Folic Acid Attenuates Behavioral Impairments Induced by Commercial Formulation of Imidacloprid in Laboratory Rats.

Master of Philosophy

in

ZOOLOGY

By

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Department of Zoology

Faculty of Biological Sciences

Quaid-i-Azam University Islamabad

2023

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A dissertation submitted in the partial fulfillment of the requirements for the Degree of

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DEDICATION

I dedicate this thesis to my unwavering source of inspiration – **my parents** – whose boundless love, relentless support, and enduring sacrifices have been the driving forces behind my academic journey. To **my siblings**, your encouragement and belief in me have been a constant reminder of the importance of perseverance. This achievement is as much yours as it is mine.

CERTIFICATE

This is to certify that dissertation entitled **"Folic Acid Attenuates Behavioral Impairments Induced by Commercial Formulation of Imidacloprid in Laboratory Rats"** submitted by **Bakhtawer Rafiq** is accepted in its present form by the Department of Zoology, Quaid-i-Azam University, Islamabad as satisfying the dissertation requirement for the degree of M.Phil. in Zoology.

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It is certified that Bakhtawer Rafiq (Registration No. 02022113003) has submitted her M.Phil. Dissertation entitled "**Folic Acid Attenuates Behavioral Impairments Induced by Commercial Formulation of Imidacloprid in Laboratory Rats**", and it has been checked on Turnitin for similarity index (plagiarism). Thesis plagiarism has been found to be 16 % that lies in the limit provided by HEC (19 %).

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DECLARATION

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

Bakhtawer Rafiq

ACKNOWLEDGMENT

First and foremost, praise is to ALLAH, the ALMIGHTY, for endowing me with health, patience, and knowledge to complete this work. Countless salutations be upon the Holy Prophet MUHAMMAD (Peace be Upon Him) who ordained every Muslim to yearn for knowledge from cradle to grave.

I would like to express my deep and sincere gratitude to my research supervisor**, Prof. Dr. Irfan Zia Qureshi** for his valuable guidance, motivation, and immense knowledge. His dynamism, vision, encouragement, and sincerity have deeply inspired me. It was a great privilege and honor to work under his guidance. I'm in debt to **Dr. Amina Zuberi** (Professor, Chairman) for her encouraging behavior and obliged to her for extending the research facilities of the department to accomplish this work.

I extend my heartfelt gratitude to my esteemed seniors (**Sumera Api, Haleema Api, Ayesha Api, Iffat Api, Tariq Bhai, Aneeqa, Momna**) whose guidance and wisdom have illuminated my path throughout this journey. Your insights have been invaluable in shaping the direction of my research. I am indebted to my wonderful batch mates (**Izhar Ahmed, Hina Afaqi, Abdul Qadeer, M Ikram, Taimoor Ahmed, Ruqayya**) for the company, intellectual exchanges, and shared moments that have enriched this experience beyond measure. My friends (**Nida Afaqi, Isha Lehraseb, and Ali Abbas**) for their motivation and support in stressful days. To my family (**Baba Jan, Walida, Rabia, Sidra, Awais, Laraib, Areeba, Manahil, Saim, Zimal, M Issa**) words cannot express the depth of my appreciation for your unwavering encouragement and understanding. This thesis stands as a testament to the collective contributions of these exceptional individuals in my life.

Bakhtawer Rafiq

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ABSTRACT

Imidacloprid (IMI) insecticide has global application in protecting crops and animals. The mechanism of IMI involves activating post synaptic nicotinic acetylcholine receptors (nAChRs), which exhibit significant affinity for insect nAChRs even though reports of toxicity in mammals also exist. This study investigates the potential role of folic acid (F.A) in ameliorating behavioral, biochemical, and hematological alterations induced by subacute exposure to commercial formulation of IMI in female Sprague Dawley (SD) rats. In this experimental design, twenty-four adult female Sprague Dawley rats was subjected to random allocation into four separate groups, each comprising six animals. The groups were defined as follows: Group 1, serving as the control, received distilled water via oral gavage; Group 2 was administered IMI orally at a dose of 45 mg/kg body weight; Group 3 was subjected to oral administration of Folic Acid (F.A) at a dose of 10 mg/kg body weight; and Group 4 underwent pretreatment with F.A one hour prior to IMI administration, both via the oral route for three weeks. Results revealed a significant reduction ($p<0.001$) in motor coordination, cognition, nociception, and fear loss. While pretreatment with F.A significantly attenuated the behavioral impairments. Rats treated with IMI showed noticeable damage to the pyramidal cells within the hippocampal regions of CA1, CA2, and CA3.Subacute IMI exposure also induced hematological alterations. There is significant decline in RBCs, WBCs, and platelets count ($p<0.05$) in IMI treated rats as compared to control. However, pretreatment with F.A counteracts the effects of IMI. Moreover, there was a significant decrease ($p < 0.01$) in the serum concentrations of reduced glutathione (GSH), superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), leading to a consequent significant increase in reactive oxygen species (ROS) and lipid peroxidation (TBARS) levels ($p < 0.05$) in the brain homogenates. The results of current study demonstrate that pretreatment with F.A has potential protective effect on behavioral, hematological, and biochemical impairments via its antioxidant, anti-inflammatory and neuro-potentiating effects against subacute IMI exposure in female SD rats.

INTRODUCTION

1.1. History of pesticides

Pesticides are chemical substances that are used to kill pests, including insects, rodents, fungus, and undesirable plants (WHO, 2020). The initial occurrences of pesticide utilization were recorded during periods when inorganic substances were employed to combat diverse insect pests in agricultural crops. They play a pivotal role in the management of pests that pose threats to crops in both the agricultural and public health domains. Up to 3.5 million tons of pesticides were reportedly used globally in 2020. Insecticides fall under this category and are becoming increasingly significant for reducing agricultural pests and disease transmitters in the interest of global health (Rezende-Teixeira et al., 2022). In principle, insecticides should only affect the targeted pest species, yet all pesticides have side effects. Pesticide exposure can affect beneficial organisms like bees and earthworms and intoxicate people via oral, cutaneous, or inhalation routes who work in agriculture (Crenna et al., 2020).

1.2. Neonicotenoids

The term "neonicotinoid" signifies a "novel insecticide resembling nicotine"(Shivanandappa and Rajashekar, 2014). Neonicotenoids are a family of synthetic insecticides which became commercially available in the 1990s. Izuru Yamamoto originally coined the word "neonicotinoid" for imidacloprid (IMI) and related insecticides to distinguish them from the more traditional pesticides (a term he had proposed in the 1960s), including the plant alkaloid (S)-nicotine, which has a comparable mode of action. Nicotine is an alkaloid substance found in the leaves of many plants, including tobacco. Neonicotinoid pesticides (NPs) are synthetic analogues of this alkaloid(Ahmed and Sajjad, 2015). The literature has previously used a range of terminology to categorize these crucial pesticides(Yamamoto et al., 1998).Insecticides make up 44% of all pesticides, and the neonicotinoid market is the largest among these (CISION, 2020). Their discovery marked a significant turning point in the development

of modern insect management and is used against a variety of sucking and specific chewing pests (Jeschke and Nauen, 2008).

In the agricultural domain, neonicotinoids are favored over alternative insecticides due to various factors, including: (1) versatile application methods such as spraying, injections, or seed treatments(McCurdy et al., 2017); (2) comprehensive insect toxicity across a wide range of species;(3) perceived minimal immediate harm to non-target aquatic and terrestrial life forms(Lewis et al., 2016); and (4) potent effectiveness against insects(McCurdy et al., 2017).

Neonicotinoids have gradually replaced traditional insecticides utilized for managing insect pests on significant crops because of the effective mode of action and lack of cross-resistance (Nauen and Denholm I, 2005). Particularly compared to other pesticides, neonicotinoids are less detrimental to species not intended as the primary target of pesticide applications, humans, and the environment. They are also renowned for their adaptable administration and systemic mode of action (Goulson, 2013).

1.3. Analogues of neonicotenoids

IMI was released to the market in 1991 as the first successful representative of this chemical class, and since then, a series of six analogues have been developed i.e. (thiacloprid , nitenpyram , acetamiprid , clothianidin , thiamethoxam, and dinotefuran have all been released) (Jeschke et al., 2011).With almost 15% of the market for pesticides, neonicotinoids are the chemical class of insecticides with the greatest rate of growth (Wollweber and Tietjen, 1999).Worldwide registrations for neonicotinoids include both agricultural and non-agricultural usage. Neonicotinoids can be administered through various methods, such as foliar application, soil drench, aerial, chemical treatment, seed treatments, and tree injection (Anderson et al., 2015).

Neonicotinoid insecticides present a favorable environmental profile and anticipated selectivity ratios of 5 to 10 times higher for insects compared to non-target organisms. As

a result, they emerge as superior substitutes for certain insecticides like organophosphates and carbamates. For instance, the EC_{50} of imidacloprid is 0.86-1μM against insect nAChRs and against mammalian nAChRs is 70μM(Thompson et al., 2020).

1.4. Mechanism of action

In plants neonicotinoids exhibit a systemic mode of action. These insecticides are consumed by the plants and subsequently transferred throughout various parts, including ground and aerial tissues(Bonmatin et al., 2015).Because of their systemic activity, these agonists of postsynaptic nicotinic acetylcholine receptors shield important crops (such as corn, soybean, wheat, sugar beet, grapes, and orchards) against harm from an extremely wide range of phytophagous insects (Jeschke, P and Nauen, R. 2008). Upon ingestion by insects, neonicotinoids interact with nicotinic acetylcholine receptors (nAChRs)(Jeschke et al., 2011). This interaction, characterized by irreversibility, initiates nerve signaling like the action of acetylcholine. Remarkably, the enzyme acetylcholinesterase, responsible for the breakdown of acetylcholine, is unable to effectively metabolize neonicotinoids. Consequently, this leads to nerve stimulation at lower concentrations, while at higher concentrations, it results in receptor blockage, paralysis, and eventual mortality. nAChRs are not limited to the intended target plant pathogens; rather, they are widely manifested in various animal species. These receptors are present in both the peripheral and central nervous systems of human beings. Their role involves transmitting signals that prompt skeletal muscles to contract(Tomizawa and Casida, 2005).

In the perspective of insects, the precise functioning of nicotinic acetylcholine receptors is not as comprehensively elucidated as in humans. Nevertheless, these receptors play a role in postsynaptic neurotransmission in insects as well(Gotti and Clementi, 2004). The nicotinic acetylcholine receptors (nAChRs) are important for learning and memory in insects and are crucial in fast neurotransmission (Bicker, 1999).Hence neonicotenoids are classified as neurotoxins and link to insects in a specific way (Kagabu & Medej, 1995). In comparison to mammals, insects possess a higher abundance of these receptors, with a

primary concentration observed in their central nervous system. Neonicotinoids share a structural resemblance to nicotine(Tomizawa and Casida, 2005). The binding mechanism of NPs to nAChRs resembles the binding process of nicotine and nicotinoids in humans. In both cases, these substances act as agonists for nAChRs(Rodrigues et al., 2010).In humans, nicotine and other nicotinoids serve as agonists for nAChRs. Notably, nicotine persists at synaptic junctions for a longer duration than acetylcholine, as the latter is broken down by acetylcholine esterase. This leads to modifications in neural signaling patterns(Changeux and Taly, 2008).

1.5. Lethal dose in varies species.

These insecticides exhibit high toxicity across various organisms, although the recorded median LC_{50} or LD_{50} values show substantial variation among species. For instance, insects show median LC_{50} values ranging from 0.01 to 2.38 mg/L and LD_{50} values ranging from 3.7 to 81 ng/bee. In comparison, crustaceans have LC_{50} values ranging from 0.59 to 37.75 mg/L, fish from 1.2 to 241 mg/L, birds from 15 to >2,000 mg/kg, and mammals from 82 to >5,000 mg/kg, indicating a gradient of toxicity from insects to mammals**.**

1.6. Hazardous effects and half life

NPs are a hazard to the environment, animals, and public health because of their systemic mechanism of action, widespread distribution, and high persistence in the environment (Thompson, D. A. et al; 2020). Comparing neonicotinoids to organophosphates, which accumulate in tissues, particularly fat, NPs show lesser bioaccumulation. Vegetable bioaccumulation is a major way that people are exposed to neonicotinoids through food, increasing the risk of environmental contamination (Han et al., 2018). They may be dispersed into the soil and water, released into the air with pollen, or remain in crops throughout time (Zhang et al., 2018). Due to their water solubility, they can have a wide range of half-lives in soils, with Clothianidin's (CLO) reaching almost 19 years (U.S. EPA, 2010). They are frequently detected in ground

water, surface water, and even tap water (Van Dijk et al., 2013). Although neonicotinoids are resistant to hydrolysis, with enough light, they can quickly degrade through photodegradation. Certain soil-based bacteria have been found to be capable of breaking down neonicotinoids like imidacloprid and thiamethoxam. These bacteria degrade neonicotinoids along a similar route to mammalian liver. NPs are difficult to get rid of water. The best method, which can remove >80% of some neonicotinoids, is the treatment utilizing granular activated carbon because conventional techniques fall short of 100% removal (Thompson et al., 2020).Since washing does not completely eliminate all of the remains, neonicotinoids are regularly found in food and pollen (Chen et al., 2014; Kapoor et al., 2013). As a result, they can also affect humans and non-target animals. Therefore major concern is that the increasing use of neonicotinoids is linked to a reduction in biodiversity (Goulson, 2013).

It has been demonstrated that the widespread usage of neonicotinoids is responsible for the regional massive decline of bees. NPs were deemed to pose an intolerably high risk to wildlife and honeybees by EFSA in 2013 (EFSA, 2018). It is formerly established that places where neonicotinoids are used have fewer insects of all kinds (Sánchez-Bayo and Wyckhuys, 2019). The use of the neonicotinoids CLO, IMI and thiamethoxam (TMX) in plant protection and seed treatment products was therefore limited by the European Union (EU). All outdoor applications of the three chemicals were prohibited in 2018 because of these restrictions, which were supported by substantial data collection. In 2020 a neonicotinoid thiacloprid's was revoked due to the possibility of endocrine disruption (Ensley, 2018). Twelve neonicotinoid-containing products were revoked by the US EPA in 2019 (U.S.EPA, 2019). The daily recommended consumption levels for a lifetime without significantly increasing the danger to health were established in the same year. These levels were determined at 10-200 g/kg body weight for a range of neonicotinoids. (Thompson et al., 2020).

About 90% of orally delivered NPs in rats are absorbed; and small intestine is the majority site where this absorption occurs (Taira, 2012). NPs that pass through the

placenta and blood-brain barrier which enter the bloodstream and circulate throughout the body(Burke et al., 2018, Taira, 2012).Limited research has investigated human exposure to neonicotinoids and the potential risks these insecticides may pose to human health(Cimino et al., 2017).A comprehensive review of the literature conducted between 2005 and 2015 identified a limited number of studies, only eight in total, that investigated the potential human health implications associated with NPs exposure. Out of these studies, four investigations concentrated on instances of acute exposure, such as deliberate cases of self-poisoning, while the remaining four studies focused on assessing the consequences of chronic environmental exposures. The outcomes derived from the chronic exposure investigations, primarily reliant on surrogate exposure data, indicated a potential association between prolonged low-level NPs exposure and adverse developmental and neurological effects. Notably, in 2013, the EU designated two NPs, ACT and IMI, as potential neurotoxic agents(Products and Residues, 2013).

1.7. Metabolism of neonicotenoids

In mammals, the metabolism of neonicotinoids takes place primarily in the liver through the action of enzymes such as cytochrome P450 enzymes and aldehyde oxidase. Additionally, these enzymes are expressed in various other tissues(Marfo et al., 2015). The metabolic processes in vertebrates involve numerous reactions, including reduction, demethylation, hydroxylation, and olefin formation. Owing to their low molecular weights and water solubility almost 95% of all NPs and their metabolites are eliminated via excretion (Taira, 2012). It is reported to produce a variety of metabolites in vivo, some of which are reportedly more poisonous than the main component. Some metabolites are IMI-NH (desnitro-imidacloprid), THI-NH (desyanothiacloprid), ACENH (desyanoacetamiprid), CLO-dm (desmethyl clothianidin), CLO-urea, and NG-F (methyl guanidine), which are produced from IMI, THI, and ACT, respectively (Ford and Casida, 2008). Subsequently, these metabolites conjugate with either glycine or glucuronic acid, resulting in their excretion through urine(Ford and Casida, 2006).Notably, these

metabolites have been identified in the urine of both mice and human(Thompson et al., 2020).

Table 1.1: Environmental standards and food standards for various neonicotinoid pesticides in Japan as of 29 June 2021 (Terayama et al., 2022).

Standard Type	Standard Name	Imidacloprid
Environmental standard (µg/L)	Registration withholding standards for agricultural chemicals with reference to prevention of toxicity to aquatic plants and animals.	1.9
	Registration withholding standards for agricultural chemicals with reference to prevention of water pollution.	150
Food standard (µg/kg)	Acceptable daily intake (ADI).	57
	Acute reference dose (ARfD).	100

IMI and TMX undergo metabolic processes leading to the production of nitrosoguanidine metabolites, subsequently transforming into less detrimental urea metabolites. Analogous metabolic processes take place in other vertebrates as well, encompassing species such as mice, rats, goats, and hens(Pandey et al., 2009). Although NPs primarily target the nAChR subtype α4β2, they also show minor effects on other subtypes too. Moreover, some NP metabolites exhibit a preference for subtypes different from those that the

parent molecules target (Taira, 2012).The mammalian nAChR subtypes α4β2 can also be found in a variety of organs, including the ovary and testis, in addition to the nervous system (IuO, 2014).

