ASSESSMENT OF IMIDAZOLE DERIVATIVE {1-(1,4,5-TRIPHENYL-1*H*-IMIDAZOLE-2-YL) NAPHTHALEN-2-OL} FOR OXIDATIVE-RELATED TOXICITY IN *DROSOPHILA MELANOGASTER*

BY

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DECLARATION

I, Elizabeth Temidayo OLUWAYEMI, an M.Sc. student in the department of Biochemistry, Landmark university, Omu-Aran, hereby declare that this thesis entitled: 'ASSESSMENT OF IMIDAZOLE DERIVATIVE {1-(1,4,5-TRIPHENYL-1*H*-IMIDAZOLE-2-YL) NAPHTHALEN-2-OL} FOR OXIDATIVE-RELATED TOXICITY IN *DROSOPHILA MELANOGASTER*' submitted by me is based on my original work. Any material obtained from other sources or work done by other people or institutions has been duly acknowledged.

Student's Full Name and Matriculation Number

Signature & Date

CERTIFICATION

This is to certify that this thesis has been read and approved as meeting the requirements of the Department of Biochemistry, Landmark University, Omu-Aran, Kwara State, Nigeria for the award of Master of Science.

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ABSTRACT

Imidazole-based compounds possess several pharmacological properties and play crucial roles in diverse biochemical processes. In addition, recent studies have shown that a new series of imidazole derivatives could be lead candidates for early drug development for parasitic diseases. Hence, this study seeks to investigate the toxicity of a new imidazole-based compound 1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol through the evaluation of selected oxidative stress and antioxidant markers.

In this study, both male and female *D. melanogaster* (3-5 days old) were fed a diet containing the imidazole derivative (20, 50, and 100 mg IMZ/kg diet) for 5 days. After cessation of imidazole treatment, half the population of the imidazole-exposed flies were further allowed to have a normal diet for an additional 5 days to see if the toxic effect would be resolved. The survival rate of flies was determined. In addition, levels of kynurenine, glutathione S-transferase, glutathione peroxidase, catalase activity, nitric oxide level, total protein level, lipid peroxidation, DNA fragmentation, and protein carbonyl were evaluated.

The imidazole derivative did not significantly affect the survival rate of flies. The survival rate of flies decreased by 5.3, 8.1 and 7.4% respectively for 20, 50 and 100 mg IMZ/kg diet relative to 2.4% in the control group. Furthermore, when compared to the control group, there was a significant ($p<0.05$) increase in reduced glutathione (GSH) levels. Also, the activity of catalase was significantly increased in flies fed with 50 mg IMZ/diet. In contrast, non-significant ($p>0.05$) increases were observed in the levels of glutathione peroxidase, glutathione transferase, total protein, nitric oxide, and lipid peroxidation for flies treated with an IMZ-supplemented diet when compared to the control. Furthermore, a significant increase was observed in the levels of protein carbonyl and DNA fragmentation of flies fed with (100 mg of IMZ/kg diet).

The findings suggest that administration of IMZ at the highest concentration (100 mg IMZ/kg diet) might have caused a mild level of oxidative-related toxicity in the flies, but this was resolved following cessation of treatment. Additional research into the safety and/or toxicity of the imidazole derivative in other animal models is recommended to advance the test compound's therapeutic prospects as an alternative anti-parasite agent.

Keywords: Antiparasitic; Imidazole-based compounds; Oxidative stress; Toxicity assessment

DEDICATION

This work is dedicated to God the Alpha and Omega and to my husband Oluwayemi Matthew, and children Esther and Nicole Oluwayemi.

ACKNOWLEDGEMENT

I give glory to God Almighty, the fountain of knowledge, for His grace, unending mercies, provision and protection upon my life, particularly in the course of my Master's programme at Landmark University, Omu-Aran, Nigeria. May his name be highly exalted. To my supervisors, Professor O.S. Adeyemi and Dr. (Mrs) O.J. Awakan, I appreciate you, Sir/Ma, for your guidance, advice, understanding, patience, encouragement, and support in the course of this research work. God bless and uphold you, Sir/Ma. I also want to appreciate my lovely husband, Oluwayemi Matthew Olanrewaju, the brain behind this program, for his love, support, encouragement, prayers, and understanding, and also for my lovely children, Esther Inioluwa and Nicole Moyinoluwa, for their support, love, prayers, and understanding. I also appreciate my parents, Mr. and Mrs. Adeyemi, for their prayers and support. To all the staff and faculty members of the Biochemistry Department at Landmark University, I appreciate you all for your encouragement and impact, and to my colleagues for their advice, love, support, and assistance rendered in the course of this programme.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Problem

Imidazole is a five-membered aromatic heterocyclic compound with variable constituents. It consists of two nitrogen atoms in which the nitrogen atoms are sp2 hybridized. The nitrogen containing heterocycles exhibit various arrays of biological activities because of the similarity they have with some synthetic and natural molecules of known biological activities (DeSimone *et al*., 2004). Imidazoles occupy a vital position in medicinal chemistry and this makes imidazole compounds of interest and of great significance, particularly in the exploration of new biologically active compounds for pharmaceutical purposes (Gaba *et al*., 2014).

Furthermore, imidazoles play a central role in the treatment of different kinds of diseases (Finar, 2009). Generally, imidazoles are colorless or pale yellowish solids. It is water soluble in other polar solvents as well. Imidazole is significantly present in some biological substances such as histamine, histidine, Vitamin B12, purines, and biotin. Imidazoles are well identified, known to possess several pharmacological properties and to play crucial roles in diverse biochemical processes (Shalini *et al*., 2010). Also, imidazoles constitute the complete or partial part of the binding sites of a vast number of metalloproteins for different transition metal ions such as Ni^{2+}, Cu^{2+}, or Zn^{2+}, and because of this, imidazoles have been explored as effective ligands to chelate these transition metals. Furthermore, imidazole's unique structural properties allow it to readily bind with a wide range of therapeutic targets, such as biological system enzymes and receptors, resulting in a wide range of pharmacological activities (Gaba *et al*., 2010; Ingle and Magar, 2011). Furthermore, imidazole constituents/derivatives are exceedingly versatile and have been used in large numbers of clinically active drugs such as antiulcer, antihistaminic, analgesic, antibacterial anti-HIV, antifungal, anticancer, antihypertensive,

antiviral, including antiparasitic, and other pharmaceutical agents with much effectiveness and huge market significance (Gaba & Mohan, 2015). Recently, a few new series of imidazole derivatives have been reported as prospective anti-parasitic agents (Adeyemi *et al*., 2020; Eseola *et al*., 2011).

One of the imidazole derivatives recently reported for prospective therapeutic potential against a variety of parasitic infections is 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2-ol (Adeyemi *et al*., 2020). Hence, this study sought to assess the toxicity profile of the imidazole derivative 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2-ol viz-a-viz oxidative stress markers, DNA fragmentation and kynurenine concentration in *Drosophila melanogaster*.

1.2 Statement of Problems

Imidazole-based compounds have been studied extensively by medicinal chemists due to their biological eminence and outstanding activity. Recently, *in vitro* and *in silico* studies have shown that the imidazole derivative1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol also has prospects for drug development against a parasitic infection known as toxoplasmosis, which is caused by the parasite *Toxoplasma gondii* and has been shown to possess selectivity against the parasite (Alkahtani *et al.*, 2012). Meanwhile, about one third of the human population is said to be infected with this disease, and it is considered one of the most successful human parasites.

In light of this, this study was carried out to further establish toxicity and safety of the promising imidazole derivative through the evaluation of the redox status and kynurenine levels in *Drosophila melanogaster*.

1.3 Justification for the Study

Imidazole compounds and their derivatives have been shown to display therapeutic potential against a range of parasitic infections, including Chagas disease and toxoplasmosis (Flores-Holguín and Glossman-Mitnik 2005; deAraújo *et al*., 2020). Hence, they may hold prospects as promising drug candidates.

Furthermore, recent *in vitro* and *in silico* studies have shown that a new imidazole derivative, 1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol, has the prospect of drug development against toxoplasmosis (Adeyemi *et al*., 2020). Altogether, the prospective properties of this imidazole derivative as an alternative anti-parasitic agent underscore the need to evaluate the toxicity profile or otherwise of the compound. The study contributes to the attainment of SDG 3 (Good health and Well-being) of the UN Sustainable Development Goals.

1.4 Aim and Objectives

1.4.1 Aim

The overall aim of this study is to assess the toxic effect of the imidazole derivative1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol) in *Drosophila melanogaster*.

1.4.2 Specific Objectives:

1.4.3 To determine whether exposure of *Drosophila melanogaster* to 1-(1,4,5-triphenyl-1*H*- imidazol-2-yl) naphthalen-2-ol will;

a) Cause oxidative stress in *D. melanogaster*.

b) Predispose to DNA fragmentation in *D. melanogaster*

c) Activate the kynurenine pathway in *D. melanogaster viz-a-viz* the measurement of kynurenine concentration.

1.5 Research Question(s)

Does exposure to the imidazole derivative1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol cause toxicity in *Drosophila melanogaster*?

1.6 Research Hypothesis

Imidazole derivative 1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol causes oxidative stress in *Drosophila melanogaster.*

1.7 Scope of Study

The present study investigated the toxicity of a new imidazole derivative 1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol in the *Drosophila melanogaster* model *viz-a-viz* the determination of oxidative stress markers, DNA fragmentation, and kynurenine concentration.

1.8 Significance of the Study

Imidazole-based compounds have been shown to possess several pharmacological properties which underscore their therapeutic prospects as drug candidates. Recent evidence demonstrated that a new imidazole derivative, 1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol, has promising prospects as an alternative anti-parasitic agent as a proof-of-concept (Adeyemi *et al*., 2020). This finding necessitates further investigation to boost the therapeutic prospects of this new imidazole compound.

Therefore, the present study is expected to provide data on the toxicity of this imidazole derivative in the *Drosophila melanogaster* model, through the determination of oxidative stress markers, DNA fragmentation, and kynurenine concentration. The study contributes to the attainment of SDG 3 (Good health and Well-being) of the UN Sustainable Development Goals.

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Imidazole

Imidazole is a polar compound having five membered rings with two nitrogen atoms existing in two tautomeric forms (Figure 2.1). It is a colourless or pale yellowish compound with a molecular formular of $C_3H_4N_2$. The imidazole ring and its derivatives have long been discovered in the 1840s, but it was first synthesized in 1858 by Heinrich Debusin, by dissolving glyoxal and formaldehyde in an ammonia solution to produce imidazole (Siwach & Verma, 2021). Also, imidazoles constitute the complete or partial part of the binding sites for different transition metal ions such as Ni^{2+}, Cu^{2}, or Zn^{2+} in a wide number of metalloproteins and have been used as effective ligands to chelate these transition metals.

According to literature, imidazole rings are part of numerous natural compounds like purines, alkaloids, histidine, nucleic acids, histamine, vitamin B_{12}, biotin, etc. and these compounds are essential to life in various ways. It also features in the chemical structure of some natural drugs and synthetic/man-made drug molecules (Figure. 2.2) such as azomycin, cimetidine, clotrimazole, miconazole, ornidazole, secnidazole, benznidazole, ketoconazole, and metronidazole (Klemaan *et al.*, 1999; Carrillo-Munoz *et al.*, 2005; Burnier and Wuerzner 2011). Imidazole rings can act as both acids and bases, i.e., they are amphoteric. As an acid, imidazole pKa is 14.5 and this makes it less acidic than imides, phenols, and carboxylic acid, but a little more acidic than alcohol. As a base, imidazole has a pKa of 7. The unique properties of imidazole have made it possible for medicinal chemists to prepare new chemotherapeutic drugs which are clinically suitable for the treatment of various types of diseases. Various techniques and methods have been established for the assemblage and modification of the imidazole ring with different functional groups. Also, the synthesis, chemical structure, and

chemical reactions of imidazole have offered several opportunities in the area of medicinal chemistry (Singh and Kapoor, 2008).

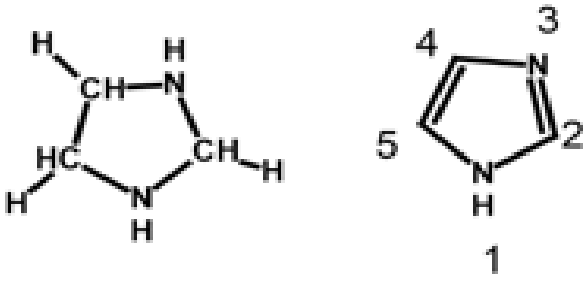
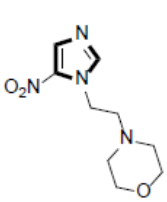
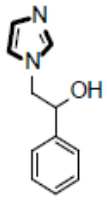


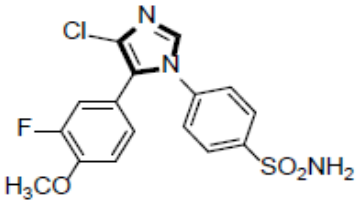
Figure 2.1: Structures of Imidazole source: (Xunan *et al*., 2020)



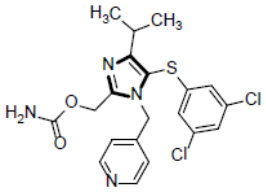
Nimorazole



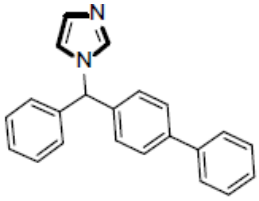
Phenethylimidazole



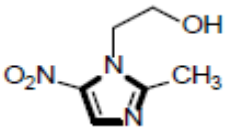
Cimicoxib



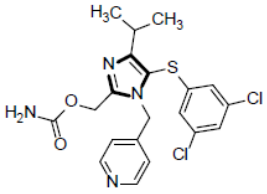
Capravirine



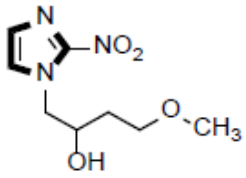
Bifonazole



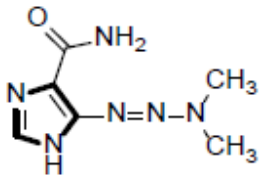
Metronidazole



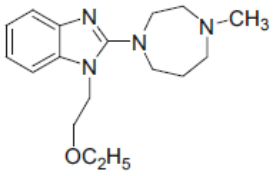
Capravirine



Misonidazole



Dacarbazine



Emedastine

Figure 2.2: Structure of some imidazole derivatives Source: (Xunan *et al*., 2020)

2.2 Chemistry of Imidazole

Imidazole compounds were first synthesized in the year 1858 (Kumar *et al*., 2017) from glyoxal and ammonia. Some of the methods that are available for synthesis of imidazole compounds are:

a) Dehydrogenation of imidazolines from alpha halo ketones
b) Radiszewski synthesis
c) Wallach synthesis

2.3 Radiszewski Synthesis of Imidazole

This involves condensation of two carbonyl compounds such as α-keto aldehyde and glyoxal with an aldehyde in the presence of ammonia to yield 2, 4, 5-triphenylimidazole (Figure 2.3).

