bodies and causing contamination in aquatic environments. This pollution adversely impacts aquatic life and poses a threat to the purity of drinking water sources as well (Bashir et al., 2020). Similarly, pesticide residues on crops can enter the food chain and harm human health with acute and long-term effects, including developmental issues, neurologic disorders, and certain cancers. Farm workers who handle pesticides are at risk of direct exposure, potentially leading to acute poisonings and chronic health problems (de-Assis et al., 2021).

Since the middle of the 1980s, the regional landscape has transformed. Sales have increased significantly in Latin America and Asia, outpacing other areas. Two critical considerations should be considered: corporations modify pricing to adapt to expanding economies, frequently giving

lower costs, while developing nations have increased demand for cheaper goods. This demand is coupled by rising pesticide use, particularly in tropical locations, which offers acute toxicity hazards. Evaluating sales only on value may mask larger volumes in certain countries (Dinham, 2010). Based on a global farming population of around 860 million, a more recent study published in 2020 found that approximately 44% of farmers worldwide are poisoned by pesticides each year. These poisonings resulted in nearly 20,000 fatalities (Boedeker et al., 2020). In Vietnam, a self-surveillance study of 50 farmers over 12 months revealed that, while only two moderate poisonings were treated at the neighborhood health service each month, 54 moderate poisonings were reported on average per month (Murphy et al., 2002).

The neurological and endocrine systems are the primary sites of action for most pesticides, making them potentially hazardous to humans and having substantial direct or indirect negative health impacts (Nicolopoulou-Stamati et al., 2016a). Pesticides can directly or indirectly affect people. Indirect exposure occurs when Food and water that have been contaminated are consumed, Droplets of pesticide are inhaled from the small airborne droplets or particles of the pesticide, and direct exposure happens during the procedure of Applying pesticides in agriculture, public health, livestock, and disinfection. Due to their physiology, behavior, and physical makeup, fetuses and children are more sensitive to pesticides than adult; even extremely low amount of exposure during critical developmental periods might have negative health impacts (Benjamin and Nosiri, 2011). Some of the major health issues as a result of pesticide poisoning/interaction are discussed as below.

1.1.1. Cancer

Numerous studies draw a direct link between cancer and toxicity, De Roos et al., (2005) conducted a thorough study in which they gathered information from pesticide sprayers and correlated pesticide contact with cancer. A bibliometric study released in 2021 discovered that pesticide exposure and toxicity has been linked to a variety of malignancies, together with breast cancer, colorectal cancer, myeloma, and non-melanoma skin cancer (Pedroso et al., 2022). Similarly, according to Beane Freeman et al., (2005), licensed pesticide applicators who were exposed to higher levels of diazinon had an increased risk of developing lung cancer and leukemia. Researchers have extensively attempted to address this issue, primarily employing statistical methods, yet their endeavors have been predominantly obscured by the absence of a definitive

solution. Nevertheless, it is worth noting that no significant correlations were detected in relation to additional cancer sites. Despite the persistent efforts of numerous researchers, the lack of clarity in resolving this matter remains prevalent (Cavalier et al., 2023).

1.1.2. Respiratory illnesses

Multiple studies have pointed out that, Agriculture workers are at an increased risk to respiratory conditions like asthma and chronic bronchitis. In agricultural practices, pesticide exposure has been linked to a higher likelihood of respiratory symptoms (Faria et al., 2005). A study by Buralli et al., (2018) also indicates that pesticides may increase the risk of developing lung conditions, morbidity and mortality issues. Apart from above reports there are many other suspected disorders that have been attributed to be caused by different pesticides such as neurological disorders (Richardson et al., 2019), diabetes, heart disorders (Mostafalou and Abdollahi, 2013), reproductive syndromes (Hanke and Jurewicz, 2004) and several others.

1.1.3. Environmental Hazards of Pesticides

Pesticides can pollute soil, grass, water, and other types of flora. Apart from eliminating weeds and insects, pesticides may also poison other animals, including birds, fish, non-target plants and helpful insects (Mahmood et al., 2015). The unchecked use of pesticides has caused a decline in various aquatic animal, terrestrial and plant species. Additionally, they have presented a threat to the survival of some endangered species, including the peregrine falcon, bald eagle and osprey (Mahmood et al., 2015) . According to a European study, seventy-six pesticide residues have been found in topsoil, according to their findings, 83% of the soil samples tested for pesticide residues contained at least one residue, and 58% of the samples tested positive for two or more residues. Notably, consistent high concentrations of glyphosate and its byproducts were found. The study also found numerous pesticides in surface water, rivers, and lakes throughout Europe, which may be dangerous for aquatic life (Silva et al., 2019). Only 0.1% of applied pesticides reach their intended target, and the remainder pollutes soil (Pimentel, 1995). Amphibians are particularly impacted by pesticide-contaminated surface waters and habitat loss (Products et al., 2018).

Pesticides are crucial for protecting crops and public health from pests, weeds, and diseases. They prevent the spread of vector-borne illnesses like malaria, dengue fever, and schistosomiasis. Examples of pesticides include herbicides, insecticides, rodenticides, fungicides, and plant growth regulators (Nicolopoulou-Stamati et al., 2016b). Pesticides can be broadly categorized based on

applications, target organisms, and chemical composition. Pesticides are also classified based on their source and target pest species. They can also be grouped according to how they function (Abubakar et al., 2020).

1.2. Classification of pesticides

- **Inorganic Pesticides**

They are made up of chemicals which are simpler and soluble in water. Inorganic salts like copper sulphate, ferrous sulphate, lime, and sulfur are examples of inorganic pesticides (Gunnell et al., 2007).

- **Natural Organic Pesticides**

Alkaloids, terpenes, and phenolic compounds, which showed pesticidal potential, are just a few examples of the phytochemicals found in plants that are classified under the category of natural organic pesticides (Hedin and Hollingworth, 1997). Due to their low mammalian toxicity, brief environmental persistence, and complex chemistry that prevents pest resistance, these natural organic are especially desirable (Abubakar et al., 2020).

1.3. Classification based on Origin

Pesticides are classified according to their sources, which include organic pesticides such as pyrethrin and neem oil, biological pesticides derived from bacteria, viruses, and fungi, inorganic pesticides such as inorganic salts, and synthetic pesticides like organophosphates, organochlorines, carbamates, and pyrethroids. Also, Plant-incorporated-protectants (PIPs) refer to pesticidal substances synthesized by plants using genetic material introduced into the plant (Garcia et al., 2012) and (Agency, 2020).

1.4 Pesticide Classification Based on Pest Species

- **Fungicides**

  This product effectively eliminates various types of Blights, mildews, molds, and rusts are all examples of fungi. Some examples of the active ingredients include azoxystrobin, benalaxyl, copper sulfate, and many others (Wood Lee, 1988).

- **Herbicides**

There are a variety of herbicides available for the control of weed, including sulfonylureas like amidosulfuron, flazasulfuron, metsulfuron-methyl, rimsulon, sulfometuron-methyl, terbacil, nicosulfuron, and triflusulfuron-methyl. These products are commercially available. The acetolactate synthase enzyme is inhibited by these broad-spectrum herbicides, which kill plants, weeds, and pest (Appleby et al., 2001) .

- **Rodenticides**

  Used to kill rodents and warfarin is a famous example.

- **Insecticides**

  These are substances used to kill insects. This category of pesticides is further sub-divided into following.

  - **Organochlorides**

Those Organic compounds containing at least one covalently bonded atom of chlorine. DDT , endosulfan, Chlordane (Rehman et al., 2016) ; (Richardson et al., 2019).

  - **Organophosphates**

This pesticide is formulated to disrupt the insect's neural functions. Organophosphates interfere with cholinesterase enzymes, causing insect to die or become incapacitated by interfering with nerve impulses (Marrs, 1993). Their use has been restricted in many countries due to their adverse effects on non-target animals. Examples include Malathion, Chlorpyrifos, Diazinon etc.

  - **Carbamates** Pests and insects that are exposed to carbamates suffer neurological damage because they prevent the activity of the acetylcholinesterase (WHO, 1986).
  - **Pyrethroids** They are axonic excitotoxins, which means their toxicity is mediated by inhibiting the closure of voltage-gated sodium channels in axonal membranes.

1.5. Neonicotinoids

The discovery of neonicotinoids in the 1990s was an important turning point in crop protection, revealing a rapidly expanding family of pesticides. Because of their effectiveness and extraordinary adaptability, neonicotinoid insecticides have subsequently become an essential component of current agricultural techniques (Jeschke et al., 2011). The neonicotinoids function as agonists of post-synaptic nicotinic-acetylcholine-receptors, which are being said to be their molecular target site, and specifically interact with the insect central nervous system (Tasman et al., 2021). Neonicotinoids can be divided into one of three chemical groups: nitromethylenes

(nitenpyram), N-cyanoamidines, and N-nitroguanidines (thiamethoxam, dinotefuran, clothianidin, and imidacloprid) (acetamiprid and thiacloprid) (Goulson, 2013).

The functional properties of insect nAChRs can be better understood through the use of neonicotinoids as helpful tools for structural investigations. Furthermore, These insecticides are highly specific to their intended targets, there is minimal risk to organisms that are not the intended target, and there are several ways to apply it (Jeschke and Nauen, 2008). Apart from their beneficial role, these insecticides have been found to have negative consequences as their presence is detected in the environment, including pollen, soil, water, and honey because of their extensive use. Aquatic insects and pollinators are especially vulnerable to neonicotinoid effects, with chronic sublethal effects more common than acute toxicity (Hladik et al., 2018). Given how frequently neonicotinoids are used as seed coatings, it is likely that crops grown from treated seeds will contain them in their pollen, leaves, and nectar. According to estimates, the crop absorbs 2–20% of the neonicotinoid coating (Sur and Stork, 2003). Similarly, neonicotinoid percentage can vary in leaves, pollen, and fruits. Additionally, neonicotinoids have been found in wildflowers close to agricultural areas, suggesting that they may disperse from the area where they were applied and be absorbed by other plants that are not the intended targets (Botías et al., 2016).

Another major problem with neonicotinoids is that they diffuse into soil from neonicotinoid-coated seeds and direct spraying. And these sprays are done each year or seasonally which further increases neonicotinoids level in soil, posing even severe threat to non-target animals and plants (Goulson, 2013).

1.6. Imidacloprid

Imidacloprid (IMI) is a chloronicotynl neonicotinoid compound which is systemic insecticide, (N-{1-[(6-Chloro-3-pyridyl) methyl]-4,5- dihydroimidazol-2-yl}nitramide). It is the most popular insecticide used to treat seeds and control insect pests globally (NCBI, 2023). It has a low soil persistence and a high insecticidal activity at a low application rate (Pandit et al., 2016). Imidacloprid binds to nicotine acetylcholine receptors (nAChR) showing significant activity on various insect species. The molecular homology found between nAChR's of insects and humans suggests that cross reaction of imidacloprid might occur in humans as well but many studies have proved their relatively low toxicity (Tomizawa and Casida, 2003); (Tomizawa and Casida, 2005).

There exist still some gaps in understanding selectivity on target and non-target animals, as overuse of imidacloprid might lead to the targeting of non-target animals such as pollinating insects like honey bees (Suchail et al., 2001a), amphibians (Campbell et al., 2022), mammals (Duzguner and Erdogan, 2010).

1.6.1. Effects of IMI on Pest animals:

Imidacloprid is very good at killing insects like aphids, leaf hoppers, plant hoppers, thrips, and hemipterans (Li et al., 2023). Furthermore, the compound is effective against some Diptera, Coleoptera, and Lepidoptera species (Easton and Goulson, 2013). There is no evidence of activity against nematodes or spider mites. Imidacloprid has high xylem mobility, making it ideal for treating seeds and putting it to soil, although it is equally effective when applied topically (Cahill et al., 1996). Imidacloprid is effective against different pests, with lethal concentrations varying from 1 ppm to 40 ppm. Even lower systemic concentrations, called sub-lethal doses, have been found to influence many pests, especially aphids. These sub-lethal doses are below 10 ppb and can change the behavioral attributes of pests, leading to decreased honeydew excretion, wandering behavior, and ultimate starvation-induced death (Cahill et al., 1996).

1.6.2. Toxicity of Imidacloprid on Humans and other animals:

Since the discovery of imidacloprid a decade ago, there have been many more cases of acute poisoning and reported fatalities worldwide. It can be nearly entirely absorbed in the gastrointestinal tract after being ingested. It then undergoes transformation into 6-chloronicotinic acid, which either naturally exits the body or is reduced to guanidine after being conjugated with glycine. Eighty percent of the administered dose may be excreted unchanged in the urine and 20% may be excreted in the feces (Wake, 2004) ; (Proença et al., 2005). After 30 minutes, a healthy 35-year-old man farmer (body weight, 85 kg) was found by his family to be sleepy with terrible nausea and copious vomiting of pesticide ingestion. In an attempt of suicide, the patient claimed to have consumed about 350 mL of imidacloprid. By the time the patient arrived at a nearby hospital (one hour after ingestion), palpitations, drowsiness, and disorientation was already present. On sixth day of his admission in hospital the patient died due to cardiopulmonary arrest (Proença et al., 2005).

Pollinating insects have declined significantly as a result of increased pesticide usage, especially IMI. Pollinators, such as honeybees, are more sensitive to poisoning than pests. Even modest pesticide concentrations have caused colony depletion and hive death (Suchail et al., 2001a).
Birds reproductive systems are believed to be adversely affected by imidacloprid, making it acutely toxic to them. In one study, when bobwhite quail were given a five-day exposure to imidacloprid, they developed hypo-reactivity, wing drop, ataxia, diarrhea, rigidity and severe arching of the back, intoxication, and immobility (CCME, 2007). According to a study published in 2018 Imidacloprid accumulated in the blood of rainbow trout (Frew et al., 2018).

1.6.3. Fate in the environment

According to several reports, imidacloprid may escape into the environment when used excessively and may persist in the soil for about 3000 days (Hussain et al., 2016). Because it is applied to crops, imidacloprid can potentially be damaging to both aquatic and terrestrial life as it enters the food chain and leaches into the water. The Environmental Protection Agency (EPA) claims that among all pesticides, imidacloprid has the greatest chance of leaching. Studying soil mobility in irrigated areas has also supported these findings Additionally, imidacloprid affects soil fertility, nitrogen fixation, various enzyme activities, and soil microflora (Bonmatin et al., 2019).

Imidacloprid can be degraded by photolysis, volatilization, hydrolysis, and microbial degradation. It can also be transported through the air and deposited onto surfaces. For example, a study found that imidacloprid can be transported through the air and deposited onto wildflowers near agricultural areas (Gervais, 2010). In a study, the insecticide imidacloprid was applied as a foliar spray in the counties of Butte, Imperial, Santa Clara, and Solano. The California Department of Pesticide Regulation immediately collected air samples after the applications and did not find any of imidacloprid residue in air (Segawa et al., 2004).

It has been demonstrated that estimates about soil adsorption and half-life depend on soil characteristics, such as imidacloprid sorption is affected by organic carbon and minerals (Liu et al., 2006). A study was conducted in California in 2012 results of which demonstrated that Imidacloprid frequently disperses off-site and contaminates surface waters at levels that may be harmful to aquatic invertebrates (Starner and Goh, 2012) . Imidacloprid has been demonstrated to

be toxic to soil microbial populations, showing that it can stay in the soil and possibly cause damage to soil health (Cycoń et al., 2013).

Numerous bacteria, fungi, and algae have been identified, to be capable of metabolizing imidacloprid using known metabolic pathways. The bacteria Azotobacter, Bacillus, Pseudomonas, Mycobacterium, Rhizobium, Ochrobactrum, and Paracoccus actively contribute to the biological remediation of imidacloprid (Pang et al., 2020).

