

**ASSESSMENT OF REDOX STATUS AND THE EXPRESSION OF *P53* AND *PARP1*
GENES IN *DROSOPHILA MELANOGASTER* EXPOSED TO ISOMETAMIDIUM
CHLORIDE**

BY

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AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA**

July, 2023

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**DEPARTMENT OF BIOCHEMISTRY,
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ZARIA, NIGERIA**

July, 2023

DECLARATION

I declare that the work of this dissertation titled ‘Assessment of Redox status and the expression of *p53* and *PARP-1* genes in *Drosophila melanogaster* exposed to Isometamidium chloride,’ has been carried out by me in the Department of Biochemistry, Faculty of Life Sciences, Ahmadu Bello University Zaria. All information derived from literature has been duly acknowledged in the text and a list of references is provided. Furthermore, no part of this dissertation has been previously presented for any academic diploma or degree in this institution or elsewhere.

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Name

(Signature)

Date

CERTIFICATION

This dissertation entitled “ASSESSMENT OF REDOX STATUS AND THE EXPRESSION OF *P53* AND *PARP1* GENES IN *DROSOPHILA MELANOGASTER* EXPOSED TO ISOMETAMIDIUM CHLORIDE” by Shadrack Dangabar APOLLOS meets the regulations governing the award of the degree of Master of Science in Biotechnology of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentations.

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DEDICATION

I dedicate this work to God Almighty for the provision and sustenance throughout the period of this programme.

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ABSTRACT

Isometamidium chloride is a drug used in the prevention and treatment of Animal African Trypanosomiasis (AAT). However, several side effects have been reported in the use of this drug. This study was, therefore, designed to evaluate its ability to induce oxidative stress and DNA damage using *D. melanogaster* as a model organism. The LC₅₀ of the drug was determined by exposing the flies (1-3 days old of both genders) to six different concentrations (0.1 mg, 1 mg, 2 mg, 4 mg, 5 mg and 10 mg per gram of diet) of the drug for a period of seven days. The effect of the drug on survival (28 days), climbing behavior, redox status, oxidative DNA lesion, expression of *p53* and *PARP1* (Poly-ADP-Ribose Polimerase-1) genes after five days exposure of flies to 0.449 mg, 0.897mg, 1.794mg and 3.588 mg/g of diet was evaluated. The interaction of the drug *in silico* with p53 and PARP1 proteins was also evaluated. The result showed the LC₅₀ of isometamidium chloride to be 3.588 mg/g diet for seven days. Twenty eight (28) days exposure to isometamidium chloride showed a decreased percentage survival in a time and concentration-dependent manner. Isometamidium chloride significantly ($p < 0.05$) reduced climbing ability, total thiol level, glutathione-S-transferase and catalase activity. The level of H₂O₂ was significantly ($p < 0.05$) increased. The result also showed significant ($p < 0.05$) reduction in the relative mRNA levels of *p53* and *PARP1* genes. The *in silico* molecular docking of isometamidium chloride with p53 and PARP1 proteins of *D. melanogaster* and *Bos taurus* showed high binding affinity of -9.4Kcal/mol, -9.2Kcal/mol and -8.1Kcal/mol and -10.3Kcal/mol respectively. The results suggest that isometamidium chloride could be cytotoxic and a potential modulator of p53 and PARP1 proteins.

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LIST OF ABBREVIATIONS

8-OHdG - 8-hydroxydeoxyguanosine

AAT - Animal African Trypanosomiasis

AChE - Acetylcholinesterase

AgNPs - Silver nanoparticles

Apaf-1 - Apoptotic protease activating factor 1

Bax –Bcl-2-associated X protein

BSA - Bovine serum albumin

CAT - Catalase

CDKs - Cyclin-dependent kinases

cDNA - Complementary DNA

CDNB - 1-chloro-2,4-dinitrobenzene

C_q - Quantification Cycle

DNA - Deoxyribonucleic Acid

dsDB - Double Strand DNA Break

DTNB - 5,5'-dithio-bis-(2-nitrobenzoic acid)

EDTA – Ethylenediaminetetraacetic acid

EFVb-HAART – Efavirenz based- Highly Active Antiretroviral Therapy

ELISA - Enzyme-Linked Immunosorbent Assay

GPDH -Glycerol-3-phosphate dehydrogenase

GSH - Glutathione

GST - Glutathione-S-transferase

H₂O₂ - Hydrogen peroxide

HDM2 - Human Double Minute 2

IR - Ionizing Radiation

kDa - kilodalton

kDNA-Kinetoplast Deoxyribonucleic Acid

LC₅₀ - Lethal Concentration

MDM2 - Murine Double Minute 2

MMEJ - Microhomology-Mediated End Joining

NAD - Nicotinamide Adenine Dinucleotide

NaF - Sodium fluoride

NCBI - National Center for Biotechnology Information

O₂⁻ - Superoxide ion

OH⁻ - Hydroxyl ion

p21 - Cyclin-dependent kinase inhibitor

p53 - Tumor protein 53

p53R2 – Tumor protein 53 ribonucleotide reductase

PAR - Poly ADP-ribose

PARG - Poly ADP-ribose glycohydase

PMAIP1 – Phorbol-12-myristate-13-acetate-induced protein 1

PUMA - p53 upregulated modulator of apoptosis

qPCR - Real Time Polymerase Chain Reaction

RNA - Ribonucleic Acid

ROS - Reactive Oxygen Species

RT-PCR - Reverse Transcriptase Polymerase Chain Reaction

SDS-PAGE - Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

ssDB - Single Strand DNA Break

TNB - 2-nitro-5-thiobenzoic acid

TSH - Total thiol

UV - Ultraviolet

CHAPTER ONE

1.0 INTRODUCTION

Animal African trypanosomiasis (AAT) encompasses different wasting diseases caused by numerous species of unicellular flagellated protozoan parasites belonging to the genus *Trypanosoma*. The parasite is transmitted actively by tsetse fly (*Glossina*) from infected to uninfected hosts (Pan *et al.*, 2006). There are three important pathogenic trypanosomes *T.vivax*, *T.congolense* and *T. brucei* belonging to the Salivaria group, so called because their transmission to the vertebrate host is mainly through the infected saliva of the tsetse fly (Giordani *et al.*, 2016). There are several clinical signs shown by the disease, ranging from fever to neuropathology which may lead to death if left untreated (Rodrigues *et al.*, 2009). The disease has high economic and social impact in many areas where transmission occurs as it affects animal production and husbandry with Africa suffering the highest burden historically (Steverding, 2008).

Of the different control measures, chemotherapy and chemoprophylaxis seem to be the most widely used in animal trypanosomiasis control in enzootic countries. Currently, only six compounds are licensed (Diaminazene, Homidium, Quinapyramine, Suramin, Melarsomine, and Isometamidium), most dating back to the first half of the 20th century (Giordani *et al.*, 2016). All the currently used drugs have small therapeutic indices and can cause local irritation at the injection site, which make their use limited (Leach and Roberts, 1981; Giordani *et al.*, 2016).

Isometamidium chloride hydrochloride is a cationic phenanthridine synthesized by linking homidium with p-aminobenzamide, a moiety of diminazene (Giordani *et al.*, 2016). It was introduced in the 1960s for use in the treatment of *Trypanosoma* species in horses and other

ruminants and sold under different trade names; Samorin®, Trypamidium®, and Veridium® (Delespaux *et al.*, 2007; Giordani *et al.*, 2016). Although the drug is used mainly in the treatment of *T. congolense* and *T. vivax*, it can also be used against *T. evansi* but not when the infection has reached the central nervous system as the drug does not cross the blood-brain barrier (Giordani *et al.*, 2016). Depending on the dosage, the drug can be used for both curative and prophylactic purposes (Desquesnes *et al.*, 2013a). There are reports of side effects in the use of the drug ranging from muscle tremor, salivation, lacrimation, increased activity of the intestinal tract, convulsion, tachycardia, hyperaesthesia, laboured breathing and cell/tissue damage (Ali and Haroun., 1984; Schillinger *et al.*, 1985). These side effects have been associated with oxidative stress and DNA damage (Uttara *et al.*, 2009; Chatterjee and Walker, 2017). In case of DNA damage, p53 and PARP1 serve in stabilizing the genome (Mishra *et al.*, 2013).

p53 is a transcription factor coded by the *p53* gene which has been implicated as the frequently mutated gene in cancer patients. p53 is known for its anti-proliferative function in response to DNA damage, hereby ensuring genetic stability of the cells exposed to mutagens. The protein exists in an inactive state in normal cells due to the presence of its negative regulator Murine Double Minute 2 (MDM2) or Human Double Minute 2 (HDM2 in human) which on binding inactivates it and enhances its translocation from the nucleus to the cytosol where proteasome degrades it (Mishra *et al.*, 2013). However, on DNA damage (by ionizing radiation, application of cytotoxic drug or chemotherapeutic agents, oxidative stress etc.) p53 is phosphorylated by protein kinases which dissociates it from mdm2 and in turn leads to its activation (Mishra *et al.*, 2013). It serves the function of arresting cell growth by regulating the expression of *p21* gene responsible for arresting the cell cycle at G1/S phase and in a manner buys time for DNA repair proteins like P53R2 to fix the damage. In cells where the repair system fails, p53 regulates the

expression of apoptotic protein (Bax, Apaf-1, PUMA and PMAIP1) and ensures cell death thereby safeguarding against accumulation of defective cells (Mishra *et al.*, 2013).