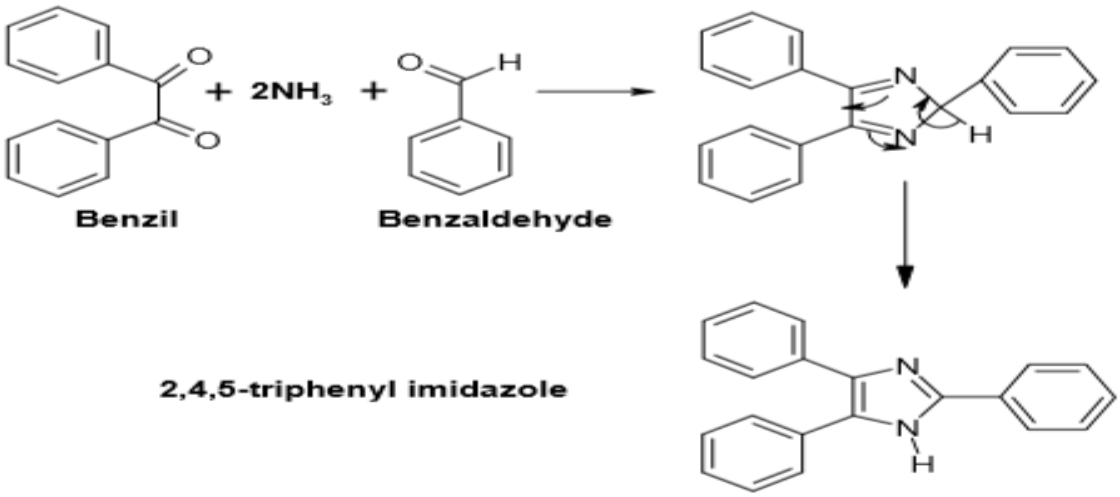
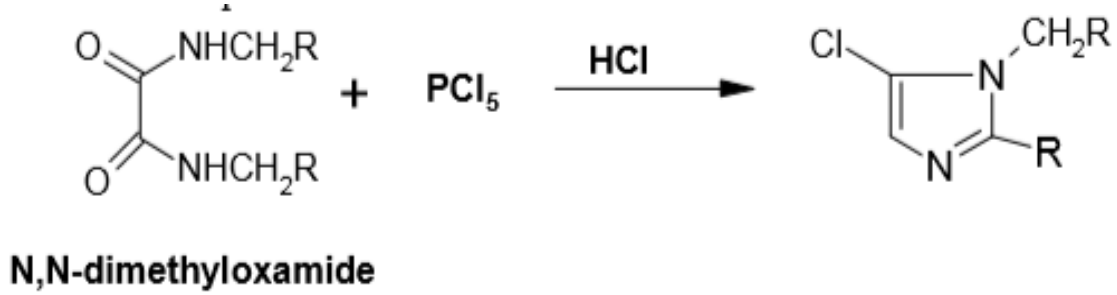


Figure 2.3: Structure of some imidazole derivatives Source: Banerjee *et al*, (2015)

2.4 Wallach Synthesis of Imidazole

This entails treating N, N′-dimethyloxamide with phosphorus pentachloride to produce a chlorine compound that, when reduced with hydroiodic acid, yields N-methyl imidazole (Figure 2.4).



N,N-dimethyloxamide

Figure 2.4: Wallach synthesis of imidazole Source: Banerjee *et al.* (2015)

2.5 Pharmacological Activities of Imidazole

Numerous reports indicate that imidazole derivatives possess various pharmacological properties, among which are:

a) Antibacterial activity (Vijesh *et al*., 2011; Lu *et al*., 2012)

b) Anticancer activity (Alkahtani *et al*., 2012; Yang *et al*., 2012)

c) Antitubercular activity (Lu *et al*., 2011)

d) Analgesic activity (Ucucu *et al*., 2001; Kankala *et al*., 2013)

e) Anti-HIV activity (Brzozowski *et al*., 2006)

f) Anti-inflammatory activity (Puratchikody *et al*., 2007, Siwach & Verma. 2021)

g) Antioxidant activity (Bhati *et al*., 2020)

h) Antiparasitic activity (de Araújo *et al*., 2019; Adeyemi *et al*., 2020)

2.5.1 Antibacterial activity

In vitro antibacterial activity of some newly synthesized imidazole derivatives was carried out by Vijesh *et al*., (2011) on some bacteria strains such as *E. coli, S. aureus, B. subtilis, S. typhimvrium, C. perfringens,* and *P.aeruginosa*. The antibacterial testing showed that some of

the compounds that were tested had good inhibitory activity against microbial strains. A new imidazole derivative (Figure 2.5) was reported to possess good activity against bacterial strains of *Pseudomonas aeruginosa* and *Clostridium perfringens* when compared with some commonly used antibacterial drugs such as streptomycin. The series of alkylimidazole was synthesized by Khabnadideh and co-workers and its antibacterial activity was investigated against *E. coli, P. aeruginosa, S. aureus, P. aeruginosa* and the imidazole derivative (Figure 2.6) is the most potent antibacterial agent.

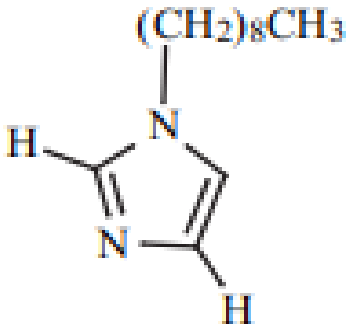


Figure 2.5: N-alkylimidazoles (Source: De luca, 2006)

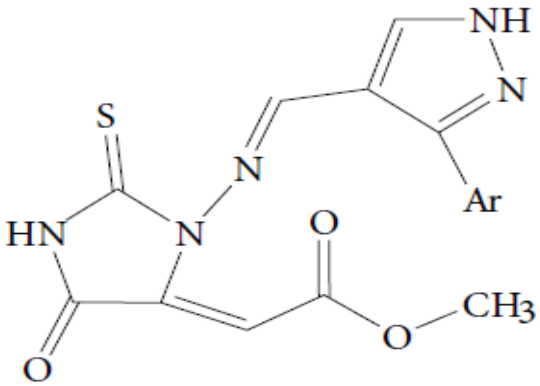


Figure 2.6: 4-(2, 6-dichlorobenzyloxy) phenyl imidazole derivatives (Source: Amita *et al.*, 2013)

2.5.2 Anti-tubercular activity

Bhati and colleagues synthesized and characterized benzimidazole 2-(pyridin-3-yl)-1H-benzimidazole (Logu *et al*., 2019). Molecular docking and *in vivo* studies were carried out on the compound in order to evaluate its anti-tubercular activities and to understand the interaction between the target protein and the imidazole ligand. The imidazole was screened against a mycobacteria tuberculosis strain (vaccine) using a known drug as a standard. The compound demonstrated a significant activity compared to the standard drug and was found to be effective against tuberculosis (Bhati *et al.,* 2020). Furthermore, a series of imidazole derivatives were evaluated for anti-tubercular activity using cytotoxicity assays. The compound (2,6-dichlorophenyl) hydrazono) ethyl)-1H-imidazole derivative appeared to be the most potent and promising antitubarcular agent (Logu *et al.,* 2019).

2.5.3 Anticancer activity

The anticancer properties of imidazole led to the development of imidazole derivatives with the expectation of making it more efficient while reducing its side effects. Evidence suggests that various types of cancer are caused by structural and functional changes in vascular endothelial growth factor, deoxyribonucleic acid, topoisomerase I and II, CYP26A1 enzyme, receptor tyrosine kinases, and other proteins (Alniss *et al*., 2012; Ketron *et al*., 2012).

The imidazole derivative 2,5,6-trihalogenobenzimidazoles was explored by Ng *et al.* (2007) explored as antagonists of the androgen receptor in prostate cancer and discovered that 2,5,6-trihalogenobenzimidazoles were the most potent androgen receptor antagonist. (Gowda *et al*., 2019) also synthesized a series of benzimidazoles that contain 5-carboxylic acids, and the compound was evaluated as an antileukemic agent due to its potential to activate apoptosis. Similarly, a sequence of benzimidazoles was reported by Hranjec *et al*. (2010) and the compound 2-substituted-N-4-(1-methyl4, 5-diphenyl-1*H*-imidazole-2-yl) phenyl acetamide

derivatives (Figure 2.7) which is linked to a quinolone was discovered to be the most effective anticancer agent on all cancer cell lines due to its capability to insert into the space between the DNA base pairs, thereby causing cleavage of DNA.

Yusuf *et al*. (2010) synthesized many imidazole (Bnz)-azole and imidazole epiperazie derivatives and tested them for anticancer activity, finding that they were very active when compared to standard anticancer drugs like cisplatin and a few others.

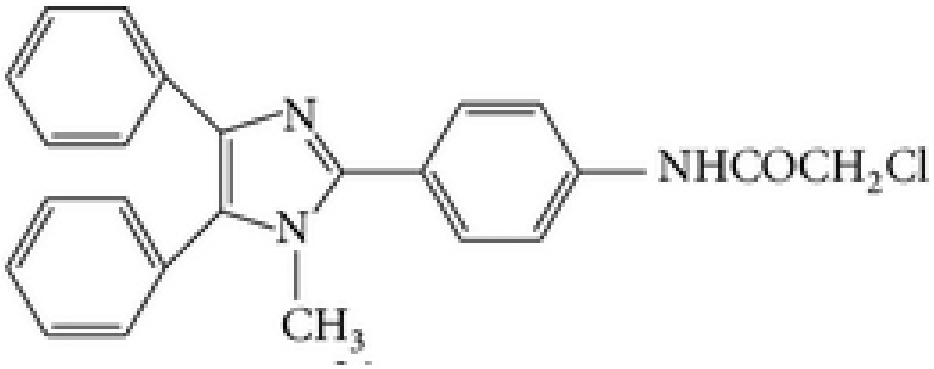


Figure 2.7: 2-substituted-N-4-(1-methyl4, 5-diphenyl-1H-imidazole-2-yl) phenyl acetamide derivatives (Source: Amita *et al*. 2013)

2.5.4 Antifungal activity

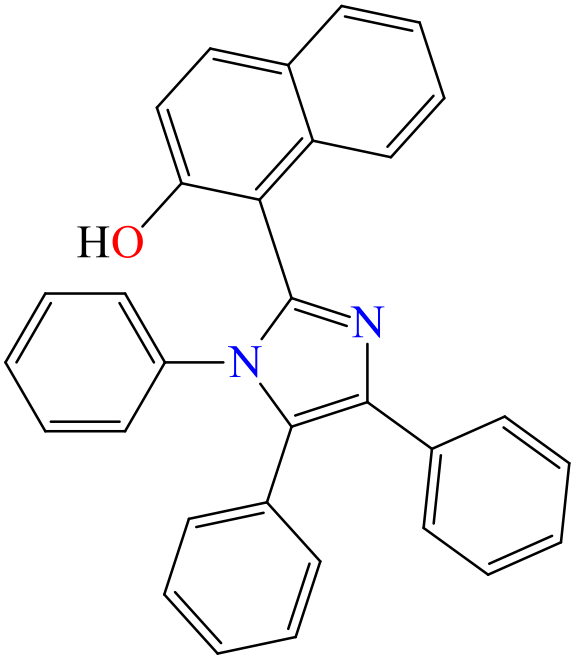
Some imidazole derivatives were synthesized by Yang and co-workers, and were screened against a few selected fungi like *Rhizoctonia solani, Dothiorella gregaria, Gibberella zeae,* and *Colletotric humgossypii*. These newly synthesized compounds were discovered to possess good activity against fungi, particularly *Rhizoctonia solani* (Amita *et al*., 2013).

2.5.5 Anti-inflammatory agent

Almasirad *et al* designed and synthesized 1, 2, 4-triazoles and methyl-imidazolyl-1,3,4-oxadiazoles. These compounds were evaluated for anti-inflammatory activity *in vivo* (Almasirad, *et al*., 2014). It was revealed that these compounds possess anti-inflammatory activity relative to the standard drug used and *in silico* studies showed that these compounds have the highest drug score and druglikeness values. Furthermore, Bukhari *et al*. (2016) investigated the anti-inflammatory property of a series of imidazole derivatives *in vitro* using Ellman's method-based assay. Some of the compounds were found to possess anti-inflammatory activity and were also potential inhibitors of IL-6 phospholipase A2, TNF-α, and lipoxygenase (Alghamdi *et al*., 2021).

2.5.6 Anti-parasitic activity

A new series of imidazole derivatives classified as phenyl-substituted I*H*-imidazoles, bis-imidazoles, and thiophene-imidazoles were reported to possess promising activity against *Toxoplasma gondii* and *Trypanosoma brucei* (Adeyemi *et al.*, 2020; 2021). In addition, a recent report by Johnson and co-workers showed that imidazole derivatives possess promising inhibitory action against SARS-CoV 2 drug targets *in silico* (Johnson *et al.*, 2021). Furthermore, the imidazole derivative (Figure 2.8) is one of the new series of imidazole derivatives shown not only to be active against *T. gondii* (Adeyemi *et al*., 2020), but also against *T. brucei* (Adeyemi *et al*., 2021).



1-(1,4,5-triphenyl-1*H*-imidazol-2-yl)naphthalen-2-ol

Figure 2.8: Molecular structure of 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2-ol, an imidazole derivative

2.6 Bioactive Properties of Imidazole Derivative1-(1, 4, 5-Triphenyl-1*H*-Imidazol-2-yl) Naphthalen-2-ol

Based on the *in vitro* studies, the imidazole derivative has been shown to possess specific anti-parasite action. Furthermore, a toxicological risk prediction ranking on protox II (a web-based system that has the ability to determine properties such as overall drug-score, toxicity prediction and risk assessment, and fragment-based drug-likeness), revealed that the imidazole-based compound belongs to toxicity class four (IV) with an oral lethal dose (LD_{50}) of 2000mg/kg in mice (Adeyemi *et al.,* 2020). On the other hand, the probability of toxicity endpoints using (Protox II) showed that the imidazole derivative is likely a non-carcinogenic, non-hepatotoxic, non-cytotoxic, non-immunotoxic, and non-mutagenic compound (Adeyemi *et al.,* 2020). The molecular descriptors of the imidazole derivative are as presented in Table 2.1, while the bioactivity and toxicological risk predictions are presented in Table 2.2.

Table 2.1: Molecular description of imidazole derivative 1-(1, 4, 5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol.

Molecular formular	**$C_{31}H_{54}N_2O$**
Molecular mass	470.42361437
Composition	C - 79%, H - 11%, O - 3.40%
Molecular weight	470.77

Source: Adeyemi *et al*. (2020)

Table 2.2: Available data on *in-silico* analyses, bioactivity and toxicological risks prediction studies on 1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol

TPSA (total polar surface area)	**35.49**
nON (number of hydrogen bond acceptors)	3
Number of violations of Lipinski rule of drug likeness	1 (violated 1 rule of Lipinski)
Number of hydrogen bond donors	2
Number of rotatable bonds	4
Mean molecular volume	501.90 (A^3)

Source: Adeyemi *et al*. (2020)

2.7 *Drosophila melanogaster*

D. melanogaster (Figure 2.9), which is also known as the fruit fly, belongs to the family *Drosophilidae* and class insecta. It is a dipteran insect and one of the most commonly used model organisms for biomedical science and has been studied extensively for decades. The first widely accepted person to use fruit flies was Charles W. Woodworth (Abolaji *et al*., 2013). They possess a complex nervous system which enables them to carry out some neuronal tasks related to those of humans. Also, some important developmental and functional features such as regulation of gene expression, cell death, and membrane trafficking in *drosophila* and in humans are the same, despite the fact that the *drosophila* brain has a different anatomical structure from that of a human (Whitworth *et al*., 2006). Also, about 75% of human related diseases have a *Drosophila* ortholog (Reza *et al*., 2015). Furthermore, humans and *Drosophila*

share related pathways for intercellular signaling and the development of tumors and metastasis (Potter *et al*., 2000). It was also documented that they are vital tools for genetic development and related fields (Sepel and Loreto, 2010). *Drosophila melanogaster* is majorly used as a model organism in biochemistry, genetics, cell biology, medicine, developmental biology, and human disease. It has also been classically used as a model of genotoxicity. Lately, *D. melanogaster* has been included as an alternative model for studying toxicology (Paula *et al*., 2013).