1.6.4. Imidacloprid mechanism of action

Nicotinic-acetylcholine-receptors (nAChRs) are ligand gated ion channels that play a role in the quick transmission of cholinergic synaptic signals in the brain at neuromuscular junctions (Amiri et al., 2008). Both mammals and insects have nAChRs subtypes but due molecular differences the imidacloprid is more specific towards insect nAChRs. The nAChRs are located on post-synaptic neuronal membrane which are main target site for neonicotinoids like imidacloprid (Buckingham et al., 1997). The majority of nAChRs exist as heteropentamers made up of α subunits that have two adjacent cysteines in loop C of the extracellular N-terminal region, or ligand binding domain, and non- α subunits (β subunits) that do not have this motif. Homopentamers with α subunits are referred to as homomeric α receptors. Different Heteromeric and homomeric neuronal nAChRs are found in both mammals and insects confirmed by study of (Thany et al., 2007). This differential distribution of nAChRs in mammals and Insects has allowed us to selectively target insect's nAChRs, but cross reaction is also possible and have been confirmed by many studies.

These post-synaptic receptors can be found on entire central-nervous-system of insects as well as mammals. Due to their role in rapid neurotransmission across a variety of species, including arthropods and mammals, nAChRs are of particular importance (Changeux et al., 1998) ; (Thany et al., 2007). At least three different subtypes of nicotinic receptors are affected by imidacloprid in cockroaches. A bi-phasic response is brought about by neonicotinoids: an initial increase in spontaneous discharge frequency, followed by a total block of nerve transmission.

Structure:

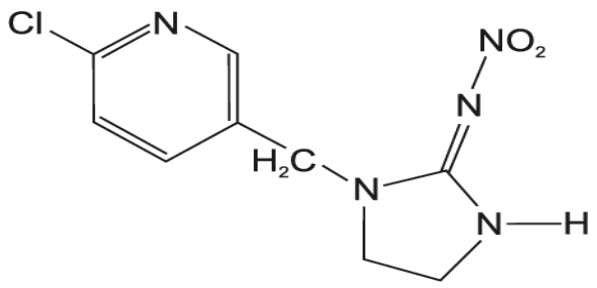

Fig 1.1 Chemical structure of Imidacloprid, (Papp, 2014).

An imidacloprid molecule is formed by connecting the 6-chloro-3-pyridyl group and the 2-nitroimino-imidazolidine group through a methylene chain. All three of these components have an impact on toxicity, binding, and activation of receptor (Kagabu, 1997). The 2-nitroimino-imidazolidine group, among these, is essential for the selectivity for insect nicotinic acetylcholine receptors (nAChRs). In comparison to compounds that contain CH-NO_2 group at this position, the presence of N-NO_2 substituent at the 2-position of the imidazolidine ring has little effect on insecticidal activity, yet, it causes a substantial decrease in receptors' binding capacity. This indicates that the increase in hydrophobicity, which improves transport to the target sites, offsets the reduction in binding caused by the nitrogen atom at the 2-position (Kagabu, 1997). Presently, researchers are employing alpha bungarotoxin to examine the interaction between imidacloprid, nicotinic acetylcholine receptors (nAChRs), and how they associate. This approach could potentially unveil specific nAChR subtypes that might be susceptible to these insecticides. By comparing α-Bgt binding with the binding profiles of imidacloprid and other neonicotinoids, valuable insights into their characteristics can be collected. This knowledge aids in an improved comprehension of how neonicotinoids, such as imidacloprid, affect insect nAChRs and their selectivity (Taillebois et al., 2014). These results obtained by Lind et al., (1999) and Zhang et al., (2004) imply that the majority of nAChR binding sites were α-Bgt-sensitive. As demonstrated in competitive experiments on native insect nAChRs, the high density of these binding sites suggests that labelled α-Bgt could be of particular interest to study the binding characteristics of other nAChR ligands, such as neonicotinoid insecticides. In competitive binding assays, different results have been observed with the neonicotinoid insecticides IMI, CLT, and thiamethoxam (TMX). melanogaster head membrane binding experiments showed that [3 H]-IMI and [3 H] α-Bgt bind

to different binding sites. The binding capacity of [3 H]-imidacloprid in Drosophila melanogaster is not affected by the addition of [3 H] α-Bgt at the same time, proving the existence of separate binding sites for imidacloprid and α-Bgt (Zhang et al., 2004). According to the data provided by Taillebois et al., (2018), while hemipterans typically have two IMI-binding sites, some species, such as dipterans and lepidopterans, have only one. There are however some exceptions, including the single-binding site Bemisia *argentifolii* (Silverleaf whitefly) although a hemipteran but still it has one binding site (Taillebois et al., 2018).

1.6.5. Metabolism of Imidacloprid

Imidacloprid is metabolized by various enzymes, including cytochrome P450 isozymes and liver microsomal and cytosolic enzymes. These enzymes facilitate the oxidation and reduction processes of imidacloprid, occurring at the imidazolidine and nitroimine segments, respectively (Schulz-Jander and Casida, 2002) ; (Lu et al., 2016).

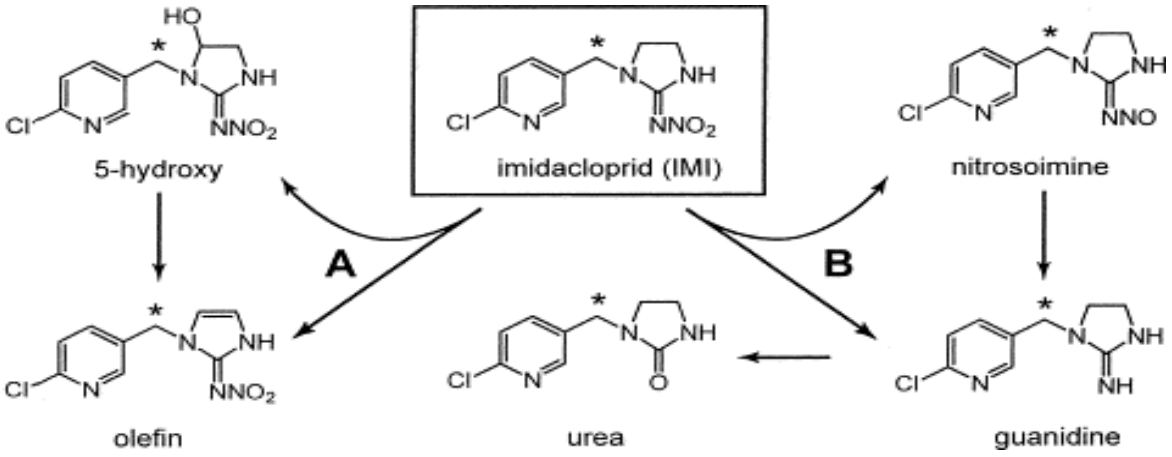

Fig. 1.2 (Schulz-Jander and Casida, 2002)

Imidacloprid remains resistant to hydrolysis under acidic or neutral conditions. However, hydrolysis becomes more noticeable over time with elevated temperatures. Esterase can also

hydrolyze imidacloprid, leading to the formation of imidacloprid olefin (Gervais, 2010). Imidacloprid olefin undergoes oxidation reactions primarily mediated by cytochrome P450 enzymes. This step can result in the formation of various metabolites, including 5-hydroxyimidacloprid and 4,5-dihydroxyimidacloprid (Lu et al., 2016). Imidacloprid is rapidly metabolized by honeybees, with a half-life of 4.5-5 hrs. In honeybees, the two primary imidacloprid metabolites are 5-hydroxyimidacloprid and olefin (Suchail et al., 2004).Imidacloprid is also metabolized in plants, with the major metabolite being the ethylene-bridge hydroxylation of the imidazolidine ring and water elimination (Sur and Stork, 2003). In mammals and insects, toxic metabolites can also be produced which can prove to be even more lethal such as IMI-ole, 5-hydroxyimidacloprid, 4,5-dihydroxyimidacloprid and Desnitro-imidacloprid which has been shown to be toxic to honey bees (Suchail et al., 2001b).

More than 90% of the dose is excreted within 24 hours, and the remainder is gone entirely by 48 hours. Eighty percent of the dose is eliminated through the urine, and 20% is passed through the feces. Imidacloprid gets transported to organs and undergoes absorption in rats within 1 hour of oral administration. The central nervous system(CNS) does not tend to build up because it do not get distributed to fatty tissues, bone, or the CNS (Baselt, 2014).

1.6.6. Investigating the NOEL Dose of Imidacloprid:

Wistar adult male and female rats received imidacloprid through their diets for 13 weeks, and at any dose level, even the maximum dose of 300 mg/kg/day, there were no clinical signs. The primary target organ was the liver, which underwent mild sporadic hepatocyte necrosis and hypertrophy in high-dose males but recovered within four weeks. In males and females, the no observed adverse effect level (NOAEL) was 14 and 83 mg/kg/day, respectively (Baselt, 2014). Imidacloprid was given to Wistar rats for 12 months and 2 years at concentrations of 100, 300, 900, and 1800 ppm. This corresponds to mean daily amounts of 5.7, 17, 51, and 103 mg/kg for males, and 7.6, 25, 73, and 144 mg/kg for females. The no-observed-adverse-effect-level (NOAEL) was established at 5.7 mg/kg per day, and there was no evidence suggesting carcinogenicity (Sheets, 2014).

1.7 Vitamin B9 (Folic-Acid)

Folic acid, often known as vitamin-B9, is an essential vitamin that actively promotes cellular health and well-being. It belongs to the water-soluble vitamin, which is crucial in many physiological functions (Shulpekova et al., 2021). Firstly, folic acid serves as a cofactor in essential one-carbon pathways, participating in biochemical reactions that donate methyl-groups to key molecules like choline phospholipids, creatine, epinephrine, and DNA. By engaging in these pathways, folic acid supports crucial cellular functions, promoting overall health. Folic acid, in particular, is required for DNA synthesis, its repair, and methylation, which ultimately contributes to healthy cell division and growth. It helps in generating new cells and contributes to red blood cell production (Crider et al., 2012). Optimal folic acid levels are particularly crucial during periods of rapid cell division, such as pregnancy and infancy. Likewise, the animal body direly needs folic acid for the generation and metabolism of neurotransmitters such as serotonin, dopamine, and norepinephrine, which govern mood, cognition, and mental well-being. Thus, it also plays a role in behavioral development as well as maintenance (McGarel et al., 2015) . Folic acid is shown to promote cardiovascular health by managing homocysteine levels in the blood. Increase in the homocysteine levels is linked to an increased risk of cardiovascular disease. By maintaining optimal homocysteine levels, folic acid promotes cardiovascular well-being. Because human bodies cannot manufacture enough folic acid, it must be obtained from supplements or diet (Li et al., 2016). Leafy green vegetables, legumes, citrus fruits, fortified cereals, and liver are excellent sources. Adequate folic acid intake is especially critical during pregnancy to avoid severe health complication.

1.7.1. Importance of Folic Acid in Maintaining Cellular Health

Folate coenzymes are essential for the cellular systems of organism because they facilitate the transfer of one-carbon units. The coenzymes in question can receive and transfer these one-carbon units, which are essential in the metabolism of nucleic acids and amino acids. This occurs in stages, with folate coenzymes absorbing and then contributing one-carbon units. Transfer of these one-carbon units is required for various key activities, including DNA synthesis, DNA repair, amino acid synthesis, and amino acid interconversion. Folate coenzymes contribute considerably to the general metabolism and functioning of nucleic acids and amino acids in the body by facilitating the transfer of one-carbon units (Shulpekova et al., 2021). Folic acid is required for cell division and cell synthesis in numerous organs and bone marrow. It is essential for the synthesis and repair

of DNA and RNA, both of which are required for cellular development and functioning (Scholl and Johnson, 2000) and (Shulpekova et al., 2021).

1.7.2. Mechanisms of Action and Protective Effects:

Folic acid ingestion occurs via diet or through supplemental intake (Medicine et al., 2000). In the small intestine under the action of dihydrofolate reductase enzyme folic acid is converted into Tetrahydrofolate (THF), which is the biologically active form of ingested folic acid.

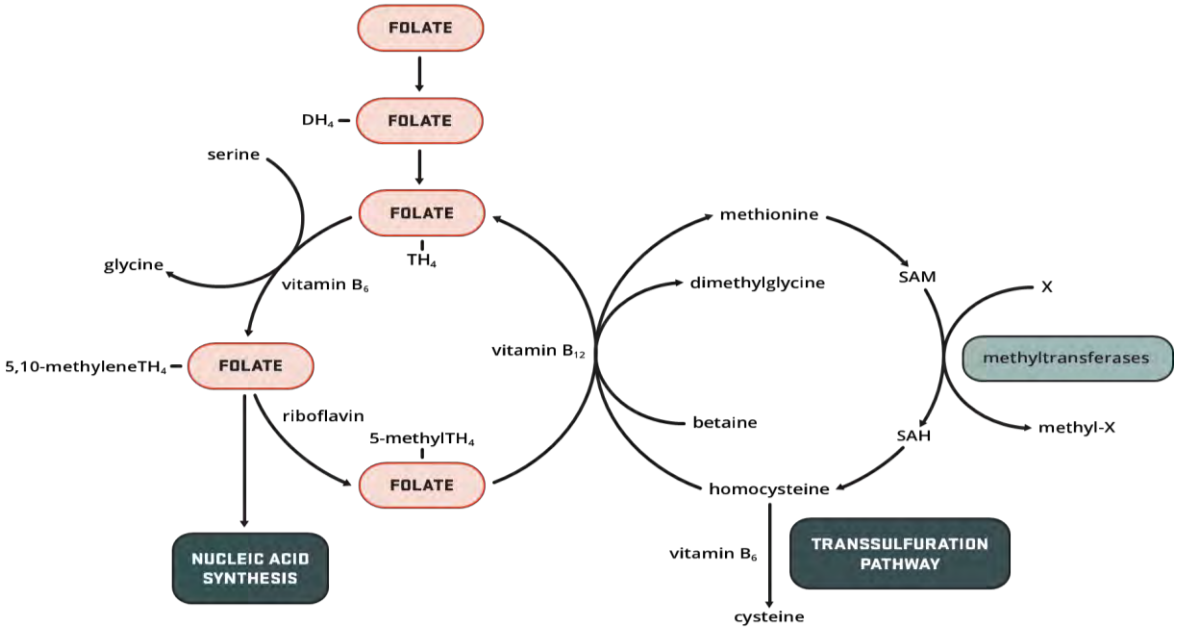

Fig. 1.3 (Higdon, 2000)

THF acting as coenzyme performs crucial role in one carbon transfer reaction. In such reactions the THF accepts one carbon atom in the form of methyl (CH3) from doner molecules (Bailey and Gregory, 1999). The step is critical where the THF donates one-carbon molecules to DNA, RNA, Lipids, and proteins. This later one controls the behavior of DNA and gene expression (Choi and Mason, 2000). Deoxyuridine monophosphate (dUMP) is methylated by THF to form thymidine monophosphate (dTMP), a crucial building block of DNA. THF also plays pivotal role in synthesis of purines and pyrimidines as well as in amino acid metabolism. Purines and pyrimidines synthesis is also facilitated by one-carbon units donation in complex chemical reactions. Homocysteine is derived from methionine metabolism THF donates methyl group to homocysteine generating methionine which is an essential amino acid used in synthesis of proteins, neurotransmitters etc.

Serotonin, nor-epinephrine and dopamine (Bailey and Gregory, 1999). S-adenosylmethionine(SAM) is synthesized partly by THF and SAM in return is responsible for neurotransmitters synthesis.

Aims and Objectives

Aims

The present research was conducted within the Laboratory of Animal and Human Physiology, Department of Zoology, to assess the in vivo toxicity of imidacloprid (IMI) and supplementary role of folic acid on body weight, biochemical, and histological parameters in female albino rats. The doses and parameters were chosen based on prior research.