Additionally, when given to laboratory animals, NPs pass the BBB, placenta, and intestinal mucosa, causing a variety of symptoms that seem to be linked to the nervous system (Taira, 2012). Both the type of NP and the animal species affect how vulnerable mature and juvenile animals are to NPs toxicity. Neonicotinoids are utilized all over the world to control insect pests, which represent a severe risk to both public health and crop protection.

1.8. Toxicity of NPs in mammals.

The ecological impact of NPs has been extensively studied, revealing potential sub-lethal effects on various species including mammals, birds, fish, amphibians, and reptiles. IMI and CLO, for instance, have been associated with adverse outcomes such as genotoxicity, cytotoxicity, compromised immune function, stunted growth, and reproductive alterations. Notably, these effects have been observed at concentrations considerably lower than those causing outright mortality(Gibbons et al., 2015).

Comparing the toxicological data of NPs to other, these pesticides generally exhibit lower acute toxicity to mammals and birds(Lewis et al., 2016). NPs have a lower tendency to accumulate in tissue while organophosphates have higher potential to accumulate (Roberts, 2013).Exposure to NPs often leads to the induction of oxidative stress, manifested through heightened lipid peroxidation, diminished levels of antioxidant enzymes (Annabi et al., 2015).

Numerous studies have documented negative impacts on the reproductive and developmental processes of mammals following exposure to NPs. These effects involve a range of adverse outcomes, such as increased risks of embryo mortality, preterm birth, reduction in pregnancy rates, sperm production impairment and function, reduced

newborn weight, and instances of stillbirth. Indeed, experimental evidence in rodent models has indicated that NPs can have an impact on the fertilization rate (Xuan et al., 2013).Furthermore, the exposure of male rats to IMI has been shown to lead to a significant reduction in serum testosterone levels(NAJAFI et al., 2010). Likewise, even at dose levels considered to have no observed adverse effects (NOAEL), IMI exposure has been linked with the reduction in testicular function in adult male rats(Bal et al., 2012).

Hepatotoxicity resulting from exposure to NPs is usually evident only at elevated doses and often coincides with reduced food intake and body weight. Serum levels of AST , ALP are frequently elevated in rats subjected to NPs exposure, which are hepatotoxicity markers (Lonare et al., 2014). Notably, liver pathology appears to be completely reversible in rats that were given time to recover following exposure to IMI (Sheets, 2010).

Based on in vitro and in vivo studies conducted during the registration process, NPs have generally been found to be non-mutagenic. Ames test which is a common method for assessing mutagenicity, generally indicates that neonicotinoids are not mutagenic(Sheets, 2010). However, it's worth noting that an IMI pesticide formulation has been shown to induce mutations in the G-C base pairs (Karabay and Oguz, 2005). In various studies, dose-dependent DNA damage in human peripheral blood lymphocytes, human lymphocytes, and HepG2 cells(Feng et al., 2005)have been documented for NPs , whether tested in their pure form or as formulations(Calderón-Segura et al., 2012),. This DNA damage has been assessed using a method known as the comet assay. NPs have a substantial impact in the micronucleus assay in various cell types, including bone-marrow cells(Bagri et al., 2016), human lymphocytes(Feng et al., 2005), and HepG2 cells. However, the results are not always consistent across different studies and cell types.

Some studies have reported induction of micronuclei and chromosome aberrations in response to NPs exposure, while others have not observed such effects. For instance, while certain studies demonstrated that neonicotinoids induce genetic alterations, not all

studies have reported these effects. Similarly, some studies have found an increase in micronuclei formation in response to neonicotinoid exposure, while others have not observed this effect in specific cell types, such as polychromatic erythrocytes in mice(Zang et al., 2000) and human lymphocytes (Demsia et al., 2007).

Overall, the effects of NPs on DNA damage and chromosome abnormalities appear to vary depending on the specific experimental conditions and cell types being studied. Carcinogenicity studies conducted with IMI and CLO have not shown any evidence of carcinogenic effects in rodents. These studies provide data that suggest exposure to these substances does not lead to the development of cancer in experimental animals(Tanaka, 2012).Considering the available research, several neonicotinoids, including ACT, CLO, DIN, IMI, and TMX, have been classified by the U.S. Environmental Protection Agency (EPA) as "Not Likely to be Carcinogenic to Humans" (Thompson et al., 2020).

1.9. Neurotoxicity of neonicotenoids

According to EPA guidelines, acute studies did not show persistent or delayed neurotoxic effects from neonicotinoid exposure. Tremors were observed in mice exposed to neonicotinoids, indicating some level of neurological impact(Sheets, 2002).While sub chronic studies suggest that NPs does not produce significant neurotoxicity after sustained dietary exposure. Rapid elimination of NPs (Yamamoto and Casida, 1999), reduced penetration across the BBB, and their preference for insect nAChRs over non-insect ones contribute to these findings(Sheets, 2010).Some studies indicate that neonicotinoids like IMI and TMX can affect motor activity in rats(Rodrigues et al., 2010).TMX may induce anxiety-like effects (Rodrigues et al., 2010), and CLO could elevate anxiety-like behavior in mice(Hirano et al., 2015).

Gestational exposure to IMI has been linked to alterations in motor coordination of newborns, and neurobehavioral parameters and cognitive function are affected after developmental exposure to CLO in rodents(Özdemir et al., 2014).Excitatory effects on

newborn rat cerebellar neurons are documented at low concentrations of ACT and IMI (Kimura-Kuroda et al., 2012).Related to the cholinergic systems following exposure to TMX and IMI, some studies in rats observed alterations in behavioral and biochemical processes (de Oliveira et al., 2010).

1.10. Adverse effects of NPs on human health

In recent times, several epidemiological studies have highlighted considerable apprehensions regarding global health, encompassing both acute effects and potential long-term effects. Instances of acute poisoning in humans associated with neonicotinoid exposure have resulted in a range of symptoms, including respiratory, cardiovascular, and neurological signs, with occasional instances leading to fatalities (Ikenaka et al., 2019). Instances of subacute intoxication resulting from the consumption of NPs contaminated food items have been identified in Japan(Taira et al., 2011). Notably, six individuals who consumed quantities exceeding 500g daily displayed a range of symptoms. These symptoms include: finger tremors, memory impairments, laziness, headaches, palpitations, muscle discomfort, weakness, and spasms also(Taira, 2014).

The documented harmful global health effects arising from exposure of NPs are limited. Nevertheless, there is a notable absence of rigorous scientific studies that comprehensively assess the associated risks, thus highlighting the imperative for further research focused on human health impacts(Cimino et al., 2017). In 2013, the EFSA Panel on Plant Protection Products and their Residues asserted that there exists substantial confirmation pointing towards the detrimental impact of two NPs on the developing human nervous system i.e ACT and IMI(Products and Residues, 2013). These effects are like the harm inflicted by nicotine. Notably, these insecticides may potentially impede the development and structure of CNS related to critical roles like cognition (Anderson et al., 2015).

1.11. Classification of insecticides wrt mechanism of action

There are following four classes of insecticides based on their specific target site or site of action which are listed below in the table:

Table 1.2: Classes of insecticides based on their mode of action (Rezende-Teixeira et al., 2022).

Target site	Site of action	Chemical classes	References
1. Nerve & muscle	Sodium channel activators	Pyrethroids and DTT	(Narahashi, 2000)
	channel Sodium inhibitors	Carbazones and piperidines	(von Stein et al., 2013)
	Chloride channel blockers (GABA agonists)	Avermectins	(Huang and Casida, 1997)
	Cholinesterase inhibitors	Organophosphates and carbamates	(Lotti ,2001)
	Acetylcholine mimics	**Neonicotinoids**	(Kathrina and Antonio, 2004)
	Calcium channel activators	Diamides	(Lahm et al., 2009)
2. Energy production	Alkylating agents	Methyl bromide	(Bulathsinghala and Shaw, 2014)
	Glycolysis inhibitors	Arsenicals	(Bencko &Yan Li Foong ,2017)
	Mitochondrial agents	Rotenone	(Isman,2006)
3. Growth and	Chitin Synthesis	Inhibitors of chitin	(Gangishetti et al.,

development	Inhibitors	biosynthesis	2009)
	Interference Growth Regulators	Juvenile Hormone Mimics	(Boudjelida et al., 2005)
4. Miscellaneous	Biological	Bacterial Larvicide	(Vachon et al., 2012)

1.12. Resistance of neonicotenoids

The NPs have emerged as a crucial asset for agricultural practitioners in effectively managing pests that are destructive for crops, primarily those belonging to Hemiptera as well as the Coleoptera order encompassing beetles. This group of pesticides has displayed its effectiveness even against pest species that had previously developed resistance to older insecticide classes. Notably, IMI has displayed remarkable resilience to the development of resistance. Nevertheless, the resistance in certain genera underscores the pests' ability to adapt and withstand the application of neonicotinoids within field settings. The continual global introduction of additional neonicotinoids, unless subjected to careful regulation and strategic coordination, appears likely to increase the exposure to this group of substances. Moreover, this trend could potentially aggravate conditions that facilitate the emergence of resistant traits among target pests(Nauen and Denholm, 2005).

However, PS are still effective in controlling many insect species, their popularity has put increasing natural selection pressure on the development of resistance to neonicotinoids. Consequently, resistance has now reached levels in numerous species that limit the effect. NPs resistance can develop as a result of target-site protein structural changes, nAChR subtype expression, or elimination processes (Thany, 2010). Pesticide resistance often involves the upregulation of detoxification enzymes, such as cytochrome P450, which facilitates the conversion of insecticides into less harmful metabolites. This process contributes to the decreased effectiveness of insecticides in controlling pest

populations(Zhou et al., 2020). As proved for mosquitoes, neonicotinoid combinations have proven to be very effective against resistant strains (Agossa et al., 2018).

Numerous studies have already shown how neonicotinoids lead to the severe declines of pollinators, notably honeybees, but until now, the consequences on human health have rarely been investigated (Thompson et al., 2020). Recent studies have raised doubt on this presumption. Chronic exposure to NPs may expose people to health hazards, such as endocrine disruption, regardless their low acute toxicity (Sun et al., 2016, 2017), neurological complications (Faro et al., 2019) and birth related defects (Sheets et al., 2016). However, the majority of nations do not have restrictions on NPs use, and farmers continue to prefer them because they are cheap , have low to moderate resistance, and powerful effects against insects (Gong et al., 2021).

1.13. Imidacloprid

Neonicotinoids, a new class of chemicals with structural similarities to nicotine, include IMI, an ectoparasiticide (Khalil et al., 2017). IMI belongs to the chloronicotinyl nitroguanidine chemical family (Tomlin, 2006). IMI, an ingredient in commercial insecticide products including Confidor, Gaucho, Prestige, Admire, and Premier that are widely utilized in agriculture, is currently the most widely used neonicotinoid (Elbert et al., 1998, Tasei et al., 2000) owing to its minimal soil persistence and strong insecticidal effects with minimal application rate (Chao and Casida, 1997).

These insecticides have sub-lethal toxic effects on non-target species, which can lead to lower activity, decreased fertility and growth rate, nervous system impacts, disturbed metabolic balance, DNA damage, and other consequences. DIN and NIT had the lowest toxicity, while IMI had the highest toxicity among them (Qi et al., 2020).

1.14. IUPAC name and structural formula

The IUPAC name of IMI is 1-[(6-chloropyridin-3-yl) methyl]-N-nitroimidazolidin-2-imine. It is an imidazolidine that is N-nitroimidazolidin-2-imine bearing a (6-chloro-3-pyridinyl) methyl substituent at position 1 having molecular weight of 255. 66100.Its molecular formula is C9H10ClN5O2. The water solubility of IMI is 0.61 g L-1 at 20°C, and its insecticidal efficacy, with an LC_{90} value, is 0.32 mg L-1 (Jeschke and Nauen, 2008). The structural formula of imidacloprid is

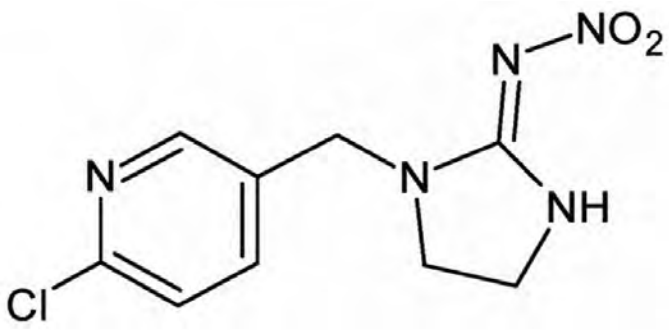

Fig.1.1. Structural formula of IMI

1.15. Lethal doses of imidacloprid in various species

The lowest median LD_{50} concentration of IMI in male Sprague Dawley rats, 440 mg/kg; female Sprague Dawley rats, 410 mg/kg; male albino mice, 100 mg/kg; female albino mice, 98 mg/kg respectively(Terayama et al., 2022). If inhaled, IMI has a range of toxicities (U.S. EPA, 2010). In rats, the inhalation LC_{50} for dust exposure was calculated to be greater than 5323 mg/m^3 and for aerosol exposure to be 69 mg/m^3. Although IMI dust is thought to be moderately harmful, the aerosol form is extremely toxic(Tomlin, 2006). IMI shows low toxic effects through dermal exposure. Rats' cutaneous LD_{50} was calculated to be more than 5000 mg/kg (Tomlin, 2006).Among the most widely employed nicotinoids, IMI stands out for its relatively lower risk to vertebrates compared to other pesticides. However, its impact on non-target invertebrates remains noteworthy

(Sohn et al., 2018). A noteworthy stride in formulating safer and more efficient products has been the emergence of eco-friendly neonicotinoid insecticides(YUANYUAN, 2019).

1.16. Mechanism of action of IMI

The interaction between nicotine and nicotinic receptors leads to swift stimulation succeeded by prolonged depolarization, inducing receptor paralysis. Nicotine was the pioneering pesticide possessing acetylcholine mimic attributes (Costa, 2008). Neonicotinoids' insecticidal effects are attributed to their ability to activate nicotinic receptors, which are still connected to postsynaptic acetylcholine receptors. The hyperexcitation of the neural system brought on by receptor activation causes paralysis and insect death (Costa, 2008). Although there are certain structural resemblances between nicotine and neonicotinoids, their modes of action diverge mainly due to nicotine having a protonated region that interacts with mammalian biological receptors at physiological pH. Neonicotinoids lack a protonated region under the same conditions, which contributes to their distinct toxicological profile within this class(Tomizawa and Casida, 2005).

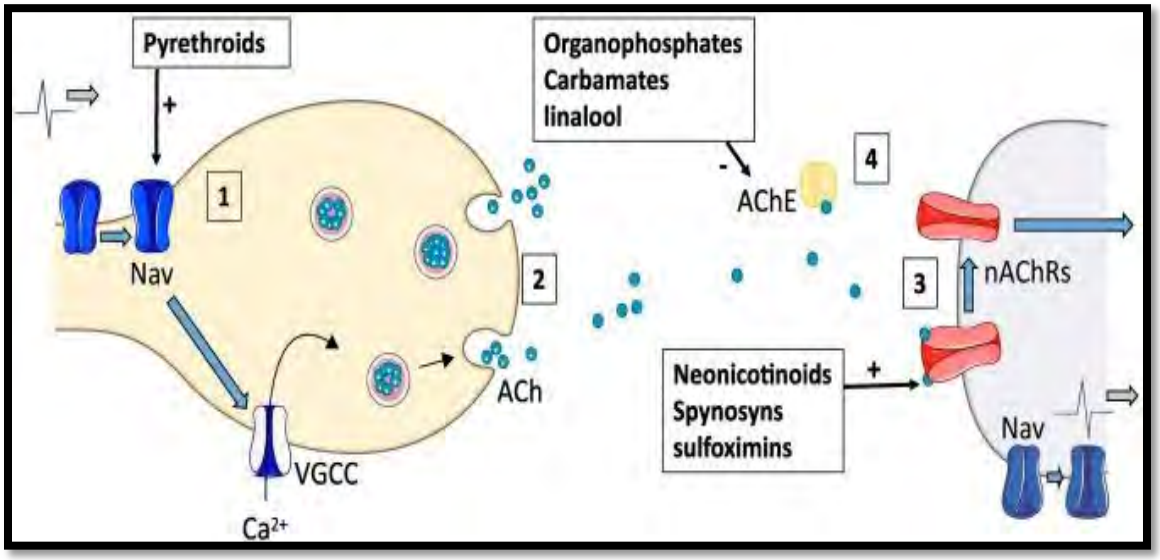

Fig. 1.2. Simplified schematic representation of the mode of action of neurotoxic insecticides at cholinergic synapse. 1: presynaptic activation and action on voltage-

dependent sodium channels (Nav). Voltage-gated calcium channels (VGCC) are activated after the depolarization by sodium channels to stimulate synaptic vesicles. Migration of vesicles containing the neurotransmitter acetylcholine (ACh) to the synapse. 2: neurotransmitter is released into the synaptic cleft. 3: ACh binds to the postsynaptic nicotinic acetylcholine receptors (nAChRs). 4: ACh is degraded by acetylcholinesterase (AChE). Neonicotinoids, spinosyns and sulfoximine act on nAChRs. Organophosphorus (including organophosphate, all named OPs), carbamates and linalools inhibit AChE. All these neuroactive molecules lead to an over-stimulation of the central nervous system resulting in insect death. « + » represents activation and « - » inhibition pathways. (Raymond-Delpech et al., 2005)

1.17. Metabolism of imidacloprid

Studies conducted on mammals indicate that IMI is absorbed rapidly resulting in the formation of 6-CNA and olefin as its fundamental breakdown products. However, the use of IMI remains a subject of debate due to apprehensions that some NPs or their metabolites may increase nAChR (nicotinic acetylcholine receptor) expression in mammals, potentially leading to toxicity in these hosts. This aspect has been indicated in human suicide case studies (Proença et al., 2005).