The D. melanogaster life cycle is rapid due to a productive mating pair that can generate numerous genetically similar progeny at room temperature (25 °C) within 10 to 12 days. Another fascinating feature of the fly is that it is a multiple model organism. Its embryo, larva, pupa and adult can therefore be used as models in diverse toxicological studies. For example, the pupa and the embryo can therefore be used as models in toxicological studies, while the larva can be used to study behavioral and physiological events (Abolaji *et al*., 2013).

In comparison to other animal models such as rats, *Drosophila melanogaster* reproduces quickly, is easy to use and maintain in the laboratory in large numbers due to their small body size and short lifespan, is less expensive, less time-consuming, and easier to culture, and human genes can be easily introduced and manipulated in order to study individual genes of interest. *D. melanogaster* has also been recoded to be in compliance with the European Centre for the Validation of Alternative Methods (ECVAM) standard: Reduction, Refinement, and Replacement (3Rs) of laboratory animal usage (Festing *et al.,* 1999) as a result of the achieved success in the use of flies in experimental studies.

Figure 2.9: *Drosophila melanogaster*. *Source* (Abolaji *et al*., 2013).

2.7.1 *Drosophila Melanogaster* Media Culture

For *D. melanogaster* to be used for toxicological studies, its culture media must be maintained due to its short life span. In order to make the monitoring, transferring, and culturing of flies easier, the use of vials and transparent bottles is preferable. Sterilization and cleaning of bottles are also of great significance in order to avoid diseases. There are several standard formulations for *Drosophila melanogaster* culture media. For example, (Hugo and Peter, 2008) maintained and reared flies on a cornmeal medium of (1%, v/w agar, 2%, v/w sucrose; 1%, v/w powdered milk; v/w nipagin, 1%, v/w brewer's yeast; 0.08%, 1%, v/w powdered milk) at constant humidity and temperature (60% relative humidity, 24 °C) under 12 hours light/ dark cycle. (Abolaji *et al*., 2013).

2.8 Toxicity

Toxicity is the series of evets initiated by exposure to different types of chemicals and its progression through metabolism, distribution and interaction with several macromolecules such as protein and DNA. It can also be defined. Toxicity is based on the concertation,

interactions, and quantity used (Tisserand and Young, 2013). Toxicity can be acute, sub-acute, chronic or sub-chronic. Toxicity can manifest systemically or locally in many ways. It may also involve the irreversible or reversible disruption of some normal biochemical processes which may lead to the impairment of cell viability. In extreme cases, the entire organ may fail, thereby leading to death (Tisserand and Young, 2013).

2.9 Oxidative Stress

Oxidative stress, which is also known as the imbalance between antioxidants and oxidants, occurs when there is an imbalance between antioxidants and oxidants. Oxidative stress has been reported to be associated with the progression of many chronic disorders and metabolic disorders such as neurodegenerative diseases, diabetes, and cardiovascular diseases, as well as alteration of several cellular structures and enzymes leading to abnormalities in gene expression (Aminjan *et al*., 2019). Immune responses are stimulated by oxidative stress, thereby causing diseases such as food allergies, allergic rhinitis, and asthma. This shows that the antioxidant defense system of patients with diseases has been compromised relative to that of healthy individuals (Sackesen *et al*., 2008). Modern lifestyles connected with lack of physical exercise, poor or unhealthy diet, exposure to different chemicals such as herbicides, pesticides, food additives, heavy metals (Tsatsakis *et al*., 2019), and environmental pollution can induce oxidative stress, thereby contributing to the increasing problem of prolonged diseases, as suggested by several human and experimental studies (Fountoucidou *et al*., 2019; Kostoff *et al*., 2020). In this study, protein carbonyl, lipid peroxidation, DNA fragmentation, and kynurenine levels were measured in this study.

2.9.1 Protein carbonyl

Protein carbonylation, part of the most detrimental irreversible oxidative protein modifications, is considered as the key hallmark of oxidative stress-related conditions. Protein carbonyl

measurements are often done to assess the level of oxidative stress to cellular damage, several age-related disorders and aging. (Hellwig, 2020).

2.9.2 Lipid peroxidation

Lipid peroxidation can be measured by the level of malondialdehyde (MDA). MDA is a mutagenic byproduct of lipid peroxidation that can react with or metabolize enzymatically on DNA and cellular proteins to form adducts that cause biological molecules to be damaged (Gaschler and Stockwell 2017). Lipid peroxidation is a process in which non-radical and free radical species attack lipids that contain carbon double bonds, most especially polyunsaturated fatty acids, resulting in hydroperoxides and peroxyl radicals (Mishra and Mishra 2017). MDA is also among the common markers of oxidative stress, and it is also involved in toxicity processes that result in cell death. Levels of protein carbonyl are elevated with age and are elevated in many diseased conditions such as diabetes, neurodegenerative diseases, and obesity (Marisa *et al*., 2012).

2.9.3 Kynurenine

The kynurenine pathway involves the oxidative breakdown of the amino acid L-tryptophan to kynurenine and other metabolites such as kynurenic acid and quinolinic, amongst others. The end-product of the route is NAD^+. The oxidative degradation of L-tryptophan is mediated by the enzyme indolamine-2, 3-dioxygenases (IDO-1). This enzyme can be induced by inflammation and oxidative stress. Its overexpression has been found to be connected to human cancer (Savitz, 2020). For example, during adaptive immune response, pro-inflammatory cytokines are produced, leading to an increase in the activity of indolamine-2, 3-dioxygenases (IDO-1) (Adeyemi *et al.,* 2019). In addition, oxidative stress has been linked with an increase in the activity of IDO-1 (Adeyemi *et al.*, 2019). Increased activity of IDO-1 leads to the

activation of the kynurenine pathway, which is thought to play a significant role in several neurological disorders (Sas *et al*., 2018; Adeyemi *et al.,* 2019).

2.10 Antioxidants

Antioxidants are substances used to neutralize or stabilize free radicals like reactive oxygen species (ROS) or other oxidants of biological molecules including proteins, nucleic acids and lipids. (Tufikul *et al*., 2015). Antioxidants play crucial roles in defense mechanisms. In healthy individuals, defense against the deleterious effects of reactive oxygen species (ROS) is attained by maintaining the balance between antioxidants and oxidants. In aerobic organisms, the constant production of free radicals must be balanced by a related rate of antioxidant consumption. Antioxidants can be classified as enzymatic or non-enzymatic (Banafsheh and Sirous, 2016). Antioxidant enzymes can be used to measure the redox status of cells. Reduced glutathione, glutathione peroxidase, glutathione transferase, catalase, total antioxidant capacity and catalase activities were determined in this study.

2.10.1 Reduced Glutathione

Reduced Glutathione (GSH), also known as -glutamyl-cysteinyl-glycine, is a tripeptide, distributed mainly in the cytosol (Banafsheh and Sirous, 2016). GSH scavenges free radicals and reactive ROS. GSH is also known to be involved in detoxification of xenobiotic resulting from ROS. Increased ROS-characterized conditions require enhanced GSH activity to maintain redox balance. However, in a prolonged oxidative stress condition when the cellular system cannot fight against the mediated oxidative insult, GSH amounts decrease, thereby leading to cell death and cell degeneration (Sharifi-Rad *et al*., 2020).

2.10.2 Glutathione Peroxidase

Glutathione peroxidase is a cytosolic intracellular enzyme that degrades hydrogen peroxides into lipid peroxides and water, and into their corresponding alcohols. GPx is mostly referred to as selenocysteine peroxidase. It plays a vital role in the inhibition of lipid peroxidation processes and shields the cell from oxidative stress, protecting hemoglobin from peroxide oxidation (Eren & Selami, 2020). It is the antioxidant enzyme system that provides the most important defense against the peroxidative damage of biological membranes in mammalian cells.

2.10.3 Glutathione S-transferase (GST)

GST belongs to the phase II family of detoxifying enzymes and consists of a cysteine-rich domain. They play important roles in xenobiotic detoxification by conjugating GSH with electrophilic endogenous molecules.

2.10.4 Catalase

Catalase is a tetrameric protein having four subunits. It is known as a first-line antioxidant defense enzyme which is found almost in all living tissues that utilize oxygen. It is located mainly in the peroxisomes with the highest activity, which are present in blood cells and the liver (Alfonso-Prieto *et al.,* 2009). Catalase breaks down hydrogen peroxide (H_2O_2) into molecular oxygen and water, thereby curtailing its free radical effect. Malfunctioning of catalase is associated with numerous diseases such as cardiovascular diseases, anemia, hypertension, and vitiligo (Nandi *et al*., 2019). Catalase plays an important role in the regulation of the hydrogen cellular level of hydrogen peroxide.

2.10.5 Nitric oxide

Nitric oxide is a diffusible gas and a short-lived molecule that rapidly recombines to produce stable metabolites such as nitrite and nitrate (Gupta *et al*., 2019). It is also known to be a pro-inflammatory mediator because of its capacity to react with superoxide anion to form a harmful anion, leading to oxidative related damage (Abolaji *et al*., 2018). NO is capable of stimulating different physiological responses, such as regulation of blood flow (Foster *et al*., 2009).

2.10.6 Total antioxidant capacity.

Total antioxidant capacity, a non-enzymatic antioxidant capacity, is defined as the moles of oxidants defused by one liter of body fluids (Knasmulle *et al*., 2008). In plasma, non-enzymatic antioxidants include endogenous and nutritional antioxidants such as ascorbic acid, phenolics, tocopherols, carotenoids, and other compounds. Different assays for total antioxidant capacity measure either their radical scavenging or reducing capacity.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Experimental Animals

Drosophila melanogaster (Harwich strain) was obtained from the *Drosophila* Laboratory, University of Ibadan. The maintenance and rearing of flies were carried out in the Biochemistry Laboratory, Biochemistry Department, College of Pure and Applied Sciences, Landmark University, Omu-Aran, Nigeria. Flies were reared on corn meal containing brewer's yeast, agar, and nipagin at a constant temperature (22–25 °C; relative humidity 60–70%) under 12-hour light/dark cycle conditions.

3.2 Reagents

All the reagents and chemicals used were of analytical grade and they were prepared in clean glass wares.

3.3 Imidazole Derivative (test compound): {1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol}

The imidazole derivative1-(1, 4, 5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol (Figure 2.8) was gifted by Dr. Eseola of the Institute of Inorganic and Analytical Chemistry, Friedrich-Schiller-Universität Jena, Humboldtstraße 8, 07743 Jena, Germany. The experimental synthesis and characterization have been reported previously (Eseola *et al*., 2018; Adeyemi *et al*., 2020).

3.4 *Drosophila melanogaster* Culture Media Preparation

Corn was purchased from Omu-Aran market (Latitude: 8°08′18″ N; longitude: 5°06′09″ E) Kwara State, Nigeria and ground into a fine powder. The corn meal, agar-agar, yeast and nipagin were weighed. (Table 3.1) separately and kept aside. Required quantity of water was

taken and divided into two parts, one part was used to dissolve cornmeal and to rinse glassware. The other part was heated on the hot plate and allowed to boil, agar-agar was added to the boiling water and stirred with a stirring rod in order to avoid lumps, the mixture was allowed to boil for 10 min and the corn meal was added. The mixture was covered and allowed to boil for 5 min, after which yeast was added. The mixture was then allowed to cool to 60 ^{o}C. Nipagin (preservative) was added and the mixture was dispersed into respective vials (Abolaji *et al*., 2014).

Table 3.1: *Drosophila melanogaster* Culture Media Ingredients

Ingredient	**Weight (g)**
Cornmeal	52
Agar-agar	7.9
Yeast	10
Nipagin	1
Water	450 mL

3.5 Experimental Design

The experimental design and treatment were in two phases.

3.5.1 Phase 1: Experimental Design

A test compound was introduced into corn meal media and flies were divided into four groups:

D. melanogaster flies (Harwich strain, 3 to 5 days old) were randomly distributed into 4 groups. Each group has fifty (50) flies per vial and three (3) replicates. For five days, the flies were fed

different concentrations of imidazole derivative (20, 50, and 100 mg) in their diet, as detailed below:

Group 1 served as control and received a normal diet without the imidazole derivative.

Group 2: were fed with diet containing 20 mg/kg diet of imidazole compound

Group 3: were fed with diet containing 50 mg/kg diet of imidazole compound

Group 4: were fed with diet containing 100 mg/kg diet of imidazole compound

The selection of dose was done based on a predicted oral LD_{50} of ≥2000 mg/kg in mice (Adeyemi *et al*., 2020).

3.6 Harvesting of *D. melanogaster* and Homogenate Preparation

The collection of flies for preparation of homogenate was done as previously described by (Abolaji *et al*., 2020). Briefly, after five days of exposure to an imidazole derivative, twenty-five (25) flies per vial from each group were randomly collected (the remaining 25 flies per vial were carried forward to the phase 2 experiment), and anaesthetized on ice, weighed, and homogenized in 0.1 M phosphate buffer, pH 7.4 (1:10 flies/volume (μL). The homogenate was centrifuged at 4,000 g for 10 min at 4 °C and the supernatant was separated into Eppendorf tubes and kept frozen until used for evaluation of the biochemical assays.

3.6.1 Phase 2

In the phase 2 of the experimental treatment, the remaining flies (25 per vial) from the phase 1 treatment were removed on day 5, and introduced to fresh corn meal media lacking the imidazole compound for another five days.

3.7 Survival Study

The survival of experimental flies was analyzed as described by Farombi *et al*. (2018), and this was done by recording the number of dead flies daily, which was thereafter used to plot the survival curve.

3.8 Assay for Total Protein Concentration

The assay was performed according to the procedure described by Gornall *et al.* (1949). The procedure for this assay is outlined in Table 3.2.

Table 3.1: Procedure for Determination of Total Protein Concentration

Sample (Homogenate)	1.0 mL	_
Blank (distilled water)	_	1.0 mL
Biuret reagent	4.0 mL	4.0 mL

The sample was appropriately diluted and the assay mixture was constituted as described above. The mixture was shaken thoroughly and left undisturbed at 25 ^{o}C for 30 min for color development. The absorbance was read at 540 nm using a UV/Vis spectrophotometer (Jenway, Stone Staff, UK).

The concentration of sample protein was estimated by extrapolation from a calibration curve of the standard protein. The calibration curve was obtained by using changing concentrations of BSA (1–10 mg/mL) as shown in Table 3.3. The values obtained were used to plot the calibration curve.

Table 3.2: Protein (BSA) concentration serial dilution for standard calibration

Chemical	Blank	1	2	3	4	5	6	7	8	9	10
Biuret reagent (mL)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Distilled water (mL)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0
BSA (mg/mL)	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Vol of Mixture (mL)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0

The reaction mixture was mixed using the vortex mixer, then incubated in the dark for 30 min at 25 ^{o}C. Absorbance was taken at 540 nm.

Concentration of protein = Cs x F

Where F = Dilution factor.

Cs = Protein concentration from the calibration curve.

3.9 Assay for Lipid Peroxidation: malondialdehyde (MDA) level

The level of lipid peroxidation was assessed by determining the malondialdehyde (MDA) level according to the method employed by Varshney and Kale, (1990).

In this assay, an aliquot (250 µL) of sample homogenate mixed with Tris-KCl 0.15 M (1.6 mL) buffer and 0.5 mL of 30% TCA was added. Half a milliliter (0.5 mL) of 0.75% TBA was added to the mixture and incubated for 45 min at 80 ^{o}C. The reaction mixture was then cooled to a normal temperature on ice. After cooling the reaction mixture on ice, it was centrifuged for 10 min at 3000 g. The absorbance of the clear supernatant was then measured at 532 nm against a blank (distilled water).