Objectives

Present research was carried out to investigate the following:

- To assess the impact of IMI exposure on the body weight of rats and determine whether FA supplementation could mitigate any observed effects.
- To investigate how IMI exposure influences biochemical markers related to liver function, oxidative stress, and antioxidant activity. Further, to determine whether FA supplementation has any modulatory effects on these parameters.
- To examine the histological changes in liver and kidney tissues caused by IMI exposure and to determine if FA administration can influence these changes.
- To explore the potential interaction between IMI and FA in altering the observed toxic effects, especially concerning oxidative stress and antioxidant mechanisms.
- Measurement of liver and kidney's total protein content.

Material and Methods

This study was designed and conducted in the laboratory of Human and Animal Physiology, Department of Zoology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan.

2.1 Study approval

The ethics committee at Quaid-I-Azam University in Islamabad, Pakistan, approved this study. All animal procedures were conducted in complete compliance with the standard rules and guidelines established by the Bioethical Committee of Biological Sciences. Study approval number is # **BEC-FBS-QAU2023-516**.

2.2. Animal maintenance

Twenty-four male laboratory rats average weight of 160 ±5g were purchased from the National Institute of Health Islamabad. Rats were housed in rodent cages made of polycarbonate plastic. Animals were acclimated for two weeks. They were provided standard laboratory conditions of 12:12 dark: light, a relative humidity of 50 to 60% and 24±3 °C temperature. Animals were provided rodent chow and fresh drinking water ad libitum. To prevent overcrowding-induced stress, the rats were accommodated six per cage. Throughout the study, the rats were carefully cared for, during experimentation.

2.3 Body weight measurement

Before beginning the experiment, the rat's body weights were recorded and regularly throughout the entire experimental period, with a two-day break each week.

2.4 Experimental design

Rats were assigned randomly into four groups. The first group was control of rats, who received distilled water. The second group received 45mg/kg/day Imidacloprid diluted in distilled water through oral gavage for 21 days. The third group was treated with oral gavage dosage of folic acid at 10mg/kg/day. The fourth group was given folic acid at a dosage of 10mg/kg/day orally one hour

before receiving the Imidacloprid dose of 45mg/kg/day. All the animals received their individual dosages throughout the 21-days of experimental period.

Table 2.1 Respective groups and their doses

Groups	Doses
Group 1	Control (Normal saline)
Group 2	Imidacloprid (IMI) 45mg/kg/day b.w
Group 3	Folic acid (FA) 10mg/kg/day b.w
Group 4	FA 10mg/kg/day and IMI 45 mg/kg/day b.w, after one hour of FA

Table represents groups and their doses with each group having number of individual n=6.

2.5 Dose preparation

Because IMI and FA are both soluble in water, distilled water was used as a solvent. Doses were appropriately mixed in separate falcon tubes for each chemical, and each tube was vortexed for 2 min. The administered solution with the chosen concentration of IMI and FA was approximately 1ml per rat.

2.6 Dose administration

The route of administration was via oral gavage. For each group, the chosen dose was administered orally using infant tubes or pigeon tubes. Initially, 1.0 ml of the solution containing the desired imidacloprid concentration was loaded into a 3 ml syringe. The infant tube was then attached to the syringe, and the tube was gently inserted through the mouth and esophagus into the stomach, releasing the solution slowly. If any resistance was encountered, indicating potential insertion into the rat's trachea rather than the esophagus, the tube was promptly withdrawn, and the animal was allowed to rest briefly.

2.7 Dose timing

The dosage schedule involved administration within the designated timeframe from 09:30 AM to 11:30 AM to avoid any stress or resistance from the animal. Dose timings were kept same

throughout the experiment. Dose synchronization is very important for animal receptiveness for dose administration.

2.8 Animal dissections

Following the completion of 21-day period, animals were dissected. All animals survived until the final day of experiment. The dissection process commenced 24 hours subsequent to their last administered dose. Animals were sacrificed and blood was collected in gel tubes (BIOTUE, BT company?) allowed to stand for 30 min and centrifuged for a duration of 15 min at 3500 rpm. Following the centrifugation, serum was collected and stored at -20°C for subsequent analysis. Liver and kidney tissue were taken and rinsed in saline. Part of organs obtained were treated with liquid nitrogen and covered with aluminum foil and then stored at -20C for antioxidant analysis, while remaining parts were preserved in 10% buffered formalin for histology.

2.8.1 Calculation of the Organ Mass Index

Organ mass index was calculated using the formula adopted from Reddy et al. (2011). The organ mass index was determined by dividing the organ-weight by the body weight and then multiplying the result by 100.

Organ mass index = Organ weight/Body weight x 100.

2.9. Biochemical analysis of tissues

Levels of oxidative stress and antioxidant enzymes were assessed within both the liver and kidney tissues. Parameters including ROS, TBARs (Thiobarbituric acid reactive substances), and key antioxidant enzymes encompassing SOD (Superoxide dismutase), POD (Peroxide desmutase), CAT (Catalase), as well as non-enzymatic antioxidant reduced glutathione (GSH), were quantified. Additionally, the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total protein were assessed.

2.9.1 Preparation of Extract buffer

The extract buffer contained 5.95g HEPES, 4.3g NaCl, sodium azide in 495ml dH_2O. Then 0.5g SDS, 4.38g NaCl, 5ml Triton-X 100 were added and the volume was raised to 500ml.

2.9.2 Tissue homogenate:

Liver and kidney tissues were sliced on frosted petri plates and minced in a handled tissue homogenizer (GPE limited UK). Subsequently, the minced tissue was homogenized in 1ml of extract buffer (Lysis buffer pH. 7.0), with the addition of 0.1mg of Phenylmethylsulfonyl fluoride (PMSF). The homogenate was then subjected to centrifugation at 9,300 g (Eppendorf 5415D) for 10 minutes. After careful aspiration of supernatant, it was transferred to labeled autoclaved 1.5ml eppendorf tubes which were then stored at -20°C.

The following biochemical parameters were determined.

2.9.3 Estimation of reactive oxygen species

To measure ROS in tissue samples, the method described by Hayashi et al., (2007) was followed. To prepare the required solutions, 4.1g of sodium acetate were dissolved in 500ml of distilled water, resulting in a 0.1M sodium acetate buffer with a pH of 4.8. Additionally, 10mg of N, N-Diethyl-p-phenylenediamine sulfate (DEPPD) were dissolved in 100ml buffer to prepare reagent 1. A ferrous sulfate ($FeSO_4$) stock solution was prepared by dissolving 50mg of $FeSO_4$ in 10ml of sodium acetate ($NaCH_3COO$) buffer, and then, 62.5µl of the $FeSO_4$ stock solution was dissolved in 125ml of the sodium acetate buffer to create reagent 2. Both reagents were then mixed in a 1:25 ratio and kept in the dark for approximately 2 min. Subsequently, 1200µl of sodium acetate buffer, 1680µl of the reagent mixture, and 60µl of the homogenate sample were combined in a 3ml cuvette. The absorbance was read at 560nm at intervals of 30 sec, with three readings taken for each sample which were then averaged.

2.9.4 Analysis of lipid peroxidation assay (TBARS)

Malondialdehyde (Majumdar et al.) in the tissue homogenates was determined by the method of Iqbal et al. (1996). The reaction between MDA and thiobarbituric acid (TBA) was employed to indirectly determine the oxidative stress caused by lipid peroxidation. The reaction mixture was prepared in a 15ml falcon tubes by combining 0.1ml of ascorbic acid (1.5 mM), 0.1ml of 50mM Tris-HCl, 0.1ml of FeSO4 (1mM), 0.6ml of distilled water, and 0.1ml of tissue homogenate. The mixture was vortexed and then incubated at 37°C for 15 min. After incubation, 0.4ml of 0.67%

TBA (thiobarbituric acid) was added to the mixture, and the tubes were heated in a boiling water bath for 15 min to allow the reaction between MDA and TBA to form a colored complex. Following this, the reaction was stopped by placing the tube in an ice bath. The mixture was then centrifuged at 3000rpm for 10 min, and the supernatants were transferred to cuvettes. Absorbance readings were recorded at 532nm, with three readings taken at 30-second intervals for each sample.

Antioxidant enzymes

2.9.5.1 Catalase Assay (CAT)

The catalase assay (CAT) is a technique for measuring the activity of the enzyme catalase. catalase protects cells from oxidative damage by facilitating the breakdown of hydrogen peroxide into water and oxygen.

The tissue's catalase (CAT) activity was determined in accordance with the protocol outlined by Maehly and Chance (1995). The reagent mixture was carefully prepared by combining 1000µl of H_2O_2 (5.9 mM), 1.9ml of 50 mM potassium phosphate buffer (pH: 7.00), and 0.1ml of the homogenate sample within a 3ml cuvette. Subsequently, three readings were captured at 240nm for each sample, and these readings were then averaged.

2.9.5.2 Superoxide dismutase Assay (SOD)

The Superoxide dismutase (SOD) test measures the enzyme's ability to neutralize damaging superoxide radicals. SOD activity was assessed using the method described by (Kakkar et al., 1984). A reagent was prepared by combining 4.5ml of 9.9 mM L-Methionine, 2.25ml of Triton X-100 (0.025%), and 3ml of 57µM Nitro Blue Tetrazolium (NBT), and the final volume was adjusted to 90ml with 50 mM phosphate-buffered saline (PBS) at pH 7.8. From this mixture, 1ml was transferred to a cuvette. The cuvettes were then exposed to illumination from a fluorescent lamp for 7 min, followed by incubation at 37°C for 5 min. After the initial steps, 10µl of the sample was added to each cuvette, initiating the process, and the contents were further incubated at 40°C for 8 min. Subsequently, three readings for each sample were taken at 560nm, with intervals of 1 min, to obtain reliable measurements of SOD activity.

2.9.5.3 Peroxide dismutase assay (POD)

The POD test assesses the ability of an enzyme(peroxidase) that plays an important role in neutralizing the hydrogen peroxide, providing vital information on antioxidant protection and defense against oxidative stress in biological samples. The tissue homogenate's Peroxidase (POD) concentration was evaluated using the method described by (Rahman et al., 2006).A reaction mixture was formed by vigorously combining 2.5ml of 50 mM phosphate buffer, 0.1 ml of enzyme extract, and 0.1ml of 20 mM guaiacol to ensure homogeneity. Subsequently, 0.3ml of 40 mM H2O2 was added to the reaction mixture, and the change in absorbance was observed at 470nm after one minute. With an interval of 30 sec three readings were taken for each sample.

2.9.5.4 Determination of reduced glutathione (GSH)

Reduced glutathione was measured using the method defined by Jallow et al. (1974). The reaction mixture was prepared by combining 100µl of disodium phosphate buffer (0.4M), 0.1ml of tissue homogenate, and 0.5ml of DTNB (Ellman's reagent). DTNB was prepared by dissolving 40mg of DTNB in 100ml of 1% tris-sodium citrate. Subsequently, the reaction mixture was subjected to analysis till the appearance of the yellow color. The absorbance of the resulting yellow color was measured at 412nm.

2.9.5.5 Estimation of total protein

Utilizing the standard Bradford assay, quantification of total protein was carried out in both liver and kidney homogenates. To create a stock solution, a combination of 25ml of methanol, 50ml of H3PO4, 50 mg of Coomassie blue, and 100ml of distilled water was meticulously blended. This stock solution was stored in darkness at 4°C until required. For the preparation of a working solution, the stock solution was diluted at a ratio of 1:4 with distilled water. Similarly, a stock solution of BSA was generated by adding 10mg of BSA in 10ml of phosphate buffer saline. Serial dilutions of BSA were subsequently prepared from the stock solution, which yielded concentrations of 100x, 50x, 25x, 12.5x, and 6.25x. To prepare a standardization curve, 2900µl of the working solution was combined with 100µl of the various BSA serial dilutions within a cuvette. The resulting change in absorbance was recorded at 595nm. Likewise, for the purpose of protein estimation, 2900µl of the working solution was mixed with 100µl of the respective sample, and

absorbance changes were observed at 595nm. This procedure was conducted thrice within the span of a minute for each reading.

2.9.5.6 Serum biochemistry

After dissection, blood was obtained through cardiac puncture, and serum was isolated using gel tubes by centrifuging at 3500 rpm for 15 minutes and stored at 10 ºC, pending analysis. Subsequently, these samples were analyzed. Serum ALT and AST levels were evaluated using photometric autoanalyzer.

2.9.5.7 Histopathology

Formalin fixed liver and kidney tissues were processed for histology.

Reagent preparation

A. Harris hematoxylin

Hematoxylin was prepared by combining 1g powder with 10ml of absolute ethanol, while 20g of potassium alum was dissolved in 200ml of distilled water. Following this, the solutions of hematoxylin and alum were mixed and rapidly boiled. Subsequently, 500mg of mercuric oxide was introduced to the mixture. Once the staining solution had cooled down, 8ml of glacial acetic acid was carefully added. The resulting stain was then filtered using 0.45µm filter paper and stored at ambient room temperature.

B. Eosin solution:

A quantity of 1g of eosin was introduced into 0.05ml of acetic acid, followed by the addition of distilled water to achieve a final volume of 100ml.

C. Acid alcohol

Acid alcohol was prepared by mixing 1ml of concentrated HCl with 99 ml of ethyl alcohol (70%).

Procedure

Tissues were dehydrated in an ascending series of alcohol concentrations ranging from 30% to 100%, each step lasting for 1 to 2h. The tissues were in two rounds of xylene. The tissues were

embedded in paraffin wax. 5µM sections were cut on microtome (Shandon, Finesse, Italy). Following the microtomy, the tissue sections were immediately immersed in a water bath (Boekel Scientific). Transferred to clean glass slides, then on a slide warmer for overnight drying. The deparaffinization stage involved two changes of xylene, with each change requiring the slides to be submerged for a duration of 5 min. Finally, a process of hydration was carried out using a descending series of alcohol grades, including 100%, 90%, 80%, 70%, and 50%. Each grade was applied for three minutes, ensuring that the slides were exposed to the appropriate level of hydration. As a concluding step, the slides were gently washed using running tap water, affirming the careful preparation of the tissue sections for subsequent analysis.

D. Hematoxylin and eosin staining

Slides containing tissue sections were stained for 5 min in Harris hematoxylin, gently washed for 2 min in running tap water and dipped into acid alcohol for 5 sec, then again washed in running tap water for 45 sec. Slides were then stained in 1% working solution of eosin for 2min, and washed for 45 sec, in ascending series of alcohol (50%,70%, 80%, 90% and 100%), slides were then dehydrated 3 min in each. Tissue was cleared in xylene for 3 min. Sections were mounted in DPX at room temperature. Under light microscope slides were observed, examined at 10x and 40x. Images were taken using Olympus camera microscope.

Statistical investigation

For the interpretation of results one way analysis of variance (ANOVA), was used, for comparison among the groups post hoc Dunnett test was applied. Results are expressed as ± SEM, and were considered statistically significant at a p value of <0.05

RESULTS

3.1 Body weight

Initial body weights were assessed on day 0, revealing negligible variations among animals in distinct experimental groups (Table 3.1). On day 21, right before euthanasia, animals in treated groups exhibited discernible body weight changes in relation to the control group as shown in Table 3.1). Notably, G2 treated with IMI at 45mg/kg displayed a significant decrease in body weight compared to the control. However, the remaining groups demonstrate little differences in body weight.