Poly ADP-Ribose Polymerase(PARP1) is a nuclear protein, the first to be identified among the 17 PARP family of enzymes and it is encoded by the *PARP1* gene. It catalyzes the addition of ADP-Ribose to acceptor proteins using nicotinamide adenine dinucleotide (NAD⁺) as substrate where nicotinamide is generated as a byproduct (Ko and Ren, 2012; Kirsanov *et al.*, 2014). Poly ADP-Ribose (PAR) which is a polymer of ADP-Ribose is negatively charged. As such, its accumulation modulates PARP1 activity as well as its substrates. Consequently, controlling many important cellular functions such as DNA repair, transcriptional regulation, and cell death(Ko and Ren, 2012). The activity of PARP1 consumes a large amount of NAD⁺. However, the PAR is short-lived as it is degraded soon after it serves its purpose by poly ADP-ribose glycohydase - PARG (Koand Ren, 2012). The accumulation of PAR is a cytotoxic signal as the disruption of PARG proved to be embryonically lethal in mice and associated with apoptotic cell death in blastocysts(Koand Ren, 2012). PARP1 interaction with NAD⁺, DNA and core histone activate it and each route of activation results in different outcomes. PARP1 serves as the first responder that detects DNA damage and facilitates the choice of repair pathway. However, it has been shown that when PARP1 is up-regulated, Microhomology-Mediated End Joining (MMEJ) is increased causing genome instability (D'Amours *et al.*, 1999; Ko and Ren, 2012).Though there is no sufficient information on the relationship between p53 and PARP1, the thought that PARP1 function upstream of p53 exists where PARP1 serves as the first responder that detects DNA damage and facilitates the choice of repair pathway, while p53 initiates cell cycle arrest, DNA repair/apoptosis (Mishra *et al.*, 2013; Ko and Ren, 2012; Fischbach *et al.*, 2018).*Drosophila*

melanogaster is a suitable model organism for studies involving *p53* and *PARP1* genes as these genes are similar in to those in mammals (Jin *et al.*, 2000).

Drosophila melanogaster commonly known as fruit fly or vinegar fly, is a small dipteran which belongs to the family Drosophilidae. It has over time become the hub of genetic and biomedical research due to the advantages shown in its use as a research tool e.g. short life cycle, large number of offspring generation, easy handling and homology of its genes to humans and other organisms (Bier, 2005; Haudry *et al.*, 2020).

1.2 Statement of Problem

For centuries, Animal African Trypanosomiasis have imposed a great economic burden on agricultural development in Africa (Steverding, 2008) and chemotherapy and chemoprophylaxis represents the main control measure so far. However, many factors like local irritancy at injection site, small therapeutic indices and appearance of resistant parasites limit the use of the six trypanocide compounds available (Giordani *et al.*, 2016).

Isometamidium, diaminazene and homidium are the main compounds used in the treatment of African Trypanosomiasis (Bengaly *et al.*, 2018). The drug Isometamidium chloride has proven to be effective in the treatment of this disease with mild side effects (Raftery *et al.*, 2019). However, adverse effects like cell damage in rabbits and death of small fish have been reported (Ali and Haroun, 1984; Ardelli and Woo, 2001). Also, there was a report of some neurological reactions when the drug was used in the treatment of *T. evansi* in camels (Ali and Hassan, 1986).

These side effects have been linked to oxidative stress which may or may not have something to do with DNA damage/genotoxicity. With these reported side effects, the acceptability and utility

of this drug by farmers seems to be at stake which may consequently lead to economic loss for both farmers and the drug manufacturers.

1.3 Justification

At lower levels, reactive oxygen species (ROS) serve important functions such as messengers in redox signaling reactions and also as immune defense against pathogens, but in excess are deleterious as they can cause DNA damage (Chatterjee and Walker, 2017).

Continuous DNA damage harms mammalian cells and contributes to severe disease conditions like cancer, aging, and neurodegenerative diseases. To ensure genome stability through the entire life cycle of an organism, the living system naturally has systems created to mediate it. Two proteins, p53 (guardian of the genome) and PARP1 (caretaker of the genome) are known to play important roles in these processes (Fischbach *et al.*, 2018) and in cells where DNA damage occurs, *p53* and *PARP1* genes were found to be up-regulated (Ke *et al.*, 2019; Ahamed *et al.*, 2010).

Drosophila melanogaster has only one PARP which is corresponding to PARP1 (Tulin *et al.*, 2002) and Dp53 homolog of p53 in human (Yun Fan *et al.*, 2010), making the use of this fly as a model organism suitable. However, based on the literature available, no work has been done to evaluate the possible genotoxicity of Isometamidium chloride with respect to “redox status and expression of *p53* and *PARP1* genes in *D. melanogaster*. This study is set to evaluate if the side effects reported with the use of Isometamidium chloride could be associated with oxidative stress and DNA damage.

1.4 Aim

To evaluate the *in silico* effect of Isometamidium chloride on p53 and PARP1 proteins as well as its effect on redox status, *p53* and *PARP1* genes *in vivo* using *Drosophila melanogaster* flies

1.5 Specific objectives

- To evaluate survival, negative geotaxis and neurodegeneration of *D. melanogaster* exposed to Isometamidium chloride
- To evaluate oxidative DNA lesion and redox status of *D. melanogaster* exposed to Isometamidium chloride
- To evaluate the effect of Isometamidium chloride exposure on the expression of *p53* and *PARP1* genes in *D. melanogaster*
- To evaluate the effect of Isometamidium chloride on p53 and PARP1 proteins *in silico*

CHAPTER TWO

2. 0 LITERATURE REVIEW

2.1 Animal African Trypanosomiasis

Animal African Trypanosomiasis also known as Samore in Hausa, nagana derived from a Zulu word N'gana which means useless, is caused by *Trypanosoma* species *T. congolense*, *T. vivax* and, to a lesser degree, *T. brucei* spp. The widespread of the disease is observed more in the sub-Saharan region of Africa and transmitted by tsetse fly of the genus *Glossina*, which also transmits human infective trypanosomes (*T. brucei gambiense* and *T. b. rhodesiense*, the two known protozoan parasites that cause Human African Trypanosomiasis, HAT, or sleeping sickness)(Giordani *et al.*, 2016). The disease causes high economic losses through anemia, loss of weight and also affects reproduction. Cattle are known to be mostly affected however, other animals like dogs are also affected and the disease can be fatal if left untreated (CFSPH, 2018). Trypanosomiasis is a widespread disease of livestock in Nigeria and a major constraint to the rural economy (Majekodunmi *et al.*, 2013). However, Nigeria is yet to comprehensively approach the process of eradication as it is at the infancy stage of data gathering and processing (Isaac *et al.*, 2017).

2.1.1 Transmission

Tsetse fly (*Glossina* spp) is the main vector that transmits the protozoan parasites. However, different modes of the transmission of the parasite are classified as follows:

Mechanical transmission

This process is nonspecific as it takes place when a blood-sucking insect is actively taking a blood meal from an infected host and interrupted by various means such as defensive movement

from the host. This insect immediately lands on another host to continue its blood meal. When insects such as *Tabanidae* and *Stomoxys* first attempt to feed on blood, their mouthparts can contain small amounts of blood via capillary strength (Desquesnes *et al.*, 2013b). The time interval between the two feeds is important for a successful transmission as the parasite dies when the blood dries (Uilenberg, 1998). Insects such as *Culicidae*, *Ceratopogonidae* are as well thought to play an important role in mechanical transmission of *Trypanosoma* during a blood meal from an infected to a noninfected host. Experimentally, Other insects such as *Aedes aegypti*, *Ae. Argenteus*, and *Anopheles fuliginosus* have been used to successfully transmit *T. evansi* (Desquesnes *et al.*, 2013b; Gill, 1977). *T. vivax* has been established to be mechanically transmitted more than other trypanosome parasites because, unlike other trypanosomes, *T. vivax* does not multiply in the tsetse midgut, but remains confined to the insect proboscis, where it completes its short life cycle (Gardiner, 1989). Mechanical transmission is known to maintain the effective transmission of *T. vivax* and *T. evansi* in South and central America, North Africa and Asia as well (Uilenberg, 1998).

Mechanical transmission can occur through iatrogenic means i.e. during treatment or vaccination. For example, when needles or surgical instruments are used on different animals within a short interval of time not sufficient for the blood to dry (Uilenberg, 1998).

Cyclical transmission

This mode of transmission is solely via the vector *Glossina* spp taking a blood meal from an infected host, where the parasite (trypanosome) continues its cycle of development and multiply in the digestive tract until the metacyclic trypanosomes (metatrypanosomes) which are the infective form of the parasite emerged. The infective form of the parasite is found either on the biting mouthparts or the salivary glands. It takes about one to two weeks between blood meal

and the appearance of the metatrypanosomes. Once the metatrypanosomes emerged, the vector remains infective until it dies. When taking a blood meal, the fly pierce the skin of the host with its proboscis. Following the puncture of small blood vessels, a pool of blood is formed in the tissues and the fly injects its saliva to prevent the blood from coagulating. Cyclical transmission of the parasite to host takes place at this stage, where the saliva injected contains the metatrypanosomes (Uilenberg, 1998).

At the site where the infected saliva is injected, the matatrypanosomes begin to develop and multiply into mature bloodstream trypanosomes (trypomastigotes); which are released into the blood circulation through lymph nodes and lymph vessels. As a result of this development at the injection site, swelling or chancre may be noticed (Uilenberg, 1998).

In this phase of development, the parasite reproduces asexually by binary fission. It also feeds from the host's nutrients and excretes its waste into the host, hence, the pathology.

During blood meal, the fly ingest the trypomastigotes which develop into epimastigotes (long and slender) in the digestive tract and finally into the infective trypanosomes; metatrypanosome and the infection cycle continues (Uilenberg, 1998).

Other means of transmission

Vampire bat *Desmodus rotundus* has recently been established to serve as a host, reservoir, and biological vector of *T. evansi*. Vampire bats are usually infected orally when they feed on the blood of an infected prey (horses or cattle). Some bats as hosts of the parasite *T. evansi* may develop clinical symptoms and die at the early phase of the disease. In situations where there are survivors of infected bats, the parasite multiplies in the blood and in turn is found in the saliva of both bats that are chronically infected and those not showing any clinical symptoms. Those

infected bats by biting, can later transmit the parasite to other reservoir hosts, livestock, and even transmission within their colony (Desquesnes *et al.*, 2013a).