Calculation

$$\text{MDA (units/mg protein)} = \frac{Volume\ of\ reaction\ mixture \times Absorbance}{E532nm \times mg\ protein/ml \times Sample\ volume} \quad (1)$$

Where:

E = Molar extinction co efficient = $1.56 \times 10^5 Mcm^{-1}$.

3.10 Determination of Glutathione S-transferase Activity

Glutathione transferase activity was carried out at 37 ^oC using the procedure employed by Habig *et al.* (1974). The medium for the estimation is presented in Table 3.4 and the reaction was made to run for 3 min and readings were taken at 60 sec intervals against a reference blank at 340 nm on a UV/Vis spectrophotometer (Jenway, Stone Staff, UK).

Table 3.3: Procedure for Glutathione S-transferase Activity

Reagent	**Blank**	
CDNB (20 mM)	150 µL	150 µL
Reduced glutathione (0.1 M)	30 µL	30 µL
Phosphate buffer pH 6.5 (0.1 M),	2820 µL	2790 µL
Sample	-	30 µL

Calculation

CDNB extinction coefficient =9.6 $mM^{-1}cm^{-1}$

Activity of GST=$Reaction\ volume\ \times \Delta A340/min \times\ dilution\ factor$ (2)

$$mg\ protein/ml \times\ sample\ volume\ \times\ 9.6$$

$$= \mu mole/min/mg\ protein$$

3.10 Assay for Catalase Activity

Catalase activity was determined using the method described by Beers and Sizer (1952). The details of the procedure are presented in Table 3.4.

Table 3.4: Procedure for determination catalase (CAT) activity

Reagent	**Test**
Distilled water	1.9 mL
Hydrogen peroxide (H_2O_2)	1.0 mL
Incubated for 5 min in the spectrophotometer in order to achieve temperature equilibrium and for the establishment of blank rate	
Enzyme source (appropriately diluted)	0.1 mL

Before use, the enzyme source (sample homogenate) was diluted in 0.05 M phosphate buffer (pH 7.0) to obtain a rate of 0.03 – 0.07 ΔA/min. The assay mixture was thereafter constituted as shown in Table 3.5.

A reduction in absorbance was supervised at 240 nm for 2 min immediately after adding the correctly diluted enzyme source. The following expression was used to calculate catalase activity:

$$\text{Catalase Activity (units/mg)} = \frac{\Delta A240/\text{min}}{43.6\ \text{X mg protein/mL rxn mixture}} \quad (3)$$

3.11 Assay for Reduced Glutathione (GSH)

The concentration of reduced glutathione level (GSH) was assayed following the procedure described by Jollow *et al*. (1974). The principle of this assay is based on the oxidation of GSH with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), a sulfhydryl reagent to yield a yellow derivative, 5-thio-2-nitrobenzoic acid TNB, measurable at 412 nm.

Initially, an aliquot (0.5 mL) of the sample was deproteinized by adding an equal volume of 4% sulfo-salicyclic acid and centrifuged at 3000 g for 15 min at 4 ^{o}C using a Uniscope Laboratory centrifuge. Thereafter, 0.5 mL of the supernatant was collected and then added to 4.5 mL of Ellman reagent. A blank was prepared with 0.5 mL of sulfo-salicyclic acid in 0.1 M phosphate buffer (twice dilution) and 4.5 mL of Ellman reagent. Measurement was made within 5 min of the generation of yellow color on the addition of 4.5 mL of Ellman reagent on a 412 nm UV/Vis spectrophotometer (Jenway, Stone Staff, UK). The concentration of GSH was extrapolated from a standard curve of known concentrations of GSH.

The calibration curve for GSH was prepared as presented in Table 3.5

Table 3.5: Serial dilution of reduced glutathione (GSH) concentration for standard calibration

Stock (mL)	Phosphate buffer (mL)	Ellman reagent (mL)
0.00	0.50	4.5
0.02	0.48	4.5
0.05	0.45	4.5
0.10	0.40	4.5

0.20	0.30	4.5
0.30	0.20	4.5
0.40	0.10	4.5

Serial dilutions of the GSH standard were prepared. Each serial solution was added to different volumes of phosphate buffer (pH 7.4) to make up a total volume of 0.5 mL. The blank contained no GSH stock solution.

Calculation:

$$\text{Units/mL enzyme} = \frac{(r\,A340nm/\min Test - rA340nm/\min Blank) \times 2 \times 3.1 \times df}{6.22 \times 0.5} \qquad (4)$$

Where:

df = dilution factor

6.22 = Millimolar extinction co-efficient of β-NADPH at 340 nm

3.1 = Total volume of assay

2 = 2 µmoles of GSH produced per µmole of β-NADPH oxidized

0.5 = Volume (mL) of enzyme used.

3.12 Assay for Glutathione Peroxidase Activity (GPx)

GPx activity was determined by employing a previously described procedure by Rotruck *et al.* (1973). The details of the procedure are presented in Table 3. 6

Table 3.6: Procedure for the determination of glutathione peroxidase (GPX) activity

Reagents	Blank	Test
Phosphate buffer		0.5 mL
Sodium azide		0.1 mL
Hydrogen peroxide		0.1 mL
Test sample		0.5 mL
Incubated for 3 min at 37 ^{o}C		
Trichloroacetic acid		0.5 mL
The reaction mixture above were then centrifuge at 3000 g for 5 min		
1 mL of supernatant		
Dipotassium hydrogen orthophosphate	2 mL	2 mL
DNTB	1 mL	1 mL
Distilled water	1 mL	

Absorbance was measured at 412 nm on a UV/Vis spectrophotometer (Jenway, Stone Staff, UK).

Calculation

Consumed GSH = GSH amount – GSH amount remaining (µg/mL × 4 mL)

Activity of GPx = GSH amount consumed/mg protein

= µg GSH/mg protein

3.13 Assay for DNA Fragmentation

The DNA fragmentation was estimated using the diphenylamine (DPA) assay previously described by Perandones *et al*. (1993). To extract cell lysate, the sample homogenate was resuspended in 4 mL of hypotonic lysis buffer (2% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl). The lysate was incubated for 2.5 hours at 50 ^{o}C and this was followed by centrifugation for 15 min at 13,800 *g*. The obtained supernatant containing small fragments of DNA was decanted immediately. Acid extraction of DNA was carried out by re-dissolving the pellet in Tris-EDTA buffer followed by thorough shaking to ensure adequate DNA fragment separation from intact DNA. An equal volume of 10% TCA was added to both the pellet and the supernatant and incubated at 25 ^{o}C for 15 min. Centrifugation was done at 2000 g for 10 min and the precipitate was re-suspended in an equal volume of 5% TCA. Another incubation was carried out for 10 min at 100 ^{o}C and was centrifuged at 1500 g for 15 min. The supernatant was cooled to 25 ^{o}C. To determine the content of DNA, to one volume of DNA extracted, two volumes of the colorimetric reagent were added and the mixture kept overnight at 30 ^{o}C until a blue color was established. Absorbance of the mixture was taken at 600 nm on a UV/Vis spectrophotometer (Jenway, Stone Staff, UK). The DNA fragmentation (%) was obtained using the formula;

$$\% \text{ DNA fragmentation} = \frac{Absorbance\ of\ supernatant}{Absorbance\ of\ supernatant + Absorbance\ of\ pellet} \times 100 \quad (5)$$

3.14 Determination of Total Antioxidant Capacity

Total antioxidant capacity was based on the procedure described by Saha *et al*. (2014). In this assay, 300 µL of sample was added to 3 mL of reagent solution (ammonium molybdate, sulphuric acid and sodium phosphate) and incubated at 95 °C for 90 min and thereafter allowed to cool to room temperature. The absorbance of the mixture was taken against a reference blank

at 695 nm on a UV/Vis spectrophotometer (Jenway, Stone Staff, UK). Total antioxidant capacity was expressed as ascorbic acid equivalent.

3.15 Determination of Nitrite (nitric oxide) Level

The amount of nitrate in sample homogenates was determined using the Griess reaction. In this assay, 0.5 mL of sample and 0.5 mL of Griess reagent were added and incubated at 37 ^{o}C for 20 min. The absorbance was spectrophotometrically measured at 550 nm on a UV/Vis spectrophotometer (Jenway, Stone Staff, UK). The concentration of nitrite was extrapolated from a standard curve of known sodium nitrite concentrations.

3.16 Assay for Protein Carbonyl Determination.

The protein carbonyl assay was based on the procedure described by Castegna *et al*. (2013).

The procedure for the protein carbonyl assay is presented in Table 3.7.

Table 3.7: Procedure for protein carbonyl determination

Reagents	**Blank**	**Test**
Sample (homogenate)	--	500 µL
10 % trichloroacetic acid	500 µL	500 µL
0.2 % DNPH	500 µL	500 µL
Reaction mixture as highlighted above was incubated at room temperature for 1 hour while vortexing at 5 min interval. Thereafter TCA was added as shown below.		
100 % trichloroacetic acid	55 µL	55 µL

The reaction mixture above was then centrifuged at 5000 g for 10 min. The pellets were centrifuged and washed three times with 500 L of ethanol-ethyl acetate mixture. Then, 600 µL of 6 M Guanidine hydrochloride was added to the already washed pellets, and absorbance was read at 370 nm on a UV/Vis spectrophotometer (Jenway, Stone Staff, UK).

Calculations:

A αE/C

Where A = Absorbance

E-Millimolar absorptivity = 22.0×10^{3}mM/cm^{-1}

C = concentration

Concentration = $\frac{Absorbance}{2200}$ (6)

3.17 Assay for Kynurenine Level

The kynurenine level was assayed based on the procedure described previously (Adeyemi *et al.* (2017). To (100 µL) of sample homogenate, 100 µL of 30% TCA was added and then incubated in a water bath at 50 ^{o}C for 30 min. The mixture was then vortexed, and centrifuged for 5 min at 8500 x *g*. An aliquot (100 µL) of the supernatant was then mixed with the same volume of Ehrlich reagent (2%; 100 mg *P*-dimethylbenzaldehyde in 5 mL of glacial acetic acid, freshly prepared). Absorbance was measured at 492 nm after a 10-min incubation at room temperature. The kynurenine level in the supernatant was extrapolated from the kynurenine standard curve.

3.18 Statistical Analysis and Data Presentation

Statistical analysis was carried out using GraphPad Prism 8.0 software (San Diego, California, USA). The data is expressed as the average or mean of three replicates with standard error of

mean (SEM). The treated and control groups were compared using one-way analysis of variance (One-way ANOVA). *P*-values less than 0.05 ($p<0.05$) were considered to be statistically significant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS OF FINDINGS

4.1 Results

4.1.1 Phase 1 Survival Study

Following a five-day administration of imidazole (20, 50 and 100 mg IMZ/kg diet) in *D. melanogaster*, the survival rate of flies decreased by 5.3, 8.1 and 7.4% (Table 4.1) respectively. However, these alterations were not statistically ($p>0.05$) significant.

Table 4.1: Survival rate (%) of *Drosophila melanogaster* after 5 days of exposure to 20, 50, and 100 mg imidazole (IMZ) derivative/kg diet

Days	Control	20 mg IMZ/kg diet	50 mg IMZ/kg diet	100 mg IMZ/kg diet
1	100±0.00	98.9±0.56	94.4±0.57	98.9±0.56
2	97.8±0.55	95.0±1.47	96.7±0.00	95.0±0.96
3	97.2±1.11	95.0±2.22	93.3±0.00	93.3±1.92
4	96.7±1.67	93.9±2.22	88.3±2.54	88.9±2.94
5	96.7±1.67	91.7±3.85	87.2±3.64	87.2±3.37
Average survival (%)	**97.6±1.37**	**94.7±2.27**	**91.9±4.01**	**92.6±2.10**
Average death (%)	**2.4**	**5.3**	**8.1**	**7.4**

Values are presented as the mean ± SEM of 3 replicates containing 50 flies per replicate.

4.1.2 Phase 2 Survival Study

A slight non-significant ($p > 0.05$) decrease (by 2, 2.6 and 4.6%) in the survival rate of the treatment group administered 20, 50 and 100 mg IMZ/diet was still observed five days post-cessation of treatment (Table 4.2).

Table 4.2: Survival rate (%) of *Drosophila melanogaster* after cessation of imidazole treatment

Days	Control	20 mg IMZ/kg diet	50 mg IMZ/kg diet	100 mg IMZ/kg diet
1	98.9±0.78	100.0±0.00	98.9±0.78	96.7±1.36
2	98.9±0.78	100.0±0.00	98.9±0.78	96.7±1.36
3	97.8±1.57	96.7±0.00	95.6±0.78	94.4±0.78
4	95.6±2.08	96.7±0.00	94.4±0.78	94.4±0.78
5	94.4±2.80	96.7±0.00	93.3±0.00	94.4±0.78
Average survival (%)	**97.1±0.90**	**98.0±0.80**	**96.2±1.44**	**95.3±0.54**
Average death (%)	**2.6**	**2**	**3.8**	**4.7**

Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate.

4.1.3 Total protein level

Imidazole administration reduced the total protein level of flies across the treatment group (Figure.4.1A), although not statistically significant ($p>0.05$). There was no statistically significant difference in the total protein level of flies fed a normal diet after cessation of treatment relative to the control group (Figure 4.1B).

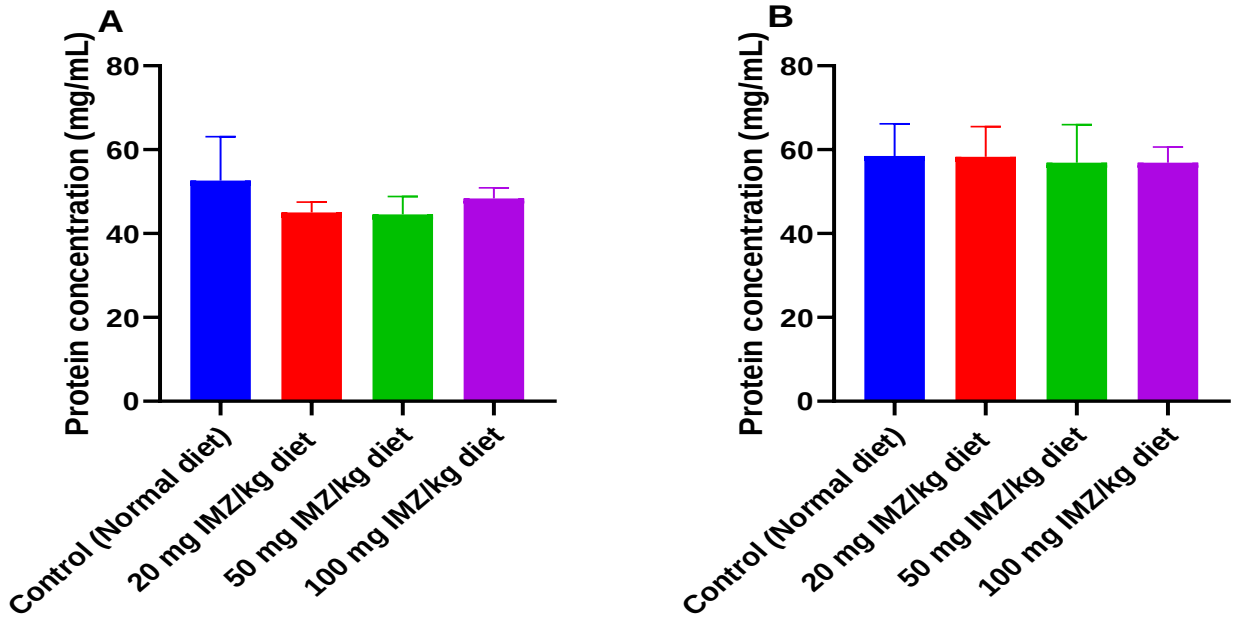


Figure 4.1: Effect of administration of imidazole (IMZ) derivative on total protein concentration in *Drosophila melanogaster* (A) Total protein concentration after exposure of flies to 20, 50 and 100 mg of imidazole/ kg diet for 5 days; (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate.