3.2 Oxidative and antioxidant stress markers of liver

3.3 Reduced glutathione (GSH)

The examination of kidney GSH levels revealed distinct patterns across the experimental groups as summarized in (Table 3.3). As shown in fig (3.2), the levels of GSH, exhibited noteworthy changes in response to different treatments. In rats treated with 45 mg/kg b.w of imidacloprid (IMI), GSH levels exhibited a significant decrease when compared to the control group (G1). Conversely, rats in G3 treated with 10 mg/kg b.w of FA displayed an increase in GSH levels, suggesting a potential enhancement of cellular antioxidant defenses. This effect was further pronounced in Group 4 (G4), where the co-administration of IMI and folic acid (IMI+FA) effectively maintained GSH levels. This preservation of GSH appeared to play a protective role against oxidative damage, a conclusion supported by histological findings. This observation aligns well with the histological results, collectively suggesting that the concurrent administration of IMI and folic acid effectively mitigated IMI-induced oxidative stress. Multiple comparisons are G1 vs G2 (**$p<0.01$),G2 vs G3 (**$p<0.01$).

3.4 Reactive oxygen species

After 21 days of exposure to imidacloprid, a significant increase in reactive oxygen species levels was observed in the tissue homogenate of group 2 (G2) compared to the control group (G1), which was treated with dH2O. In group 3 (G3), which was treated with folic acid, a different pattern emerged, with ROS levels being lower than those in group 1. In group 4 (G4), where both imidacloprid and folic acid (IMI+FA) were administered, folic acid seemed to play a supplementary role by further decreasing ROS levels compared to group 2. Significant increases in liver ROS were observed between groups 1 and 2 (**p<0.01), 2 and 3 (***p<0.001), and 2 and 4 (**p<0.01), figure (3.1).

3.5 Thiobarbituric acid-reactive substance (TBARS)

TBARS levels significantly increased in G2 tissue homogenate sub-acute imidacloprid exposure, compared to the dH_2O control group (Fig 3.3). The folic acid G3 demonstrated a contrasting trend with a decrease in TBARS levels. Whereas G4 compared to G2 had decreased TBARS levels showing supplementary role of folic acid. G1 vs G2 (****p<0.0001); G2 vs G3 (****p<0.0001) and G2 vs G4 (***p<0.001) Fig (3.3).

3.6 Superoxide dismutase (SOD)

The concentration of SOD exhibited a significant reduction in groups exposed to oral IMI over a 21-day period. This reduction occurred in a dose-dependent manner when compared to G1. Likewise, G3 demonstrated a noticeable increase in SOD levels, while G4 showed that SOD levels were sustained with the help of FA, G1 vs G2 (****p<0.0001), G1 vs G4 (****p<0.0001); G2 vs G4 (****p<0. 0001) Fig (3.4).

3.7 Peroxidase (POD)

The concentration of POD showed a notable reduction (**p<0.01) in G2 as compared to G2(IMI) after 21 days of oral IMI exposure. This reduction followed a dose-dependent pattern as can be seen in fig (3.5). Contrasting with the significant increase (***p<0.001) in POD levels observed in G3. Furthermore, in G4, a positive supplementary effect was observed, showing a reduction in

IMI toxicity (Fig 3.5). Significant multiple comparisons are G1 vs G2 (**p<0.01), 2 vs G3 (***p<0.001).

3.8 Catalase (CAT)

CAT concentration was significantly lower (**p<0.01) in the G2 receiving an oral dose of IMI for 21 days compared to the control group that received distilled water, Multiple comparisons are G1 vs G2 (**p<0.01) and G2 vs G3 (***p<0.001) Fig (3.6).

3.9 Protein quantification

Total protein concentrations were considerably decreased in IMI groups in dose dependent manner and a significant increase (*p<0.01) in G3. The outcomes in G4 offer insights into the supplementary role of folic acid, Multiple comparisons are G1 vs G2 (**p<0.01); G2 vs G3 (***p<0.001) and G2 vs G4 (*p<0.05) (Fig 3.7).

❖ **Oxidative and antioxidative stress markers of the Kidney**

3.10 Reactive oxygen species

ROS levels in kidney homogenate were significantly enhanced (**p<0.01) in G2 rats given a daily dosage of IMI 45 mg/kg b.w for 21 days Comparison of significant values G1 vs G2 (**p<0.01); G2 vs G3 (**p<0.01), G2 vs G4 (*p<0.05), Fig (3.14).Absorbance was measured in absorbance unit(AU), which is amount of light absorbed in sample.

3.11 Thiobarbituric acid reactive substance (TBARS)

Imidacloprid (IMI) treatment for 21 days resulted in a significant increase in TBARS concentration in group 2 (G2) receiving IMI, as compared to the control group (G1). This is evident in Figure 3.8, which shows that the TBARS levels in G2 were significantly higher (***p<0.001) than in G1. In contrast, the TBARS levels in group 4 (G4), which received IMI and folic acid, were significantly lower than in G2 (***p<0.001). This suggests that folic acid may have a protective effect against the toxicity of IMI (Fig 3.8).

3.12 Superoxide dismutase (SOD)

In the context of the 21-day IMI regime, the treatment groups showed a significant reduction (**p<0.01) in SOD levels when contrasted with the control group (G1) (Fig 3.9). However, a noticeable increase was evident in G3 and G4, where the levels were lower but under the controlled influence of folic acid supplementary ability, Overall statistics are G1 vs G2 (**p<0.01), G2 vs G3 (**p<0.01), G2 vs G4 (*p<0.05) Fig (3.9).

3.13 Catalase (CAT)

In the study, CAT concentration in rat kidneys showed significant differences between groups. The IMI-treated group (G2) had notably lower CAT levels than the control group (G1), G3, and G4. Statistically, the G1 vs G2 difference was highly significant (**p < 0.01), the G2 vs G3 difference was extremely significant (***p < 0.001), and the G3 vs G4 difference was significant (*p < 0.05).

3.14 Peroxidase (POD)

The study revealed a notable decline in kidney POD levels in IMI-treated rats (G2) compared to both the control G1 (*p < 0.05) and G3 (**p < 0.01), as shown in Figure (3.10). This decrease in POD levels indicates potential oxidative stress in the kidneys due to IMI exposure.

3.15 Reduced glutathione (GSH)

The study showed a marked decrease in kidney GSH levels in the IMI-treated group (G2) compared to G3 (***p<0.0001), as presented in Figure (3.12). In contrast, G4 displayed higher GSH levels, underscoring the significant supplementary effect of folic acid (FA). Additionally, the comparisons G2 vs G4 (*p<0.05) and G1 vs G2 (*p<0.05) were statistically significant.

3.16 Total protein

Exposure to IMI in rats resulted in considerable reduction in kidney protein, multiple comparison of different group are G1 vs G2 (****p<0.0001); G1 vs G4 (****p<0.0001); G2 vs G4 (****p<0.0001); G3 vs G4 (****p<0.0001) Fig (3.13).

❖ **LIVER AST and ALT**

3.17 Serum alanine aminotransferase (ALT)

The study found that rats in the IMI-exposed group (G2) exhibited a significant increase in serum ALT levels, suggesting potential liver damage, as shown in the figure (3.18) and corroborated by liver histology. However, compared to G2, the G4 group showed notably lower ALT levels, indicating the potential protective effect of folic acid against IMI-induced liver damage. The statistical comparisons were highly significant: G1 vs G2 (****$p<0.0001$), G2 vs G3 (****$p<0.00001$), and G2 vs G4 (****$p<0.0001$), as detailed in Figure (3.15) and table (3.4).

3.18 Serum aminotransferase (AST)

Rats exposed to imidacloprid showed a significant increase in serum AST levels (***$p<0.0001$), suggesting potential liver damage. Statistical comparisons revealed highly significant differences between G1 and G2 (***$p<0.001$), G2 and G3 (***$p<0.001$), and a significant difference between G2 and G4 (*$p<0.05$), as indicated in Figure (3.16).

3.19 Histopathological changes in Liver

The histopathology findings in the liver sections are consistent with hepatic injury. The presence of lipofuscin, dilation, and congestion of hepatic sinusoids, leucocytic infiltration, Kupffer cells hyperplasia, granuloma formation, and eosinophilic infiltration in G2 are all signs of hepatic injury. The presence of pyknotic nuclei in section D (G4) suggests that there is some degree of cell death in the central vein. The severity of the histopathology findings in section B (treated with IMI) is greater than that in section A (G1). This suggests that IMI treatment is associated with hepatic injury. Also, the G3(FA) were seen to have healthy central vein and hepatic cellular health and in G4 there was comparatively less damage showing possible protective role of folic acid as shown in fig (3.18).

3.20 Histopathological changes in kidney

The histological analysis of rat kidney sections under different treatments reveals distinct responses as shown in figure (3.17). In the Control group (G1), the renal morphology is normal, with intact glomeruli and unchanged renal tubules, serving as a baseline. In contrast, the IMI

Exposed group shows clear pathological changes, including an enlargement of Bowman's space and noticeable damage to the tubules. This group also exhibits dilation in the distal tubules and hypertrophy in the proximal convoluted tubules, accompanied by local hemorrhage and necrosis near vascular structures, indicating the negative impact of IMI. Conversely, the Folic Acid Exposed group demonstrates renal structures similar to the control group. The normal appearance of glomeruli and tubules suggests a protective effect of Folic Acid against renal damage. The combination treatment of IMI and Folic Acid (IMI + FA) presents an intermediate response. Mild necrosis is observed near the distal convoluted tubules, yet the overall damage to the renal structure is less than in the IMI-only group. This points to a significant reduction of IMI-induced renal damage due to Folic Acid, indicating a noteworthy mitigation of IMI-induced renal damage by Folic Acid.

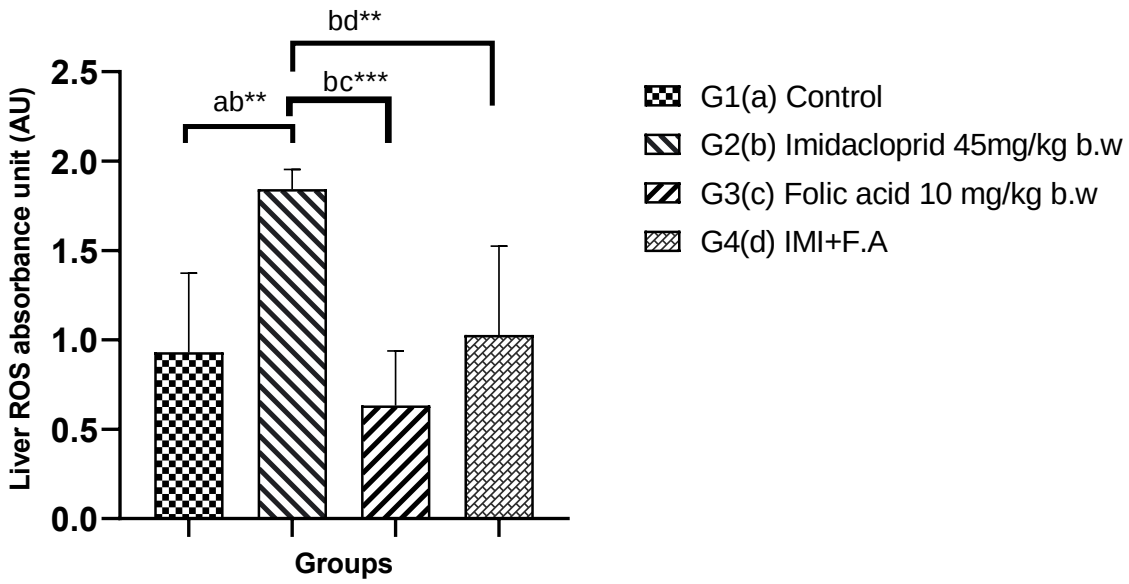

Fig 3.1 Graph represents Significant increase in liver ROS specially in G1 vs G2 (**p<0.01), G2 vs G3 (***p<0.001), G2 vs G4 (**p<0.01). Common letter a, b, c, d represents G1, G2, G3, G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

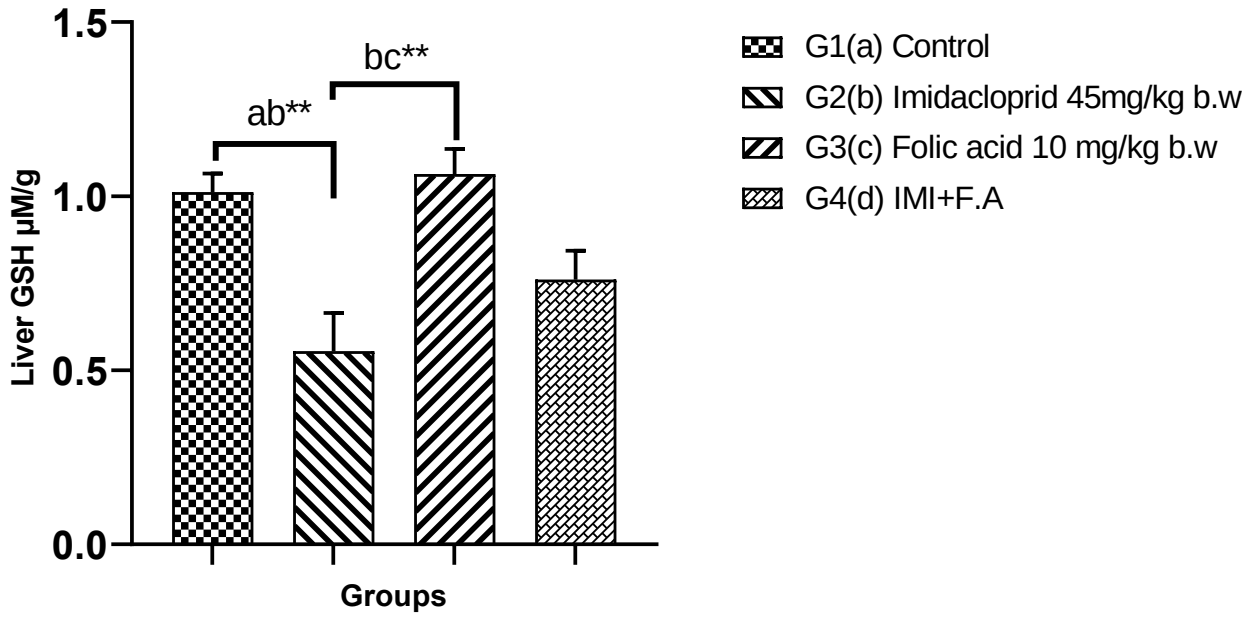

Fig 3.2 Liver reduced glutathione level (GSH) was decreased in G2 whereas, G3 showed a notable positive influence of increasing GSH levels and in G4 neutralizing IMI toxic effect. Multiple comparisons are G1 vs G2 (**p<0.01), G2 vs G3 (**p<0.01). Common letters a, b, c, d represents G1, G2, G3, G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Values expressed as mean + SEM (n=6).