In some studies, transplacental infection with *T. equiperdum* and *T. brucei* has been demonstrated in horses (Gardiner & Mahmoud, 1992; Sina *et al.*, 1979). In a review on transplacental transmission of trypanosomes, vertical transmission of *T. evansi* has been established in several instances such as in calves and mice (Ogwu & Nuru, 1981).

2.1.2 Control

The control of any vector associated disease is classically achieved in two ways; vector and pathogen control. Other traditional means of controlling transmission such as distancing and separating animals exist and can be combined to prevent infection.

Vector control

Vector control has proven to be an effective means of reducing the pressure of trypanosome transmission being that the parasite is majorly tsetse-transmitted in Africa. Strategies like the use of insecticides, impregnated screen and insect sterilization techniques have been used in a limited livestock breeding area to control the transmission of the parasite (Cuisance, 1984). One of the typical traditional methods of controlling the vector and other biting insects that can mechanically transmit the parasite is by the use of smoke released by slow fire, since smoke is traditionally known to repel insects. Nets can also be used to protect livestock from biting insects but this is not often used because of the cost (Desquesnes *et al.*, 2013a). Another integrated alternative of vector control is the use of nets impregnated with insecticides which help to reduce biting insects/vector population (Desquesnes *et al.*, 2013a). The vampire bat has been shown to act as a vector, host and reservoir of *T. evansi* in Latin America. Thus, the control of vampire

bats have been an important part of surra control. In this method of control, the Japanese nethas been used to catch the vampire bats or used as a screen to protect livestock. In this case, the net is normally set up at night to create a screen between the animal farm and the bat colony refuge (forest area). Alternatively, the Japanese nets are used to catch a few bats. These few bats are then coated with drops of anticoagulant which contains excipient, such as lanolin, before they are released. Once these bats return to the colony, they spread the chemical to the entire colony by licking and contacts. Within a few days anticoagulant like chlorophacinone can kill the bats (Desquesnes, 2004).

Chemical control

Since the use of vector control strategies have shown to not be always sustainable and expensive when practiced in a large scale, the use of trypanocidal drugs for the prevention and treatment of this disease has been the most utilized in endemic areas, ensuring maximum effect at relatively low cost (Van den Bossche and Delespaux, 2011).

The chemical treatment and prevention of trypanosome infections currently relies solely on six compounds (Diaminazene, Homidium, Melarsomine, Suramin, Quinapyrimine and Isometamidium). These six compounds all have small therapeutic indices and cause local irritation at injection sites. More so, because most of these compounds were introduced back around the first half of the 20th century; they are over used and this led to rising reports of resistance in the field (Giordani *et al.*, 2016). Two compounds (Isometamidium and Quinapyrimine) are used for both treatment and prevention of these infections, but ever since the withdrawal of Quinapyrimine due to high cases of resistance, Isometamidium became the only compound used for chemoprophylaxis purpose (Uilenberg, 1998).

Isometamidium chloride: Isometamidium chloride has been used in the field for several decades and ever since its introduction in the 1960s, it has remained the only drug used for the prevention of Animal African Trypanosomiasis (AAT), after quinapyramine was withdrawn due to rising reports of problems associated with toxicity and resistance (Peregrine, 1994).

The drug isometamidium injected intramuscularly and intravenously for the purpose of prevention and treatment respectively, is used solely to treat and prevent *T. congolense* and *T. vivax*. Its efficacy against *T. brucei* spp. is less reported, but it can also be used against some *T. b. evansi* strains, but not when the parasite has reached the central nervous system as the drug cannot cross the blood-brain barrier (Giordani *et al.*, 2016). In cattle the drug is administered at a single doses of 0.25–1.0 mg kg⁻¹ for cure, and at doses of 0.5–1 mg kg⁻¹ for prophylaxis (Leach and Roberts, 1981). The dosage recommended for the treatment of *T. evansi* infection is between 1–2 mg kg⁻¹, but due to toxicity problems it is recommended not to exceed 0.5 mg kg⁻¹ in (Uilenberg, 1998; Desquesnes *et al.*, 2013a).

The plasma concentration of isometamidium was observed to reach its peak within 1 (one) hour of injection and fall more rapidly within the first week and more slowly thereafter (Kinabo, 1993; Eisler *et al.*, 1994). In a study conducted by (Eisler *et al.*, 1994), the half-life of the drug's plasma concentration was established to be approximately 25 days in cattle. However, in another study (Eisler, 1996) the elimination half-life was shown to be about 9-19 days. The elimination of isometamidium seems to be more quickly in sheep and goats than in cattle (Wesongah *et al.*, 2004). The kidney, liver, spleen and the injection site where the drug is released slowly into the plasma to exert its preventive effect are where the drug is known to accumulate more (Kinabo and Bogan, 1988). Because the time taken for isometamidium residues to get eliminated is much

longer when compared to diminazene, 30 days was established to be the withdrawal period before consuming any produce from the cattle treated with the drug (FAO, 1990). However, the withdrawal period is product-specific (Giordani *et al.*, 2016). Excretion of the drug is solely through bile and amounts in cattle milk are usually extremely low (Kinabo, 1993).

Isometamidium has an intrinsic fluorescence property through which its accumulation in the kinetoplast was noted (Wilkes *et al.*, 1995; Boibessot *et al.*, 2002) and shown to bind to it in an uncustomary sideways geometry (Dougherty and Waring, 1982). Its high affinity for the kDNA is thought to be its main mode of action as a trypanocide by cleaving kDNA-topoisomerase complexes, leading to the disruption of the minicircle network within the kinetoplast (Shapiro and Englund, 1990).

Though the drug was first introduced about half a century ago, it still shows good therapeutic potential. However, there are increasing reports of treatment failure (Kinabo and Bogan, 1988). Through *in vivo* testing of cloned trypanosome isolates obtained from the field, the resistant phenotypes were authenticated (Delespaux *et al.*, 2005). For *T. congolense* in particular, the mechanism of resistance to isometamidium was proposed to be attributed to diminished mitochondrial potential (Wilkes *et al.*, 1997).

In a study conducted, isometamidium showed superior treatment effect to melarsomine dihydrochloride though limiting side effects such as sweating and skin wrinkling were observed on administration of the drug in donkeys (Raftery *et al.*, 2019).

2.2 Oxidative Stress and DNA Damage

Based on the cause, DNA damage can be classified into two; endogenous and exogenous. DNA damage can also be classified based on the type of damage caused; single base alteration

(deamination, alkylation, mismatch base), double base alteration (pyrimidine dimers), chain breaks (single strand and double strand breaks), and cross linkage(Chakarov *et al.*, 2014). Endogenous DNA damage occurs when DNA chemically reacts with water (hydrolytic and oxidative reactions) and reactive oxygen species (ROS) that are found within the cell. On the other hand, exogenous DNA damage is caused by environmental factors like; ultraviolet (UV) and ionizing radiation (IR), alkylating agents, and crosslinking agents (Chatterjee and Walker, 2017).

Reactive oxygen species are chiefly generated from the electron transport chain in the process of cellular respiration and are also generated by catabolic oxidases, anabolic processes and peroxisomal metabolism (Henle and Linn, 1997). At lower levels, ROS serve some important biological functions including fighting against pathogens, but are dangerous to cells at higher levels as they can cause DNA damage (Chatterjee and Walker, 2017). The negative effects of ROS are usually moderated in the cell through restriction of respiration in the mitochondria, formation of DNA complexes with histone proteins and actions of antioxidant enzymes (superoxide dismutase, catalase, and peroxiredoxin) in quenching overwhelming levels of ROS in the cell (Mates and Sanchez-Jimenez, 1999). In spite of the regulation by the cell to lower the levels of ROS, developments of diseases like cancer and diabetes are found to be associated with higher levels of ROS (Giacco and Brownlee, 2010; Liou and Storz, 2010).

Superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) are the most marked ROS (Tropp, 2011). Hydroxyl radical $\cdot OH$ is the most reactive among the three most marked ROS. It is produced as a byproduct of a reaction called Fenton's reaction when H_2O_2 reacts with Fe^{2+} . The radical is known to damage DNA, proteins and lipids (Imlay *et al.*, 1988; Dizdaroglu *et al.*, 1991). $\cdot OH$, an electrophilic radical, is capable of reacting with the DNA

bases by increasing their double bonds, attacking sugar moieties in their surroundings and attracting the hydrogen atom on their methyl groups (Breen and Murphy, 1995; Winterbourn, 2008).

Mammalian cells are constantly harmed by genotoxic stress causing different pathological conditions such as cancer, aging, and neurodegenerative diseases. Out of the several responses and repair mechanisms that evolved via evolution to ensure the integrity of the genome and physiological stability, p53 (guardian of the genome) and PARP1 (caretaker of the genome) are two proteins that play key role in these processes in mammals (Fischbach *et al.*, 2018). PARP1 serves as the first responder that detects DNA damage and facilitates the choice of repair pathway, p53 initiates cell cycle arrest, DNA repair/apoptosis (Koand Ren, 2012; Mishra *et al.*, 2013).

2.3 p53

p53 was first identified in the year 1979 and the *p53* gene was first cloned from mice by Peter Chumakov in 1982 (Chumakov *et al.*, 1982), while in 1984 the human *p53* gene was cloned (Matlashewski *et al.*, 1984). It was first thought to be an oncogene but finally characterized as a tumor suppressor by Bert Vogelstein in 1989 (Baker *et al.*, 1989).