4.1.4 Glutathione transferase activity

Imidazole administration caused an increase in glutathione transferase activity (Figure 4.2A), although not statistically (p>0.05) significant. Also, there is no significant difference in the group fed a normal diet after cessation of imidazole treatment relative to the control (Figure 4.2B).

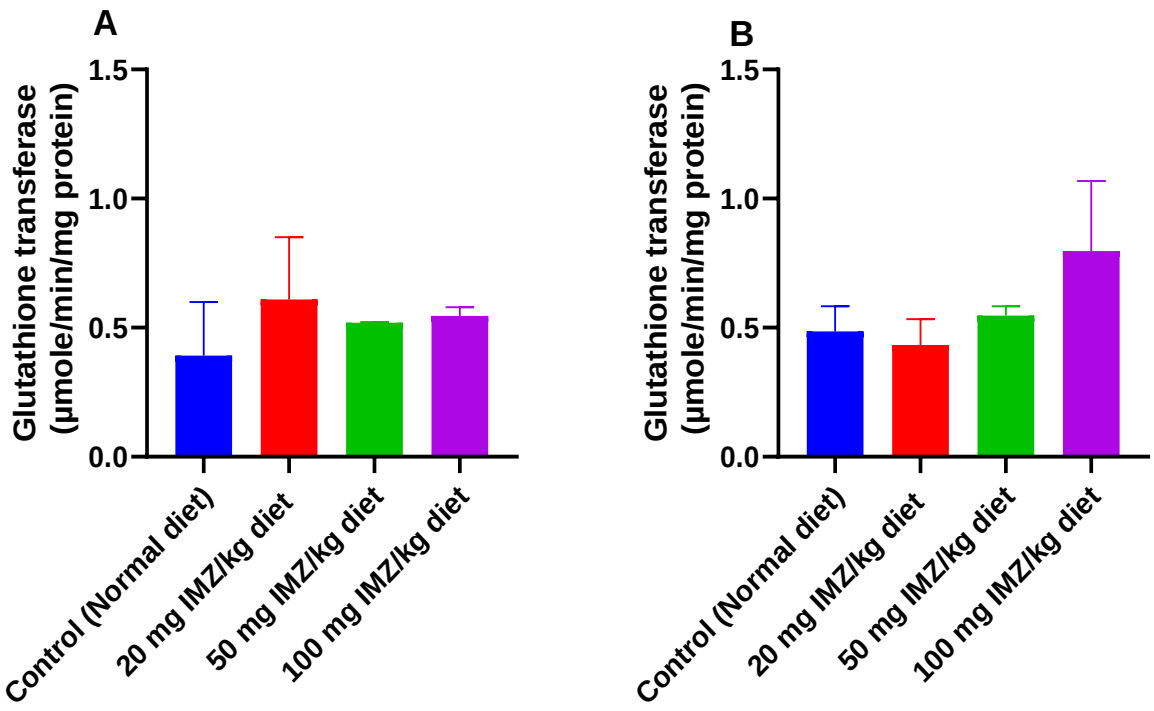


Figure 4.2: Effect of administration of imidazole (IMZ) derivative on the activity of glutathione transferase (GST) in *Drosophila melanogaster*. (A) glutathione transferase concentration after exposure of flies to 20, 50 and 100 mg of imidazole / kg diet for 5 days; (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate.

4.1.5 Reduced glutathione level

Imidazole administration led to an increase in the level of GSH of flies across the treatment groups (Figure 4.3A). Also, for the group fed a normal diet after cessation of imidazole treatment, there is no significant difference ($p>0.05$) relative to the control (Figure 4.3B).

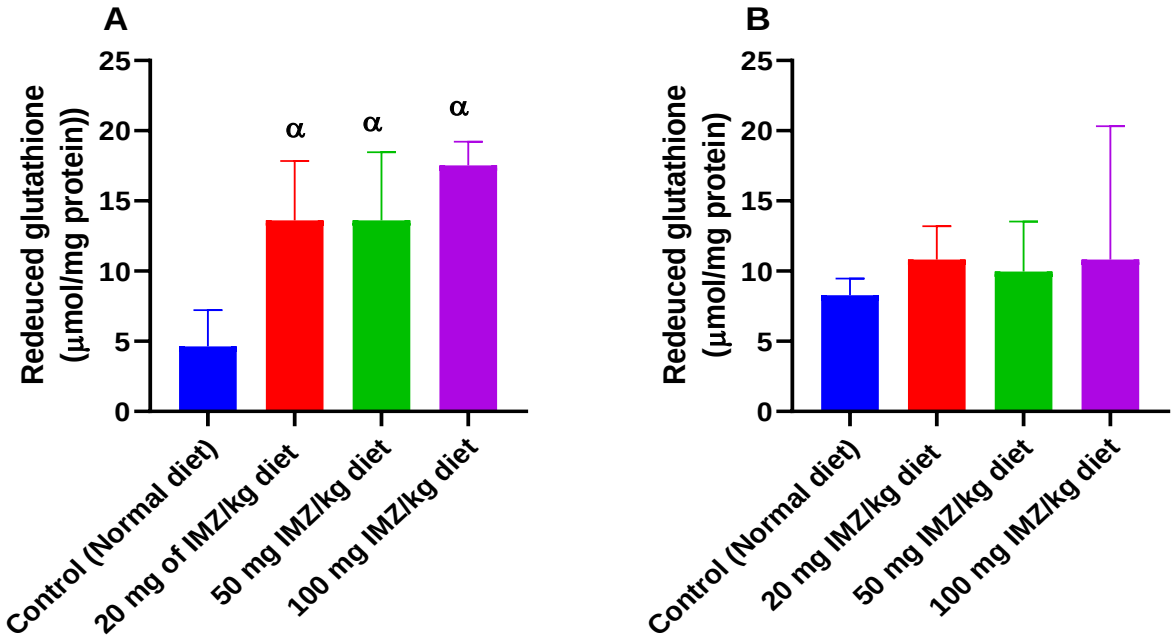


Figure 4.3: Effect of administration of imidazole (IMZ) derivative on reduced glutathione (GSH) concentration in *Drosophila melanogaster*. (A) Reduced glutathione concentration after exposure of flies to 20, 50 and 100 mg of imidazole per kg diet for 5 days; (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate. α is significant at ($p<0.05$) against the control.

4.1.6 Glutathione peroxidase (GPx)

Imidazole administration increased the glutathione peroxidase activity of flies fed with 20 and 50 mg of IMZ/kg diet, although not statistically ($p>0.05$) significant, relative to the control group (Figure 4.4A). There is no statistically ($p>0.05$) significant difference in the glutathione peroxidase activity of flies fed a normal diet after cessation of treatment relative to the control (Figure 4.4B).

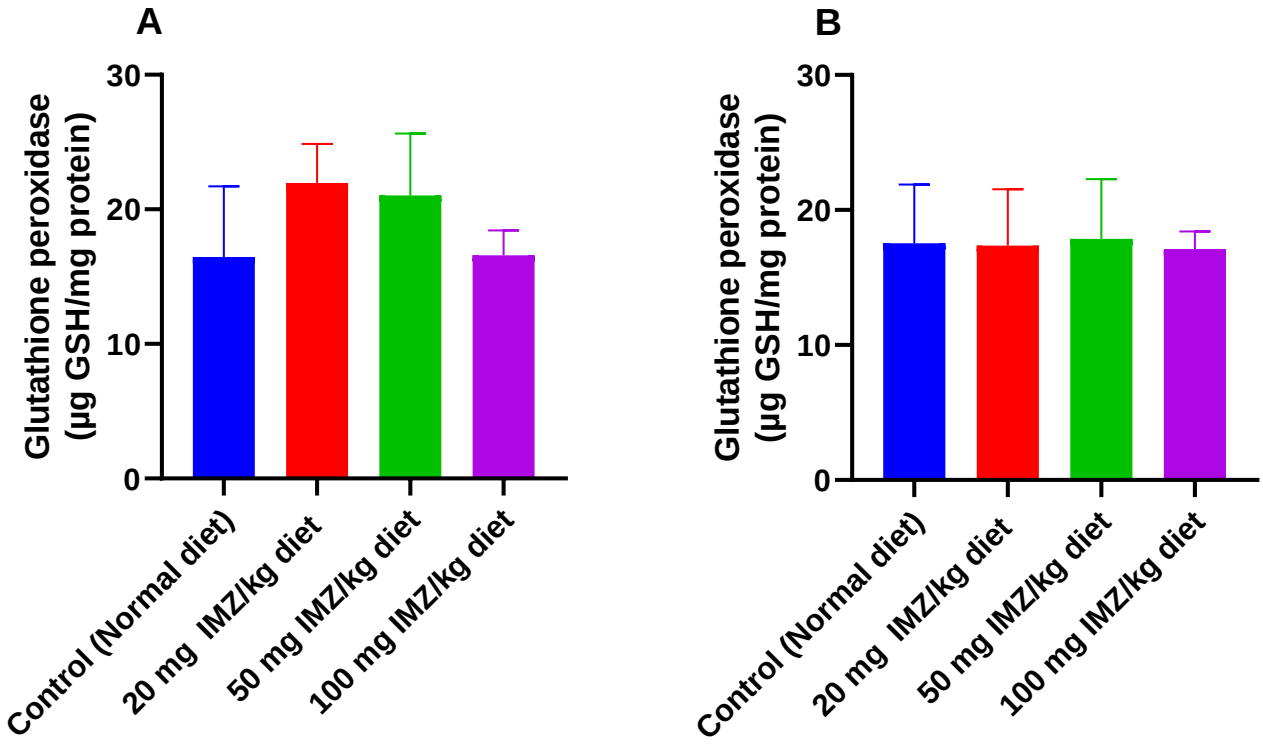


Figure 4.4: Effect of administration of imidazole derivative on glutathione peroxidase (GPX) activity in *Drosophila melanogaster*. (A) Glutathione peroxidase concentration after exposure of flies to 20, 50 and 100 mg of imidazole per kg diet for 5 days; (B) 5 days exposure to normal diet after cessation of treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate.

4.1.7 Catalase activity

Imidazole administration led to a significant increase ($p<0.05$) in the catalase activity of flies fed with a 50 mg IMZ/kg diet (Figure 4.5A) relative to the control group. There is a significant difference ($p<0.05$) in the catalase activity of flies fed a normal diet after cessation of imidazole treatment relative to the control group (Figure 4. 5B).

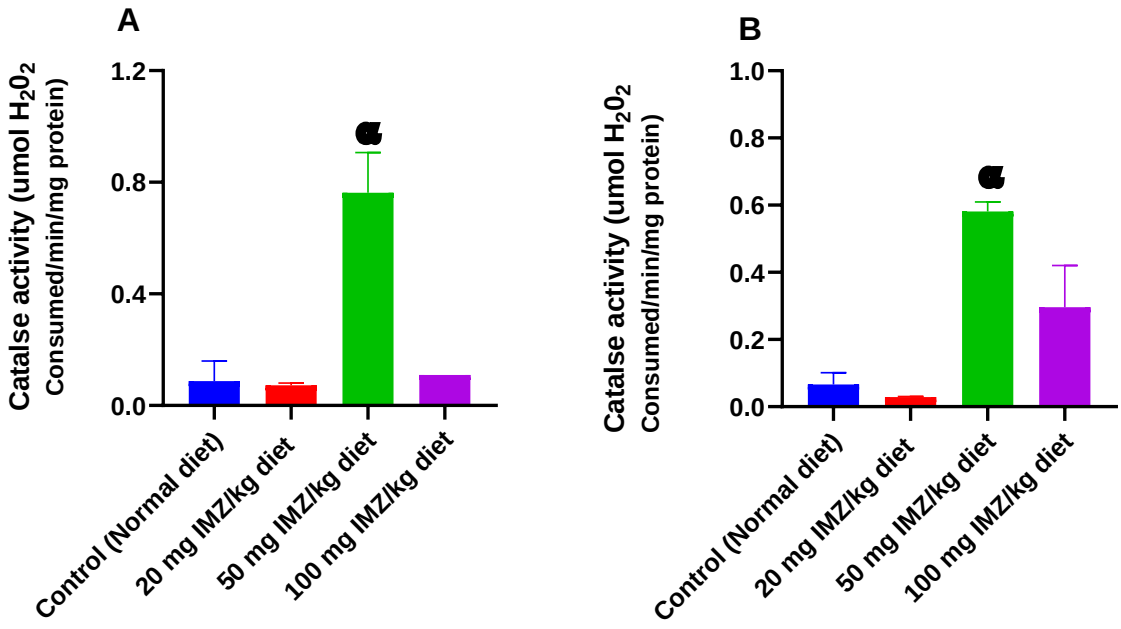


Figure 4.5: Effect of administration of imidazole derivative activity of catalase in *Drosophila melanogaster*. (A) catalase activity after exposure of flies to 20, 50 and 100 mg of imidazole per kg diet for 5 days; (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate. α is significant at ($p<0.05$) against the control

4.1.8 Nitric oxide concentration

Administration of imidazole in all treatment groups caused a dose-dependent increase in nitric oxide concentration; the lowest dose yielded the highest increase and the highest dose yielded the least increase (Figure 4.6A). Changes, however, were not statistically significant (*p>0.05*). Following treatment cessation, non-significant decreases were observed in the treatment groups administered 50 and 100 mg IMZ/kg diet relative to the control group (Figure 4.6B).

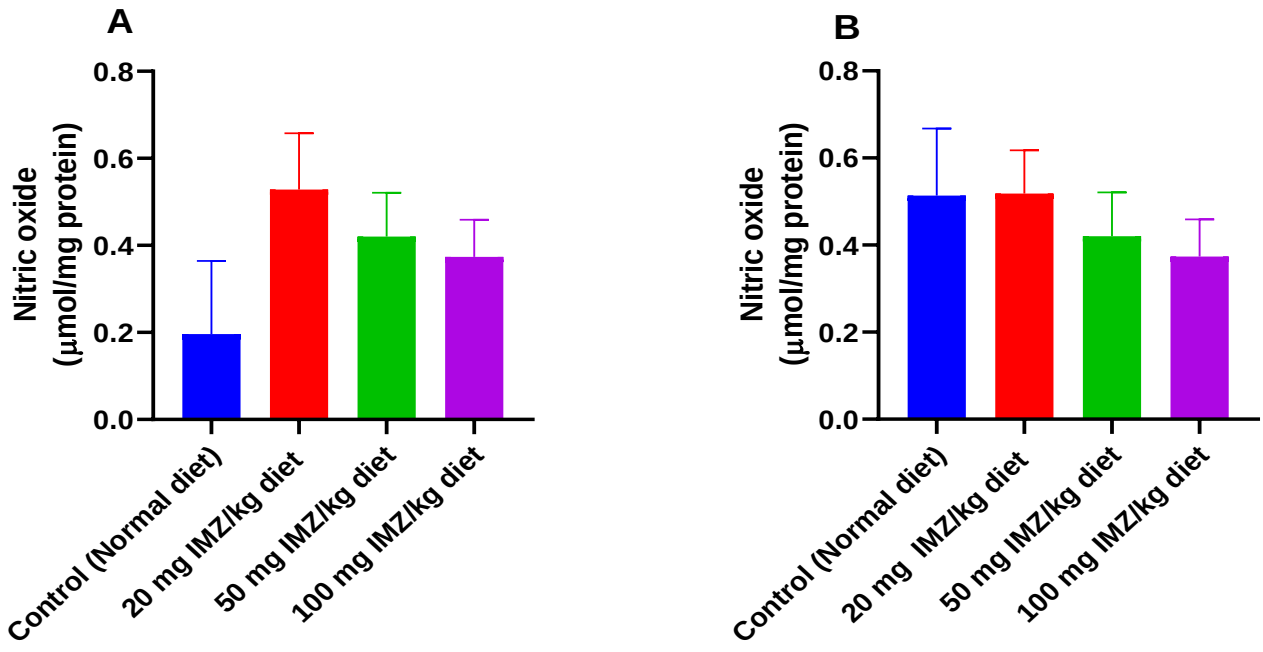


Figure 4.6: Effect of administration of imidazole (IMZ) derivative on nitric oxide concentration in *Drosophila melanogaster*. (A) Nitric oxide level after exposure of flies to 20, 50 and 100 mg of imidazole per kg diet for 5 days (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate.