IMI undergoes several chemical transformations including oxidation of the imidazolidine moiety, reduction of the nitroimine substituent, and oxidative cleavage of the chloro-pyridinyl from the imidazolidine moiety. These transformations have been extensively studied in various environmental contexts including soils, plants, and animals(Roberts, 1999). While CYP450is believed to mediate reactions involving carbon hydroxylation, other processes such as nitro reduction or secondary amine oxidation might be facilitated by CYP450, flavin monooxygenase , or other enzymatic pathways.(Parkinson and Ogilvie, 2008).

1.18. Detoxification routes of IMI

The liver is the primary detoxification site for insecticides, where they undergo biotransformation. However, this process can occasionally lead to the generation of even more toxic metabolites. The kidneys also function as a secondary route for detoxification. Notably, the nervous system is highly vulnerable to the effects of toxic substances and a range of compounds such as solvents, pharmaceuticals, substances and specially

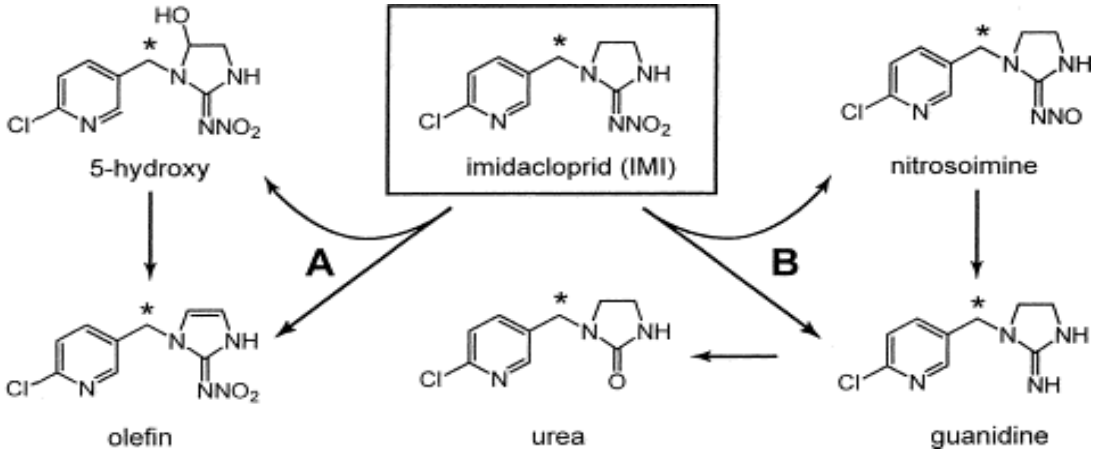

Fig. 1.3: IMI metabolism by human CYP450 isozymes involves alternative pathways. (A) Hydroxylation and desaturation of the imidazolidine moiety to give the 5-hydroxy and olefin derivatives. (B) Reduction and cleavage of the nitroimine substituent to form the nitroimine, guanidine, and urea derivatives. Asterisks designate positions of tritium labeling (Schulz-Jander and Casida, 2002).

pesticides, have shown neurotoxic properties. Moreover, research on insects has uncovered several metabolites associated with IMI's toxicity, with the olefin metabolite standing out as more toxic than IMI itself. Another significant metabolite is 5-hydroxyimidacloprid (Suchail et al., 2004). Furthermore, the desnitro metabolite demonstrates limited toxicity to the nervous system of insects, but it exhibits higher toxicity to the nervous systems of mammals compared to IMI itself(Tomizawa and Casida, 2005). The soil metabolite 2-imidazolidone has been found to cause tumors in

conjunction with nitrate and cause genetic damage (Tomizawa and Casida, 2003). These metabolites may function independently or jointly with parent chemical residues. As a result, kinetic analyses are crucial to evaluate the toxicity of neonicotinoids and the distribution of their metabolites throughout the body.

It is well-established that various classes of pesticides can lead to oxidative stress, which may contribute to their toxicity as xenobiotics (foreign substances).The antioxidant defense mechanism counteract the harmful effects of oxidative stress (Hennig et al., 2007). Additionally, dietary intake of antioxidants, such as vitamins A, B, C, and E, can further enhance this defense mechanism. These dietary antioxidants play a vital role in mitigating the damage caused by ROS and contribute to overall cellular health.

1.19. Folic Acid

Folic acid (FA) belongs to the vitamin B family and has a significant impact on cognitive function by elevating the levels of vitamins B12 and B6. Insufficient levels of folate and vitamin B12 could potentially raise likelihood of impairment in cognition (Ma et al., 2016). According to clinical data, cognitive impairment has been detected in approximately two-thirds of patients experiencing anemia or deficiencies in folate and vitamin B12(Reynolds, 2002). Homocysteine, an amino acid containing sulfur, is under the regulation of blood levels by FA, along with vitamins B12 and B6(Modaghegh et al., 2016). However, a direct relationship exists between increased homocysteine levels and cognitive impairment (Garcia and Zanibbi 2004).

The shortage of F.A leads to an elevation in homocysteine levels, which subsequently causes degeneration in the hippocampal neurons. Consequently, F.A exposure enhances memory function by regulating homocysteine levels and serving a potent antioxidant (Singh et al. 2011). Furthermore, research indicates that FA enhances both short-term and long-term memory. An experiment involving hypothyroid rats demonstrated that folic acid administration improved oxidative stress and hypothalamic monoamines (Ibrahim et al., 2012). For the brain system to develop normally, dietary folic acid is essential, as it

controls neurogenesis and apoptosis. For instance, the telencephalic region of the forebrain of fetal mice exhibits impaired neurogenesis and increased apoptosis in response to maternal diets lacking in folate (Craciunescu et al., 2004). Additionally, current study has connected folic acid intake with a general boost in memory. Reduced cognitive function is more likely to occur in people and mice with low blood folate levels (Chen et al., 2010).

1.20. Folic acid and cognitive functions

This shows that therapies aimed at boosting neurogenesis may have the ability to improve post-stroke cognitive performance. Moreover, folate is closely linked to choline metabolism and contributes to acetylcholine synthesis and release (Crivello et al., 2010). Insufficient folate, either due to genetic factors or dietary deficiency, diminishes ACh levels and may impact cognition (Chan et al., 2008). Additionally, the nutrient boosted the expression of Notch1, Hes1, and Hes5 genes, as well as the quantity of the developing hippocampus neurons. Folic acid decreases the impairment of cognitive abilities that happens after an experimental stroke and increases the stimulation of Notch signaling and hippocampus regeneration by ischemia in adult brains (Zhang et al., 2012).

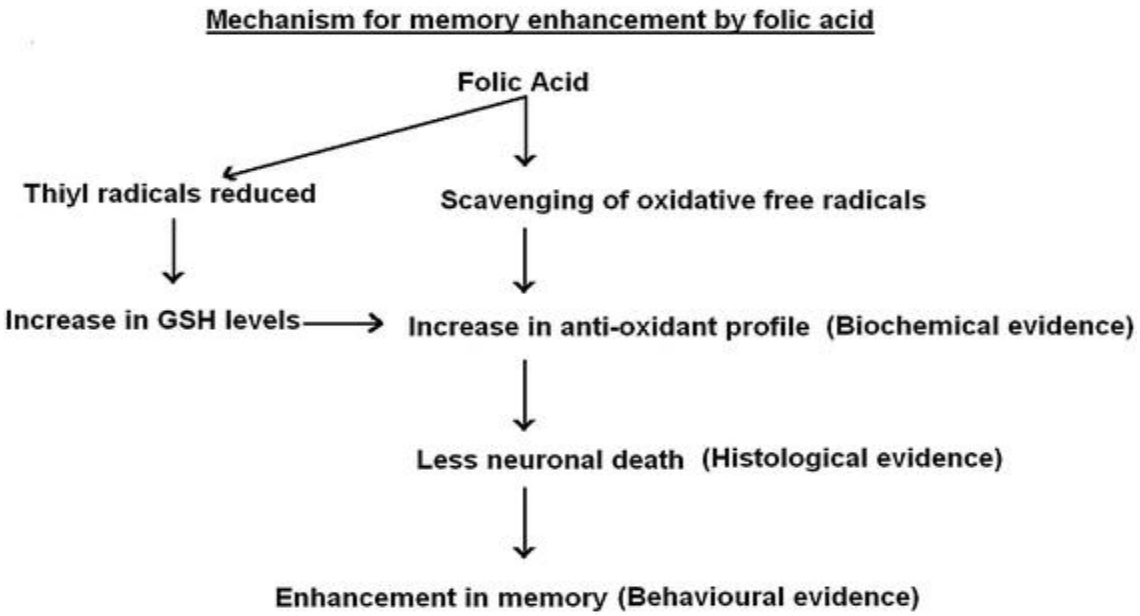

Fig. 1.4. Mechanism for memory enhancement by folic acid in aged rats (Singh et al., 2011).

Hence, this study presents an overview of the detrimental effects resulting from sub chronic exposure to IMI and their potential risks to the health of animals and humans. These effects include alterations in behavior, blood parameters, oxidative stress markers of brain as well as histopathological changes in brain hippocampal area, and the potential protective role of FA is highlighted in counteracting these hazards. Therefore, it will be interesting to investigate whether folic acid supplements can help those who are susceptible to cognitive deterioration brought on by pesticide exposure. In the current work, we examined the cognitive function of folic acid-exposed rats and further identified the antioxidant defense mechanism.

MATERIAL AND METHODS

This study was designed and executed within the controlled environment of Physiology laboratory of Department of Zoology at Quaid-i-Azam University, Islamabad, Pakistan.

2.1. Approval of ethical committee

This study was certified by the ―Bioethical committee of the Faculty of Biological Sciences" Quaid-i-Azam University, Islamabad, Pakistan. The study adhered to the guidelines outlined by the Bioethical committee.

2.2. Experimental Animals

In the current study a total of twenty-four (n=24) healthy female rats, body weight ranging from 160-164g were provided by National Institute of Health (NIH) and were housed in the animal house. Rats were placed in rodents cages. Room conditions were, temperature 24°C±2°C, humidity 50-60% and photoperiod with light-dark cycle of 12h each. Standard rodent diet and tap water were provided at libitum. To prevent overcrowding and reduce stress, a maximum of six rats were placed within each cage. Before commencing the experimental procedures, a two-week acclimatization period was ensured for the animals. Throughout the entire duration, the rats received humane and considerate treatment, both prior to and during the experimentation phase, as well as after the experiment were completed.

2.3. Experimental design

The rats were randomly allocated and categorized into four distinct groups. First group was control received distilled water. The second group was subjected to a treatment regimen of Imidacloprid pesticide at a dosage of 45mg/kg/day, administered via oral gavage in distilled water for total 21 days. The third group received folic acid

orally at a dose of 10mg/kg/day. In contrast, the fourth group was subjected to a pre-treatment protocol involving oral administration of folic acid at 10mg/kg/day, one hour prior to receiving Imidacloprid at 45mg/kg/day through oral gavage. All groups x received their respective doses for 21 consecutive days.

To assess behavioral outcomes, all rats across the four groups underwent behavioral testing during the last two days of the dosing schedule.

Table 2.1: Shows experimental design.

Groups	Doses
Group 1	Control (Distilled water via oral gavage).
Group 2	Treated (Imidacloprid 45mg/kg/day via oral gavage).
Group 3	Treated (Folic acid 10mg/kg/day via oral gavage).
Group 4	Protected (Pre-treated folic acid 10mg/kg/day and then after 1-hour imidacloprid 45mg/kg/day orally).

2.4. Measurement of body weight

The body weights of rats were measured before the initiation of the experiment. With the interval of one week, animals were weighed to administer proper dose according to b/w and to observe weight loss or gain because of the dose administered.

2.5. Chemicals

All the chemicals used in this study were procured from the Sigma Aldrich Company (Sigma, St. Louis, Missouri, USA). These include formaldehyde, chloroform, NBT, ferrous sulphate, commissive blue, triton-x 100, BSA, sodium chloride, SDS, PMSF, DEPPD, sodium azide, sodium hydroxide, hydrogen peroxide, sodium acetate, Tris-HCl, TBA, TCA, riboflavin, potassium phosphate monobasic, L-methionine, potassium phosphate dibasic, sodium phosphate dibasic, guaicol, sodium phosphate

monobasic, tri-sodium citrate, eosin, DTNB, phosphoric acid, hematoxylin, and methanol, among others.

2.6. Dose preparation

Since imidacloprid (IMI) and folic acid (F.A) both are water soluble, the dose was prepared in distilled water. The volume administered to each rat was 1ml. amount of solution containing the chosen concentration of IMI and F.A to be administered was almost 1ml per rat. Each day doses were administered at a fixed time from 10:30 am to 11:30am.

2.7. Animal dissections

After the completion of 21st days of daily dosing and observation of behavioral changes in all groups, animals were dissected. All the rats were weighed properly at the end of the experiment. Animals were anesthetized with sodium pentobarbital. Blood was collected by cardiac puncture with 3ml heparinized syringes. The brain, liver and kidney tissues were dissected out and washed with saline. Subsequently, brain tissues were stored at -20°C for biochemical investigations. For histological analysis, half of the brain tissues were fixed in 10% formalin.

2.8. Calculation of the Organ Mass Index

At the day of dissection, brains were weighed, and organ mass index was found using formula followed by Reddy et al. (2011).

2.9. Hematology

For complete blood picture 2ml of blood was collected in EDTA-coated vacutainers. Blood samples were drawn directly from the heart of the rats in heparinized

syringes. Subsequent analysis was carried out on an automated hematology analyzer (URIT-2900Vet Plus, China).

Hematology included blood components, including total white blood cells (WBCs), neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Furthermore, key parameters such as Total Erythrocyte Count, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin Concentration, Hemoglobin concentration, Mean Corpuscular Hemoglobin, and Hematocrit were quantified for each sample.

2.10. Histological analysis of brain

For tissue histology, conventional Eosin and Hematoxylin (H & E) staining method was used. Soon after dissection, brain tissues were excised, rinsed in fresh 0.9% saline, and preserved overnight in 10% buffered formalin. The steps in tissue histology include processing (fixation and dehydration), microtomy (tissue embedding and sectioning), and slide preparation (mounting, staining and microscopy).

2.10.1. Reagent preparation

Hematoxylin (1.0g) was dissolved in 10ml ethanol (absolute), while 20g of potassium alum was mixed in 200ml of distilled water (dH_2O). Both hematoxylin and alum solutions were mixed thoroughly and boiled on a hot plate after which 0.5g mercuric oxide was added. The staining solution was then allowed to cool after adding 8ml of glacial acetic acid. Staining solutions were filtered and preserved at room temperature. Eosin (1.0g) was added to 0.05ml of acetic acid and then dH_2O was added to get a final of 100ml.This staining solution was then filtered. For the preparation of acid alcohol, 1ml of concentrated HCl was added to 99ml of 70% ethyl alcohol.

2.10.2. Tissue processing

Fixed tissues underwent dehydration through an ascending ethanol series, each step lasting 1-2 hours. Subsequently, the tissues were cleared using xylene I and xylene II. They were then immersed in preheated paraffin wax at 59°C for fixation. Slices of 5µm thickness were generated using a rotary microtome (Shandon, Finesse, Italy). The tissue sections were deparaffinized in Xylene II for 5 minutes, followed by immersion in a descending alcohol concentration series of 100%, 90%, 80%, 70%, and 50%, with each concentration lasting 3 minutes. Afterward, the sections were rinsed with running tap water and prepared for staining.

2.10.3. H&E staining and light microscopy

Tissue sections were stained in Harris's hematoxylin solution for 5 min, then washed gently for 2 min in running tap water and immersed for 5 sec in acid alcohol, washed again for 45 sec in running tap water. Staining was done in 1% working solution of eosin for 2 minutes, rinsed in running tap water for 45 sec and dehydrated in an leading series of alcohol (50%, 70%, 80%, 90% and 100%) for 3 min each. Tissues were then placed in xylene for 3 min. Sections were then mounted in DPX. Sections were then observed at a magnification of 10X and 40X. magnification and photographed data were prepared with adobe photoshop (Version 7.0, Microsoft Inc. USA).

2.11. Behavioral analysis

To assess various aspects of behavior, including motor coordination, fear conditioning, spatial learning, and navigation, as well as learning and memory, a series of tests were conducted. These tests included tasks such as horizontal bar test, parallel bar test, and passive avoidance test. For the evaluation of nociception, the hot plate test, tail-flick test, and the application of electric foot shock were executed. For testing memory

impairments novel object recognition and contextual and cued fear conditioning test were performed.