The protein p53 which was first described from identified in mice, was so called because it runs as a 53-kilodalton (kDa) protein on SDS-PAGE. While its actual molecular mass based on the amino acid residues is 43.7 kDa, it appears heavier on SDS-PAGE due to the large number of proline residues in the protein that makes its migration slower (Ziemer *et al.*, 1982). This same feature is observed with p53 from different species, like humans, rodents, frogs, and fish (Mishra *et al.*, 2013).

p53 gene in humans is located on the short arm of chromosome 17 (Kern *et al.*, 1991). The human *p53* protein is a nuclear protein encoded by a 20-kb gene consisting of 11 exons and 10 introns (Lamb and Crawford, 1986). In vertebrates, exons 2, 5, 6, 7 and 8 show higher degree of conservation in most cases, but in invertebrates, the sequence found only shares distant similarity to mammalian *p53* (May and May, 1999). A common polymorphism indicating higher cancer susceptibility in humans is the substitution of arginine for proline at codon 72, but this school of thought has been conflicting in some instances (Mishra *et al.*, 2013). In a study conducted in the year 2011, the result showed no significant relationship between *p53* codon 72 polymorphisms and both colorectal and endometrial cancer risk (Wang *et al.*, 2011; Jiang *et al.*, 2011).

p53 is a transcription factor that regulates the expression of many genes so as to initiate cell cycle arrest, DNA repair or apoptosis in cases where DNA damage seems irreversible. In normal cells, the protein is inactivated by binding of a protein called MDM2 (Mishra *et al.*, 2013). MDM2 on binding to *p53* inactivates it and facilitates its transportation out of the nucleus to the cytosol where it is degraded by proteasomes (Mishra *et al.*, 2013). In stressed cells, *p53* dissociates from MDM2 through phosphorylation by protein kinases and as a result is activated (Harms and Chen, 2005). When *p53* is activated, it binds to the DNA and initiates the expression of several genes. In particular, is the *p21* gene (Mraz *et al.*, 2009). *p21* arrest cell cycle by binding to G1-S/CDK (CDK2) and S/CDK complexes, which are key molecules in the progression of cell cycle from G1-S phase. In cases where damaged cells crossed the *p21* G1-S phase checkpoint, *p53* initiates the expression of Apaf1 and Bax. Bax induces the release of Cytochrome c, which together with Apaf1 acts as binding site for Caspase 9. Caspase 9 induces apoptosis (Strachan and Read, 1999).

2.4 PARP1

Poly(ADP-ribose) polymerase1 (PARP1) is the most characterized enzyme among the 17 members belonging to the Poly(ADP-ribose) polymerases (PARPs) or ADP-ribosyltransferases (ARTs) family of enzymes expressed in humans (Gupte *et al.*, 2017). The enzyme is well known for its ribosylation activity where it utilizes NAD^+ as a donor and catalyzes the transfer poly ADP-ribose (PAR) to receptor proteins in a process called PARylation (Gibson and Kraus, 2012). The enzyme is activated on binding to single-strand and double-strand DNA breaks (ssDB and dsDB respectively). Its binding is necessary as it initiates the process of PARylation which is key for DNA repair by assembling and activating multiprotein complexes required in DNA repair (Palazzo and Ahel, 2018).

PARP1 found in eukaryotes is multifunctional and highly conserved. From its structural characterization, it is shown to have three main domains: DNA binding domain (DBD), Automodification domain and Catalytic domain which together moderate the response of the protein to different kinds of DNA damage. These functions include: repair of ssDB, dsDB, bulky lesions on DNA, stabilization of replication fork and chromatin modification (Ray and Nussenzweig, 2017).

2.5 *Drosophila melanogaster*

Since 1910 when Thomas Hunt Morgan introduced the fruit fly (*Drosophila melanogaster*) as a model organism for research, the fly has been used for over 50 years to study genetics where the results obtained paved ways to discover different biological systems and subsequently used to study human diseases and therapeutic strategies (Bellen *et al.*, 2010; Pandey and Nichols, 2011). Recently, *Drosophila* has been shown to serve as a model organism in studying toxicity (Ong *et*

al., 2015). Biomedical research with this organism is easier since the entire genome of the fly has been sequenced; the sequence has about 60% homology to that of humans and about 75% of the disease associated genes in humans have a homolog in the fly. Several advantages offered by the use of the fly which include their small size (2-3mm), short generation time, powerful genetic tool and easy/inexpensive way to culture them in the laboratory have made *Drosophila* to be one of the leading model organisms for education and biomedical research (Allocca *et al.*, 2018).

2.5.1 Life cycle of *Drosophila*

Drosophila melanogaster is a holometabolous insect belonging to the order Diptera and class Insecta. As shown in Figure 2.1, the life cycle of the fly progresses from egg laying by the adult female fly after mating, to larva, pupa and finally the emergence of the adult fly. The entire life cycle through the four stages (egg, larva, pupa and adult) of development takes about 10-12 days at 25°C (Ong *et al.*, 2015). After eggs are laid in the food, the embryogenesis established by two morphogens (*bicoid* and *dorsal* genes) takes place within or less than 24 hours. These two proteins; bicoid and dorsal are expressed and diffused across the embryo in gradients to form the anterior-posterior axis and the dorso-ventral axis respectively (Allocca *et al.*, 2018). The varying levels of each protein will activate the transcription of specific cascades of genes including *gap genes*, *pair-wise genes*, *segment polarity genes*, and *hox genes* that will divide the embryo into segments, regions, and eventually structures (Allocca *et al.*, 2018). Upon the completion of embryogenesis, the first instar larva emerges and begins massive feeding for 4 days which is not just important for growth but also for storage purposes to help sustain the larva through metamorphosis. Through a process called molting (shedding of exoskeleton) controlled by three important hormones: ecdysone (master regulator of the developmental transitions), juvenile and the prothoracicotropic hormone, the larva grow through three instar larval stages (first, second

and third instar larvae) before the final molting into pupa (Allocca *et al.*, 2018). Towards the end of the third instar larva stage, it is called ‘wandering larva’ so called because the larva leaves the food and wanders about in search of a place to pupate. During the pupa stage which takes about four days before the adult fly eclose, the animal feed through a process called developmental-autophagy, a “self-eating” mechanisms that signals for the breakdown of the stored fat deposited during the larval stage to

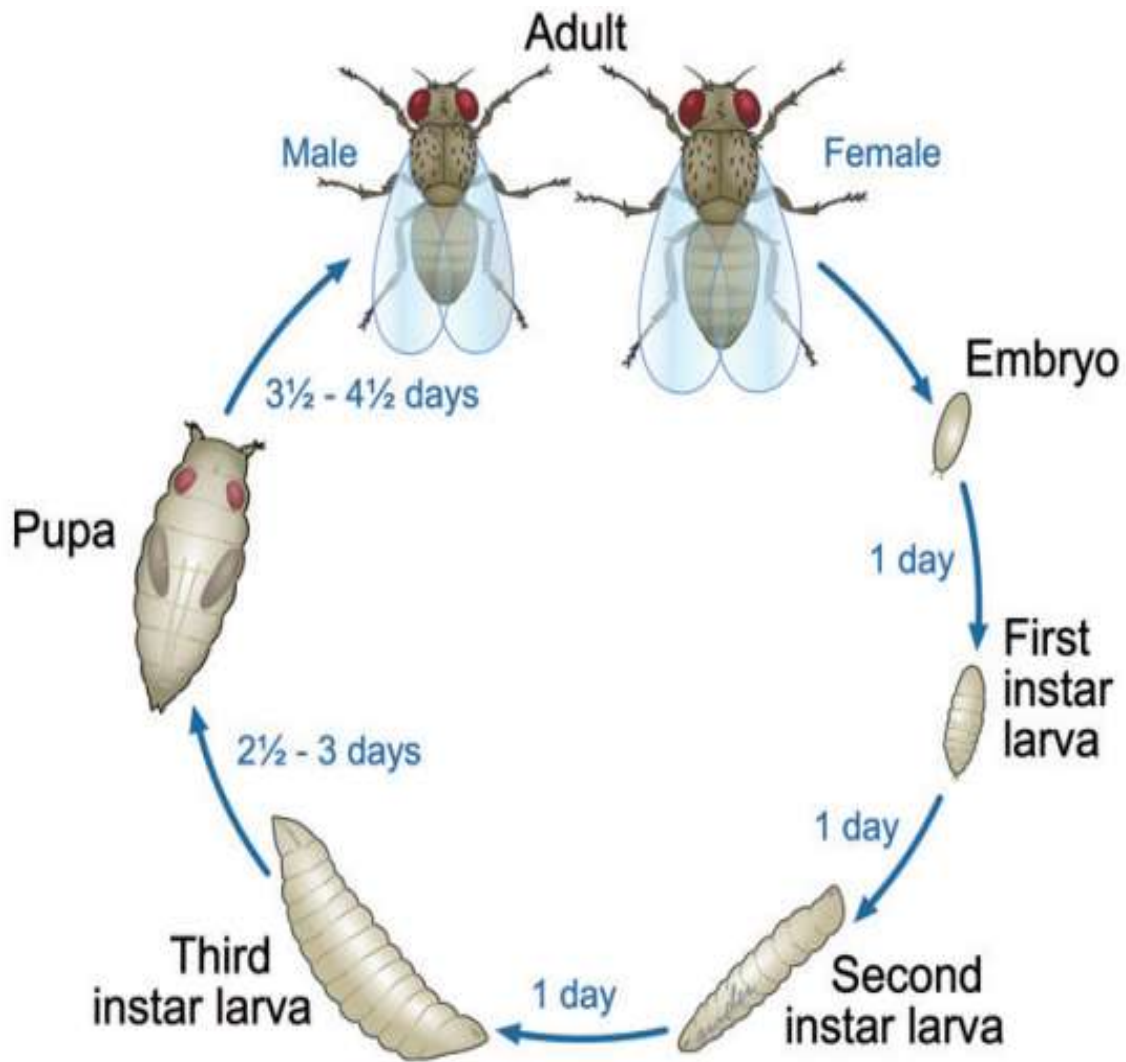


Figure 2. 1. Life cycle of *Drosophila melanogaster* (Ong *et al.*, 2015)

provide nutrients required by the developing organism since it cannot eat from the external environment(Allocca *et al.*, 2018).