4.1.9 Total antioxidant capacity

Imidazole administration caused a slight decrease in the total antioxidant capacity of flies, although not statistically significant (Figure 4.7A) relative to the control group. Also, there was a dose-dependent increase in the total antioxidant capacity level of flies across the treatment groups fed with a normal diet after cessation of imidazole treatment relative to the control group (Figure 4.7B).

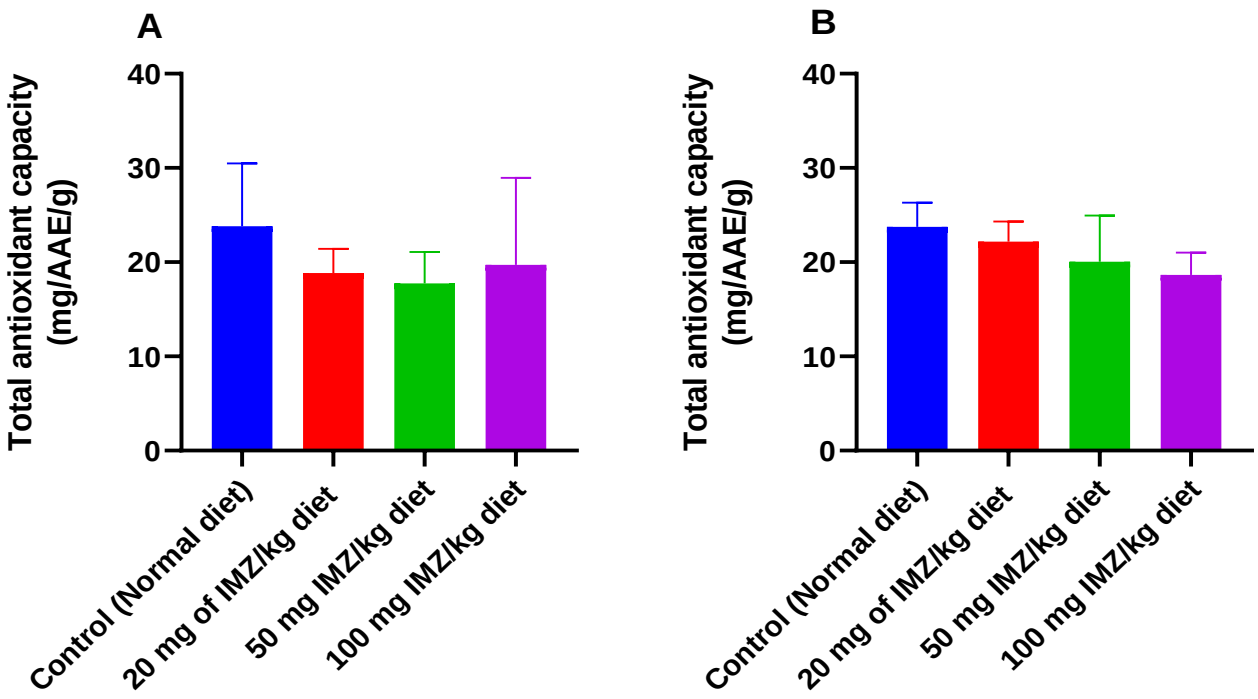


Figure 4.7: Effect of administration of imidazole (IMZ) derivative on total antioxidant capacity in *Drosophila melanogaster*. (A) Total antioxidant capacity after 5 days of exposure of flies to 20, 50 and 100 mg of imidazole per kg diet; (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate. AAE - Ascorbic acid equivalent**.**

4.1.10 DNA fragmentation

Imidazole administration increased the % DNA fragmentation of the flies fed with a 100 mg IMZ/kg diet (Figure 4.8A). The increase is statistically ($p<0.05$) significant relative to the control group. However, there is no significant difference in the percentage of DNA fragmentation of flies fed a normal diet after cessation of imidazole treatment relative to the control group (Figure.4.8B).

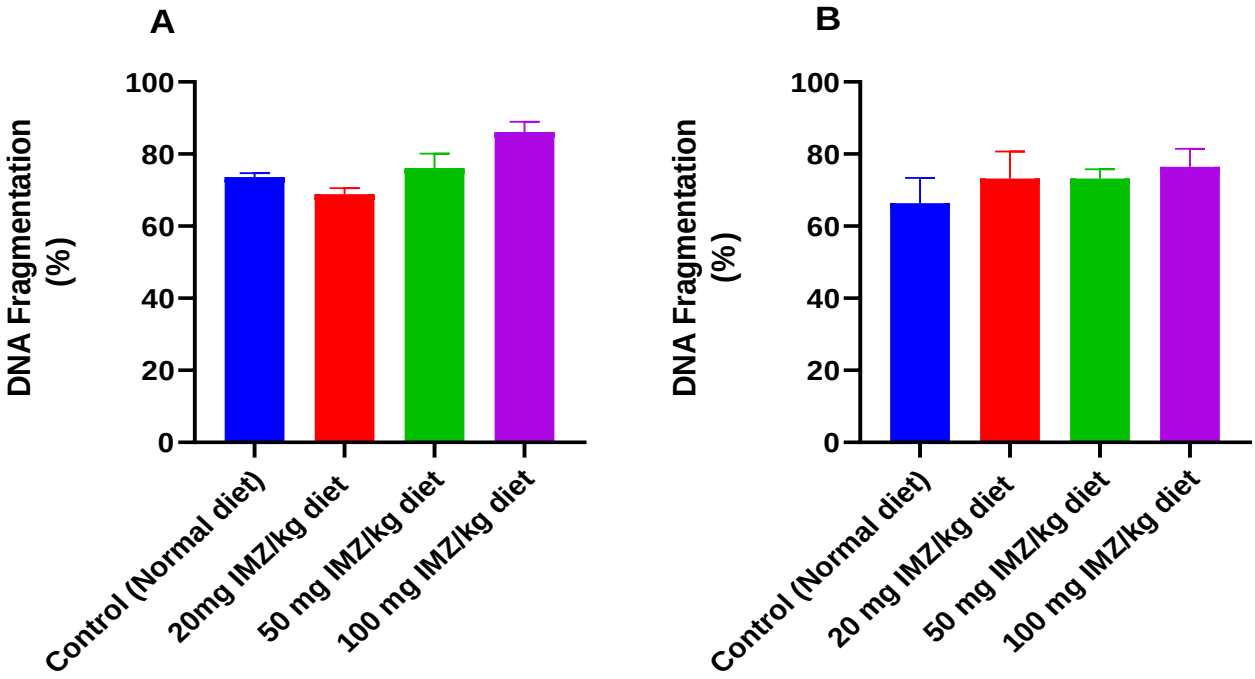


Figure 4.8: Effect of administration of imidazole (IMZ) derivative on DNA fragmentation level in *Drosophila melanogaster*. (A) DNA fragmentation after 5 days of exposure of flies to 20, 50 and 100 mg of imidazole per kg diet; (B) 5 days exposure to normal diet after cessation of treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate. α is significant at ($p<0.05$) against the control.

4.1.11 Lipid peroxidation

Imidazole administration led to an increase in the level of malondialdehyde for flies fed with 50 and 100 mg IMZ/kg diets, although not statistically ($p>0.05$) significant relative to the control group (Figure 4.9A). Also, there was no significant difference ($p>0.05$) in the malondialdehyde level of flies fed a normal diet after cessation of imidazole treatment relative to the control group (Figure 4.9B).

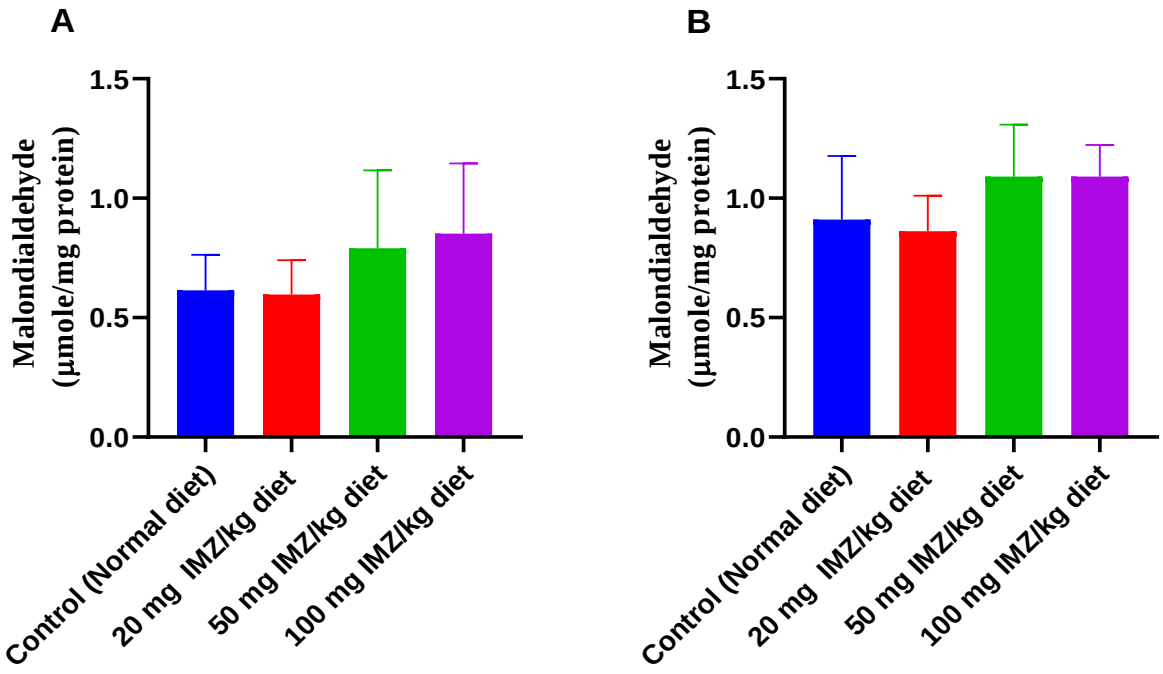


Figure 4.9: Effect administration of imidazole (IMZ) derivative on malondialdehyde (MDA) concentration in *Drosophila melanogaster*. (A) MDA concentration after 5 days of exposure of flies to 20, 50 and 100 mg of imidazole per kg diet; (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate.

4.1.12 Protein carbonyl level

Imidazole administration increased ($p<0.05$) the level of protein carbonyl for flies fed 100 mg IMZ/kg diet relative to the control group (Figure 4.10A). However, there is no significant difference in the group fed a normal diet after cessation of imidazole treatment relative to the control group (Figure 4.10B).

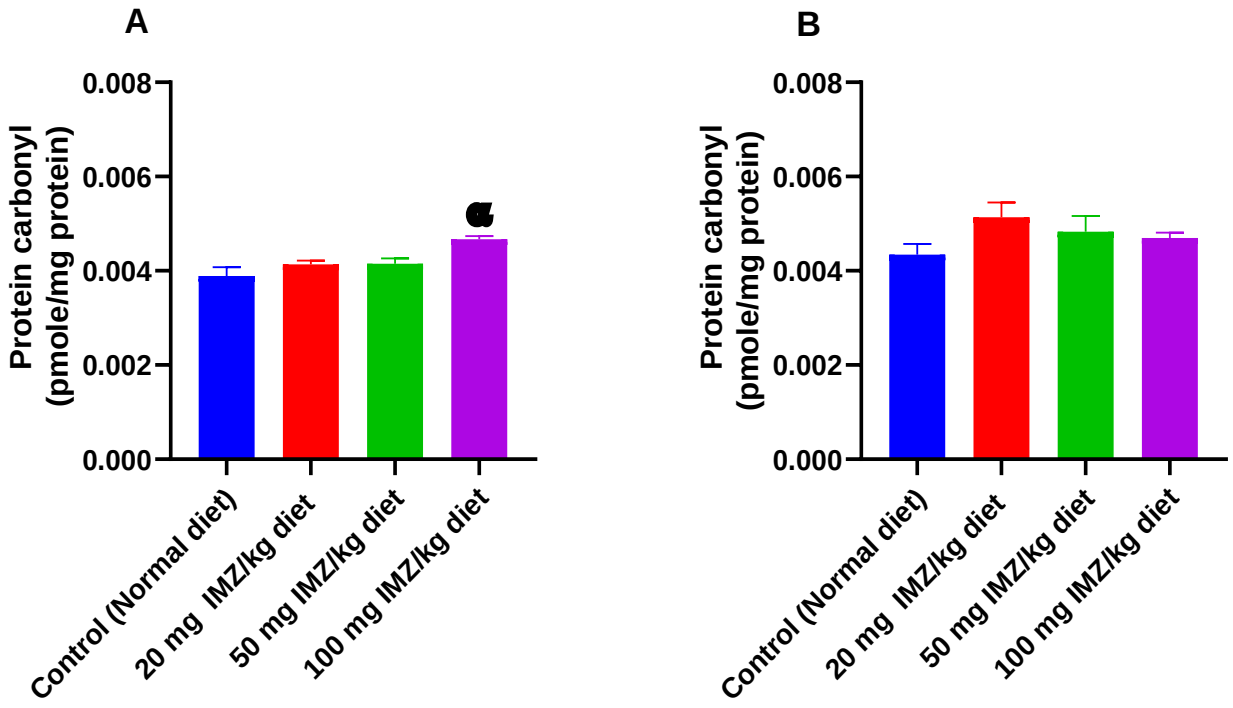


Figure 4.10: Effect of administration of imidazole (IMZ) derivative on protein carbonyl level in *Drosophila melanogaster*. (A) protein carbonyl level after 5 days of exposure of flies to 20, 50 and 100 mg of imidazole per kg diet; (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate. α is significant at ($p<0.05$) against the control.

4.1.13 Kynurenine level

Imidazole administration caused a decrease in the kynurenine level of flies fed with a 50 mg IMZ kg diet (Figure 4.11A). There was no significant difference *(p*>0.05) in the group fed a normal diet after cessation of imidazole treatment (Figure 4.11B) relative to the control group.