2.12. Horizontal bar test

Forelimb strength and coordination were assessed with the "string test", also recognized as the "coat hanger test" or "horizontal bar" assessment. The rats' capacity to grip the bar manifested an inverse correlation with the bar's diameter. For consistency, a standard bar with a 2mm diameter was used. The bar's dimensions were length of 91.4cm, elevation 73.6cm above the floor by means of wooden support columns.

The testing involved delicately holding the rat by its tail and placing it on the table near the apparatus. The rat was then promptly drawn back by approximately 20cm, facilitating its alignment perpendicular to the bar. Swiftly the rat was elevated and permitted to grip the horizontal bar solely with its forepaws, securing its position at the bar's midpoint and allowing its tail to relax fully. Time was recorded with a handheld stopwatch.

The test assessed the duration until the rat descended from the bar prior to traversing the entire length of the apparatus and the interval until one of the rat's forepaws contacted the supporting pillar. The predetermined optimal time frame for these observations was set at 30 sec.

2.12.1. Scoring procedure

The scoring was carried out as follows: if the rat failed to grasp bar before the first 5 sec, this phenomenon was attributed to inadequate positioning, and this decline was excluded from the analysis.

If the rat fell within 1-5 sec score = 1

If the rat fell within 6-10 sec score = 2

If the rat fell within 11-20 sec score = 3

If the rat fell within 21-30 sec score = 4

If the rat fell in duration exceeding 30 sec score = 5

2.13. Parallel bars test

Two parallel steel bars, each with a length of 91.4cm and a diameter of 4mm, were affixed to wooden supporting columns at their respective ends, maintaining a separation of 2.5cm between them. The apparatus was elevated to a height of 73.6cm overground level. Throughout the experiment, two key parameters were carefully recorded, with the test duration limited to 120 sec.

The rat was handled with care, held by its tail, and gently positioned on the bench facing the apparatus. A swift motion was employed to draw the rat approximately 20cm backwards, ensuring its alignment perpendicular to the bar. With precision, the rat was raised and allowed to grasp the horizontal bar exclusively with forepaws, while tail was allowed to relax completely. A suitable duration was granted for the rat to achieve stability, thereby minimizing potential stress. Subsequently, the stopwatch was activated to commence recording the duration of the test. The initial parameter entailed measuring the duration the rat required to orient itself by 90° from the starting point. The second parameter involved recording the time it took for the rat to traverse the entire length of the bar and reach its end.

2.14. Fear conditioning test

Fear conditioning tests were conducted using a fear conditioning test box consisting of light and dark compartments. The dark compartment was equipped with an electric bell for cued fear conditioning, and a steel wire floor connected to an electric supply for contextual fear conditioning. Each chamber had dimensions of 73cm in length and width,

with individual compartments measuring 36.8cm each. Both compartments were fitted with windows of 10cm in diameter. Before the actual experiment, a 30-minute acclimatization period was provided to the rats in the testing room.

For the contextual fear conditioning test, the rats were positioned within the testing chamber and exposed to a 2-second electric foot shock of 0.14mA. Following the shock, the rats were swiftly transferred from the shock compartment to the alternate compartment, and their freezing behavior was monitored for a duration of 5 minutes. During the cued fear conditioning test, the rats were placed in the same testing compartment, where the electric bell was activated and the electric current through the steel wires was deactivated. This elicited a 30-second white noise tone at a volume of 90dB. Subsequently, the rats were promptly relocated from the bell-equipped compartment to the other compartment, and their freezing behavior was observed for a period of 5 minutes.

In both the cued and contextual fear conditioning tests, the rats were placed in the conditioning test box and given a 2-minute acclimatization period. Subsequently, each rat was subjected to a 30-second noise tone at 90dB, followed by a 2-second electric foot shock of 0.14mA. There was a 2-minute interval between the tone and shock. After the last shock exposure, the rats remained in the chamber for an additional 20 seconds before being taken out. The freezing behavior of the rats was monitored for a period of 5 minutes.

2.14.1. Evaluation of shock threshold for various responses.

The assessment of the threshold for flinching, jumping, and vocalization in response to an electric shock was carried out using a passive avoidance test box. This test box was equipped with adjustable current levels in milliamperes (mA). The measurements were performed on the control group, the IMI group, and the pre-treated groups that received a combination of F.A and IMI.

2.14.2. Passive avoidance test

The passive avoidance (PA) test was utilized to investigate non-spatial memory. On day first of experiment, the rats were placed in an illuminated chamber, and the time for entrance into dark chamber was considered as the latency. Following this, an electric shock of 0.2mA intensity was administered for a duration of 2 sec, after which the rat was removed from the chambers.

On the second day, the rats were once again placed in the illuminated compartment, and the latency for them to enter the dark compartment was measured. The maximum time allowed for this measurement was set at 5 min.

2.15. Hot plate test

The hot plate test is a well-established (Eddy and Leimbach, 1953) and commonly employed experimental method for evaluating nociception in rodents (Le Bars et al., 2001). It involved placing the animal on an enclosed hot plate and calculating the time the animal takes to either lick a hind paw or jump out (Bannon and Malmberg, 2007).

2.15.1. Procedure

The experiment started with the activation of the hot plate, allowing it to reach a temperature of 60°C. A cylindrical jar was positioned atop the hot plate, and prior to use, the cylinder was sanitized using a 70% ethanol disinfectant. A waiting period of 2 min was observed to ensure the restoration of the desired temperature before the commencement of testing.

Subsequently, the first rat was gently positioned onto the hot plate, and the stopwatch was quickly switched on to record parameters including hind paw withdrawal latency, hind paw lick duration, walk sniff latency, walk sniff duration, freezing latency, and freezing duration. The stopwatch ceased as soon as the rat exhibited any reaction to the heat

stimulus, such as paw licking, among others. In instances where rats did not display any heat-induced response within 30 sec, a latency value was assigned by default.

All forms of aversive behavior were precisely observed, documented, and the latencies or durations for all groups were carefully quantified. Following the test completion, the rats were returned to their respective housing rooms, and the apparatus underwent a thorough cleaning process.

2.16. Novel object recognition test

The novel object recognition test is a commonly used behavioral test for the assessment of various aspects of learning and memory in rodents. Because rats have an innate preference for new objects as compared to familiar ones, if the rats recognize the familiar object, it will spend more time with novel object. The duration of retention intervals can be altered, allowing for the assessment of short-term memory with shortened intervals or the exploration of long-term memory with prolonged intervals(Lueptow, 2017, Ennaceur and Delacour, 1988).

2.16.1. Procedure

An open-field experimental setup was employed. It comprises of a wooden box with dimensions of 40 x 95 x 95 cm. Objects of comparable dimensions, but varying shapes were utilized as stimuli. The rat underwent a habituation phase over two consecutive days, during which they were placed within the box for 5 minutes and subsequently returned to their respective cages. On the third day, a pair of identical objects (A1 and A2) were introduced to the box, allowing the rat to interact with them for a 5 min period. After 24h of training session with identical objects (A1 and A2), one of the objects was exchanged with a new object (B). The rats were placed into wooden box for 5 min, providing them an opportunity to interact with the objects. To remove any sign

of previous exposure 70% ethanol was used after each trial. The spatial configuration of the objects remained consistent throughout both the training and assessment phases.

2.17. Hot Water Immersion Tail-Flick Test

The tail-flick test is an extensively employed method for quantifying the level of nociception perceived by laboratory animals (D'Amour and Smith, 1941). The hot water tail-flick immersion test stands as one of the alternative methodologies. Due to its simplicity, cost-effectiveness, and consistent replicability, the tail-flick test finds extensive application in research focused on nociception, analgesia (antinociception), and the exploration of opioid drug tolerance.

2.17.1. Procedure

To familiarize the rats with the laboratory setting and alleviate stress, the rats were permitted to move freely within a towel with their tails extended outside. Over the course of two days prior to the actual experiments, the rats were gently held within the towels for a few min each day. During this preliminary stage, the rats' tails were not exposed to hot water.

At the commencement of the tail-flick tests, the water temperature was carefully maintained at 52 ± 0.5°C throughout the experiment. 3 cm proximal to the tail tip of each rat, a mark was positioned, corresponding to the exact length of the tail that would be dipped into the hot water. The tail immersion was carried out in one rapid motion. The latency of the response to the heat stimulus, indicated by a quick tail flexion known as the tail-flick reflex, was measured. The time interval between the immersion of the tail and the occurrence of the tail-flick reflex was recorded using a stopwatch with a precision of 0.01 seconds.

2.18. Biochemical analysis of tissues

Biochemical analysis of the brain included the estimation of reactive oxygen species (ROS) activity, as well as the indirect assessment of lipid peroxidation through thiobarbituric-acid-reactive substances (TBARS). Antioxidant enzymes, including the superoxide dismutase (SOD), peroxide dismutase (POD), catalase (CAT), and non-enzymatic reduced Glutathione (GSH) levels.

2.19. Preparation of Extract buffer (Lysing buffer)

To formulate the extraction buffer, a mixture was prepared by adding 5.95g of HEPES, 0.1g of Sodium azide, 0.5g of SDS, and 4.38g of NaCl to 495ml of distilled water. Subsequently, 5ml of Triton-X-100 was introduced to the mixture, effectively attaining the desired volume of 500ml for the extract buffer.

2.20. Method of preparation of tissue homogenate

The brain tissue, weighing 100mg, was carefully minced on frosted petri plates. Utilizing a handheld manual homogenizer (GPE Limited, UK), the tissue was homogenized. A total of 1ml of extract buffer (Lysis buffer pH. 7.0), supplemented with 0.1mg of PMSF, was employed for homogenization. Following homogenization, the resultant mixture underwent centrifugation at 93,000 g (Eppendorf 5415D) for a duration of 10 minutes. This procedure effectively separated the supernatant, which was subsequently aspirated into properly labeled and autoclaved 1.5ml Eppendorf tubes. The collected supernatant was then appropriately stored at -20°C to facilitate subsequent biochemical investigations.

2.21. Oxidative profile parameters

Oxidative parameters include the following assays:

2.21.1. Estimation of reactive oxygen species (ROS)

The determination of ROS concentration in tissue homogenates the protocol by Hayashi et al. (2007) was followed. Initially, 4.1g of sodium acetate was dissolved in 500ml of distilled water, yielding a 0.1M sodium acetate buffer with a pH of 4.8. Subsequently, 10mg of DEPPD was dissolved in 100ml of this buffer to create reagent 1. Preparation of reagent 2, a FeSO4 stock solution was generated by dissolving 50mg of ferrous sulphate in 10ml of sodium acetate buffer. From this stock solution, 62.5µl of $FeSO_4$ was extracted and dissolved in 125ml of sodium acetate buffer. The two reagents were then combined in a 1:25 ratio and placed in darkness for an incubation period of approximately 2 minutes. For the subsequent procedure, 1200µl of sodium acetate buffer, 1680µl of the reagent mixture, and 60µl of the homogenate sample were carefully measured and combined within a 3ml cuvette. Absorbance of sample was measured at 560nm, with three readings taken for each sample at intervals of 30 sec and then averaged.

2.21.2. Analysis of lipid peroxidation assay (TBARS)

The quantification of TBARS within the homogenate was performed according to the procedure outlined by Iqbal et al. (1996). This method involves its reaction with thiobarbituric acid and serves as an indirect determination of oxidative stress resulting from lipid peroxidation.

In a 15ml falcon tube, a reaction mixture was generated 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. Following vortex, the mixture was incubated at 37°C for a duration of 15 min. Subsequently, the mixture was subjected to centrifugation at 3000rpm for 10 minutes. The resulting supernatants were carefully transferred into cuvettes, and absorbance measurements were taken at 532nm.For each sample, a total of three readings were collected at intervals of 30 sec. This approach allowed for the

determination of TBARS concentration and, consequently, provided insights into oxidative stress arising from lipid peroxidation.

2.22. Antioxidant enzymes

Enzymatic antioxidants are Catalase (CAT), Superoxide dismutase (SOD), Peroxide dismutase (POD), and Reduced glutathione (GSH).

2.22.1. Catalase Assay (CAT)

The Catalase (CAT) activity was determined in the tissue in accordance with the protocol established by Chance and Maehly (1995). Reagent mixture was prepared by combining 1000µl of H_2O_2 (5.9 mM), 1.9ml of potassium phosphate buffer (50 mM) with a pH of 7.00, and 0.1ml of the homogenate sample within a 3ml cuvette. For each sample, three readings were recorded at 240nm and subsequently averaged.

2.22.2. Superoxide dismutase Assay (SOD)

Superoxide Dismutase (SOD) activity was determined according to the procedure outlined by Kakar et al. (1984). 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). To achieve a final volume of 90ml, 50 mM phosphate buffer saline (PBS) with a pH of 7.8 was added.

For analysis, 1ml of the prepared mixture was transferred to a cuvette. These cuvettes were then exposed to illumination under a fluorescent lamp for a duration of 7 min, followed by incubation at 37°C for 5 min. Subsequently, 10µl of the sample was introduced into each cuvette from the mixture to initiate the process. The contents were then incubated at 40°C for 8 min. For each sample, a total of three readings were recorded at 560nm at intervals of 1 min and ten averaged.

2.22.3. Peroxide dismutase assay (POD)

The quantification of Peroxidase (POD) concentration in the tissue homogenate was conducted in accordance with the method established by Chance and Maehly (1995). Prepare reaction mixture, 2.5ml of 50 mM phosphate buffer, 0.1 ml of enzyme extract, and 0.1ml of 20 mM guaicol were combined. The contents were vigorously mixed to achieve a uniform solution. To this reaction mixture, 0.3ml of 40 mM H2O2 was introduced. Following the addition of H2O2, the absorbance change of the reaction mixture was monitored at 470nm after one minute. Peroxidase activity was expressed in units, where one unit of peroxidase activity corresponds to an absorbance change of 0.01 units per min.

2.22.4. Determination of reduced glutathione (GSH)

Reduced glutathione was determined according to Jollow et al. (1974). The reaction mixture was prepared by combining 0.1ml of disodium phosphate buffer (0.4M), 0.1ml of tissue homogenate, and 0.5ml of DTNB (Ellman's reagent). The preparation of DTNB involved 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 this yellow color was measured at 412nm.

2.23. Statistical Analysis

The obtained results were presented as mean ±SEM. A one-way analysis of variance (ANOVA) was conducted, followed by Tukey's post hoc test, utilizing GraphPad Prism Software Inc. (Version 7, California, USA). The significance level was set at $p < 0.001$.

RESULTS

3.1. Histological evaluation

The outcomes of the Passive Avoidance Test and the Novel Object Recognition Test have revealed that subacute exposure to IMI impairs cognitive capabilities in female Sprague-Dawley rats. Given that the learning processes in both tests are associated with the integrity of the hippocampus, our investigation was directed towards assessing the histopathological changes in the hippocampus prompted by IMI treatment. The hippocampus can be categorized into distinct regions based on the morphology of pyramidal neurons, which encompass CA1, CA2, CA3, and CA4. Our study employed staining techniques to evaluate changes within the hippocampus.

Our results substantiate that subacute IMI exposure triggers alterations in multiple hippocampal regions. It is well-established that the function of pyramidal cells in the CA1 region is linked to long-term memory, and the degeneration of CA1 pyramidal cells can precipitate substantial memory impairment. Consequently, our focus was on analyzing histopathological changes and ultrastructural variations in response to IMI exposure, with a specific emphasis on the CA1 and CA3 regions of the hippocampus, assessed through Hematoxylin and Eosin (H&E) staining.