2.5.2 Sex of the Fly

The adult female flies are usually bigger than the adult male flies with the females weighing about 1.4 mg and males about 0.8 mg (Ong *et al.*, 2015). Basically, the females and males are distinguished physically due to the presence of black patch on the males' abdomen which is not usually noticed in the newly emerged flies. The males also have hair-like structures on their first leg and around the reproductive parts used to attach to the female during mating (Va *et al.*, 2009). Normally, in less than 24 hours after eclosion, the females are ready to mate and can lay about a hundred eggs daily. The complete life span of the adult flies is about 40-60 days (Ong *et al.*, 2015).

D. melanogaster have four pairs of chromosomes, where the first (sex chromosomes) is either X and Y depicting a male, or X and X depicting a female. The Y chromosome is very small and carries very few genes. Of the remaining chromosome pairs: second, third and fourth, the fourth is the smallest among them and less commonly manipulated because of the difficulty to insert foreign genes (Allocca *et al.*, 2018). The knowledge about the fly's genetic makeup and the fact that the entire genome has been sequenced, makes it easier for scientists to manipulate the fly genome using different techniques (Allocca *et al.*, 2018).

2.5.3 Drosophila as a Model in Toxicology Study

Even with the increase in the *in vivo* nanomaterial toxicity studies, many questions such as what the long-term exposure of a toxicant could cause and how or what exactly the toxicant's molecular mechanism of toxicity is, still remain unanswered. Many *in vivo* models are expensive and raise ethical concerns (Ong *et al.*, 2015). Considering the advantages such as; short generation time, unique developmental stages, completely known genome sequence and physiological similarities with humans offered by *Drosophila melanogaster*, the European

Centre for the Validation of Alternative Methods recommendations indicated it as an ideal model organism to study nanomaterial-mediated toxicity (Ahamed *et al.*, 2010).

One of the best and direct ways to check for toxicity in *Drosophila* is through the evaluation of survival and as shown by Abolaji *et al.*(2019) the survival and life span of flies exposed to different concentrations of Sodium fluoride (NaF) were reduced when compared to the control group. In this same study, NaF was shown to disrupt the activities of antioxidant enzymes and increase the levels of reactive oxygen species (ROS). However, on treatment with resveratrol, those effects were ameliorated.

Negative geotaxis (climbing) is an innate escape response where flies ascend the wall of a cylinder after being tapped to its bottom. *Drosophila* innately tends to move opposite to the earth's gravitational vector when disturbed in a certain manner (Gargano *et al.*, 2005). The ability of the fly to climb against gravity is naturally affected by aging or exposure to toxins which cause neurodegeneration. Hence, negative geotaxis assay is being used to study aging, neurodegeneration and toxicity in *Drosophila*(Miller and Keller, 1999; Abolaji *et al.*, 2015).

Recently, an easy and reliable method of studying acute toxicity using *Drosophila* as a model organism was shown where LC₅₀ was determined by exposing *Drosophila* to different concentrations (10-1200mg/10g diet) of Efavirenz-based highly active antiretroviral therapy (EFV_b-HAART, Efavirenz/Lamivudine/Tenofovir) for seven (7) days (Iorjiim *et al.*, 2020). Furthermore, the flies from the Iorjiim *et al.*, (2020) study after LC₅₀ determination, were exposed to varying concentrations (100%, 50%, 25%, and 12.5% of LC₅₀) of EFV_b-HAART where the results showed increased mortality, locomotor deficits and reduced reproductive capacity in *Drosophila melanogaster* when compared to the control group.

Another approach to studying toxicity in an organism is through assessing genotoxicity and *Drosophila* has shown to be a good model in assessing acute and/or chronic genotoxicity since it has a short lifespan, high homology to humans and the whole genome has been sequenced (Ong *et al.*, 2015). One important molecule in DNA damage repair, cell cycle and apoptosis is p53 (Marcel *et al.*, 2011). The expression of p53 and p38 was found to be up-regulated upon Silver nanoparticles (AgNPs) ingestion during the third-instar larval stage, indicating the effects of AgNPs on DNA integrity and cell viability (Ahamed *et al.*, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemical reagents used in this study were of molecular analytical grade. Isometamidium chloride (Veridium®, LOT: 310A1), 1-Chloro-2,4-dinitrobenzene, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (Sigma Aldrich), and TRIzol® (Invitrogen).

3.1.2 Equipment and Apparatus

Centrifuge (Eppendorf), ELISA plate reader, fly vials, 0.5 ml centrifuge tube and piston, ELISA Microplate Reader (Apex Scientific, South Africa), Fly vials, Rectangular cylinder, Pipette (Eppendorf), qPCR Thermal cycler (Biorad), SpectraMax plate reader (Molecular Devices, USA), UV-Spectrophotometer (Unico) and Weighing balance.

3.1.3 Collection, and Maintenance and Fly grouping

D. melanogaster used in this study were obtained from *Drosophila* and Neurogenetic Laboratory Department of Zoology, Ahmadu Bello University Zaria and maintained at room temperature in fly vials. Flies were maintained at room temperature under 12h dark/light cycle.

With each groups in three replicates, flies were grouped as follows:

Group 1: Thirty (30) flies exposed to 0.000 mg (12.5% LC₅₀) Isometamidium chloride per g diet.

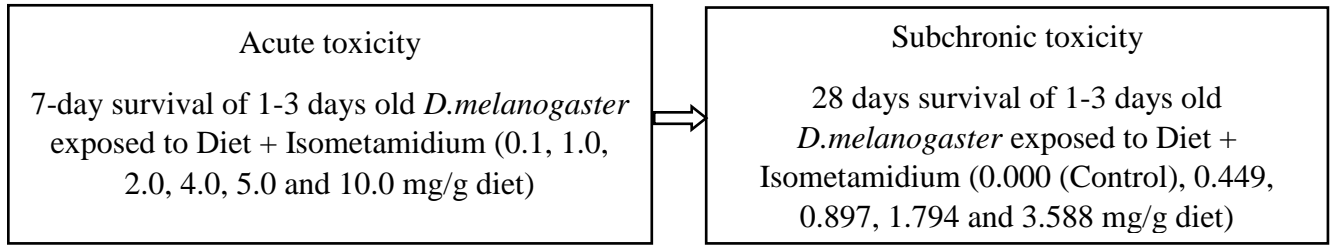
Group 2: Thirty (30) flies exposed to 0.449mg (12.5% LC₅₀) Isometamidium chloride per g diet.

Group 3: Thirty (30) flies exposed to 0.897mg (25% LC₅₀) Isometamidium chloride per g diet.

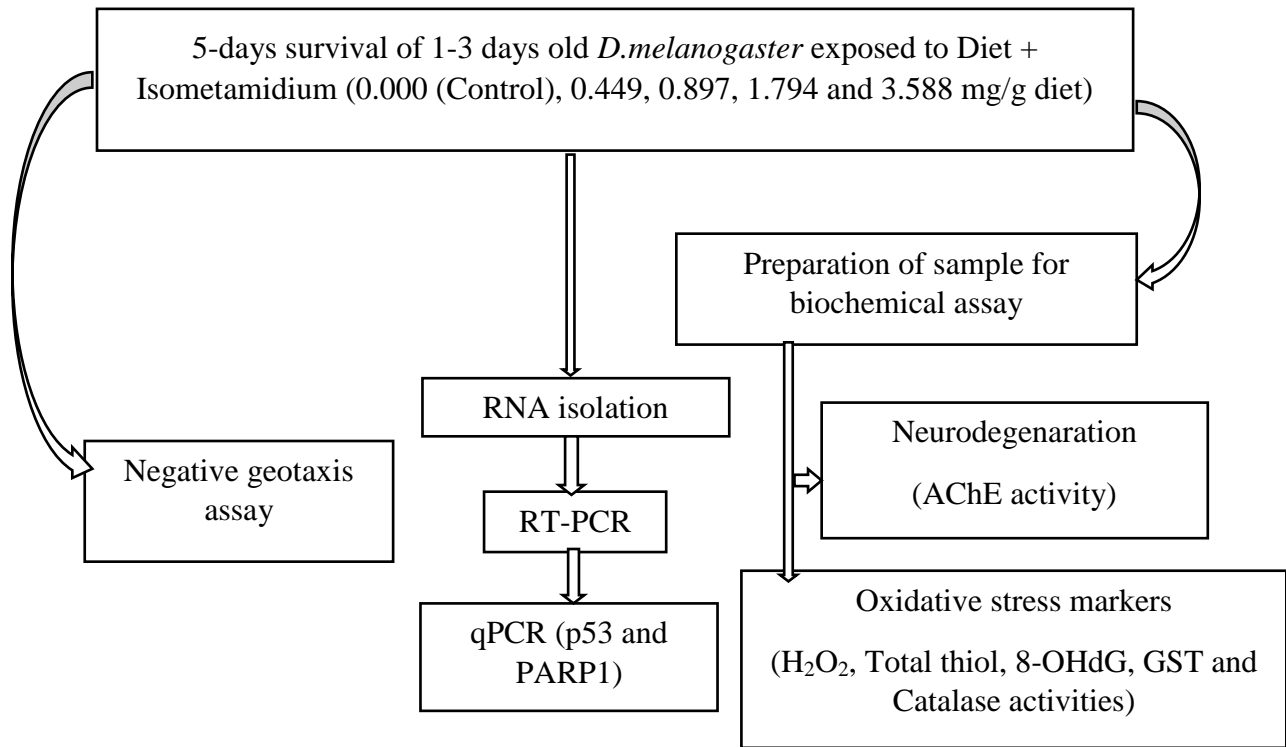
Group 4: Thirty (30) flies exposed to 1.794mg (50% LC₅₀) Isometamidium chloride per g diet.

Group 5: Thirty (30) flies exposed to 3.588mg (100% LC₅₀) Isometamidium chloride per g diet.