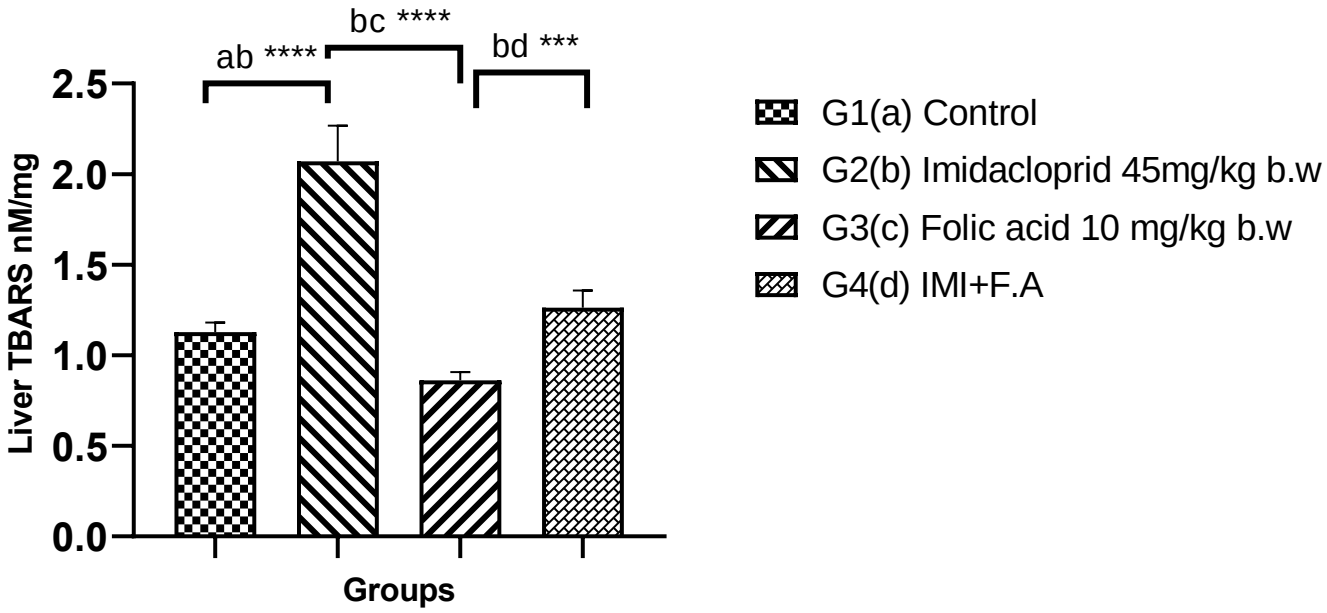

Fig 3.3 Graph shows a significant increase in TBARS of G2 as compared to controlled (G1), G3 and G4. G1 vs G2 (****p<0.0001); G2 vs G3 (****p<0.0001) and G2 vs G4 (***p<0.001). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

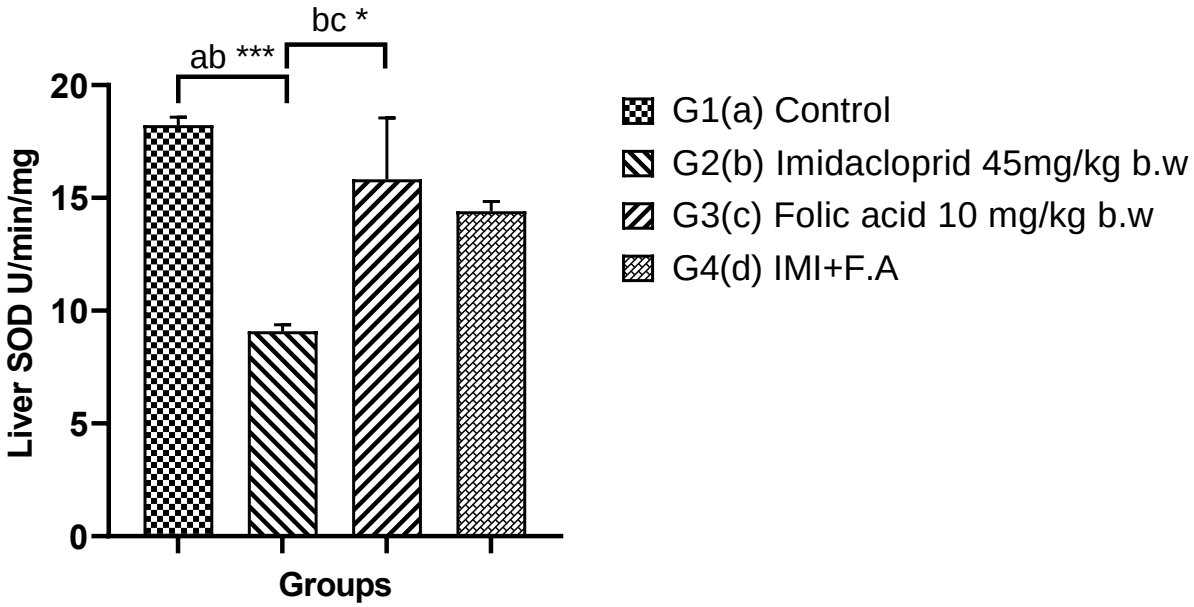

Fig 3.4 The concentration of liver SOD was found to be considerably lower in G2 compared to G1, G3 and G4. G1 vs G2 (***p<0.001), G2 vs G3 (*p<0.05); G2 vs G4 (ns). Common letters a, b, c, d represents G1, G2, G3, G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Values expressed as mean + SEM (n=6).

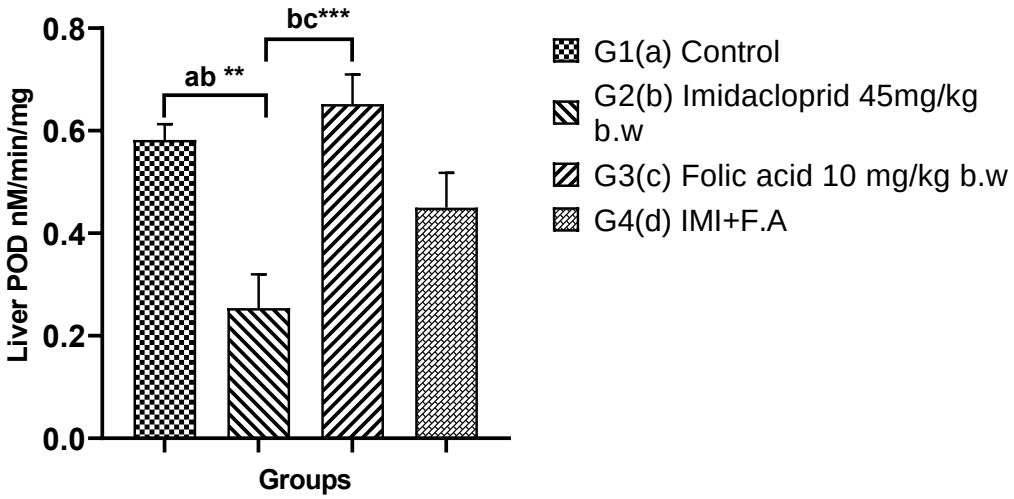

Fig 3.5 The level of concentration of liver POD in G2 is observed to be significantly lower than G1 and G3. Multiple comparisons are G1 vs G2 (**p<0.01), 2 vs G3 (***p<0.001). Common letters a, b, c, d represents G1, G2, G3, G4. Values expressed as mean + SEM (n=6), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

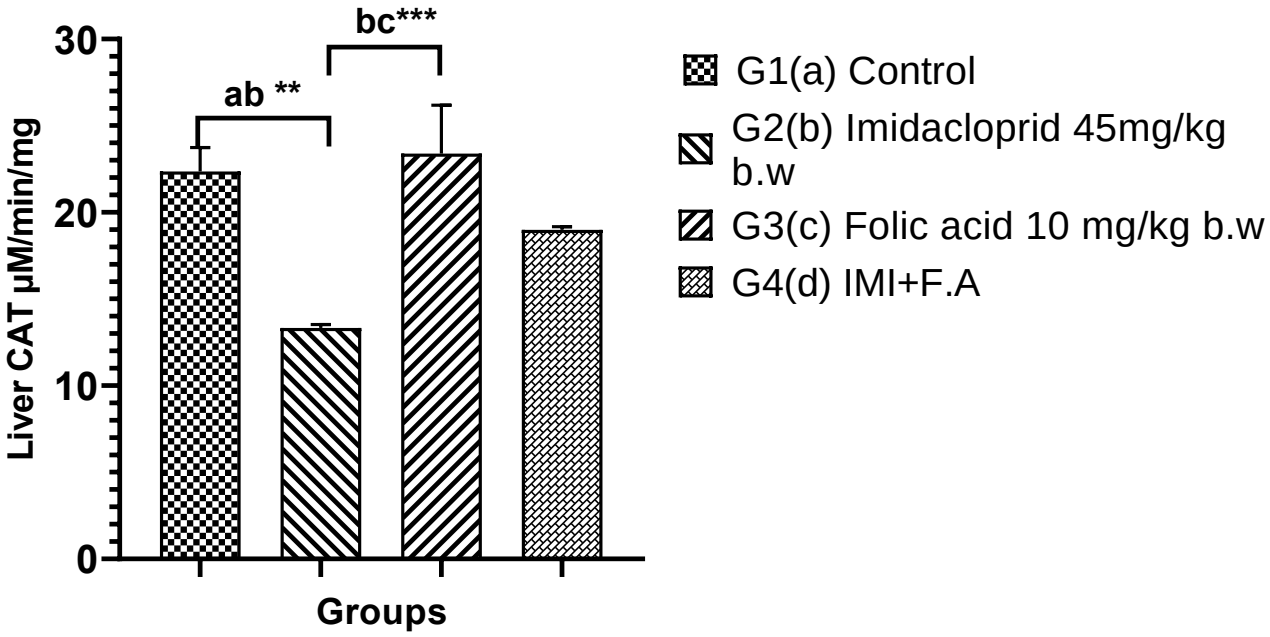

Fig 3.6 Graph represents the level of liver CAT concentration in G2 was found to be considerably lower than G1, G3 and G4. Multiple comparisons are G1 vs G2 (**p<0.01); G2 vs G3 (***p<0.001). Common letters a, b, c, d represents G1, G2, G3.G4. Values expressed as mean + SEM (n=6), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

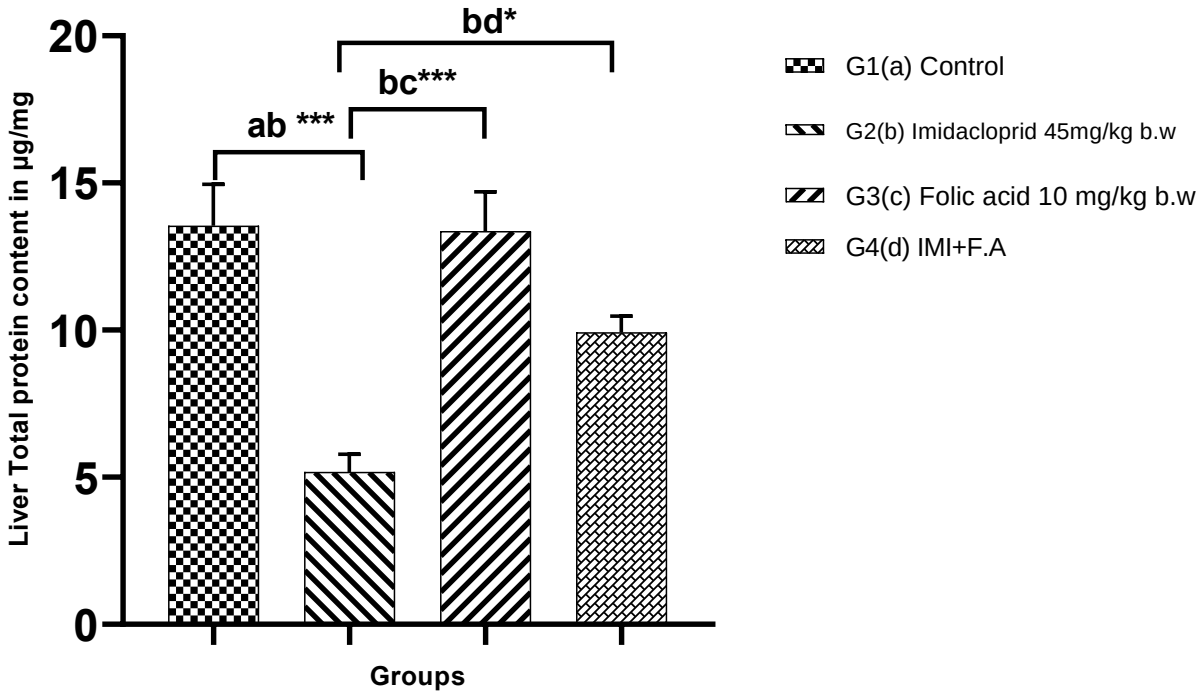

Fig 3.7 Total protein content of liver was decreased notably in G2 samples and a significant increase in G3. Multiple comparisons are G1 vs G2 (***p<0.001); G2 vs G3 (***p<0.001) and G2 vs G4 (*p<0.05). Common letter a, b, c, d represents G1, G2, G3 and G4 respectively and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

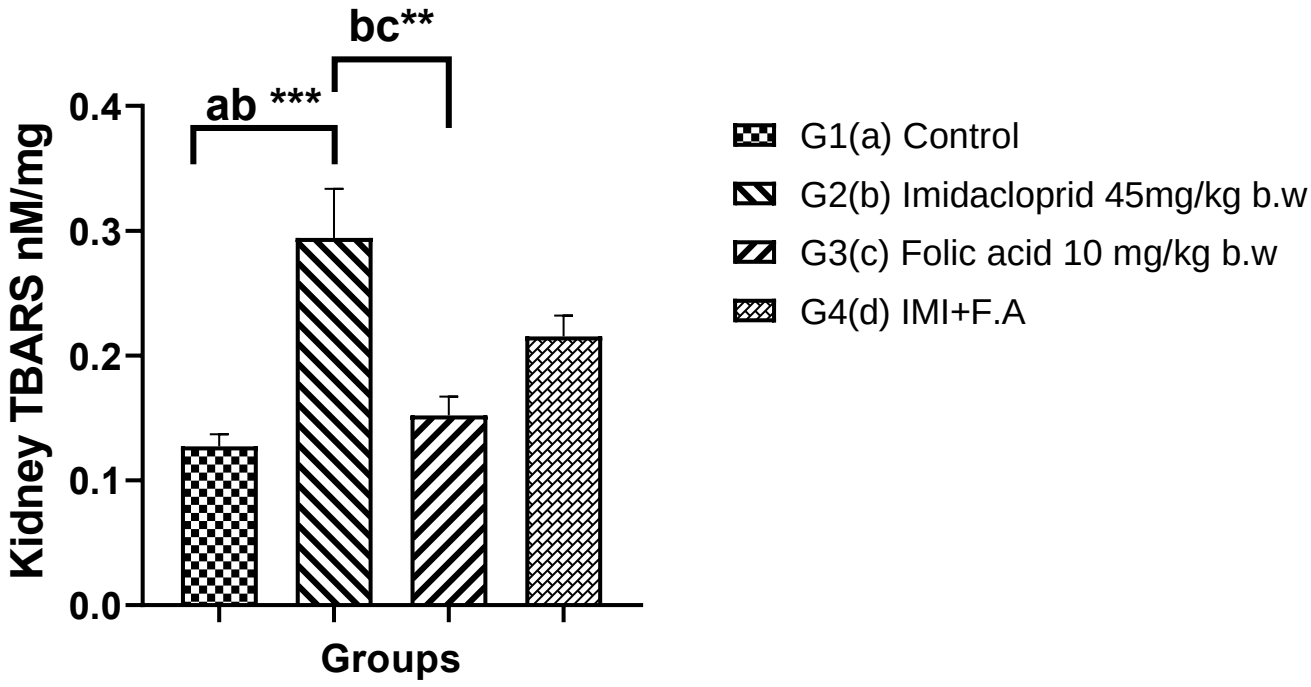

Fig 3.8 TBARS levels were significantly higher in the IMI group (G2) than in the control group (G1) (***p<0.001) and (**p<0.01) for G1 vs G3. Common letter a, b, c, d represents G1, G2, G3 and G4 respectively and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