H&E staining of the control group illustrated the orderly arrangement of pyramidal cells with clear, dark, blue-stained intact nuclei. However, staining of the hippocampus from rats exposed to subacute IMI treatment revealed an increased presence of degenerative neurons, with pyramidal cells exhibiting granular vacuolar alterations and nuclear pyknosis. Degeneration was also evident in cell junctions and basement membranes. In contrast, the pyramidal cells in the group pretreated with F.A exhibited improved morphology compared to those subjected only to IMI treatment. These cells displayed intact cell junctions and a compact cellular structure, with only a limited number of cells

displaying degenerative changes. These observations distinctly underscore the mitigating effects of F.A against IMI-induced neurotoxicity. **Fig.3.1**

3.2. Body and organ mass

After 21 days of treatment weight gain is observed in all groups. However, wrt weight of the brain a non-significant change is observed. There was no mortality throughout the experiment. **Table: 3.1.**

3.3. Evaluation of motor coordination using horizontal bar test.

Motor coordination was evaluated, and results were compared among experimental groups. **Table: 3.2**. A significant difference was observed in their motor coordination. The forelimb and hindlimb grip strength (sec) of G2 (IMI) decreased significantly as compared to G1 (control) ($p < 0.001$). In G4 (IMI+F.A), impairments in forelimb and hindlimb grip strength were significantly counteracted by folic acid ($p<0.001$) as compared to G2 (IMI) (**Fig.3.2).**

A significant increase was observed in time taken to orient 90°(sec) in G2 (IMI) as compared to G1 (control) ($p=0.0006$). While there was non-significant change in G3 (F.A) as compared to G1 (control). Compared to G3 (F.A) there occurred significant decrease in G2 (IMI) ($p<0.001$). In G4(IMI+F.A), folic acid pretreatment attenuated the impairments in time taking to orient 90° on horizontal bar as compared to G2(IMI alone) with ($p<0.001$). **Fig 3.3**

As compared to G1 (control), there was a significant decrease in distance covered (cm) by rats on horizontal bar in G2 (IMI) ($p<0.001$). As compared to G1 (control), a nonsignificant change occurred in G3 (F.A). In G3 (F.A), a significant change ($p=0.0001$) was found in the distance covered as compared to G2 (IMI). In G4 (IMI+F.A), pretreatment with F.A improved significantly ($p=0.001$) the distance covered on horizontal bar as compared to G2(IMI). **Fig 3.4**

3.4. IMI induced deficit of contextual and cued memory reversed by F.A pretreatment.

Rats were trained for behavior testing. Two types of stimuli were administered to rats, mild foot shock and an associated auditory cue. After the training session, it was found that imidacloprid treated rats exhibited significantly less freezing ($p<0.001$) as compared to control and pretreated folic acid and imidacloprid exhibit non-significant change as compared to control. These results suggest that pretreated folic acid counteracts the loss of fear conditioning caused by acute exposure of imidacloprid. **Table:3.3.** Initially % freezing in cued fear conditioning and contextual fear conditioning were evaluated and then % freezing in combined cued and contextual fear conditioning was also evaluated. Similar trends in % freezing was observed as described previously. **Fig 3.5-3.7**

3.5. IMI induced impaired passive avoidance improved by pretreatment of folic acid.

In the passive avoidance test, the rats learned to reduce their natural tendency to avoid from light compartment and step into dark compartment of training chamber. The rats were trained by placing them in a lighted compartment. Latency (sec) to enter the dark compartment was measured after which they received a mild electric foot shock. Next day, latency to enter the dark compartment was measured. **Table: 3.4** the results showed that despite receiving foot shock on day 1, the latency to enter the dark compartment was significantly reduced ($p <0.001$) in G2 (IMI) as compared to G1 (control) on day 2. In G4 (IMI+F.A), the pretreatment with folic acid significantly increased ($p <0.001$) the latency to enter the dark chamber after prior electric foot shock on day 1. These findings suggest that the loss of sensory reception in sub-acute imidacloprid exposure and their reversal by pretreatment of folic acid. **Fig 3.8**

3.6. Quantification of shock threshold for flinching, jumping and vocalization.

Based on results presented in the **table: 3.5**, a significant increase in shock threshold (milliampere) for flinching, jumping, and vocalization was observed in imidacloprid treated rats ($p<0.001$) as compared to control. This indicates that the loss of general sensory perception. Pretreated folic acid G4 (IMI+F.A) rats indicate significant decrease ($p<0.001$) as compared to G1 (control) and G2 (IMI) alone. This shows that pretreatment with folic acid attenuates the impairments in threshold caused by imidacloprid exposure in female rats.

3.7. Nociception evaluation using hot plate test.

The imidacloprid treatment G2 (IMI) led to a significant decrease in latency and duration of following parameters on hot plate test i.e. hind paw licking($p<0.001$), walk sniffing ($p<0.001$) and freezing ($p<0.001$). **Table: 3.6** However, pretreatment of folic acid in G4(IMI+F.A), caused a significant increase ($p<0.05$) in above mentioned parameters of the hot plate test. The results indicated that pretreatment of folic acid attenuates the impairments in nociception of female rats induced by subacute imidacloprid exposure. **Fig 3.9-3.14**

3.8. Nociception evaluation using hot water immersion tail flick test.

The imidacloprid treatment G2(IMI) led to a significant decrease ($p<0.01$) in the latency of tail flick in hot water immersion tail flick test as compared to G1(control). **Table:3.7** There was a highly significant ($p<0.001$) increase in latency of tail flick response in folic acid alone G3(F.A) treated rats as compared to imidacloprid alone G2(IMI) treated rats. While pretreatment with folic acid resulted an increase ($p<0.001$) in the latency of tail flick response in G4(IMI+F.A). This indicates that F.A has analgesic effect against IMI administration in female SD rats **(Fig 3.15).**

3.9. Memory assessment using NOR test.

Novel object recognition test was used to assess memory impairments caused by subacute imidacloprid exposure and its reversal by pretreatment of folic acid in female SD rats. A slightly significant change ($p<0.005$) was observed in time spent with novel object (B) as compared to familiar object (A) in G1 (control). There was a non-significant change in time spent with novel object (B) as compared to familiar object (A) in imidacloprid administered rats G2 (IMI). This indicates that subacute imidacloprid exposure causes memory impairment in female rats. While the pretreatment of folic acid G4 (IMI+F.A) attenuates the memory impairments ($p<0.05$) caused by imidacloprid exposure. **Table: 3.8**

3.10. Hematological evaluation

Hematological results show that imidacloprid treated rats G2 (IMI) had a significant decrease ($p<0.01$) in WBCs count, and platelets and nonsignificant decrease in RBCs count as compared to G1(control). While pretreatment with folic acid G4 (IMI+F.A) showed improvement ($p<0.05$) in their counts. There is a significant change($p<0.001$) in MCH and MCHC in imidacloprid treated rats G2(IMI) as compared to G1(control). While in G4 (IMI+F.A), pretreatment of folic acid brought the levels near to G1 (control). **Table: 3.9**

3.11. Oxidative stress evaluation

The biochemical analysis showed significant increase ($p<0.01$) in oxidative stress markers (ROS and TBARS) and consequent decrease in antioxidant enzymes (SOD, POD, CAT and GSH) in IMI treated rats as compared to control. While pretreatment with folic acid brought their levels near to control.

Table:3.1 Effect on body and brain weights in female rats exposed to IMI, F.A and their combination.

Groups	Initial body weight(g)	Final body weight(g)	Brain weight(g)
G1(Control)	161.50±15.42	163.00±15.67	29.63±0.63
G2(IMI)	160.33±29.62	153.33±16.28[c]	29.24+1.24
G3(F.A)	163.67±17.13	169.83±20.76	29.98±2.53
G4(IMI+F.A)	160.33±17.63	157.50±21.55[d]	29.52±0.95

Data expressed as mean±SEM. Superscript c, d indicates significant p value < 0.01 and 0.05, respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table:3.2 Effect on latency of forelimb and hindlimb grip strength, distance covered by rat and time taken to orient 90° on horizontal rod test in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Groups	Latency of forelimb and hindlimb grip strength (sec)	Distance covered by rat (cm)	Time taken to orient 90° from start (sec)
G1 (Control)	23.08±1.49	72.71±2.98	2.56±0.16
G2 (IMI)	11.5±1.176[b]	45.37±1.61[a]	4.08±0.13[b]
G3 (F.A)	27.22±0.92	64.83±2.65[b]	2.21±0.15
G4(IMI+F.A)	24.55±1.34[a]	59.33±2.97[c]	2.56±2.24[a]

Data expressed as mean±SEM. Superscript c, d indicates significant p value < 0.01 and 0.05, respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table:3.3 Effect on %freezing in contextual fear conditioning, cued fear conditioning and contextual and cued fear conditioning in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Groups	Contextual fear conditioning	Cued fear conditioning	Contextual and cued fear conditioning
G1 (Control)	56.67±1.78	47.33±1.78	60.67±1.52
G2 (IMI)	28.17±0.87[a]	37±1.78[a]	43±1.37[b]
G3 (F.A)	51.5±1.12	56.5±1.57	71.17±1.66
G4(IMI+F.A)	38.17±1.82[b]	49.17±0.95[b]	63.33±1.69[a]

Data expressed as mean±SEM. Superscripts a, b indicates significant p value < 0.0001, and 0.001, respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table:3.4 Effect on passive avoidance latency at day 1 and day 2 (sec) in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Groups	Day 1 latency (sec)	Day 2 latency (sec)
G1	8.83±0.60	175.17±5.22
G2	10.33±0.49	93.67±2.40[a]
G3	9.67±0.49	189.67±3.57
G4	11.5±0.43	168±3.98[b]

Data expressed as mean±SEM. Superscript a, b indicates significant p value < 0.0001 and 0.001, respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table:3.5 Effect on shock threshold for flinching, jumping and vocalization (milli ampere) in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Groups	Flinching	Jumping	Vocalization
G1	0.16±0.04	0.25±0.02	0.31±0.01
G2	0.33±0.02[b]	0.31±0.02[c]	0.44±0.02[d]
G3	0.12±0.01	0.24±0.02	0.21±0.03
G4	0.17±0.01[a]	0.27±0.02[b]	0.32±0.02[b]

Data expressed as mean±SEM. Superscript a, b, c, d indicates significant p value < 0.0001, 0.001, 0.01and0.05 respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table: 3.6 Effect on various parameters on hot plate test for 30 sec in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Parameters	G1(Control)	G2 (IMI)	G3 (F.A)	G4(IMI+F.A)
Hind paw lick latency(sec)	12.06±0.74	17.68±0.59[b]	14.17±0.91	15.46±0.33[c]
Hind paw lick duration(sec)	4.95±0.19	6.00±0.26[d]	4.03±0.24	4.65±0.14[c]
Walk sniff latency(sec)	0.38±0.19	2.96±0.19[a]	1.24±0.19	1.77±0.13[b]
Walk sniff duration(sec)	4.80±0.14	6.94±0.20[a]	4.45±0.15	4.72±0.09[b]
Freezing latency (sec)	0.91±0.07	1.77±0.08[a]	0.49±0.02	0.94±0.06[b]
Freezing duration (sec)	1.83±0.04	0.6±0.06[a]	2.08±0.09	1.59±0.05[b]

Data expressed as (Mean±SEM). Superscripts a, b, c, d indicates significant p value < 0.0001, 0.001, 0.01 and 0.05 respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table:3.7 Effect on tail flick latency in hot water immersion tail flick test in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Groups	Tail flick latency (sec)
G1 (Control)	19.52±0.68
G2 (IMI)	10.18±0.13[c]
G3 (F.A)	21.75±1.24[a]
G4 (IMI+F.A)	19.75±1.53[b]

Data expressed as mean±SEM. Superscripts a, b, c indicates significant p value < 0.0001, 0.001 and 0.01, respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table:3.8 Parameters evaluated in the final session of novel object recognition test in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Final session (5 min)	G1 (Control)	G2 (IMI)	G3 (F.A)	G4 (IMI+F.A)
Time spent with object A1 (sec)	3.17±0.13	4.92±0.76	4.52±0.23	4.90±0.36
Time spent with object B (sec)	4.75±0.07	4.52±0.73[a]	7.18±0.39[b]	6.68±0.31[c]
Number of lines crossed	7.2±0.58	9.16±0.79	7.9±1.06	8.16±0.79
Defecation	0.4±0.24	0.5±0.34	0.5±0.34	0.17±0.17
Urination	0.2±0.2	0.3±0.3	0.16±0.15	0.17±0.17

Data expressed as mean±SEM. Superscripts a, b, c indicates significant p value < 0.0001, 0.001 and 0.01, respectively as compared to control (one way ANOVA followed by post hoc Tukey's test).

Table:3.9 Effect on hematological parameters in female rats exposed to IMI, F.A and their combination (IMI+F.A).

Parameters	Units	G1(control)	G2(IMI)	G3(F.A)	G4(IMI+F.A)
WBCs count	10^3 /µL	10.43±1.80	5.03±1.59[b]	3.97±0.09	7.50±2.74[c]
RBCs count	10^6 /µL	7.57±0.28	7.53±0.15	6.77±0.15	6.83±0.35
Hemoglobin	g/dL	13.70±0.12	14.40±0.20[d]	13.47±0.48	12.63±1.12[c]
PCV/HCT	%	39.03±0.38	42.63±0.20[c]	38.50±1.08	35.37±2.74[d]
MCV	fL	50.40±0.52	50.77±0.13	51.67±0.71	50.13±1.14
MCH	Pg	18.60±0.46	19.10±0.21	19.37±0.20	18.33±0.95
MCHC	g/dL	36.00±0.58	37.60±0.31	37.60±0.06	36.47±0.92
Platelet count	10^3/µL	1091.33±20.90	1064.67±34.22[b]	1099.00±57.50[d]	1082.00±35.08[c]
Neutrophils	%	6.67±0.08	10.00±0.02[c]	7.11±0.31	8.45±0.15[d]
Lymphocytes	%	83.00±0.88	79.00±3.21[c]	82.67±1.20	79.67±1.36[c]
Monocytes	%	8.33±0.88	9.13±2.73	8.67±1.86	7.93±0.88
Eosinophils	%	3.00±0.58	5.67±0.67	2.67±0.05	3.83±0.02

Data are expressed as mean ± SEM. Superscripts b, c, d indicates significant p values < 0.001, 0.01 and 0.05 as compared to control (one-way ANOVA followed by Tukey post hoc test). (MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, HCT=hematocrit).

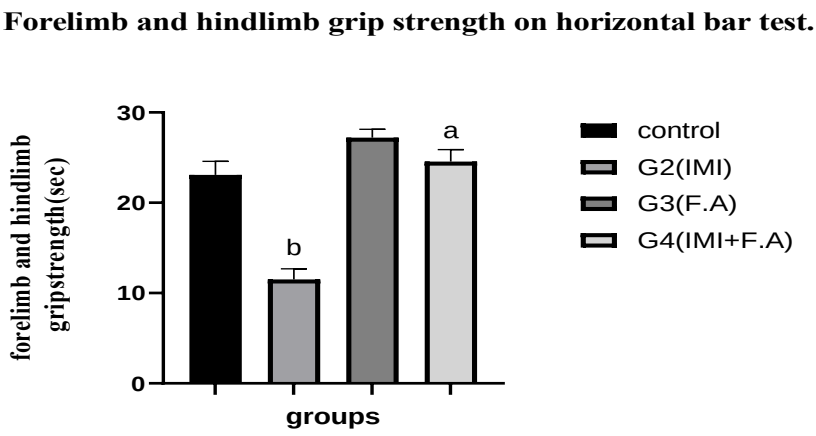

Fig 3.2 Graph represents forelimb and hindlimb grip strength on horizontal bar test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean± SEM (n = 6). Superscripts a, b indicates significant p values 0.0001 and 0.001 (~~–a~~" as compared to G2 and ~~–b~~" as compared to G1).

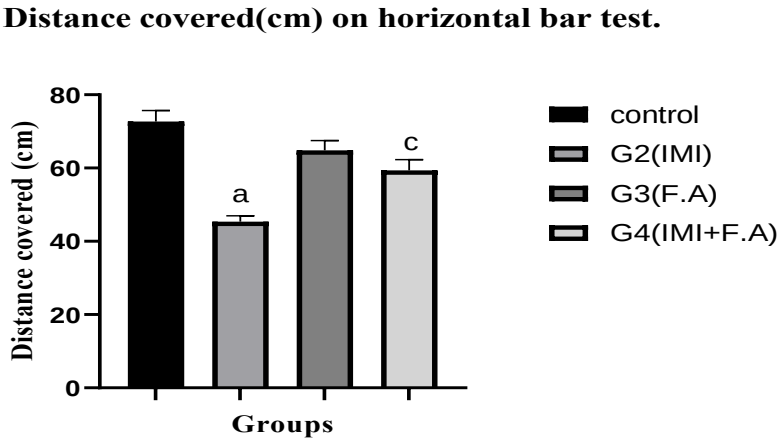

Fig 3.3 Graph represents the distance covered (cm) on horizontal bar test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n =6). Superscript a, c indicates significant p value < 0.0001 and 0.01 compared to G1 and G2 respectively.

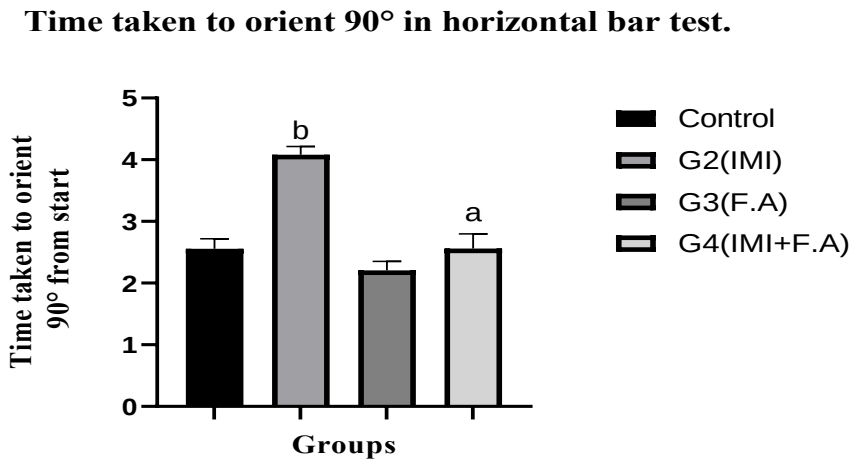

Fig 3.4 Graph represents time taken to orient 90° on horizontal bar test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001 (~~a~~" as compared to G2 and ~~b~~" as compared to G1).