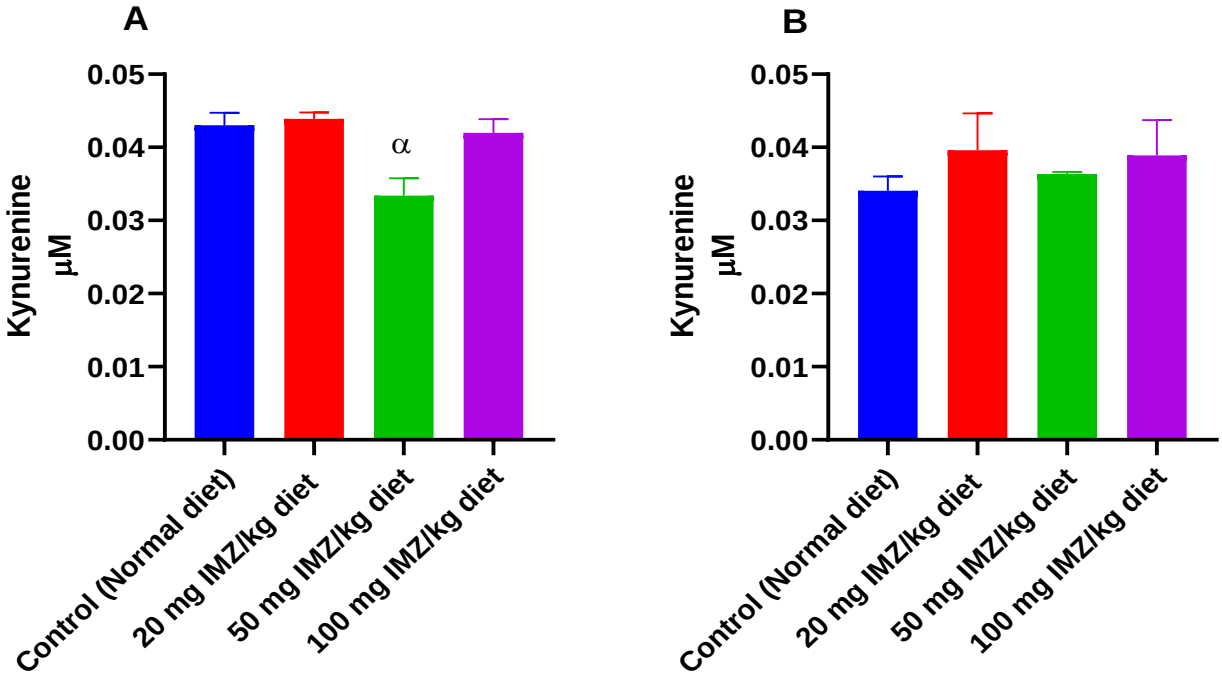


Figure 4.11: Effect of administration of imidazole (IMZ) derivative on kynurenine level in *Drosophila Melanogaster*. (A) Kynurenine level after 5 days of exposure of flies to 20, 50 and 100 mg of imidazole per kg diet (B) 5 days exposure to normal diet after cessation of imidazole treatment. Values are presented as mean ± SEM of 3 replicates containing 50 flies per replicate. α is significant at (*p<0.05*) against the control.

4.2 Discussion

4.2.1 Effect of imidazole derivatives on survival rate of flies

In this study, the effect of an imidazole derivative at different dosages was evaluated. Imidazole treatment might not have adversely affected the survival of the flies. Only a few deaths (averagely 10%) were recorded following imidazole treatment compared with the control. In addition, after cessation of imidazole treatment, fewer deaths (average <4%) were recorded five days post-imidazole treatment. Together, this may suggest the relative safety of the compound.

4.2.2 Total protein concentration

In the present study, the treatment of flies with imidazole after 5 days reduced the total protein concentration, although not statistically significant when compared to the control (Figure 4.3A). However, the reduced total protein concentration was restored after imidazole cessation of treatment. Imidazole treatment might not have adversely affected total protein turnover in the present study.

4.3 Effect of imidazole derivative on antioxidant enzymes

Antioxidants are substances used to neutralize or stabilize free radicals like reactive oxygen species (ROS) or other oxidants of biological molecules including proteins, nucleic acids and lipids. (Sharifi-Rad, *et al.*, 2020). Antioxidant enzymes can be used to measure the redox status of cells.

4.3.1 Glutathione S-transferase

Glutathione S-transferase (GST) belongs to the phase II family of detoxifying enzymes and consists of a cysteine-rich domain. They play important roles in xenobiotic detoxification by conjugating GSH with electrophilic endogenous molecules. This antioxidant enzyme also

protects the cell from oxidative stress and redox cycling (Abolaji *et al*., 2018). This antioxidant enzyme protects the cell from the harmful effects of oxidative stress (Frat & Aytekin, 2018), while also assisting in the regulation of cellular processes involved in oxidative stress. Under a diseased condition, GST levels are usually low, and are high under normal physiological conditions (Maduaguana *et al.,* 2020). In this present work, a non-significant increase was observed in the total activity of GST and this may suggest the activation of the phase II detoxification system to metabolically cope with imidazole exposure. This may indicate an attempt and capacity of the flies to detoxify the imidazole derivative.

4.3.2 Reduced glutathione

GSH, which is also known as -L-glutamyl-L-cysteinyl-glycine is a cellular non-enzymatic antioxidant known to protect cells against assault caused by free radicals. It is also needed for various cellular processes that are connected with alteration in the regulation and maintenance of the redox status of the thiol group because of its ability to exist in several redox species (Katia *et al*., 2014). GSH is also known to be involved in detoxification of xenobiotics resulting from ROS. Increased ROS-characterized conditions require enhanced GSH activity to maintain redox balance. However, in a prolonged oxidative stress condition when the cellular system cannot fight against the mediated oxidative insult, GSH amounts decrease, thereby leading to cell death and cell degeneration. In the present study, exposure of flies to the imidazole derivative significantly ($p<0.05$) raised the GSH levels across the treatment groups. The increased GSH level could be as a result of an adaptive response mechanism in response to imidazole. It could also be to counteract likely oxidative stress due to the treatment. Therefore, increased GSH levels in *D. melanogaster* might be to protect against oxidative stress resulting from imidazole exposure.

4.3.3 Glutathione peroxidase (GPx)

Glutathione peroxidase is a cytosolic intracellular enzyme that degrades hydrogen peroxides into lipid peroxides and water, and into their corresponding alcohols. GPx is mostly referred to as selenocysteine peroxidase. It plays a vital role in the inhibition of lipid peroxidation processes and shields the cell from oxidative stress (Eren & Selami, 2020). Its clinical importance has been emphasized in some studies (Ighodaro & Akinloye, 2018). Individuals deficient in GPx are predisposed to increase vascular oxidative stress, impaired antioxidant protection, oxidative damage to functional proteins and fatty acids (Rayman, 2005). GPx also protects the cell against free radical effects and oxidative stress, but the role it plays in flies is not fully understood (Jo & Imm 2017). In this present study, imidazole administration increased glutathione peroxidase levels of flies fed with 20 and 50 mg/kg diets, although not statistically significant when compared to control (Figure 4.6), the increase in GPx level may indicate an attempt by the cell to shield against the toxicity caused by the imidazole derivative. The observed increase in GPx level in this study is in agreement with a previous report by Zeferino *et al*. (2020).

4.3.4 Catalase (CAT)

Catalase is a tetrameric protein having four subunits. It is known as a first-line antioxidant defense enzyme which is found almost in all living tissues that use oxygen. Catalase breaks down hydrogen peroxide (H_2O_2) into molecular oxygen and water, thereby curtailing its free radical effect. Although at low concentration, H_2O_2 plays a role in some physiological processes such as carbohydrate metabolism, cell death, platelet activation and maintenance of redox balance. Also, at high concentrations, H_2O_2 has been reported to be toxic (Ighodaro & Akinloye 2018). The mutation or deficiency of CAT has been linked with different kinds of diseases. ROS have the ability to indirectly or directly damage biological molecules, including DNA, lipids and proteins.

CAT activity also helps to reduce deleterious effects associated with ROS, such as ageing (Butterfield *et al.* 2002). In this study, imidazole administration significantly increased the catalase activity of flies fed with 50 mg of IMZ/kg diet compared to the control. The increase in catalase may suggest the election of adaptive mechanisms to tackle increased production of hydrogen peroxide (H_2O_2). Increased CAT activity has been reported to be an adaptive response to oxidative stress challenges (Likidlilid *et al*., 2010). Moreover, the results of the present work showed that there was a non-significant differential in TAC. TAC, which measures the capacity to scavenge free radicals, is considered one of the useful antioxidant status markers (Suresh *et al*., 2009).

The increase in the antioxidant enzymes can be linked to the induction of ROS production in *D. melanogaster* likely caused by oral exposure to an imidazole derivative. This finding seems consistent with Oboh *et al*. (2018), in which dietary inclusion of Garcinia kola (GK) seed increases antioxidant enzymes. The increased MDA level in *D. melanogaster* may indicate a state of ensuing oxidative stress. This may explain the reason for the increased level of antioxidant enzymes as an adaptive response mechanism to oxidative assault by exposure to the imidazole derivative. Adaptive response has been recognized in *D. melanogaster* (Abolaji *et al*., 2017) and it is the ability of an organism to resist damage to cellular tissues induced by exposure to toxic agents. In addition, such an increase in the activity of antioxidant enzymes as an adaptive response to cytotoxic agents in *D. melanogaster* has been reported previously (Bayliak *et al.,* 2018). This result corroborates previous reports by Liu *et al*., 2019 where imidazole-based compounds enhanced fish antioxidant capacity.

4.4 Effect of imidazole derivative on oxidative stress markers

4.4.1 Nitric oxide

Nitric oxide is a diffusible gas and a short-lived molecule that rapidly recombines to produce stable metabolites such as nitrite and nitrate (Gupta *et al*., 2019). It is also known to be a pro-inflammatory mediator because of its capacity to react with superoxide anion to form a harmful anion, leading to oxidative related damage (Abolaji *et al*., 2018). The NO radical plays an important role as a physiological messenger, but its accumulation could result in a reaction with superoxide anion to generate a nitrite anion, thereby causing damage to tissues. NO is formed from L-arginine by nitric oxide synthase, which exists in several isoforms such as *i*NOS, *n*NOS and *e*NOS. Overproduction of NO can occur in different pathological conditions such as ischemia, endotoxic shock and inflammation (Friederic *et al*., 2009). Studies have indicated that NO functions in the immune response of flies to parasites and pathogens (Eleftherianos *et al*., 2014). Though there is no information on the role NO plays in the physiology of adult flies, NO production has been reported to be more narrowed to the brain of adult flies (Oboh *et al.*, 2017). In this present study, there is a non-significant increase in NO level when compared to control. Thus, imidazole administration did not affect the nitric oxide level of flies at the administered doses. These findings could also imply that the non-significant increase in NO levels protects antioxidant enzymes and has the potential to prevent inflammation in *D. melanogaster*.

4.4.2 Lipid peroxidation

Lipid peroxidation can be measured by the level of malondialdehyde (MDA), a mutagenic end-product of lipid peroxidation (Mishra and Mishra 2017). MDA is also among the common markers of oxidative stress, and it is also involved in toxicity processes that result in cell death (Marisa *et al*., 2012). Oxidative stress may induce the peroxidation of polyunsaturated fatty

acids. Lipid peroxidation plays a major role in the pathophysiology of diseases such as infectious nutritional and inflammatory diseases (Gentile *et al.,* 2017). In this study, the administration of imidazole did not have any significant effect on the MDA level of *D. melanogaster* compared to the control. This may be as a result of improved antioxidant response by the flies as imidazole exposure led to increases in the level of GSH, GPx, GST and CAT as well as TAC.

4.4.3 Protein carbonyl level

Protein carbonyl is known as one of the most important and commonly used oxidative stress markers (Hecker & Wagner, 2018). Protein carbonyl is a result of protein oxidation. Protein oxidation is involved in the regulation of physiological events. It is also known to function in pathophysiological events of aging as well as damage to tissues (Gonos *et al.,* 2018). In the present study, our data suggest that the effects of the imidazole derivative on protein carbonyl might be related to oxidative stress. In the present study, the administration of the imidazole derivative at lower doses of 20 and 50mg of IMZ/kg diet did not significantly affect protein carbonyl, but at the highest concentration (100 mg of IMZ/kg diet), a significant increase was observed in protein carbonyl level. This could indicate that *D. melanogaster* is experiencing oxidative stress. The results may also suggest that imidazole treatment at the highest dose (100 mg of IMZ/kg diet), temporarily caused toxicity by elevating the protein carbonyl level of *D. melanogaster* compared with the control. However, the toxicity caused by imidazole exposure was ameliorated following 5 days post-cessation of imidazole treatment.

4.4.4 DNA fragmentation

DNA fragmentation may be used to assess the extent of oxidative stress. The administration of the imidazole derivative at lower doses of 20 and 50 mg of IMZ/kg diet did not significantly alter the level of DNA fragmentation, but at the highest concentration (100 mg of IMZ/kg diet),

a significant increase was observed in the level of DNA fragmentation. This may indicate ensuing oxidative stress in *D. melanogaster*. The results may also suggest that imidazole treatment at the highest dose (100 mg of IMZ/kg diet) temporarily caused toxicity by elevating the level of DNA fragmentation level of *D. melanogaster*, and this fact agrees with the previous report by Zeferino *et al*. (2020). However, the toxicity caused by imidazole exposure was ameliorated following 5 days post-cessation of imidazole treatment.

4.5 Effect of imidazole derivative on kynurenine level

The kynurenine pathway involves the oxidative breakdown of the amino acid L-tryptophan to kynurenine and other metabolites such as kynurenic acid and quinolinic, amongst others. The end-product of the route is NAD^+. The oxidative degradation of l-tryptophan is mediated by the enzyme indolamine-2, 3-dioxygenase (IDO-1). This enzyme can be induced by inflammation and oxidative stress. For example, during adaptive immune response, pro-inflammatory cytokines are produced, leading to an increase in the activity of indolamine-2, 3-dioxygenases (IDO-1) (Adeyemi *et al.,* 2019). In addition, oxidative stress has been linked with an increase in the activity of IDO-1 (Adeyemi *et al.,* 2019). Increased activity of IDO-1 leads to the activation of the kynurenine pathway, which is thought to play a significant role in several neurological disorders (Sas *et al*., 2018; Adeyemi *et al.,* 2019). In this study, administration of an imidazole derivative did not affect the level of kynurenine differently from the control except in the group fed with 50 mg IMZ/kg diet (Figure 13A), for which a decrease ($p<0.05$) in kynurenine level was observed. The finding may further support the absence of oxidative stress. This is plausible if we consider the kynurenine result alongside the data on oxidative stress markers. The oral exposure to the imidazole derivative might not have caused oxidative stress, possibly as a result of improved antioxidant responses by the flies.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The findings from this study revealed that the imidazole derivative did not cause strong toxicological effects as the antioxidant enzymes were increased, likely as an adaptive response to oxidative stress consequent upon exposure to the test compound. The imidazole derivative exposure in flies led to mild alteration of oxidative stress markers which was more prominent at the highest concentration of the test compound. However, the mild oxidative-related toxicity was restored to normal 5 days post-cessation of imidazole, suggesting a reversal of the effect. Moreover, the survival rate of flies after exposure to the imidazole derivative was well above 85% across the treatment groups, thus reinforcing the fact that the test compound might have caused strong toxicity in *D. melanogaster* at the doses used in this study.

5.2 Contribution to Knowledge

This study contributed the following:

a) Five days of exposure to the imidazole-based compound 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2 had no effect on the survival rate of melanogaster.
b) The test compound 1-(1,4,5-triphenyl-1*H*-imidazol-2-yl) naphthalen-2-ol) altered the redox parameters of *Drosophila melanogaster* in differentially.
c) The imidazole derivative 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2 might have caused a mild oxidative related toxicity in *D. melanogaster*, but this effect was resolved after 5 days post-cessation of imidazole treatment.

5.3 Recommendation

Based on the findings of this research work, it is recommended that

a) Additional research into the safety and or toxicity of the imidazole derivative 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2-ol in other animal models is recommended to advance the therapeutic prospects of the test compound, 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2-ol as an alternative anti-parasite agent.
b) Future research should focus on the molecular mechanism of action of this imidazole derivative 1-(1,4,5-triphenyl-1*H*-imidazole-2-yl) naphthalen-2-ol in animal models.