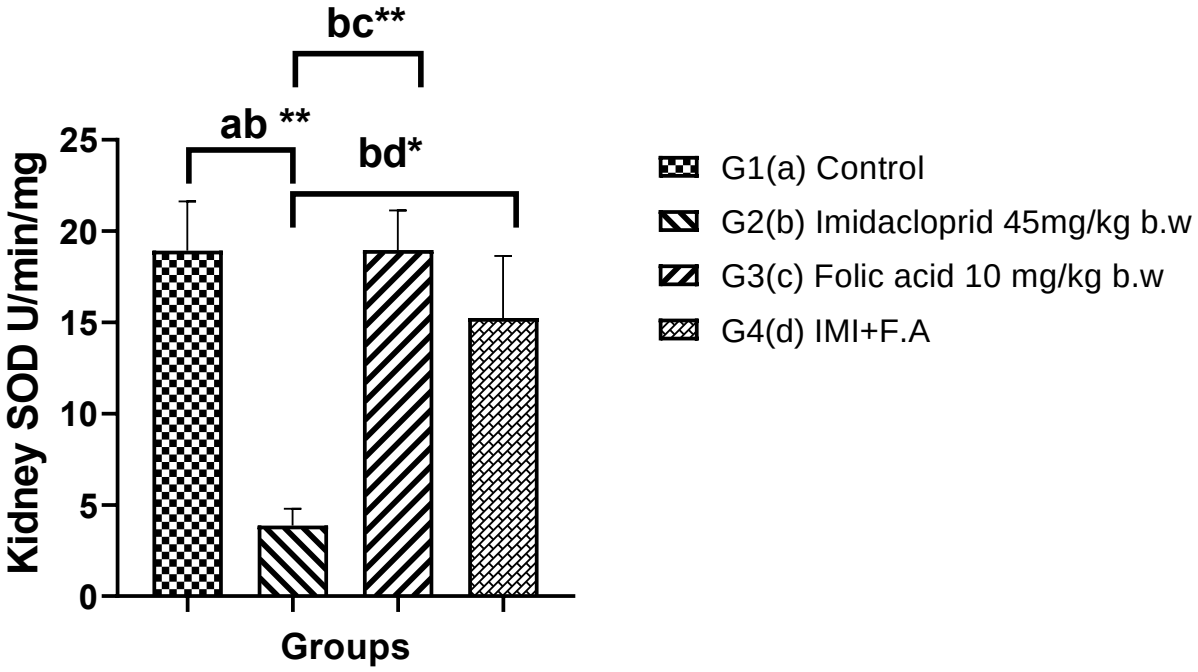

Fig 3.9 Kidney SOD level was found to decrease significantly in G2 treated with IMI compared to G1 and G3. Similarly, G1 vs G2 (**p<0.01), G2 vs G3 (**p<0.01), G2 vs G4 (*p<0.05). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

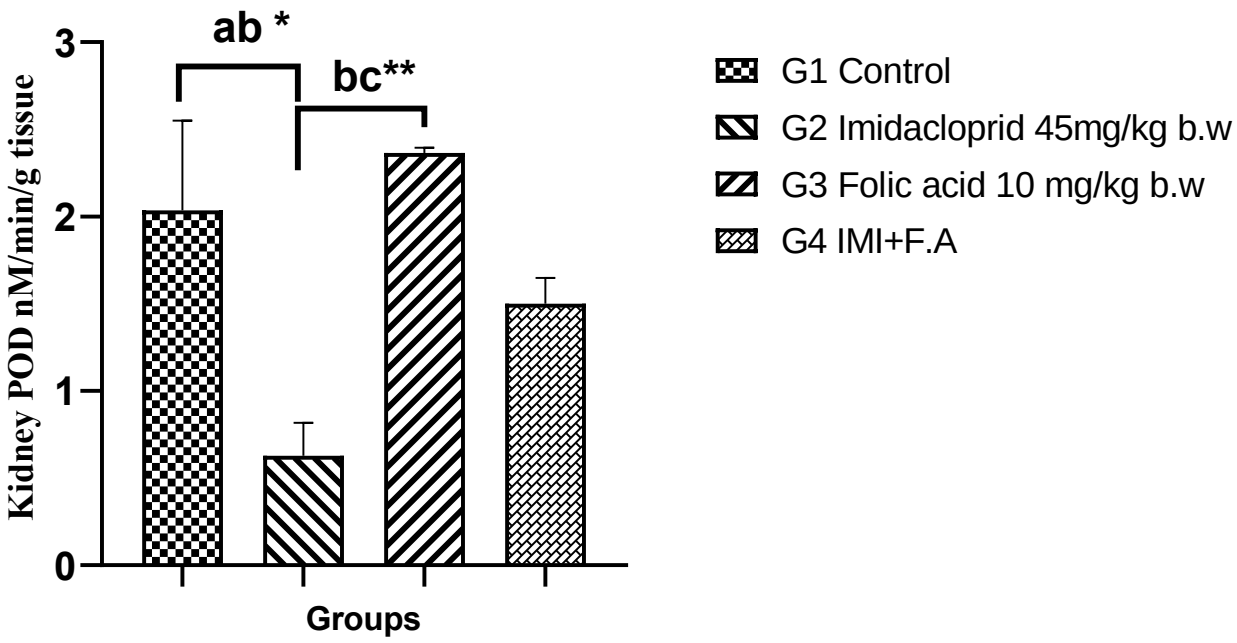

Fig 3.10 A significant decrease in kidney POD levels was observed in group 2 (G2), which was treated with IMI, compared to groups 1 (G1), 3 (G3), and 4 (G4). Pairwise comparisons showed significant differences between G1 and G2 *(*$p < 0.05$) and G2 and G3 (**$p < 0.01$). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

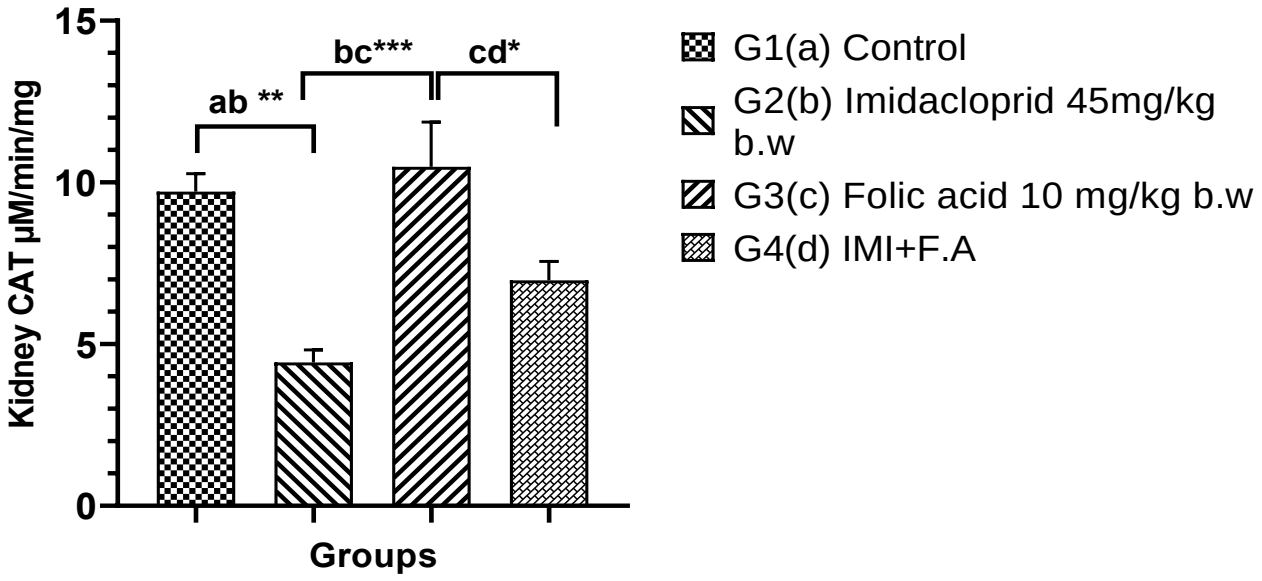

Figure 3.11 Significant difference in CAT concentration was observed in IMI-treated group 2 (G2) compared to groups 1 (G1), 3 (G3), and 4 (G4). Pairwise comparisons showed significant differences between G1 and G2 (**p < 0.01), G2 and G3 (***p < 0.001), and G3 and G4 (*p < 0.05). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

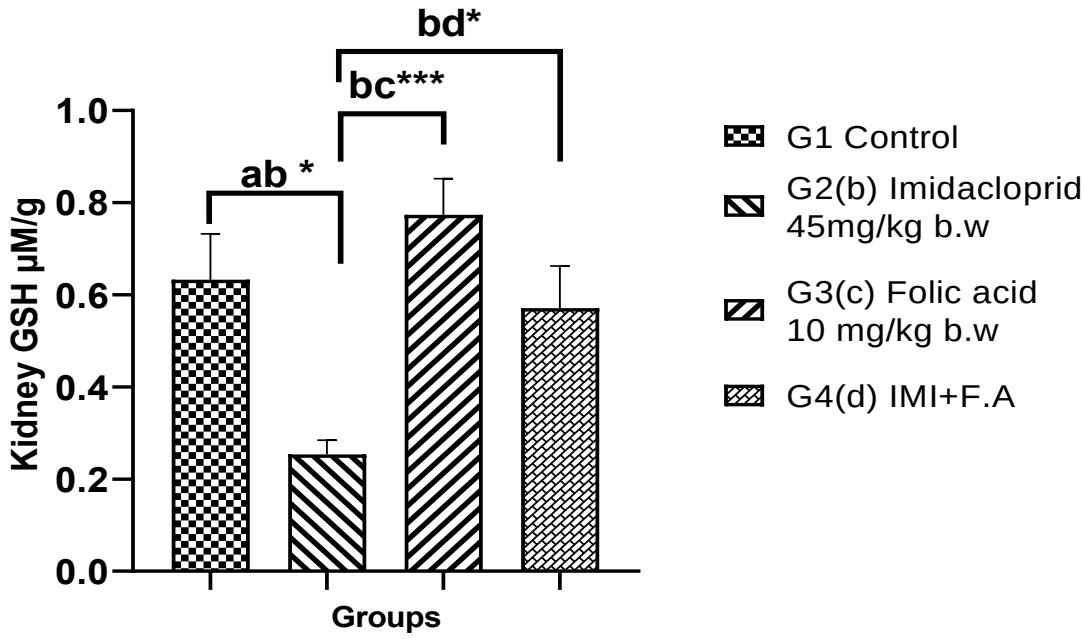

Fig 3.12 Reduced glutathione (GSH) levels in the kidneys of rats exposed to imidacloprid (G2) and folic acid (G3). (***p<0.001) for G2 vs. G3**;** (*p<0.05) for G1 vs. G2**;** (*p<0.05) for G2 vs G4. Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

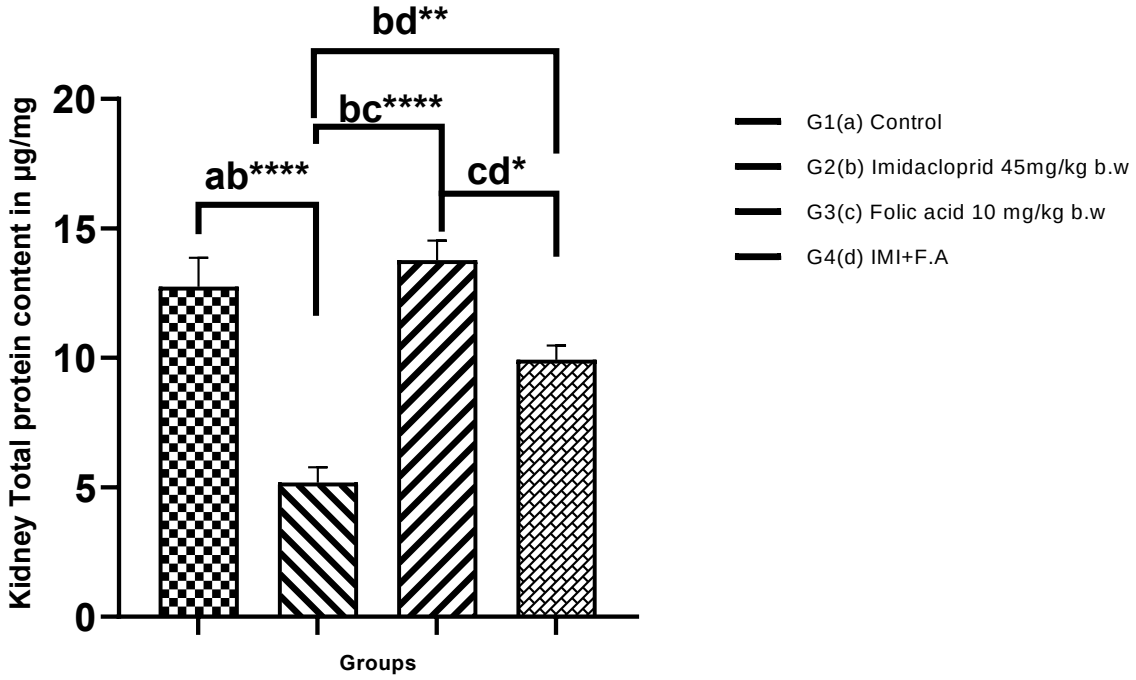

Fig 3.13 A notable fall in total protein content of kidney can be seen in Imidacloprid exposed G2 compared to G1, G3 and G4. G1 vs G2 (****p<0.0001); G2 vs G4 (**p<0.01); G3 vs G4 (*p<0.05). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

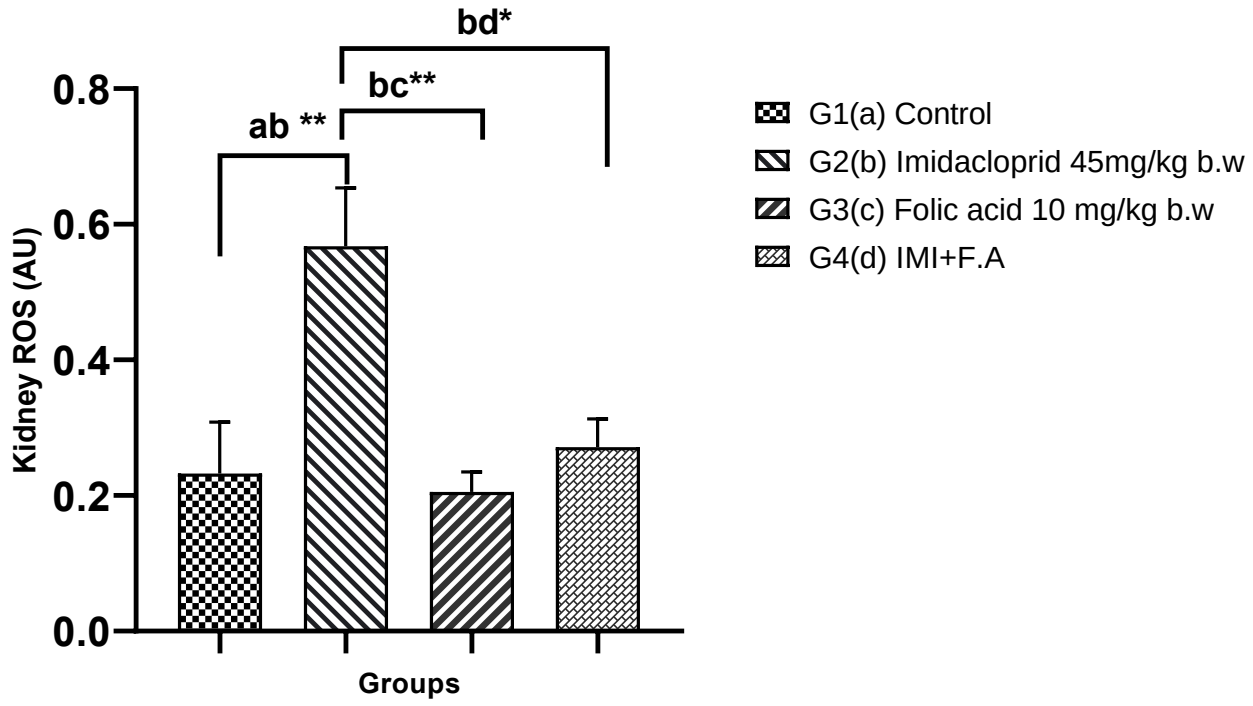

Figure 3.14 A noteworthy difference in the data can be seen in kidney ROS AU (Absorbance Unit). Group 2 (G2) exhibits a significant peak compared to the control group, Comparison of significant values G1 vs G2 (**p<0.01); G2 vs G3 (**p<0.01), G2 vs G4 (*p<0.05). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance is interpreted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns= non-significant change. Values expressed as Mean + SEM (n=6).