Phase I



Phase II



Phase III

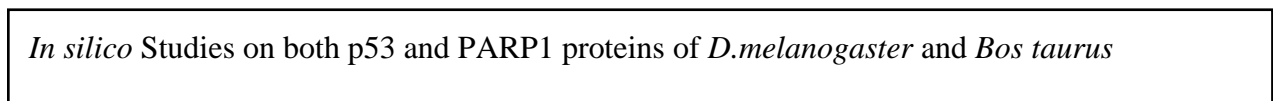


Figure 3. 1. Experimental Flowchart of the Study

3.2 Methods

3.2.1 Determination of 7-Day (164hrs) LC₅₀

The LC₅₀ was determined according to the method described by Iorjiim *et al.* (2020) with some slight modification. Newly eclosed (1-3 days old) flies of both genders were anesthetized using ice, from which 30 flies were counted and exposed to each of the different graded concentrations of Isometamidium chloride 0.0 (as control) 0.1, 1.0, 2.0, 4.0, 5.0 and 10.0 mg each per gram fly diet for seven (7) days (168hrs). Number of live and dead flies was recorded daily (24hrs interval) for the 7 days duration. The LC₅₀ was determined using Graphpad prism 8.0.2 by subjecting the survival percentage at day 7 to dose-response simulation.

3.2.2 Twenty eight (28) Days Survival assay

Thirty (30) flies each per group were exposed to 0.00 (Control), 0.449 (12.5% LC₅₀), 0.897 (25% LC₅₀), 1.794 (50% LC₅₀) and 3.588 mg (100% LC₅₀) of Isometamidium chloride per gram diet as summarized under fly grouping in page 23. The number of live and dead flies were recorded daily for the period of 28 days and used to plot survival curves as described by Abolaji *et al.* (2015).

3.2.3 Negative geotaxis assay

As described by Abolaji *et al.* (2015), the locomotor (climbing) performance of Isometamidium chloride-exposed and control flies was examined using the geotaxis assay after 5 days exposure. Briefly, ten (10) Isometamidium chloride-exposed and control flies were immobilized under mild ice anaesthesia and placed separately in labelled vertical glass columns (length, 15cm; diameter, 1.5cm). After the recovery period, the flies were gently tapped to the bottom of the column. After 6s, the number of flies that climbed up to 6cm mark of the column, as well as those that remained below this mark were recorded. Data was expressed as the percentage of flies that

escaped beyond the 6cm mark in 6s. The score of each group in triplicate was recorded in an average of two trials.

3.2.4 Sample preparation for biochemical assays

As adopted from Abolaji *et al.* (2015), 15 flies from each group of control and Isometamidium chloride-exposed flies were anesthetized in ice, weighed and homogenized in 0.1M phosphate buffer, pH 7.0(1mg:10 μ L) and centrifuged for 10min at 4000 x g (temperature, 4°C). The supernatants obtained was used to determine the activities of Glutathione-S-transferase (GST), Catalase (CAT) and Acetylcholinesterase (AChE), Total thiol content, Hydrogen peroxide (H₂O₂) and 8-hydroxydeoxyguanosine (8-OHdG).

Measurement of H₂O₂ Level

The principle is based on the fact that, in dilute acid, ferrous (Fe²⁺) is oxidized to ferric (Fe³⁺) ion by hydroperoxides. The ferric ion formed binds selectively to Xylenol orange to form a blue-purple colour with maximum absorbance at 560nm (Wolff, 1994).

According to the method described by Wolff (1994), H₂O₂ was measured by adding 10 μ L of sample to 290 μ L of FOX reagent (10 μ L Xylenol orange, 10 μ L sorbitol, 50 μ L Amonium ferrous sulfate, 50 μ L and 50 μ L distilled water), incubated at room temperature for 30mins and absorbance taken at 560nm using SpectraMax plate reader (Molecular Devices, USA).

Total thiol determination

Ellman's reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) also known as DTNB, on reacting with a free sulfhydryl in solution yields a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB). DTNB in the reaction targets the conjugate base (R—S-) of a free sulfhydryl group. The TNB produced is coloured and has a high molar extinction coefficient in visible range. By comparison to a

standard curve composed of known concentrations of a sulfhydryl-containing compound such as cysteine/Glutathione (GSH), the sulfhydryl groups in a sample can be estimated using Colorimetry (Ellman, 1959; Rucha and Gregory, 2018).

The reaction mixture contained a 170 μ L potassium phosphate buffer (0.1M, pH 7.0), 20 μ L of sample as well as 10 μ L of DTNB (10mM). After incubation for 30min at room temperature, the absorbance was measured at 412nm and used to calculate the total thiol levels of the sample (in μ mol/mg protein) using GSH as standard (Abolaji *et al.*, 2015).

Glutathione-s-transferase (GST) activity

This spectrophotometric assay depends on the change in the absorbance of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) when it is conjugated with Glutathione (GSH), reaction catalyzed by Glutathione-S-transferase (GST). The more the enzyme activity, the more conjugate formed and the more the increase in absorbance per unit time (Habig and Jakoby, 1981).

The activity of glutathione-s-transferase was determined as described by Abolaji *et al.* (2015) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay reaction mixture contained 190 μ L of solution A (20mL of 0.25M potassium phosphate buffer, pH 7.0 with 2.5mM EDTA, 10.5mL of distilled water and 500 μ L of 0.1M GSH at 25 $^{\circ}$ C), 20 μ L of sample (1:5 dilution) and 10 μ L of 25mM CDNB. An increase in absorbance was measured at 340nm for 2min at 30s interval using SpectraMax plate reader (Molecular Devices, USA). The data expressed in mmol/min/mg of protein.

Catalase (CAT) activity

Catalase decomposes H₂O₂ to yield H₂O and O₂. In ultraviolet range with decreasing wavelength, H₂O₂ shows continual increase in absorption. Therefore, the more the enzyme

activity, the more the decomposition of H₂O₂ and as a result; decrease in absorbance at 240nm (Aebi, 1984).

The reaction mixture composed 10µL of 300mM H₂O₂, 10µL of sample (in 1:50 dilution) and 290µL of potassium phosphate buffer (pH 7.0). The disappearance of H₂O₂ was measured at 240nm for 2min at intervals of 30sec using a SpectraMax plate reader (Molecular Devices, USA). H₂O₂ disappearance was used as an index for catalase activity and the result will be represented as µmol of H₂O₂ consumed/min/mg of protein (Abolaji *et al.*, 2015).

Acetylcholinesterase (AChE) activity

Acetylcholinesterase cleaves acetylthiocholine to thiocholine and acetate. Thiocholine reacts with DTNB to yield TNB which is yellow in colour and the colour change can be detected using Colorimetry (Ellman *et al.*, 1961).

AChE activity was determined by the method described by Abolaji *et al* (2015). To the reaction mixture containing 135µL of distilled water, 20µL of 100mM potassium phosphate buffer (pH 7.4), 10mM DTNB and 5µL of sample, 20µL of 8mM acetylthiocholine was added. The change in absorbance was monitored at 412nm for 2min at 30s intervals, using a plate reader. The data was calculated against blank and sample blank. The enzyme activity was expressed as µmol/min/mg of protein.

Protein quantification

The principle of the Lowry method of protein quantification is based on the fact that under alkaline condition, the peptide nitrogen(s) of a protein molecule react with copper II ions and the reduction of Folin-Ciocalteay phosphomolybdic phosphotungstic acid (Folin-Ciocalteau solution)

to heteropolymolybdenum blue with maximum absorbance at 660nm wavelength (Lowry *et al.*, 1951).

Bovine serum albumin (BSA 1mg/ml) was used as standard to estimate the total protein contents, where different concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of the BSA were prepared by diluting with distilled water. From the different dilutions and samples, 0.2ml was pipetted out to different tubes after which, 2ml of alkaline copper sulphate reagent was added, mixed thoroughly and incubated for 10mins. To each tube, 0.2ml Folin-Ciocalteu solution was added and incubated for 30mins. Distilled water was used as blank and absorbance was measured at 660nm. Standard calibration curve was plotted as absorbance against protein concentration. The total protein in the samples was extrapolated on the standard curve (Lowry *et al.*, 1951).

8-Hydroxydeoxyguanosine (8-OHdG) Assay

The principle of 8-OHdG quantification is based on the competitive inhibition Enzyme-Linked Immunosorbent Assay where sample's 8-OHdG and 8-OHdG precoated on the microtiter plate, compete for the binding sites of 8-OHdG monoclonal antibody. Unbound antibodies are washed while secondary antibodies linked with enzymes bind to the bound primary antibodies. Addition of a chromogen results in the development of colour (reaction catalyzed by the linked enzyme) in proportion to the number of antibodies bound and quantified using colorimetry (Yin *et al.*, 1995).

Manufacturer's protocol for the 8-OHdG ELISA kit (ELK Biotechnology) was used as thus; 50µL of standard or sample was mixed with 50µL Biotinylated-conjugate (1x) in each well, covered and incubated for 1hr at 37°C. Each well was washed three times using 200 µL Wash buffer and blotted against clean paper towels. To each well, 100µL of Streptavidin-HRP(1x) was

added, covered with adhesive film and incubated for 1hr at 37°C. Then each well was washed five times using 200µL Wash buffer and blotted against clean paper towels. To the empty wells, 90µL each of Substrate solution was added and incubated for 20min at 37°C. Finally, 50µL of Stop solution was added to each well and absorbance determined at 450nm using a microplate reader.

3.2.5 Isolation and quantification of mRNA levels

Nucleic acid extraction is based on the principle that under acidic conditions, DNA and proteins on centrifugation settle at the interphase or lower organic phase, while RNA remains at the upper aqueous phase. Then utilizing the precipitation of RNA using absolute alcohol (Isopropanol) to recover the total RNA in the aqueous phase (Ali *et al.*, 2017). However, RNA quantification is based on the reverse transcription and the quantification of the cDNA based on the C_q (quantification cycle) values (Bustin *et al.*, 2009).