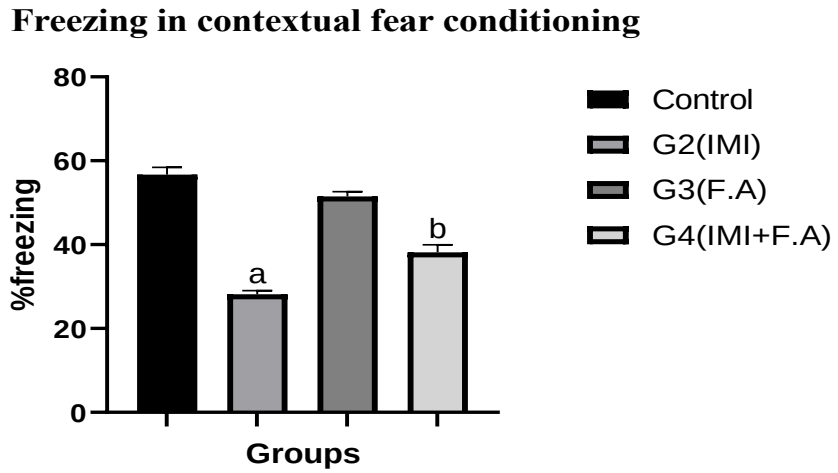

Fig 3.5 Graph represents % freezing in contextual fear conditioning test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean± SEM (n= 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001 compared to G1 and G2 respectively.

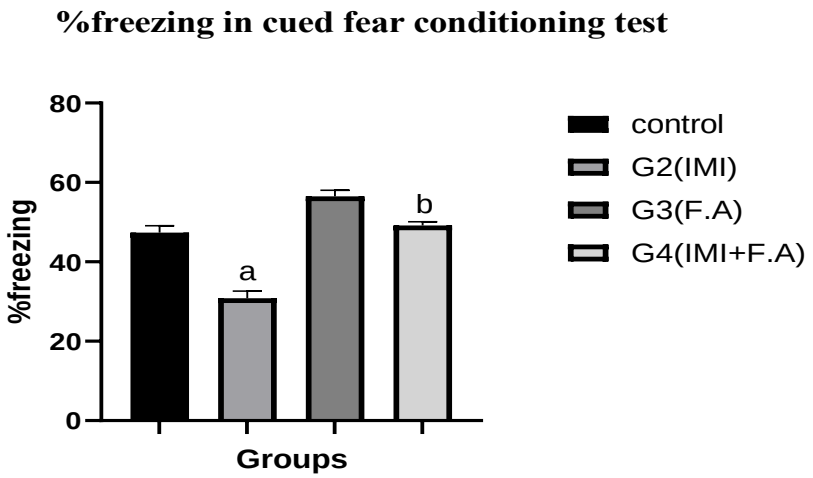

Fig 3.6 Graph represents % freezing in cued fear conditioning test in SD female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean ± SEM (n = 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001 compared to G1 and G2 respectively.

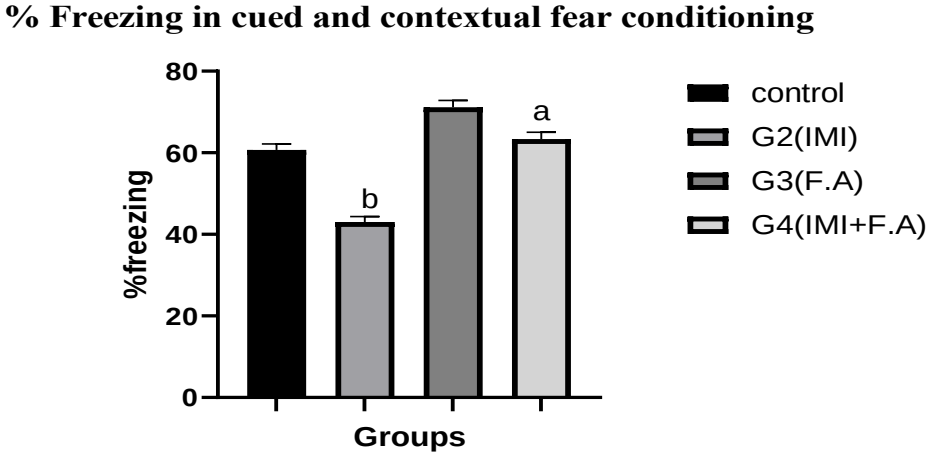

Fig 3.7 Graph represents % freezing in cued and contextual fear conditioning test in SD female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001 compared to G2 and G1 respectively.

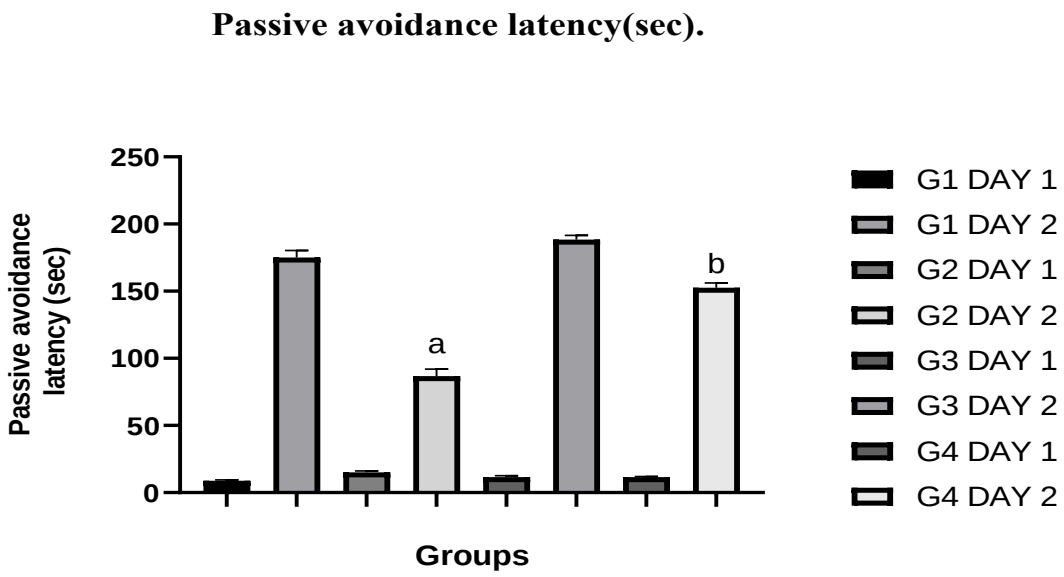

Fig 3.8 Graph passive avoidance latency(sec) after electric shock at day 2 in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001, respectively ("a" as compared to G1 and "b" as compared to G2).

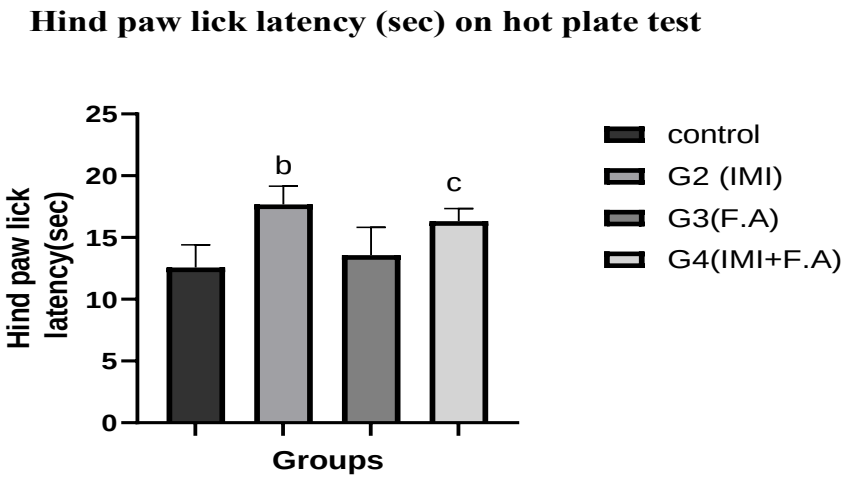

Fig 3.9 Graph represents hind paw lick latency(sec) of hot plate test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts b, c indicates significant p value < 0.001 and 0.05 as compared to G1 and G2 respectively.

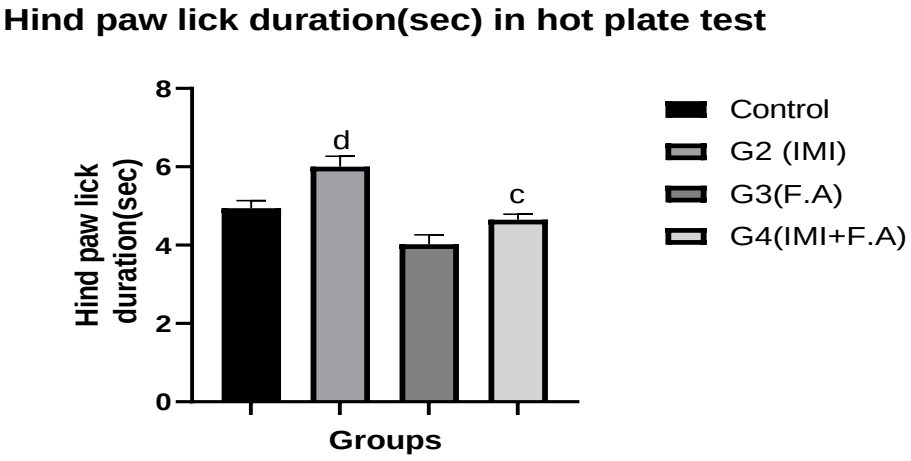

Fig 3.10 Graph represents hind paw lick duration(sec) of hot plate test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts d, c indicates significant p value < 0.01 and 0.05 compared to G1 and G2 respectively.

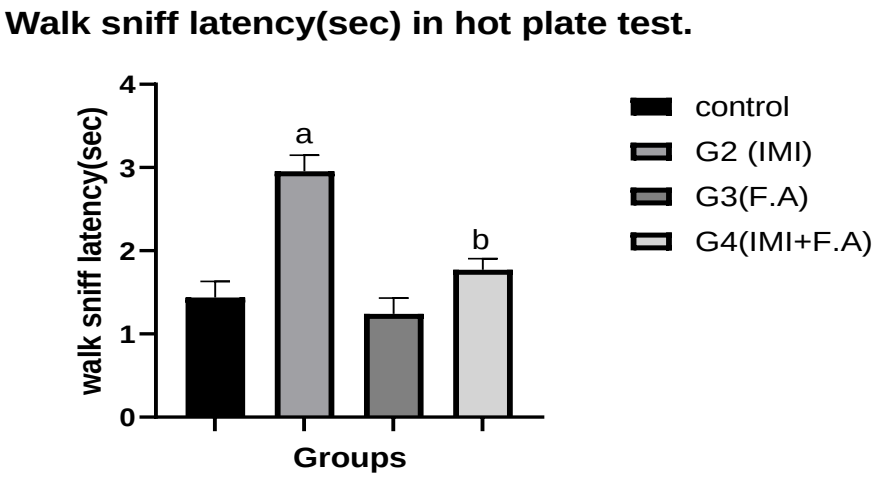

Fig 3.11 Graph represents walk sniff latency(sec) of hot plate test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a, b indicates significant pvalue < 0.0001 and 0.001 compared to G1 and G2 respectively.

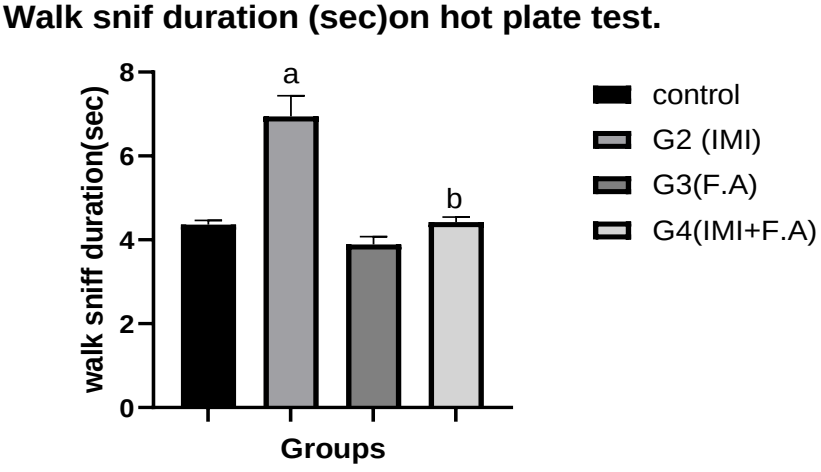

Fig 3.12 Graph represents walk sniff duration(sec) of hot plate test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001 compared to G1 and G2 respectively.

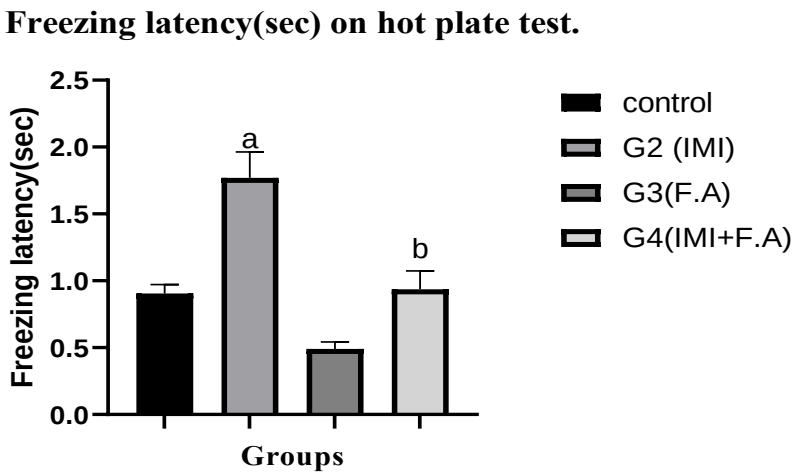

Fig 3.13 Graph represents freezing latency (sec) of hot plate test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001 compared to G1 and G2 respectively.

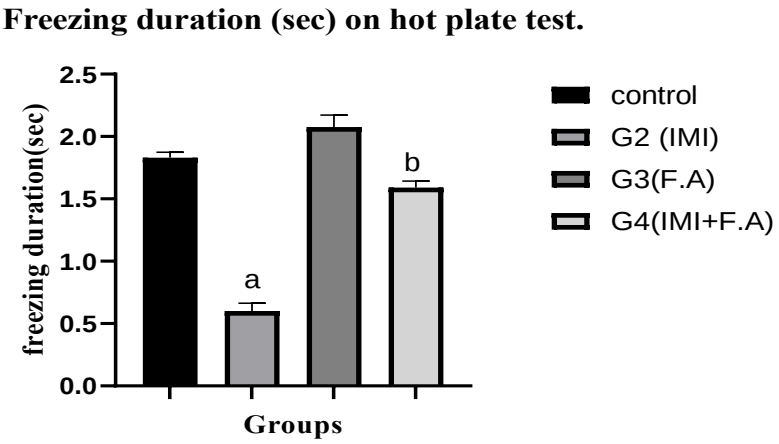

Fig 3.14 Graph represents freezing duration (sec) on hot plate test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a, b indicates significant p value < 0.0001 and 0.001 compared to G1 and G2 respectively.

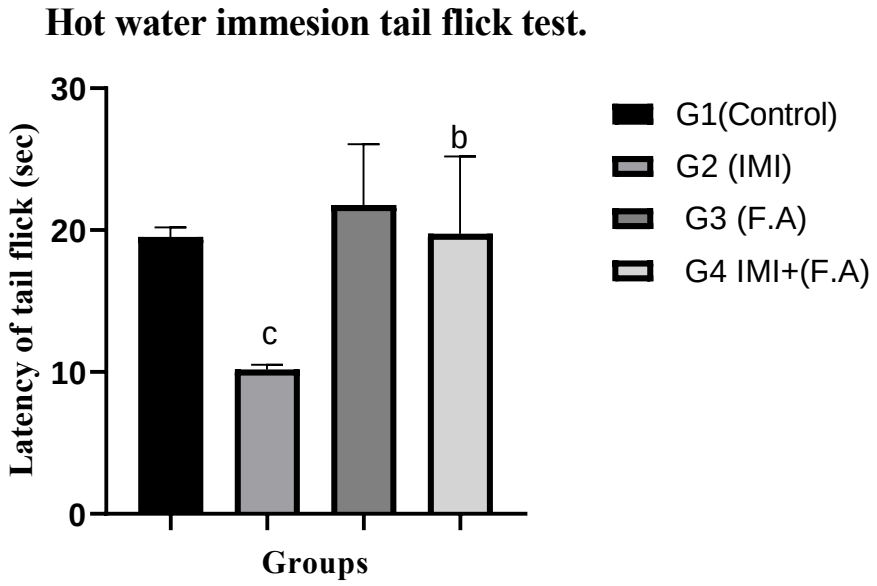

Fig 3.15 Graph represents latency of tail flick (sec) in hot water immersion tail flick test in female rats. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts c, b indicates significant p value < 0.001 and 0.01 compared with G1 and G2 respectively.

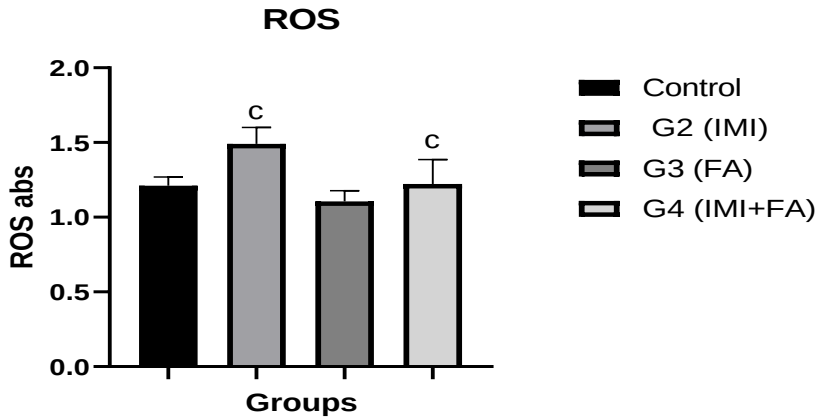

Fig. 3.16 Effect on ROS levels in brain homogenates of female rats treated with IMI, F.A and combination. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts c indicates significant p value < 0.01 compared with G1 and G2 respectively.