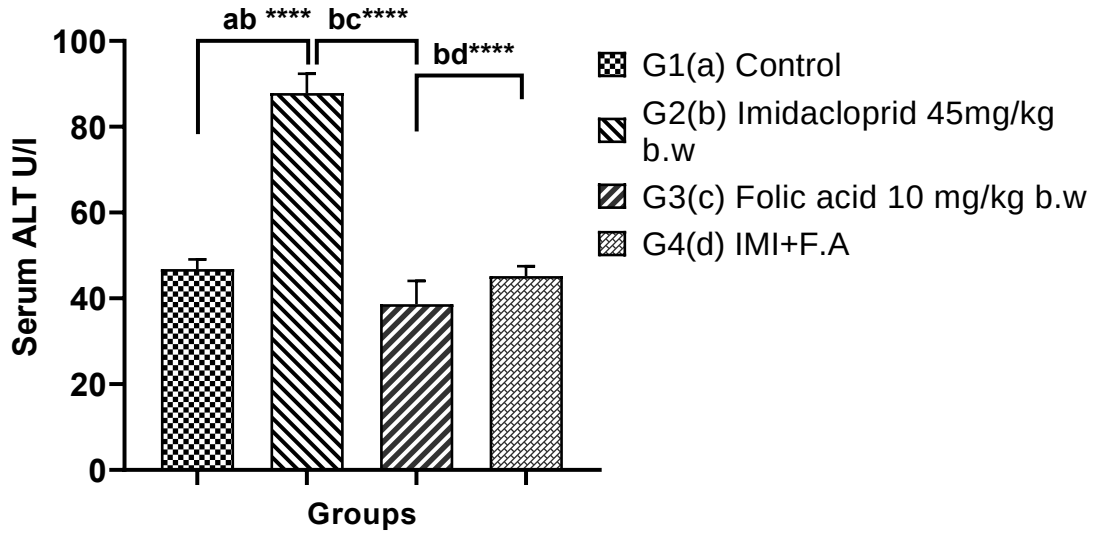

Fig 3.15 There was a significant increase in serum alanine aminotransferase (ALT) levels in Group 2 (G2), while G3 and G4 showed a noticeable decrease. G1 vs G2 ($p<0.0001$); G2 vs G3 ($p<0.00001$); G2 vs G4 ($p<0.0001$). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance is interpreted as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns= non-significant change. Values expressed as Mean + SEM (n=6).

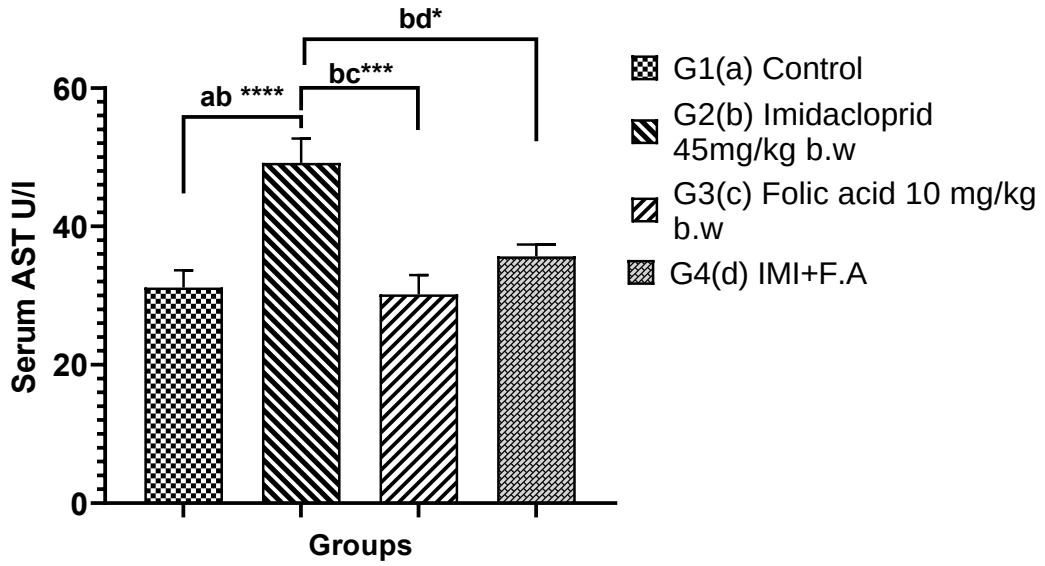

Fig 3.16 A significant rise can be clearly seen in aspartate aminotransferase (AST) of imidacloprid-exposed G2, while G3 AST levels are significantly lower. G1 vs G2 ($p<0.001$); G2 vs G3 ($p<0.001$); G2 vs G4 ($p<0.05$). Common letters a, b, c, d represents G1, G2, G3 and G4 respectively and significance interpreted as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns= non-significant change. Values expressed as Mean + SEM (n=6).

Table 3.1 Effect of imidacloprid on body weight of female albino rats as compared to control(G1)

Name of Tissue/organ	Control (G1)	G2 Imidacloprid	Folic acid	Imidacloprid +F.A
Av. feed intake (g/rat/day)	21.73 ± 1.06	17.13 ± 0.716	20.38 ± 1.62	21.65 ± 1.15
Initial weight (g)	160.7±6.77	160.3±12.09	163.7±6.99	160.3±7.196
Final weight (g)	174.3 ±7.297	144.8±17.24	174.8±8.47	166.5±5.94
Net body weight gain (g/100 g bw)	10.6±7.56	-15.5 ±17.23	11.1± 8.475	6.2±6.19

Data expressed as Mean ± SEM of 6 animals in each group.

Table:3.2 Presents the measured values of key biochemical parameters of Kidney antioxidants

Parameters(K)	G1 (Control)	G2(IMI)45 mg/kg b.w	G3(FA)10mg/kg b.w	G4 (IMI + FA)
ROS(AU)	0.567±0.086	0.205±0.029	0.271±0.041	0.273±0.041
TBARs	0.228±0.031	0.351±0.04	0.183±0.028	0.215±0.016
CAT	9.713±0.554	4.4373±0.388	10.48±1.308	6.966±0.589
SOD	0.074±0.011	0.033±0.013	0.087±0.004	0.051±0.015
POD	2.034±0.515	0.628±0.188	2.365±0.03	1.499±0.147
GSH	0.632±0.099	0.253±0.031	0.773±0.078	0.572±0.091
Protein content	14.95±0.392	5.18±0.597	14.36±0.395	9.924±0.546

Data expressed as mean +SEM. Notably, the comparison of G2, G3, and G4 with the control group (G1) reveals the impact of IMI and FA, both individually and in combination, on kidney antioxidant parameter.

Table:3.3 Presents the measured values of key biochemical parameters of liver antioxidants

Parameters(L)	G1 (Control)	G2(IMI)45 mg/kg b.w	G3(FA)10mg/kg b.w	G4 (IMI + FA)
ROS(Abs)	0.93±018	1.84±0.11	0.632±1.027	1.027±0.203
TBARs	0.250±0.016	0.393±0.032	0.249±0.021	0.266±0.031
CAT	22.36±1.374	13.33±0.197	23.4±2.787	19.01±0.176
SOD	18.23±0.348	9.085±0.294	18.66±0.351	14.41±0.423
POD	0.581±0.030	0.253±0.065	0.651±0.058	0.449±0.068
GSH	1.011±0.053	0.555±0.109	1.063±0.072	0.760±0.082
Total protein	20.27 ±0.681	9.576±0.337	22.03±0.906	15.62±1.215

Data expressed as mean + SEM. Notably, the comparison of G2, G3, and G4 with the control group (G1) reveals the impact of IMI and FA, both individually and in combination, on kidney antioxidant parameter

Table 3.4 ALT and AST Levels in Rat Livers, this table shows the ALT and AST levels in the livers of 6 individuals in each of 4 groups.

Parameters	G1(Control)	G2(IMI)	G3(Folic acid)	G4(FA+IMI)
ALT	46.83±2.227	87.83±4.534[a]	38.67±5.395[a]	45.17±2.344[a]
AST	31.17±2.469	49.17±3.506[a]	30.17±2.798	35.67±1.706c

All values are presented as mean ± standard error mean (SEM), Superscripts a, b and c indicate significant differences from the control at $p < 0.0001$, <0.001 and <0.01.

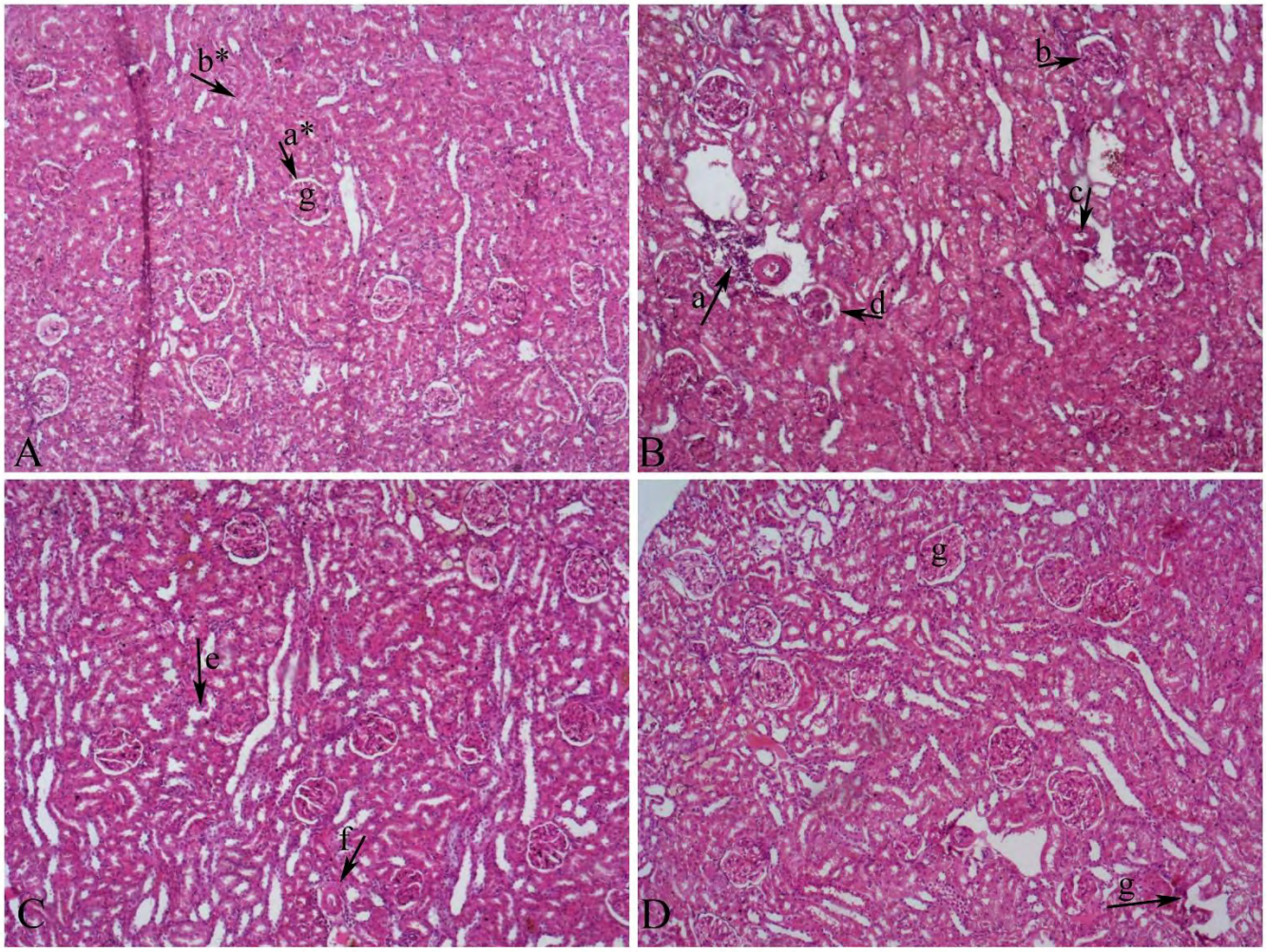

Fig 3.17 Photomicrograph showing a section of the rat kidney, section A: control(G1), B: IMI exposed, C: Folic acid exposed, D: IMI + FA. **Section A** Normal glomeruli(g)=a*, and normal Renal tubules(b*) from the renal cortex. **Section B** Increased bowman space(d), Dilation of distal tubules(b & d), possible hypertrophy seen in proximal Conv tubules, Hemorrhage and clear tubular injury(c), clear necrosis in blood vessels vicinity(a), glomerulosclerosis. **Section C** Healthy glomerular histology observed **Section D** Normal glomerulus, mild necrosis observed near DCT area(g), overall, FA seems to have maintained damage in G4.

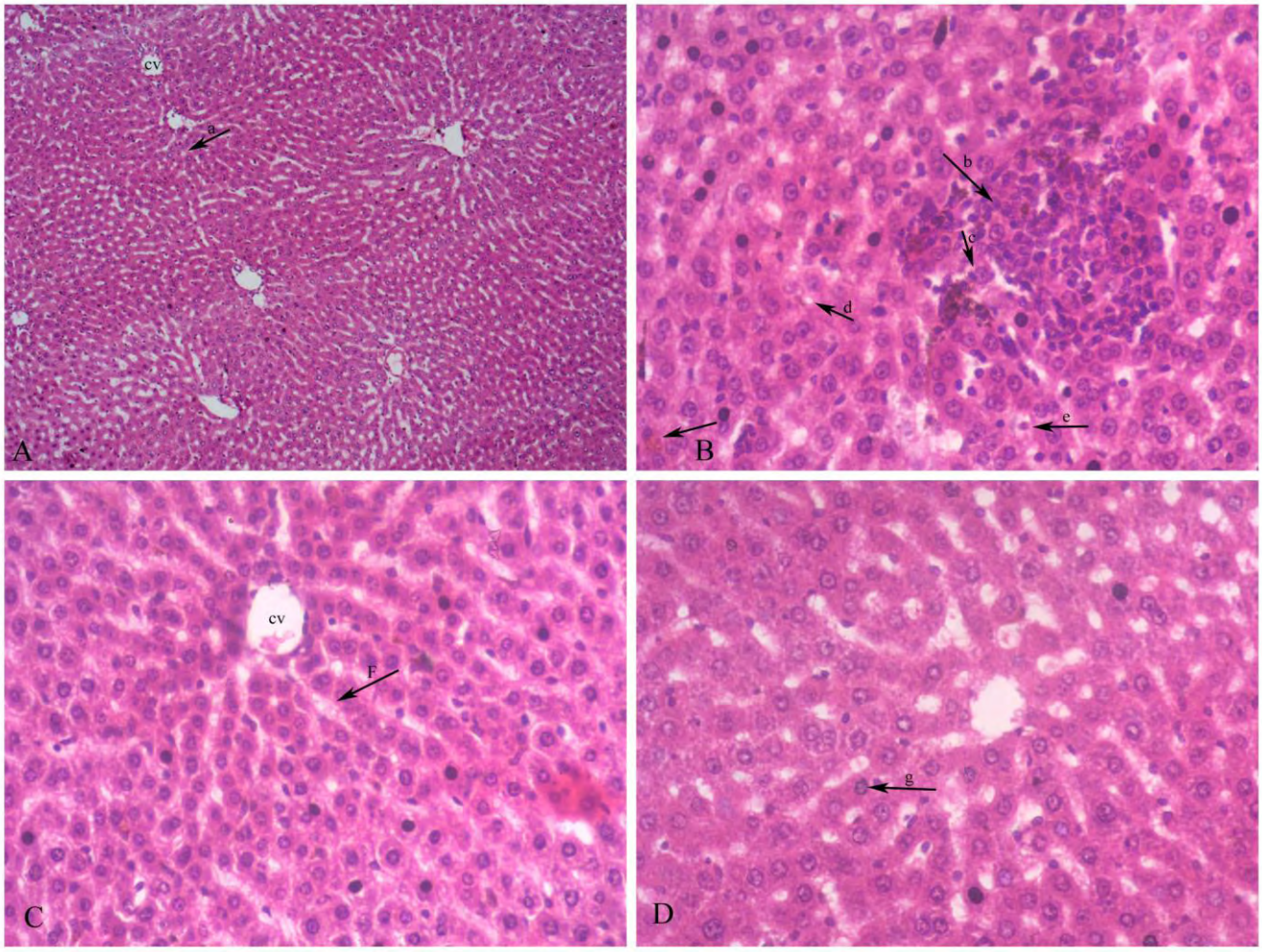

Fig 3.18 Photomicrograph showing section of rat liver normal cellular architecture in **section A** control(G1), **Section B(G2),** showing alterations in the histological structure of hepatic lobule in its entirety treated with IMI, presence of Lipofuscin(arrow) (lipochrome formation), Clear dilation and congestion of hepatic sinusoids(d), leucocytic infiltration(e), Possible Kupfer cells hyperplasia and granuloma formation along with eosinophilic infiltration (b & c). **Section C** normal sinusoidal space maintained, intact and normal hepatic lobular physiology (F), Central vein preserving intact histological architecture (cv). **Section D** normal central vein(cv), however sparsely distributed pyknotic nuclei(g) observed.