Total RNA was extracted separately from both Isometamidium-exposed and non-exposed group flies using Thermo Fisher TRIzol® (Invitrogen) according to the manufacturer's protocol. To avoid DNA contermination, the total RNA extracted was treated with DNase. Thereafter, its integrity and quantity was determined using NanoDrop. cDNA was synthesized using EntiLink™ (ELK Biotechnology) in line with the manufacturer's protocol. Then, qPCR was carried out with a final volume of 20µL consisting; 10µL qPCR master mix (EnTurbo™ SYBRGreen PCR SuperMix), 0.4 µL (10µM) each forward and reverse primer, 8.4µL molecular grade water, 0.8µL cDNA template. A real time PCR system(Biorad) was used for the amplification with the following reaction condition; 95°C initial activation for 5min, 35 cycles of 15s at 95°C denaturation (denaturation cycle), 15s at 60°C (annealing cycle) and 25s at 72°C (extension cycle) then final hold temperature for 1min 72°C and 4°C for infinity.

Primers

The primers for the genes of interest were designed according to the published sequences the NCBI (<https://www.ncbi.nlm.nih.gov/>) database, design using Primer3Plus (<https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and supplied by InqabaBiotec while sequences of the two reference genes were adopted from Abolaji *et al.* (2015). The primer sequences are shown in Table 3.1 below.

3.2.6 Retrieval and Modelling of p53 and PARP1 Proteins

The proteins p53 (NP_996267.1) and PARP1 (NP_001104452.1) of *D. melanogaster* as well as the *Bos taurus* p53 (CAA57348.1) and PARP1 (NP_777176.1) amino acid sequences were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/>) (accessed January 25, 2022). To model the proteins, the SWISS-MODEL server (<https://swissmodel.expasy.org/>) (accessed January 25, 2022) was used and the structures were saved in PDB format. The MolProbability server (<http://molprobability.biochem.duke.edu/>) (accessed January 25, 2022) was used to further confirm the correctness of the modelled proteins. To prepare the modelled proteins for docking, the saved PDB formats were taken to the PyRx virtual screening software (<https://pyrx.sourceforge.io/>) where they were prepared as autodock molecules and saved in pdbqt formats. The 3D structure of Isometamidium chloride (CID_72552) was retrieved from PubChem data base (<https://pubchem.ncbi.nlm.nih.gov/>) (accessed January 25, 2022) and saved in SDF format. The ligand (Isometamidium) was transferred to the PyRx virtual screening software (<https://pyrx.sourceforge.io/>) where it was prepared as an autodock molecule and saved in pdbqt formats.

Table 3. 1 Primer sequences

	Primer sequences
P53Forward primer	5'- TGTATCGGGCGAAAAGAAAC -3'
P53 Reverse primer	5'- CTCGGCTATCATTGCTCTCC -3'
PARP1Forward primer	5'- GATTTGCCGATCAAAAGGAA -3'
PARP1 Reverse primer	5'- AAAGGTTGGCCTCCGTACTT -3'
GPDHForward primer	5'-ATGGAGATGATTCGCTTCGT-3'
GPDH Reverse primer	5'-GCTCCTCAATGGTTTTTCCA-3'
TubulinForward primer	5'-ACCAATGCAAGAAAGCCTTG-3'
TubulinReverse primer	5'-ATCCCAACAACGTGAAGAC-3'

Molecular Docking of Isometamidium chloride against p53 and PARP1 Modelled Structures

The ligand (Isometamidium) was docked against the proteins. The Vina wizard was commanded to start the docking process, then Auto Grid box centers (*D. melanogaster* p53; coordinates-X: 225.815, Y: 149.2223, Z: 237.4365 and dimensions-X: 52.5220, Y: 59.0137, Z: 52.8612. *D. melanogaster* PARP1; coordinate-X: -3.7423, Y: 34.5067, Z: -25.2430 and dimensions-X: 74.7218, Y: 60.5665, Z: 80.6555 as well as *B. taurus* p53; coordinates-X: -3.306, Y: 16.6457, Z: -3.8092 and dimensions-X: 82.3294, Y: 65.7094, Z: 67.3784. *B. taurus* PARP1; coordinate-X: 1.2921, Y: 33.2974, Z: -24.9082 and dimensions-X: 63.5398, Y: 59.3528, Z: 82.6151) were maximized without the ligand to allow the ligand free movement in selecting the best binding site. The binding energy of interaction which shows the best binding modes (Dallakyan and Olson, 2015) between the ligand (Isometamidium) and the two modelled proteins (p53 and PARP1 of *D. melanogaster* and *B. taurus*) was obtained in the PyRx software.

3.3 Statistical analysis

Where appropriate, the result was represented as Mean \pm SEM (Standard Error of Mean) and one-way ANOVA was used followed by Duncan's post-hoc test to assess the significant differences at $p < 0.05$ among multiple groups of the different treatments.

CHAPTER FOUR

4.0 RESULT

4.1. Acute exposure of *Drosophila melanogaster* to Isometamidium chloride

Almost 100% survival of *D. melanogaster* exposed to 0.1, 1.0 and 2.0 mg/g diet was observed throughout the exposure time while less than 40% survival was recorded in the groups exposed to 4.0 and 5.0 mg/g diet (Figure 4.1). The flies exposed to higher concentration (10.0 mg/1g diet) showed 100% mortality after exposure (Figure 4.1). Extrapolation from Figure 4.1 revealed a 3.588 mg/g diet to be the LC₅₀ of Isometamidium chloride in *D. melanogaster*.

4.2. Sub-chronic exposure of *D. melanogaster* Flies to Isometamidium chloride

The percentage survival in the flies exposed to 3.588 and 1.749 mg/g diet significantly ($P < 0.05$) reduced compared to the non-exposed flies (Figure 4.2). Similarly, a significant ($P < 0.05$) reduction in the percentage survival was also observed in the 0.897 mg/g diet exposed flies while only 0.449 mg/g isometamidium chloride exposed animals showed a non-significant ($P > 0.05$) reduction in the survival compared with the non-exposed *D. melanogaster* (Figure 4.2). Eighty (80) percent survival was observed across all the groups exposed to isometamidium chloride within the first five (5) days of exposure (Figure 4.2).

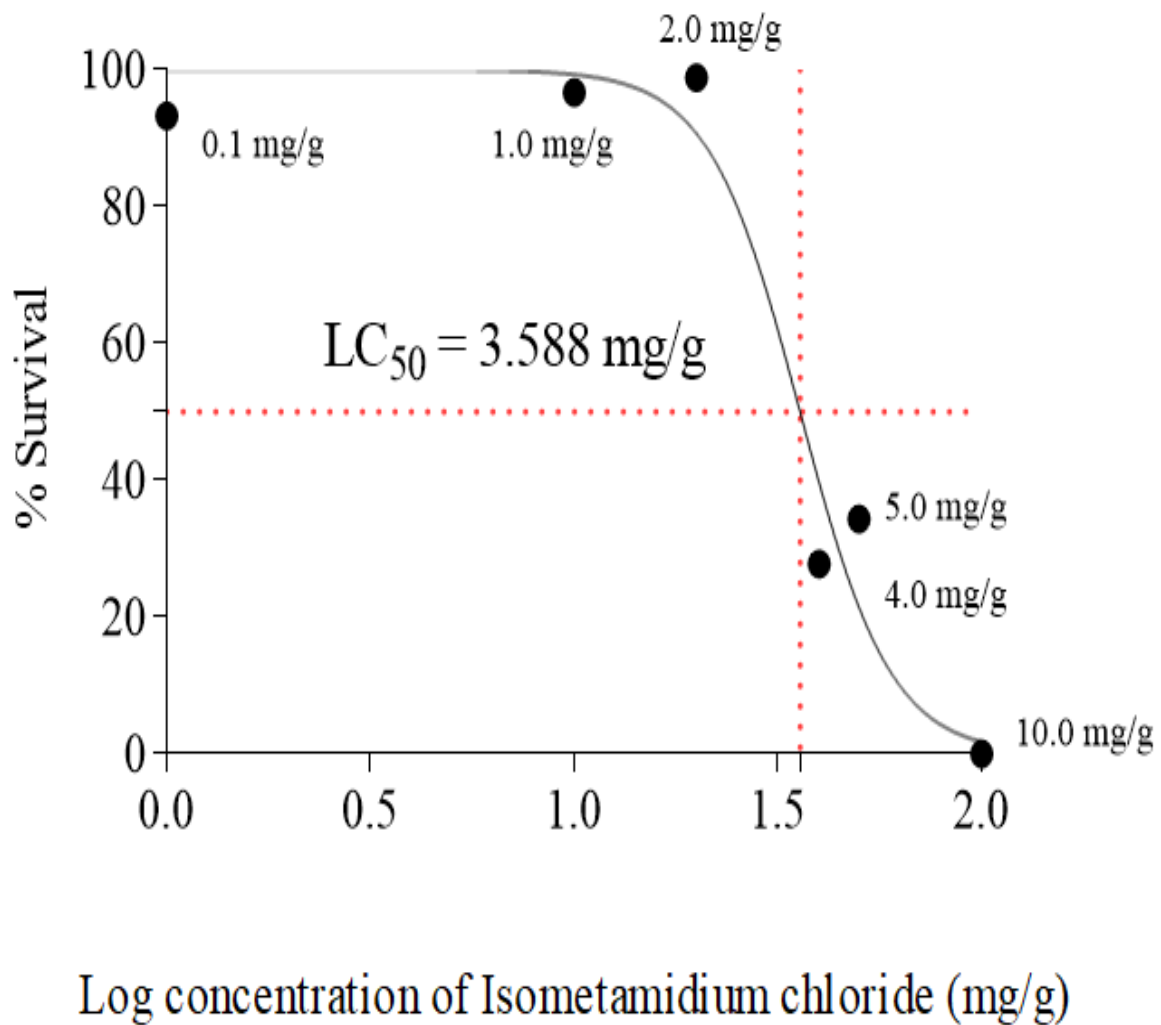


Figure 4. 1: Acute toxicity study of Isometamidium chloride in *Drosophila melanogaster*