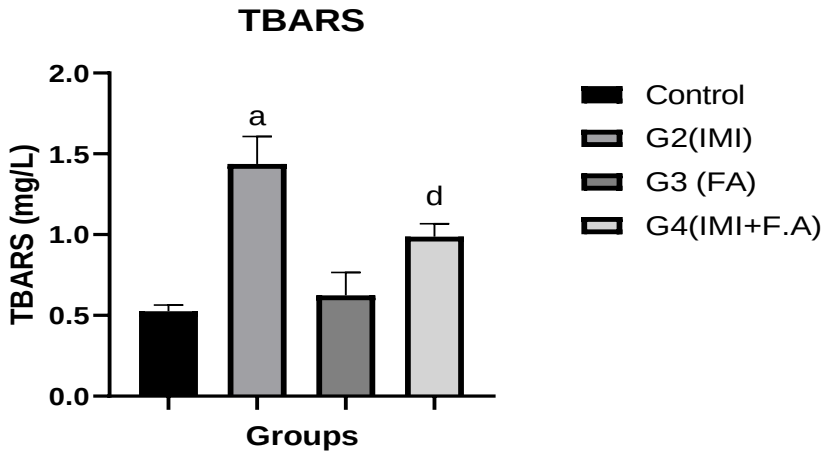

Fig. 3.17 Effect on TBARS levels in brain homogenates of female rats treated with IMI, F.A and combination. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a and d indicates significant p value < 0.0001 and 0.05 compared with G1 and G2 respectively.

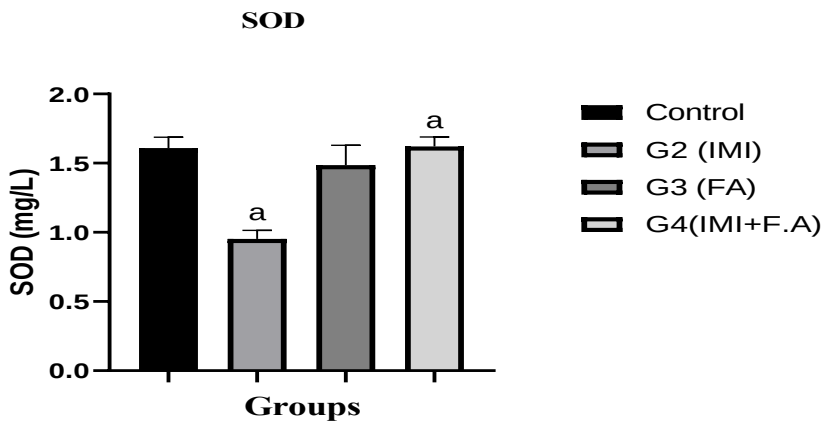

Fig. 3.18 Effect on SOD levels in brain homogenates of female rats treated with IMI, F.A and combination. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a indicates significant p value < 0.0001 compared with G1 and G2 respectively.

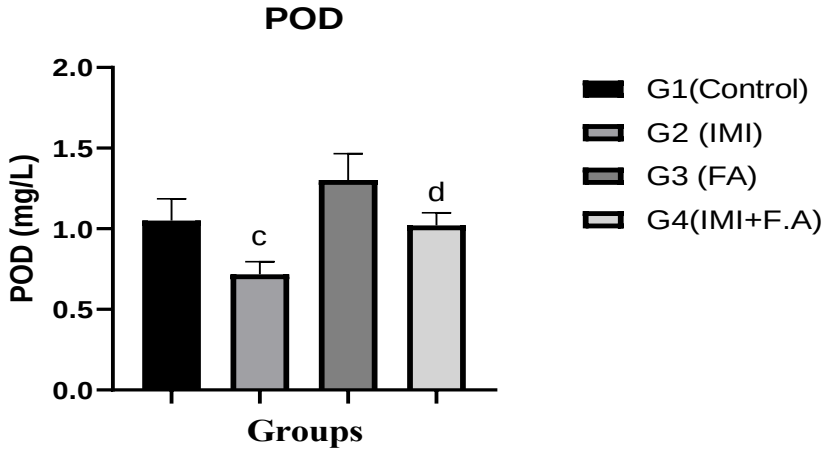

Fig. 3.19 Effect on POD levels in brain homogenates of female rats treated with IMI, F.A and combination. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts c and d indicates significant p value < 0.01 and 0.05 compared with G1 and G2 respectively.

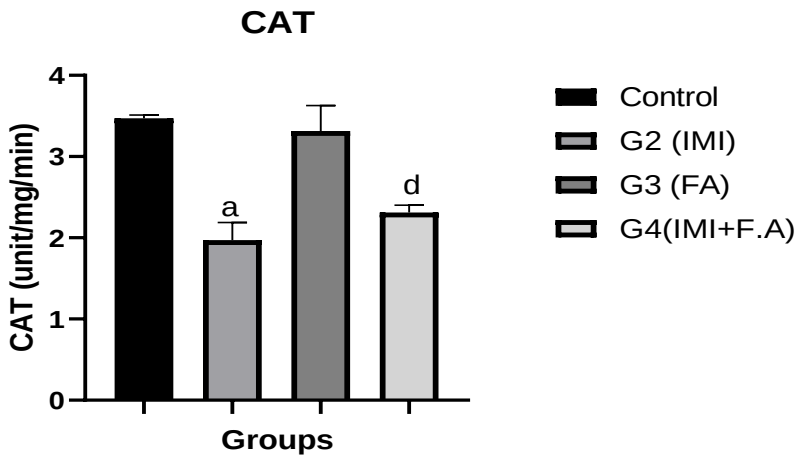

Fig. 3.20 Effect on CAT levels in brain homogenates of female rats treated with IMI, F.A and combination. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts a and d indicates significant p value < 0.0001 and 0.05 compared with G1 and G2 respectively.

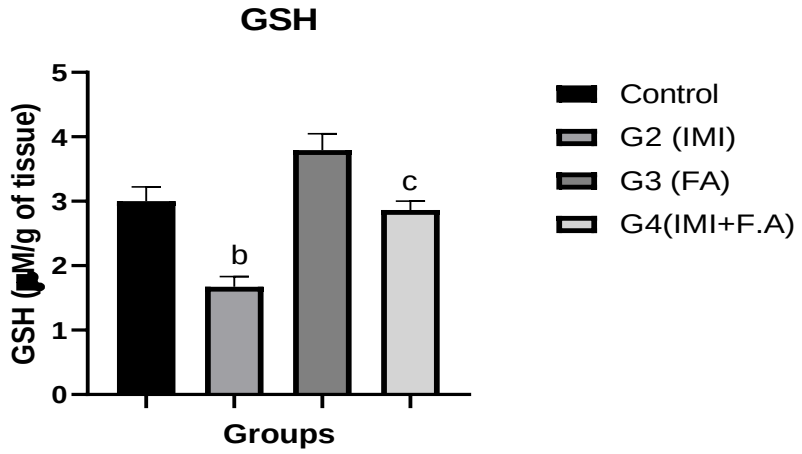

Fig. 3.21 Effect on GSH levels in brain homogenates of female rats treated with IMI, F.A and combination. Data was statistically analyzed by one way ANOVA followed by post hoc Tukey's test. Each column represents mean±SEM (n = 6). Superscripts b and c indicates significant p value < 0.001 and 0.01 compared with G1 and G2 respectively.

DISCUSSION

Neonicotinoid pesticides (NPs) constitute an emerging category of synthetic pesticides that currently command the largest global market share. Presently, NPs are distributed across over 120 countries and are recognized as highly potent insecticides, particularly effective against sucking insects and specific chewing species, both in agricultural and pet contexts(Jeschke et al., 2011).NPs exert their effects by specifically targeting neuronal nicotinic acetylcholine receptors (nAChRs). Their effectiveness stems from this mechanism of action, serving as strong activators for insect nAChRs while exhibiting minimal binding affinity to vertebrate nAChRs. This characteristic reduces possible toxicological hazards and improves protection for non-target organisms. However, even though NPs are acknowledged for their safety, their presence in the environment might increase the likelihood of exposure and subsequent toxicity. On the other side, although neonicotinoids exhibit decreased affinity for mammalian nAChRs, the prevalence, variety, and extensive dispersion of these receptors, coupled with their diverse roles, warrant an investigation into the possible impacts of these insecticides on non-target organisms (Costas-Ferreira and Faro, 2021).

Folic acid (F.A), a component of the vitamin B complex, plays a crucial role in cognition by modulating the levels of vitamins B12 and B6. Deficiencies in folate and vitamin B12 have been linked to an increased vulnerability to dementia and memory impairment(Ma et al., 2016). Studies indicates that about two-thirds of individuals afflicted with anemia or deficiencies in folate and vitamin B12 manifest cognitive deficits(Reynolds, 2002). Homocysteine, a thiol amino acid, is subject to regulatory influence by F.A, as well as vitamins B12 and B6, within the circulatory milieu (Modaghegh et al., 2016). Insufficiency of F.A precipitates enhanced homocysteine concentrations, thereby inciting DNA impairment and apoptosis within hippocampal neurons. Consequently, exogenous administration of F.A engenders amelioration in cognitive faculties by harmonizing homocysteine levels and engendering robust antioxidative mechanisms(Singh et al., 2011). Furthermore, the symbiotic association

between folate and choline is pivotal, participating in biosynthesis and stimulation of acetylcholine(Crivello et al., 2010).Diminished folate availability, either due to genetic predisposition or nutritional constraints, culminates in attenuated acetylcholine levels, thereby potentially impinging upon cognitive domains(Chan et al., 2008).The current study contributes to our comprehension of the potential neuroprotective role of F.A in mitigating behavioral impairments induced by exposure to a commercial formulation of IMI in laboratory rats.

The current investigation highlights that subacute exposure to IMI precipitated a reduction in spontaneous locomotor activity, diminished cognitive functions and elicited heightened pain sensitivity, concurrently with observable pathological alterations in the brain. In agreement with our findings there are several studies that confirm that pesticides exposure could alter locomotor activities, impair cognitive functions and consequently pathological alterations in various brain regions. Moreover, IMI can also cause oxidative stress in brain as indicated by increase in ROS and TBARS levels and decrease in GSH levels, SOD and POD also. There was significant reduction in WBCs, RBCs, Hb, HCT and platelets count in IMI treated rats while pretreatment with F.A causes reduction in their levels as compared to control. Our findings align with previous research indicating that imidacloprid exposure is associated with significant behavioral deficits in animals (Singh et al., 2011; Jeschke et al., 2011)

The neurotoxic results imparted by IMI within the cerebral domain are contingent upon their aptitude to traverse the blood-brain barrier (BBB), a safeguarding interface overseeing the transference of intrinsic and exogenous agents amidst the blood and the extracellular milieu of the brain (Von Wedel-Parlow et al., 2009). Based on the detection of pesticides and their metabolites in the brains of exposed animals, there are studies that confirm that NPs can cross BBB(Burke et al., 2018; Shamsi et al., 2021).Various studies show modifications in neuromotor activity coupled with cognitive effects, along with a reduction in hind limb grip potency, consequent to the exposure to insecticides encompassing parathion, chlorpyrifos, and tebupirimphos(Terry et al., 2003).The

subacute exposure of IMI (90 and 45 mg/kg b.w, orally) causes behavioral, biochemical alterations, neurotoxic effects, and its attenuation by curcumin (100g/kg b.w, orally) in male rats. There was significant increase in pain threshold after IMI exposure which was significantly attenuated by curcumin treatment(Lonare et al., 2014). A study by Tonietto et al. (2022) shown similar findings in which adult male administeredwith IMI suspension orally at doses of 1.5, 5, and 15 mg/kg for 45 days daily. There were no discernible changes in horizontal spontaneous locomotor activity at low doses. However, the group administered with 15 mg/kg of IMI exhibited an augmentation in the duration spent at the periphery as well as an elevated frequency of rearing and a reduction in time spent to finish the OX maze task.As reported previously, ACT has been shown to negatively affect cognitive function in exposed animals, particularly at higher dosage levels(Mondal et al., 2014).

Kara et al.(2015) studied potential toxic effects of IMI at three low doses. Results indicated that learning functions were substantially impaired at doses of 2 and 8 mg/kg in rats. However, IMI, administered at the dose of 0.5 mg/kg which is at the Maximum Residue Level (MRL) did not lead to any decline in cerebral functions, even in newborn rats. da Silva et al.(2018) has revealed the adverse impact of abamectin on spatial memory and behavior in animals following chronic exposure to a dose equivalent to 1/10 of the LD_{50} over a 90-day period. Despite being safe for vertebrates due to its specific mode of action and the presence of P-glycoproteins in the blood-brain barrier, abamectin, a biopesticide, demonstrated neurotoxicity even at low doses. In one study by Hossain et al.(2015) showed that animals were exposed to pyrethroid near the Lowest Observed Adverse Effect Level dose (3 mg/kg every 3 days for 60 days), resulting in hippocampal endoplasmic reticulum anxiety and memory deficits. In another study, rats were subjected to oral administration of deltamethrin (DM) at doses lower than 1.0 mg/kg/day from postnatal day 3 to 20, following a split-litter design. This exposure resulted in impairments in both allocentric and egocentric learning and memory(Pitzer et al., 2019).

An investigation by Katić et al. (2021) stated that the hat 28-day rat exposure to IMI resulted in detectable levels of IMI in the plasma and brain tissue. Quantifying pesticide concentrations in plasma and brain tissue could hold significance in the evaluation of neurotoxicity. This is because past research has indicated a connection between pesticide levels and the extent of toxicity and neurotoxic symptoms, with higher pesticide concentrations in the brain being linked to increased severity(Nagata et al., 1996). IMI exposure led to the occurrence of Purkinje cell necrosis, accompanied by dendritic loss and granular layer disruption in the cerebellum of female rats, as reported previously by (Bhardwaj et al., 2010). Moreover, gestational exposure resulted in the manifestation of neurobehavioral impairments and an elevated expression of glial fibrillary acidic protein (Abou Donia et al., 2008; Ndonwi et al., 2020). Several studies have indicated that exposure to organophosphates, known neurotoxic compounds, during both pre- and postnatal stages, has been linked to cognitive and neurobehavioral impairments in children (Bouchard et al., 2011; Rohlman et al., 2016). A study revealed an association between repeated exposure to pesticides at high and medium levels, along with heightened anxiety and symptoms of stress. This connection was particularly notable among individuals who stored pesticides, including pyrethroids, within their homes (Kori et al., 2018).

The obtained data of IMI indicates a significant decrease in locomotor activity of female rats after IMI exposure while pretreatment with F.A attenuated the impairments in G4(IMI+F.A) as compared to control animals. It has been reported previously that F.A reduces impairments in locomotor activities as documented by (Amirahmadi et al., 2021). Khalil et al.(2017) demonstrated that rats exposed to doses of 0.5 and 1 mg/kg of IMI for 60 days displayed reduced time spent in the center of the open field arena. This behavior was consistent with the findings in groups treated with 1.5 and 5 mg/kg of IMI, indicating the induction of anxiety-like behavior by IMI exposure(Tonietto et al., 2022). A similar anxiogenic effect was also observed in the hole-board test in rats treated with a standard IMI dose of 1 mg/kg for 30 days, as reported by (Abd-Elhakim et al., 2018). Notably, Lonare et al.(2014) documented a reduction in both horizontal and vertical

locomotor movements in rats administered higher doses (45 and 90 mg/kg) of a commercial pesticide containing IMI for 28 days, indicating reduced rearing behavior. Similarly, (Bhardwaj et al., 2010) found that female rats subjected to technical grade IMI at doses of 5, 10, and 20 mg/kg for 90 days exhibited decreased distance traveled and less time in spontaneous locomotor activity.

A study byRahman et al.(2023) revealed distinct behavioral responses in C. *gariepinus* following the consumption of a diet enriched with HTF (highly unsaturated fatty acids) and subsequent exposure to IMI. No significant changes were observed in the behavior of the HTF treated fish and the control fish. However, the IMI-exposed fish exhibited pronounced signs of distress, including heightened aggression, restlessness, impaired swimming, surface air gasping, and loss of equilibrium. Interestingly, the inclusion of HTF in the diet of IMI-exposed fish mitigated these alterations in behavior, resulting in their reduction to moderate or weak levels. This study is in support of our current findings in which IMI subacute exposure led to decrease in locomotion.

A study by Kammon et al.(2010) documented that oral administration of IMI at a dosage of 139 mg/kg induced signs of toxicity within a 15-minute timeframe. These indications encompassed lethargy, closed or drooping eyes, chickens assuming a sitting position on their hocks, breathing with an open mouth, muscular tremors, chickens lying on one side, and instances where, upon being prompted to move, they laid down again. Some of the chickens also displayed paralysis and exhibited watery diarrhea. Berheim et al.(2019) conducted a study in which the presence of IMI was detected in the bodies of all deer, encompassing both treated subjects and the control group. These findings underscore the environmental ubiquity of NPs, particularly within food sources and vegetation. Although IMI was detected in various organs of white-tailed deer, minimal or undocumented levels were noted in the brain. This outcome was attributed to the apparent inability of the pesticide to traverse the blood-brain barrier (BBB) in these animals. A study demonstrated that bats exposed to IMI exhibited disorientation and erratic flight trajectories indicating a potential disruption of their echolocation capabilities (Hsiao et

al., 2016; Wu et al., 2020). The above documented studies support the results of current study that IMI exposure can cause behavioral impairments.