Discussion

Pesticides have been used for thousands of years to control insects and mites. Ancient civilizations like the Sumerians and Egyptians used natural compounds such as sulfur and arsenic to protect their crops from pests (Stănescu, 2014). As pests developed resistance, humans developed new pesticides. This ongoing battle persisted until World War 2 when famines and extreme food shortages threatened humans. This time marked a sudden rise in the use of pesticides to increase crop yields to deal with food shortages. Due to poor selectivity pesticides came and got replaced by others as time went by. The proliferation of diverse pesticides, varying in environmental persistence, poses a significant risk of bioaccumulation, thereby posing threats to both human health and wildlife (Bernardes et al., 2015). Pesticides not only persist in the environment and act as endocrine disruptors but also cause oxidative stress at the cellular level, leading to various secondary abnormalities with continuous exposure. This is the case when environmentally stable pesticides accumulate in organisms, causing oxidative stress on a cellular level in their bodies. Oxidative stress arises when the production of reactive species surpasses their decomposition rate within antioxidant systems, leading to heightened oxidative damage across various cellular components (Jabłońska-Trypuć, 2017). Oxidative stress, induced by both pesticides and their metabolic byproducts, is recognized as a prominent contributor to conditions including atherosclerosis, diabetes, cataracts, neurodegenerative ailments, autoimmune disorders, and cancer (Etemadi-Aleagha et al., 2002); (Abdollahi et al., 2004).

The aim of this study was to evaluate the toxicity of imidacloprid in vivo and the potential protective role of folic acid. The study measured body weight, biochemical parameter and histopathological activity in Sprague-Dawley rats. The rats were divided into groups and orally exposed to imidacloprid alone (G2), folic acid alone (G3), and both imidacloprid and folic acid (G4). Doses and other parameters in this study were selected based on earlier research on imidacloprid and the supplementary role of vitamins (Kapoor et al., 2010); (Soujanya et al., 2013); (Toghan et al., 2022). Although they used mild doses of toxicant and supplement, the present study used relatively higher doses for a shorter duration.

The present study showed a decline in body weight and feed intake of animals exposed to imidacloprid, particularly with reference to untreated controlled group and on the other hand G3 and G4 were observed to have no or little decrease in body weights. Our results were in agreement with Vohra and Khera, (2015a). Similarly Bhardwaj et al., (2010) and Kapoor et al., (2010) in their respective work has proved that body weights were decreased in those groups of rats exposed to imidacloprid. This study conclude that the body weight loss might be due to the anorexic effect of imidacloprid which has been pointed out in an earlier study of Kapoor et al., (2010). Kidney and liver weights of imidacloprid exposed rats were decreased notably in this study which are also observed by Kapoor et al., (2010). The diminished food intake and decreased liver weight and kidney observed in animals exposed to a high dose could likely be attributed to the toxic effects of imidacloprid (Soleski, 2001).

Serum alanine transaminase (ALT) is a specific marker for evaluating liver function, released into the bloodstream upon liver damage. AST (aspartate aminotransferase) levels similarly rise in the bloodstream upon damage to the liver, heart, and muscles, albeit with less liver specificity compared to ALT. In the present-study, the toxic effects of imidacloprid were observed for both ALT and AST. Both showed significantly higher concentrations. A significant increase in ALT and AST levels was observed in group 2, also evidenced by Bhardwaj et al., (2010). However, group 3, which was given only folic acid, showed notably stable levels of ALT and AST (Lee et al., 2011). Possible liver damage caused by necrosis could be one of the reasons for the significant increase in serum ALT and AST levels. The present study is unique in its understanding of the supplementary role of folic acid in reducing the harmful effects of imidacloprid, as indicated by the slight increase in ALT and AST levels compared to imidacloprid alone G2. Moreover, there has been carried out population-based study by Chen et al., (2022) correlating ALT and AST levels with chronic diseases like cancer and have shown significant positive correlation.

The impact of imidacloprid and folic acid on oxidative & antioxidative parameters in the kidneys and liver of rats was investigated. The study evaluated the effects of imidacloprid alone, folic acid alone, and a combination of both. Reactive oxygen species and thiobarbituric acid reactive substances (TBARS) were used to evaluate oxidative stress induced by imidacloprid whereas, folic acid's potential supplementary role. Antioxidative status was gauged by peroxidase (POD),

superoxide-dismutase (SOD), catalase (CAT), and reduced glutathione (GSH). The results indicated a significant increase in ROS and TBARS concentrations, while POD, CAT, SOD and GSH reduced substantially in imidacloprid exposed group of rats. The folic acid alone group showed positive results as they exhibited decrease in ROS and TBARS, whereas increasing antioxidant activity of SOD, CAT, POD and GSH. The folic acid supplementary role was clearly observed in G4 by significantly reducing ROS and TBARS levels and maintaining higher levels of SOD, POD, CAT and GSH. These results are similar with those of Gallego-Lopez et al., (2022) for folic acid supplementation and Mahajan et al., (2018) for imidacloprid induced toxicity. Thus, from previous literature we now know that in a healthy cell, we need lower levels of ROS and TBARS, but elevated levels of SOD, POD, CAT and GSH. When considering ROS sources, it's important not to view them solely as isolated enzymatic systems. Biological processes encompass the concept of ROS-induced ROS, where reactive oxygen species initiate subsequent ROS generation. The term "ROS" serves as a broad categorization, as it encompasses various types, some of which can lead to ROS production, not necessarily harmful. While all radicals are classified as ROS, it's important to note that not all ROS are radicals (Davies, 2016). Mammalian mitochondria maintain ROS levels in form of oscillations as balancing factor and this promotes resistance to oxidative stress (Calabrese et al., 2013). The present study, guided by obtained results and informed by existing literature, helps clarify that imidacloprid and its metabolites could potentially disrupt the balanced ROS dynamics maintained through mitochondrial biology. This proposition finds reinforcement in the research of Bizerra et al., (2018).

Cell membrane integrity and function is compromised and harmed due to lipid peroxidation. ROS can lead to oxidation of polyunsaturated fatty acids in cell membranes leading to acceleration of lipid peroxidation (Ammendolia et al., 2021). The formation of lipid hydroperoxides takes place, then these lipid hydroperoxides can further decompose to form various breakdown products, including malondialdehyde and other aldehydes, which are collectively referred to as TBARS. Higher levels of TBARS indicated increased oxidative damage to lipids in cell membranes, TBARS acts as an indicator to gauge lipid peroxidation and oxidative stress within cell membranes, offering insights into potential harm induced by reactive oxygen species and the effectiveness of antioxidant interventions (Li et al., 2015); (Egea et al., 2017). The present study

confirmed peak levels of TBARS in imidacloprid (IMI) treated rats, compared to folic acid (F.A) treated group and combined IMI + F.A group (G4). A significant difference was observed between group G2 and G4, suggesting supplementary role of folic acid, which is also pointed out by Gallego-Lopez et al., (2022). Likewise, in a recent meta-analysis, Asbaghi et al., (2021) have proposed that dietary supplementation with folic acid enhances the antioxidant defense system by elevating serum concentrations of glutathione (GSH) while concurrently diminishing lipid oxidation. During the microscopic examination of liver sections, our ongoing investigation revealed formations resembling tumors. Consequently, the surmise emerges that the conspicuous emergence of growths akin to tumors could plausibly stem from escalated TBARS and ROS levels observed within the IMI-treated groups. As a result, there's a thought that IMI might indirectly contribute to increasing the development of tumors. A DNA damaging activity was detected by Tariba Lovaković et al., (2021) in their work on male wistar rats, this study shows direct role of IMI exposure.

Superoxide-dismutase (SOD) is a vital antioxidant enzyme that shields cells from oxidative stress provoked by ROS. SOD helps transform superoxide radicals into hydrogen peroxide, which is then further broken down into water and oxygen by other antioxidant enzymes such as CAT and GSH. SOD occupies the frontline in a cascade that effectively neutralizes the impact of ROS. Pesticide exposure, such as imidacloprid, can diminish SOD activity in various organs of rats, as demonstrated by the present study indicating notably lower SOD levels in both kidney and liver tissue homogenates. This observation is additionally supported by (Kapoor et al., 2010). Conversely, the treated group solely with folic acid (FA) displayed heightened SOD levels, indicative of FA's beneficial effect role in enhancing and upregulating SOD activity. These results echo the outcomes of Li et al., (2022), aligning with our study's findings that rats administered FA solely exhibited significantly elevated SOD levels. Similarly, in Group 4 (IMI + FA), folic acid mitigated the toxic repercussions of IMI, suggesting a promising role played by folic acid in countering the adverse effects of IMI and its metabolites.

CAT plays a crucial role in neutralizing hydrogen peroxide, efficiently breaking it down into water and oxygen. This pivotal function intriguingly aligns with the interplay of CAT and imidacloprid. Although CAT activity is not significantly changed when low doses of IMI are given but at high

doses its levels are drastically effected (Mahajan et al., 2018). The current study parallels the findings of Mahajan et al., (2018) concerning the IMI-alone group, where CAT levels exhibited a significant drop. Conversely, the fourth group (IMI+FA) demonstrated reduced CAT levels, reaffirming folic acid's capacity to alleviate the effects of IMI, as observed previously in SOD case. Once more, the FA-treated group has displayed elevated CAT levels, reinforcing its supportive role in fortifying the antioxidative mechanism.

Peroxide dismutase (POD), an enzyme with a heme component, catalyzes the oxidation of diverse substrates such as hydrogen peroxide (H_2O_2) generated by SOD, thereby curbing the excessive buildup of H_2O_2 produced through regular cellular processes. In our study, POD concentration demonstrated a noteworthy decline in the IMI-alone group, while registering a considerable rise in the FA-alone group. Remarkably, in Group 4 (IMI+FA), folic acid appeared to counteract IMI's toxic influence, potentially by preserving POD levels from drastic reduction (Mudgal et al., 2023).

In the current study, the total protein levels were determined in the kidney and liver. Our findings revealed a notable decrease in both organs among the IMI-exposed group. This decline in protein content could potentially be attributed to elevated oxidative stress marked by ROS and TBARS, which might have led to DNA damage. Consequently, this cellular impairment could have adversely impacted the cells' ability to synthesize proteins. Protein content results of present study are in agreement with the work of Nasr, (2018).

In the current study, histological changes induced by IMI in liver and kidney were also evaluated. The results showed histological alterations in histological architecture of tissue of IMI exposed groups in contrast to controlled group (G1), our results are in agreement with Vohra et al., (2014) and Vohra and Khera, (2015b). Histology of the control group kidney showed no histological change in overall structure of renal corpuscles and renal tubules. Similarly, the abnormalities observed in the IMI-exposed group can be ascribed to IMI's toxic effects on renal structures. Shrinkage of the glomerulus likely arises from direct IMI exposure, influencing renal function and potentially contributing to the widening of Bowman's space through increased filtration pressure or altered permeability. The degeneration of proximal convoluted tubules correlates with toxic impacts on tubular epithelial cells, influencing their function. Concomitantly, distal tubule dilation may stem from disrupted electrolyte balance or altered transport mechanisms, affecting tubular

morphology. An observed hypertrophic response in the proximal convoluted tubules could be a reactive adaptation to cellular injury or heightened workload. Hemorrhages may develop because of IMI-induced vascular injury or changes in coagulation factors, resulting in bleeding. Direct IMI poisoning may have caused clear tubular injury, impairing the integrity and function of tubular structures. Furthermore, the presence of interstitial nephritis indicates an immunological response to IMI exposure, resulting in interstitial tissue inflammation. Finally, glomerulosclerosis, or scarring of the glomeruli, may be the result of long-term IMI exposure. These changes highlight the negative impact of IMI on renal structures as well as its potential toxicity. These interpretations align with the findings of Vohra et al., (2014).

Likewise, the histopathological variations in liver samples aligned with shifts in biochemical indicators. Evaluation of liver sections from the untreated group displayed the anticipated cellular structure. Conversely, in rats treated with IMI, the presence of lipofuscin accumulation pointed towards cellular aging. This could potentially be connected to IMI and its metabolites burdening lysosomes within Kupffer cells. This phenomenon could contribute to observed lipofuscin formation, thereby revealing an intricate connection between IMI exposure and cellular dynamics. Dilation and congestion of hepatic sinusoids points towards compromised blood flow, possibly influenced by vascular changes triggered by IMI. Furthermore, leucocytic infiltration implies an activated immune response, likely initiated by IMI-induced inflammation. Hepatic steatosis, indicative of lipid buildup, could be a result of disrupted metabolism associated with IMI's impact. Moreover, Kupffer cell hyperplasia may be attributed to immune activation or inflammation, accentuating the adverse effect of IMI on liver structure and function. These interconnected changes collectively underscore the unfavorable consequences of IMI exposure on liver health.

In conclusion, this study demonstrated the impact of IMI exposure and the potential protective role of folic acid on various biological parameters. The results presented a comprehensive understanding of how IMI's toxic effects can disrupt cellular balance, leading to oxidative stress, altered biochemical markers, and histopathological abnormalities in both kidney and liver. The study also shed light on the potential mitigation of IMI's effects by folic acid, offering promising insights into avenues for intervention. However, it's important to acknowledge the limitations of this study. The dosages and exposure durations used may not encompass complete mechanisms

underlying the interaction between IMI and folic acid require further investigation. But, while real-world scenarios may indeed introduce various complexities, the controlled experimental approach taken in this study allows us to isolate and comprehend the specific interactions between IMI and folic acid. This controlled environment provides valuable insights into the mechanisms underlying their effects, paving the way for more comprehensive investigations in future research. Moreover, the study primarily focused on short-term effects, while the long-term consequences of continued exposure remain a topic for future exploration. In summary, this research provides a foundation for understanding the intricate interplay between IMI exposure, oxidative stress, and the potential protective effects of folic acid. Future studies could get deeper into the molecular mechanisms underlying these interactions and explore the long-term consequences of both human health and the environment exposure to pesticides.

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