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APPENDIX 1

Timeline for project work

Time	Description of activity
Week 1-4 15th June- 13th July, 2020	**Hands on *drosophila* training**
Week 5-7 20th July-10th August, 2020	**Administration of imidazole to *drosophila***
Week 8-16 17th August -5th October,2020	**Biochemical assay**
Week 16-24 12th October-7th December, 2020	**Analysis of data, interpretation of data and drawing conclusion**
Week 24 – 14th December 2020-	**Writing and submission of dissertation**

APPENDIX 2

Preparation of solutions and buffers

Reagents for lipid peroxidation

30% Trichloroacetic acid (TCA)

4.5 g of TCA (CCl_3COOH) was dissolved in distilled water and made up to 15 mL with the same.

0.1 M Hydrochloric acid (HCl)

13 µl of concentrated HCl (36.5-38%) was added to distilled water and the volume made up to 15 mL with the same.

0.75% Thiobarbituric acid (TBA)

0.1125 g of TBA was dissolved in 0.1 M HCl and made up to 15 mL with the same. Dissolution was aided by stirring in a hot water bath (50˚C).

0.15 M Tris-KCl buffer (pH 7.4)

0.559 g of KCl and 0.909 g of Tris base were dissolved in 45 mL of distilled water, the pH was then adjusted to 7.4 with HCl and the volume made up to 50 mL with the same.

Reagents for reduced glutathione

GSH stock solution preparation

40 mg of GSH was dissolved in 0.1 M phosphate buffer, pH 7.4 and made up to 100ml with the same.

Phosphate buffer (0.1 M, pH 7.4)

Dipotassium hydrogen phosphate trihydrate (2.098 g) and potassium dihydrogen phosphate (0.791 g) were dissolved in 120 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 150 ml with distilled water.

Ellman's Reagent

60 mg of Ellman's reagent was in dissolved in 0.1 M phosphate buffer, pH 7.4 and made up to 150 ml with the same.

Sulphosalicylic Acid (4% solution)

0.8 g of sulphosalicylic acid was dissolved in 20 ml of distilled water.

Reagents for Glutathione transferase

Reagents

1-Chloro-2,4-dinitrobenzene (20 mM)

16.85 mg of 1-chloro-2,4-dinitrobenzene (CDNB) was dissolved in 5 ml of absolute ethanol.

Reduced Glutathione (0.1 M)

30.73 mg of glutathone (GSH) was dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.5).

Phosphate buffer (0.1 M, pH 6.5)

Dipotassium hydrogen phosphate trihydrate (0.381 g) and potassium dihydrogen phosphate (1.134 g) were dissolved in 90 ml of distilled water, the pH adjusted to 6.5 and the volume made up to 100 ml with distilled water.

Reagents for glutathione peroxidase activity

Sodium azide *(10* mM*)*

3.25 mg of sodium azide was dissolved in 50 mL of distilled water.

Reduced glutathione (4 mM)

12.3 mg of GSH was dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4.

Hydrogen peroxide (2.5 mM)

14 µl of 30% hydrogen peroxide was added to distilled water and the volume made up to 50 ml with the same.

Trichloroacetic acid (10%)

2 g of TCA was dissolved in distilled water and the volume made up to 20 ml with the same.

Dipotassium hydrogen orthophosphate (0.3 M)

4.11 g of $K_2HPO_4.3H_2O$ was dissolved in distilled water and the volume made up to 60 ml with the same.

Ellman's reagent (DTNB)

19.8 mg of DTNB was dissolved in 50 ml of 0.1 M phosphate buffer, pH 7.4.

Phosphate buffer (0.1 M, pH 7.4)

Dipotassium hydrogen phosphate trihydrate (1.399 g) and potassium dihydrogen phosphate (0.527 g) were dissolved in 90 mL of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

Reagents for nitric oxide

Griess Reagent

1 g of sulphanilic acid and 0.1g of N-(1-naphthyl) ethylenediamine dihydrochloride was dissolved in 5 mL of phosphoric acid H_3PO_4 and made up to 100ml. The solution was kept in the dark for 8hrs and the filtered and properly transferred into an amber bottle.

0.02 M sodium nitrate ($NaNO_2$)

13.8 mg of sodium nitrate was dissolve in distilled water and made up to 10mL. Serial dilutions of the stock was prepared and used as standard.

Reagents for protein determination

Biuret reagent

1.5 g of $CuSO_4.5H_20$ and 6 g of sodium potassium tartrate was dissolved in 500 mL of distilled water to give solution A. 30 g of NaOH was dissolved in 500 mL of distilled to give solution B.

Solution A was added to solution B slowly with continuous stirring and resulting solution transferred to non- litre standard flask and the volume made up to the marked level with distilled water. The solution was stored in a polythene bottle.

BSA stock solution (10 mg/ml)

1 g of BSA (Bovine Serum Albumin) was dissolved in a little quantity of distilled water and made up to 1000 cm^3.

Reagents for DNA fragmentation

Hypotonic lysis buffer at pH 8 (0.2% TritonX-100, 10 mM Tris, 1 mM EDTA).

Tris-EDTA (TE) buffer (10 Mm Tris-HCL at pH 8, 1 mM EDTA at pH 7.4).

10% Trichloroacetic acid (TCA)

5% Trichloroacetic acid (TCA)

Colorimetric solution (98 % V/V of 1.5g Diphenylamine (DPA) in 100 mL glacial acetic acid .98 % V/V 1.5 mL sulphuric acid, 0.5% VV acetylaldhyde)

Reagents for catalase

Potassium phosphate buffer (0.05 M pH 7.0)

0.05 g of phosphate buffer pellets was dissolved in 200 mL distilled water equivalent to 0.05 M.

0.059 M hydrogen peroxide (H_2O_2)

4.0 mL of stock solution of hydrogen peroxide was added to distilled water to make 100 mL solution equivalent to 0.059 M

Calibration curves

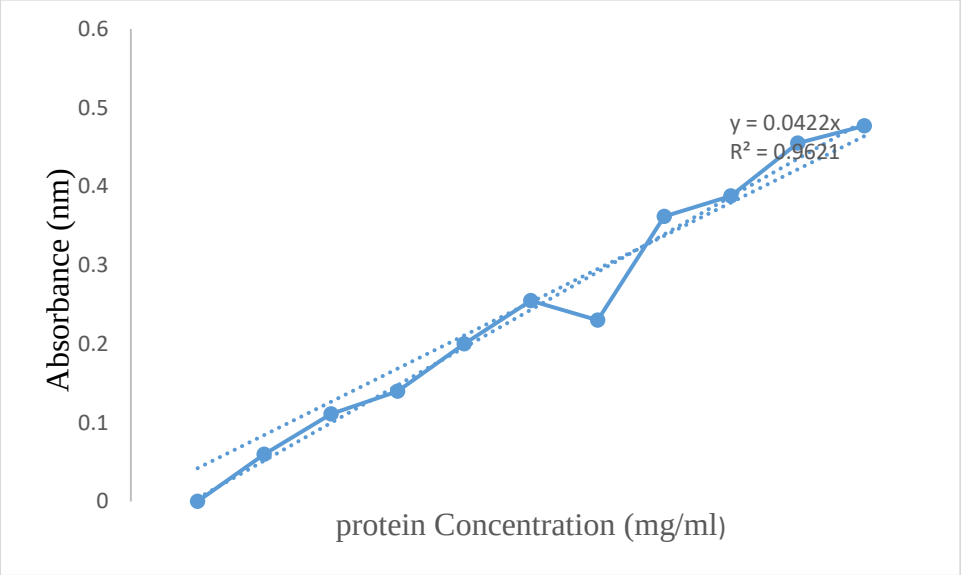

Figure 5.1: Standard curve for assay of protein concentration

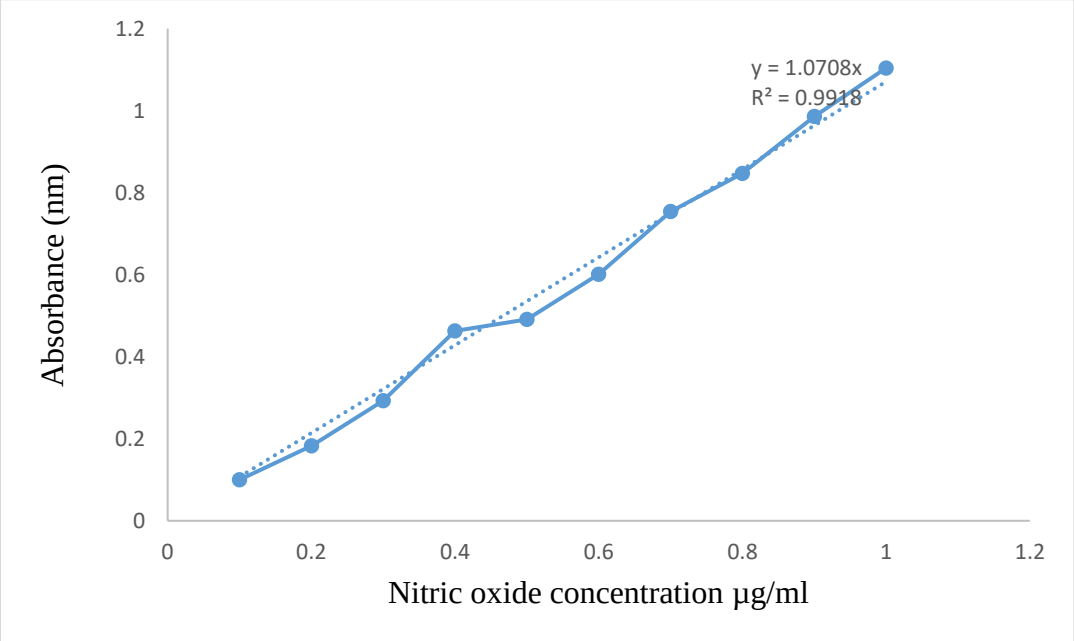

Figure 5.2: Standard curve for assay of nitric oxide

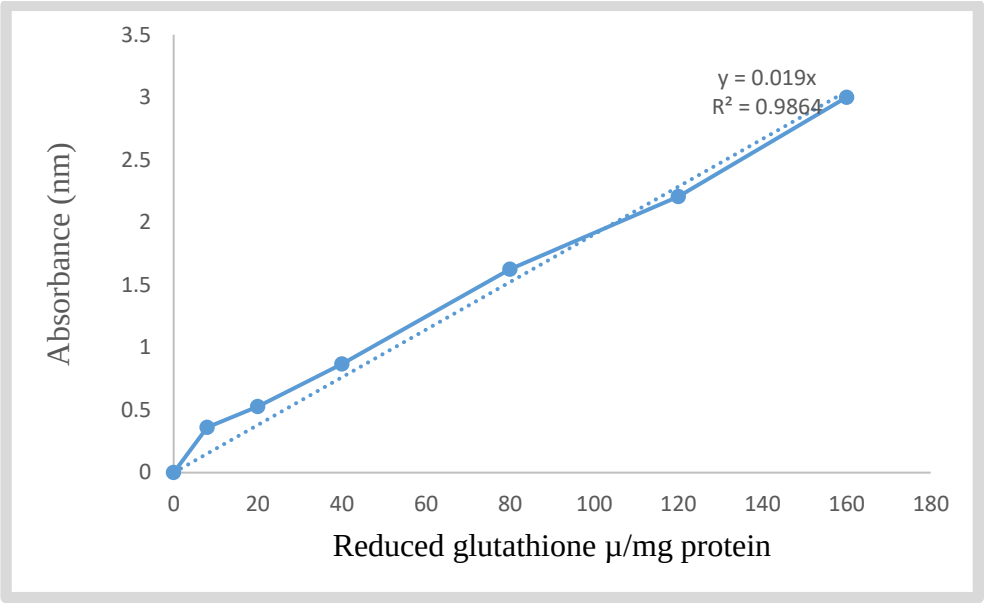

Figure 5.3: Standard curve for assay of reduced glutathione

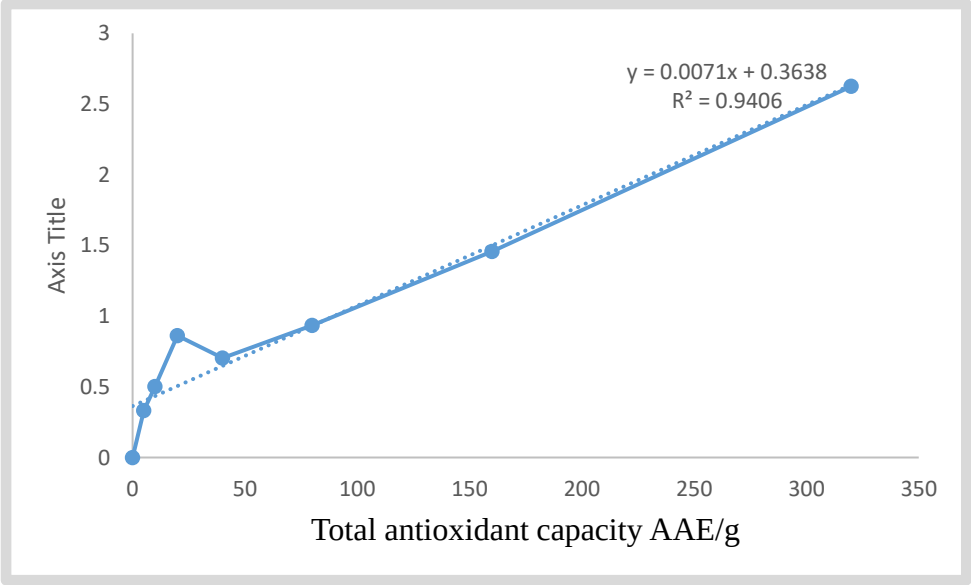

Figure 5.4: Standard curve for assay of total antioxidant capacity

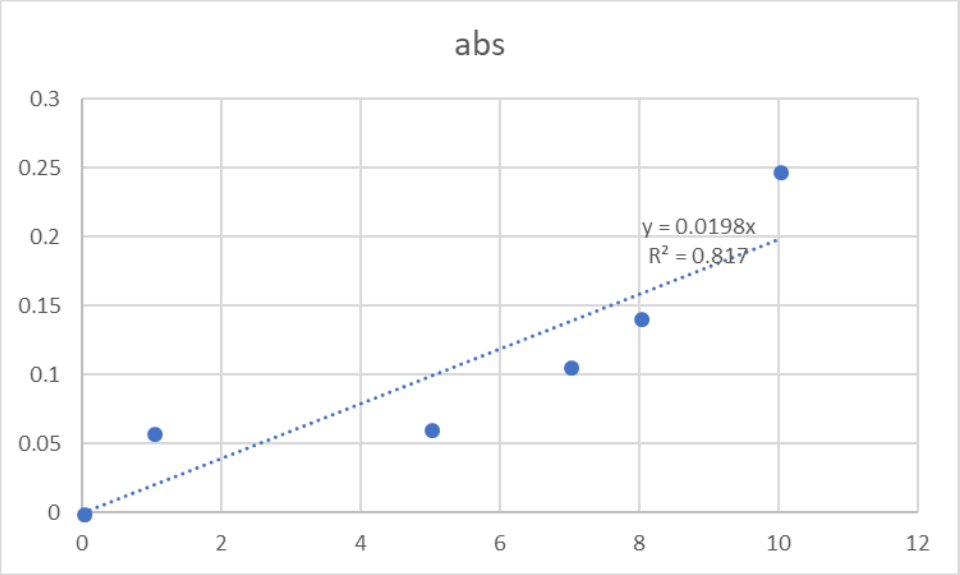

Figure 5.5: Standard curve for assay of kynurenine