The horizontal bar test is a simple test that evaluates grip strength, balance, and locomotion. Administration of IMI caused sensory-motor impairments by significantly reducing the distance covered and forelimb and hind limb grip strength while time taken to orient 90° from start increased in rats. Pre-treatment with F.A, however, was able to restore motor coordination as suggested by the increased fore limb and hind limb grip strength and distance covered on horizontal bar.

Similarly, a study by Akinrinde and Adebiyi, (2019) supports this finding in which a similar significant decrease in the meantime spent by the rats in hanging wire in the cobalt chloride group compared with the control. Rats that received oral administration of DM through gavage at different doses (1.0, 0.5, 0.25, and 0 mg/kg/day) from postnatal day 3 to 20 displayed diminished locomotor activity in the open field maze(Pitzer et al., 2019). Moreover, Nieradko-Iwanicka and Borzęcki, (2015) demonstrated a decline in locomotor activity in mice following intraperitoneal injection of DM at doses of 8.3, 20.75, and 40.5 for a duration of 28 days. Similarly, a slight decrease in locomotor activity is reported by (Khalifa et al., 2022; Akinrinde and Adebiyi, 2019) in open filed test. These varying outcomes could potentially be attributed to differences in dosages, methods of administration, and the species under investigation.

In the passive avoidance test, the outcomes indicated that despite receiving a foot shock on the first day, rats treated with IMI exhibited a significant decrease in the latency to enter the dark compartment compared to the control group. However, rats that were pretreated with folic acid demonstrated a noteworthy increase in the latency to enter the dark compartment after receiving an initial electric foot shock on the first day.Similar findings were reported by Amirahmadi et al. (2021), where they administered PTU (0.05% in drinking water) and F.A (at doses of 5, 10, and 15 mg/kg through oral gavage) to rats over a 7-week period. In this study, the latency to enter the light chamber

decreased, and the time spent in the light chamber reduced significantly in rats induced with hypothyroidism by PTU. This group also showed a significant increase in the time spent and the frequency of entry into the dark chamber at 3, 24, 48, and 72 hours after the electric shock. However, the hypothyroid rats treated with various doses of F.A demonstrated an increased latency to enter the dark chamber compared to the control group, particularly at the 3-hour mark following the electric shock.The outcomes of the passive avoidance test further revealed that the utilization of F.A as a treatment intervention extended the elapsed time prior to entering the dark compartment. Additionally, F.A administration led to a reduction in the duration of stay within the dark compartment and a decrease in the frequency of entries into the same compartment. It is worth noting that investigations concerning the impact of F.A on IMI-associated deficits in learning and memory have not been previously documented in the scientific literature. Prior research has established that the administration of vitamin B contributes to the enhancement of cognitive capabilities in individuals exhibiting low F.A levels, primarily through the reduction of homocysteine levels, as elucidated by(Shooshtari et al., 2012). Conversely, a deficiency in F.A has been associated with elevated homocysteine levels and has been correlated with cognitive impairments, as declared in studies conducted by (Dam et al., 2017). Similarly (Singh et al., 2011; Menegas et al., 2020) also reported the role of F.A in enhancement of cognitive functions in aged rats. The enhancement of cognitive functions of current study are supported by reduction in reactive oxygen species and increase in GSH, CAT, SOD and POD levels in brain.

Recognition memory, categorized as declarative memory in humans and explicit memory in animals, pertains to the capacity to discern whether various stimuli have been encountered previously. This memory type involves the ability to identify and acknowledge the familiarity of previously experienced stimuli. Our findings demonstrate that IMI treated rats spend equal time with both familiar (A) and novel object (B). This indicates that IMI exposure led to impairments in cognitive abilities as proven by our findings of NOR test. While pretreatment with F.A attenuates IMI induced memory impairments. Previous studies by Broadbent and colleagues conducted an extensive

investigation into the involvement of the hippocampus in recognition memory. Their study revealed impairment in object recognition, both 15-16 days after hippocampal removal surgery and during a postoperative test with a 3-hour interval(Broadbent et al., 2010). In a 24-hour object recognition test conducted in mice, the induction of c-fos expression, potentially indicative of hippocampal activation (alongside the prefrontal cortex and Para hippocampal regions), was observed during the formation of object recognition memory(Tanimizu et al., 2018). Notably, inhibiting proteasome activity in the CA1 region during the post-sample phase resulted in impaired long-term memory (Figueiredo et al., 2015). Similarly, intrahippocampal micro infusions of muscimol led to memory impairment (Cohen et al., 2013). Specifically, mice that received muscimol injections into the hippocampus immediately after the sampling phase exhibited compromised retrieval of object recognition memory. After 24 hours, the same mice managed to learn a task in the Morris Water Maze, suggesting that the effect of muscimol is transient (Stackman Jr et al., 2016). These studies demonstrate that any impairments in hippocampal structure affect cognitive function as in our current study.

Synaptic transmission serves as the foundation for the perception of stimuli, irrespective of their nature. Nociception, which is the perception of harmful stimuli, plays a crucial role in an organism's ability to evade hazardous surroundings. In the current research treatment with IMI leads to a decline in thermal nociception in rats as compared to control. While F.A pretreatment improved their sensory perception on hot plate test and tail flick test. A study by Liu et al. (2022) revealed that IMI led to a reduced responsiveness of fruit flies towards mechanosensitive, nociceptive, and photogenic stimuli. Notably, the chronic effects of IMI were diminished when the antioxidant osthole was administered. Additionally, the study highlighted a decrease in the expression levels of transient receptor potential cation channel subfamily M (TRPM) in IMI-treated fruit flies. The TRP channel family, found from invertebrates to humans, plays a crucial role in sensing various environmental stimuli(Fowler and Montell, 2013). TRPM falls under the category of temperature-sensitive channels and is known to play a role in thermal nociception(Turner et al., 2016). Hence, in addition to influencing neuroplasticity, the

modification of temperature-sensitive channel expression might be the basis for the IMI-induced reduction in nociception.

Hematological findings of Toghan et al. (2022) showed that ACP treated group displayed a notable decrease in both red blood cells (RBCs) and hemoglobin (Hb) levels compared to the control group. The authors additionally suggested that this decline could be linked to a potential failure in red cell production or an elevation in erythrocyte breakdown. Such factors could contribute to the lowered Hb concentration and subsequent anemia associated with the exposure to these insecticides (Yousef et al., 2003).In a recent study conducted by Rahman et al. (2023), it was observed that exposure to IMI resulted in a substantial decrease in white blood cells (WBCs), red blood cells (RBCs), hematocrit (HCT), and hemoglobin (Hb) levels in Clarias *gariepinus* when compared to the control group. Conversely, diets enriched with HTF exhibited a noteworthy increase in the count of both WBCs and RBCs, along with elevated levels of Hct and Hb concentrations in the HTF-fed fish in comparison to the control group.

Similarly (El-Bouhy et al., 2021; Abd El-hameed et al., 2021) documented similar findings. In the present study, exposure to IMI was associated with a significant decrease in key hematological markers, specifically RBCs and Hb, indicative of an anemic state. This decline may be attributed to the direct toxic effects of IMI on blood cells, which could hinder the process of erythropoiesis. Additionally, the IMI exposure resulted in a notable reduction in total WBCs, indicating a suppression of the nonspecific immune response. These reductions could potentially stem from the cytotoxic effects of IMI, possibly stemming from oxidative stress triggered by excessive reactive oxygen species generation. Interestingly, pretreatment with Folic Acid appeared to mitigate the negative effects on the hematological parameters. These findings align well with previous research conducted in this field (Singh et al., 2011; Kataria et al., 2016; Rahman et al., 2022).

Our findings suggested that there is an increase in oxidative stress as indicated by brain antioxidants levels. There is an increase in ROS and TBARS level while significant

decrease in GSH and SOD, POD levels in IMI treated rats as compared to control in brain homogenates. While pretreatment with F.A significantly reduced the ROS and TBARS and there was a significant increase in GSH, SOD, POD, and CAT levels. ROS are byproducts of regular metabolic processes, and their production is increased by accidental and work-related interactions with substances such as pesticides. ROS presents a significant way for the toxic effects of numerous pesticides (Servat-Medina et al., 2015, Satpute et al., 2017). Among all bodily organs, the brain is particularly susceptible to oxidative stress (Servat-Medina et al., 2015) due to its elevated oxygen consumption and relatively limited antioxidant defenses(Ogaly et al., 2015).The observed neurotoxicity of IMI in this study aligns with findings in literature, where deltamethrin has been shown to decrease enzymic and non-enzymic antioxidants while increasing levels of thiobarbituric acid reactive substances in brain homogenates(Mani et al., 2014).The increase in oxidative stress after IMI exposure in our study showed similar results with (Katić et al., 2021). This highlights a significant association between oxidative damage and IMI induced neurotoxicity.

In instances of acute exposure to 10 μM IMI, an increase in plasma MDA content and heightened GSH-Px activity in the brain were observed, while brain SOD and CAT activities remained unaffected(Duzguner and Erdogan, 2010).On the other hand, chronic oral administration of 1 mg/kg b.w./day of imidacloprid for 30 days led to elevated brain and plasma MDA levels, along with increased CAT activity in the brain and no variations in SOD and GSH-Px activities(Duzguner and Erdogan, 2012). The decline in brain GSH concentration noted in both investigations might indicate GSH conjugation or oxidation to glutathione di-sulfide due to the generation of oxygen free radicals and their derivatives caused by pesticide exposure.

In a study conducted by (Lonare et al., 2014), elevated oxidative stress indicators were observed in the brains of male rats exposed orally to doses of 45 and 90 mg/kg b.w. of IMI for 28 days, suggesting a plausible mechanism of neurotoxicity. Enhanced lipid peroxidation, indicated by higher brain MDA levels, was evident in all IMI-treated rats.

Moreover, brain GSH levels were notably reduced at the higher IMI dose, and CAT activity was diminished at both doses. Oral exposure of male and female mice to a dosage of 15 mg/kg/day of IMI for a duration of 21 days resulted in significant elevations in both blood and brain MDA levels. Additionally, blood GSH levels were observed to be diminished, and erythrocyte SOD activity was reduced, while brain SOD and CAT activities remained unchanged. These observations collectively signify the presence of oxidative stress induced by IMI exposure in the tested mice(Ince et al., 2013).Elevated concentrations of MDA were observed in both plasma and liver tissue within a 2-hour timeframe following the exposure of a single dose of technical grade IMI (26 mg/kg) via the intravascular route in adult female Wistar rats (Duzguner and Erdogan, 2010, Barrera et al., 2018).

In a study conducted by (Rahman et al., 2023), it was reported that the African catfish exposed to IMI exhibited the lowest levels of catalase and superoxide dismutase, along with the highest level of malondialdehyde. Notably, the combination of HTFwith IMI led to an increase in the antioxidant enzyme levels and a concurrent reduction in MDA levels compared to the group exposed to IMI alone.A study by (Ge et al., 2015) documented that IMI exposure inhibited SOD and GST activity in zebrafish, induced excessive ROS and accumulation of MDAin zebrafish. Similar results are documented by (El-Bouhy et al., 2021, Naiel et al., 2020) in various fish species after IMI exposure.

A study by (Yang et al., 2020) stated similar findings that IMI exposure in lizards caused oxidative stress in them by increasing the levels of ROS and reducing SOD, POD , GSH and CAT levels. The administration of cobalt chloride at 150 mg/kg for 7 days daily in rats showed substantial increases in the levels of nitric oxide NO, MDA, and H_2O_2 within the brain, concurrently leading to a reduction in GSH, GPx, and SOD in comparison to the control rats (Akinrinde and Adebiyi, 2019). A study by (Lahouel et al., 2016) examined how pesticide mixtures influenced the redox status within the hippocampus. Conventionally, it is understood that pesticides trigger oxidative stress in exposed organisms; however findings by (Ghasemnejad-Berenji et al., 2021) suggested

that exposure to a minimal dose of pesticide mixtures might lead to enhancement in certain oxidative stress biomarkers. Based on earlier studies, it is known that pesticides incite the generation of ROS, prompting the activation of diverse antioxidant mechanisms aimed at safeguarding crucial macromolecules like proteins, lipids, and DNA against oxidative harm (Docea et al., 2018).

Our findings indicate that subacute exposure of IMI could potentially trigger an adaptive reaction to counteract the detrimental effects of oxidative stress. The reduction in the activities of antioxidant enzymes observed in the rats treated with IMI in our study, along with the decreased activities of brain antioxidant enzymes mentioned earlier, could be attributed to the increased consumption of these enzymes in response to heightened oxidative stress. This consumption may be associated with the metabolic breakdown of ROS and TBARS. The antioxidant defense system serves as a crucial safeguard against cellular damage caused by free radicals and oxidative stress resulting from various external substances(Yang and Lee, 2015). Taking into consideration the widely recognized role of oxidative stress as a key factor in the toxicity of pesticides and its contribution to neurobehavioral impairments.

Our findings also demonstrate that F.A reduces TBARS levels while concurrently enhancing GSH and SOD levels in hippocampus. Similar results are reported by (Calderon Guzman et al., 2020). A study by (Toghan et al., 2022) revealed that total antioxidant capacity were substantiallyreduced in ACT (10mg/kg b.w, orally) treated rats. Co-administration of F.A attenuated the effects of ACT in liver and kidney. Previous research has indicated that F.A augments the concentration of GSH and concurrently leads to a noteworthy reduction in cerebral MDA levels, underscoring its role in suppressing lipid peroxidation, as elucidated by (Singh et al., 2011, Abdel-Rahman and Abdel-Baky, 2021).

The findings of (Li et al., 2021) demonstrated that exposure to 0.2% lead acetate led to an elevation in oxidative stress, which in turn triggered the overexpression of NrF2 and HO-

1 proteins. However, upon the administration of 0.4 mg/kg/d F.A, the levels of NrF2 and HO-1 protein expression decreased, approaching those of the control group. This observation indicates that F.A has the potential to mitigate lead-induced oxidative stress. Another investigation conducted by Gupta and colleagues revealed that elevated concentrations of F.A, despite being an antioxidant itself, could induce a pro-oxidative state, leading to diminished protection against oxidative stress due to the depletion of antioxidant enzyme activity (Nikolic et al., 2020).

The administration of F.A to individuals suffering with Alzheimer's disease holds the potential to enhance cognitive function and diminish inflammatory factors, as indicated by studies by (Chen et al., 2016, Calderon Guzman et al., 2020). It's important to highlight that a deficiency in Folic Acid can enhance vulnerability to a range of neurological disorders, including stroke. This susceptibility is linked to the initiation of oxidative DNA damage, coupled with structural abnormalities and an intensified cellular autophagy functionas discussed by (Zhao et al., 2016).

Furthermore, in a clinical context, the short-term administration of F.A for a duration of 7 weeks to metformin-treated type 2 diabetes patients showed considerable reduction in serum MDA, as documented by(Aghamohammadi et al., 2011). Notably, F.A, alone or in combination with vitamin B12, administered over a span of 30 days, demonstrated the capacity to prevent mitochondrial dysfunction and DNA damage triggered by short-term arsenic trioxide treatment in rat models, as demonstrated by (Majumdar et al., 2009). In the domain of stress-related responses, the application of F.A in rat models subjected to stressors yielded a reduction in depressive-like behaviors and cerebral oxidative damage, concurrently ameliorating the antioxidant imbalance within the hippocampus, as corroborated by studies conducted by (Budni et al., 2013, Réus et al., 2018, Menegas et al., 2020). A study by (Troen et al., 2008, Singh et al., 2011) reported that Inadequate levels of folate have been shown to negatively affect cognition, as evidenced by decreased performance in a sensitive assessment of spatial learning and memory.

In conclusion, our study highlights a potentially ameliorating effect of F.A supplementation on these IMI-induced behavioral impairments. Rats that received concurrent F.A supplementation alongside IMI exposure displayed improved performance in various behavioral tasks compared to those exposed solely to IMI. These findings corroborate studies that suggest F. A's neuroprotective properties, possibly attributed to its antioxidative and anti-inflammatory effects on neural tissue. The observed behavioral improvements in the F.A-treated group suggest a possible interaction between F.A and IMI's impact on neurotransmitter pathways, oxidative stress, or other mechanisms involved in behavioral regulation.

It is important to acknowledge the limitations of our study, including the use of a specific rat model and the employed dosages of IMI and F.A. Additionally, while our study suggests a positive effect of F.A supplementation, further mechanistic investigations are warranted to elucidate the exact pathways through which F.A may mitigate IMI-induced behavioral impairments. Future studies could explore different dosages and administration regimens of F.A, as well as potential interactions with other neuroprotective agents to provide a more comprehensive understanding of its therapeutic potential in pesticide-induced neurotoxicity.

The implications of this research extend to the realm of environmental and public health, where the development of interventions to mitigate neurotoxin-induced behavioral deficits assumes paramount significance. Overall, this study provides a steppingstone for further exploration in this domain, thereby advancing the scientific knowledge pertinent to neurotoxicology and neuroprotection.

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