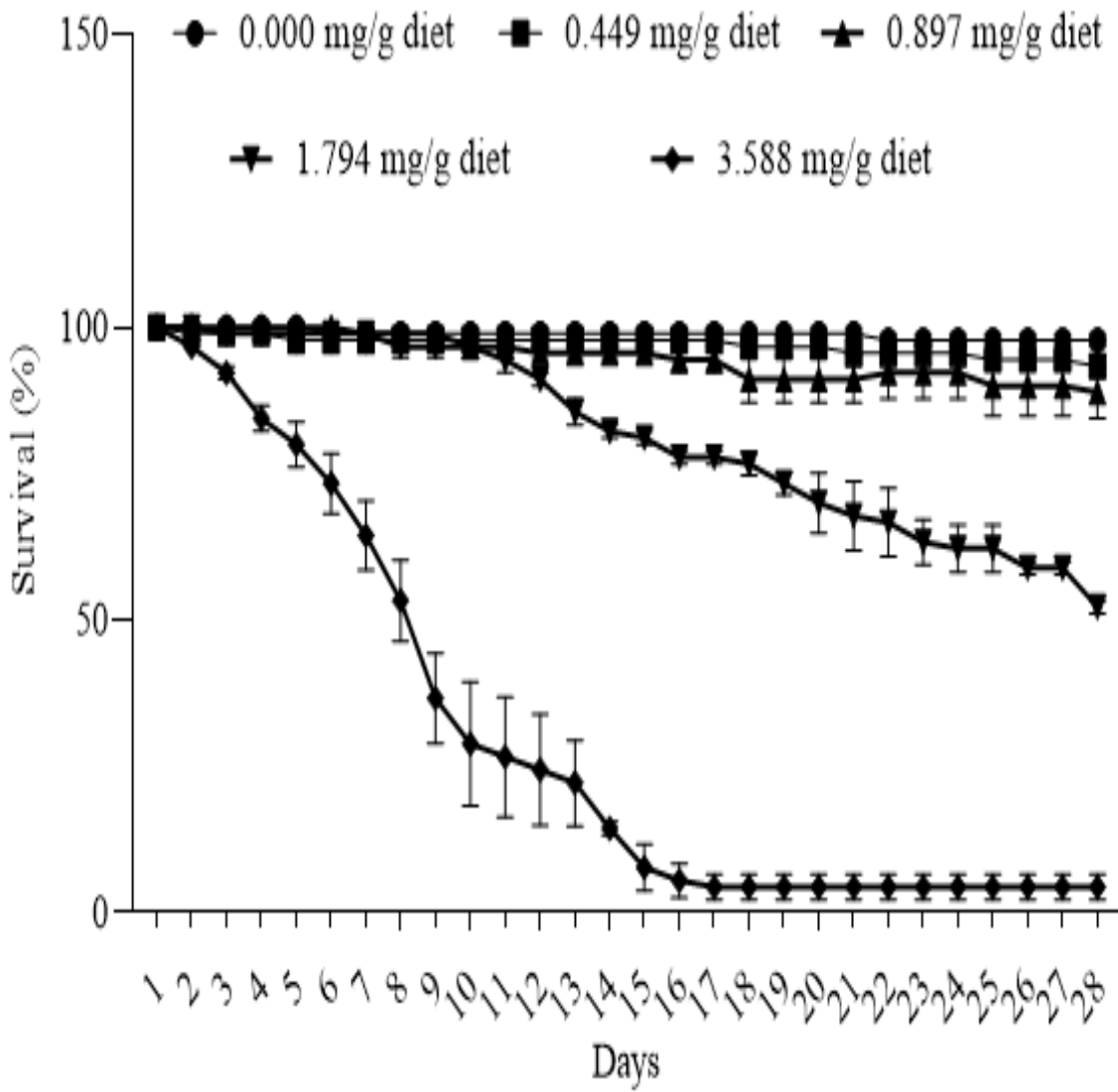


Figure 4. 2: Twenty eight (28) days survival of *Drosophila melanogaster* exposed to Isometamidium chloride

Result presented as Mean±SEM for three independent treatments

4.3 Survival, Negative Geotaxis (Climbing performance) and Neurodegeneration of *D.*

melanogaster exposed to Isometamidium chloride

The survival analysis after the five days exposure showed that the survival percentage of flies exposed to 3.588mg/g diet was significantly ($P < 0.05$) reduced while the other groups exposed to (0.449, 0.897, and 1.794) mg/g diet showed a non-significant reduction in the percentage survival compared to the non-exposed *D. melanogaster* flies (Figure 4.3). This showed a threshold concentration between 1.794 and 3.588 mg/g diet above which, caused death in *D. melanogaster*.

The ability of the flies exposed to the varied concentrations of the isometamidium chloride to climb (negative geotaxis) six (6) cm mark after six (6) seconds was investigated (Figure 4.4). The non-exposed flies showed significantly ($P < 0.05$) higher negative geotaxis with 96.67% of the flies climbing when compared with the isometamidium chloride exposed flies (Figure 4.4). However, the 1.794 mg/g of the diet showed significantly higher geotaxis when compared with the other concentrations although not significantly higher compared with 0.897 and 3.588 mg/g diet exposed groups (Figure 4.4). Moreover, the lower concentration showed lower negative geotaxis (Figure 4.4).

There was no significant ($P > 0.05$) difference in the activity of acetylcholinesterases (AChE) in the isometamidium chloride exposed groups compared to the non-exposed groups (Figure 4.5). Although not significantly ($P > 0.05$) different, there was increase in the level of AChE activity from 0.449, 0.897 and particularly at 1.794 mg/g diet. However, at 3.588 mg/g diet, AChE activity was reduce.

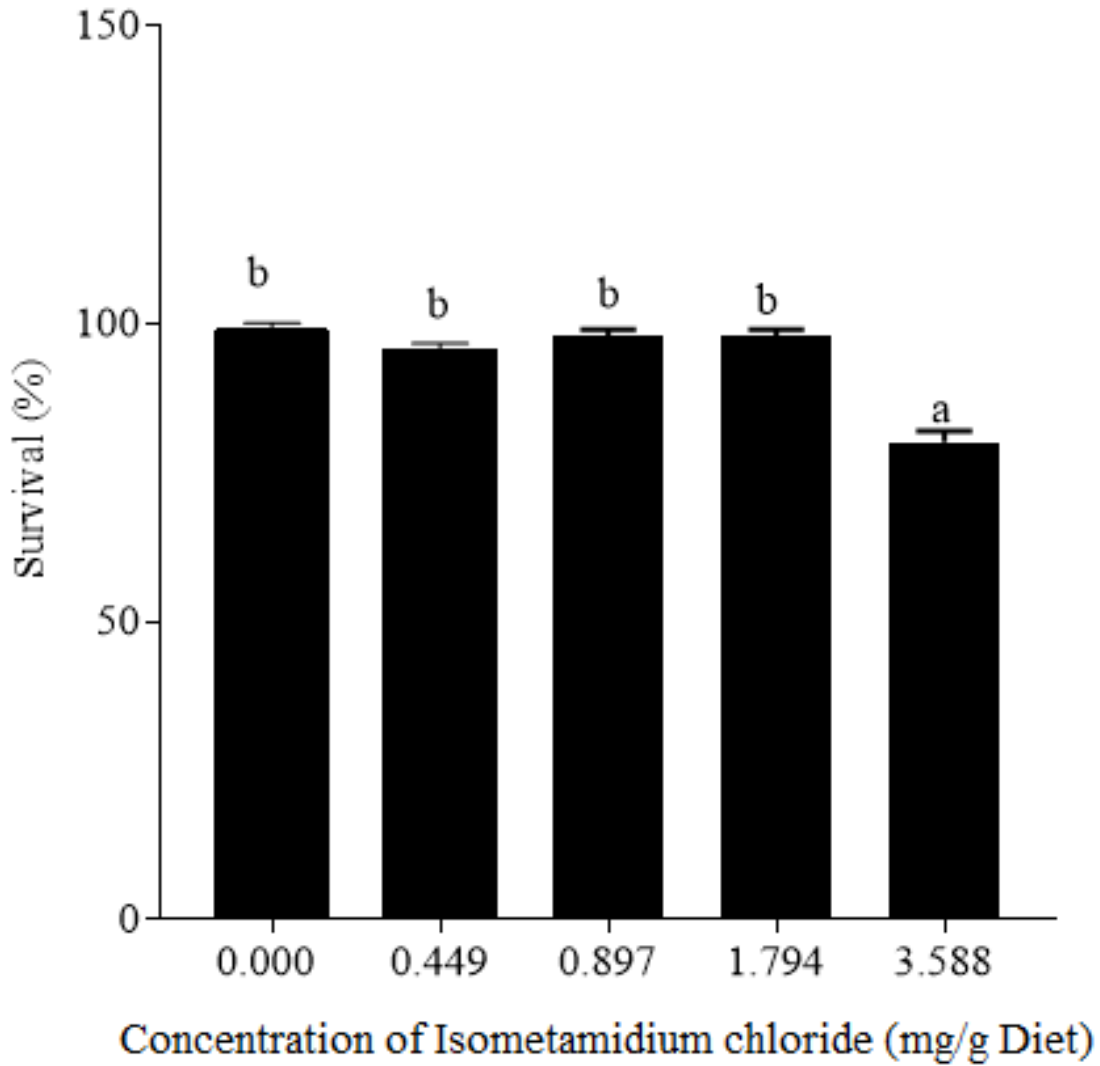


Figure 4. 3: Determination of the earliest day of noticeable alteration in survival of *Drosophila melanogaster* exposed to Isometamidium chloride

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are significantly different from each other

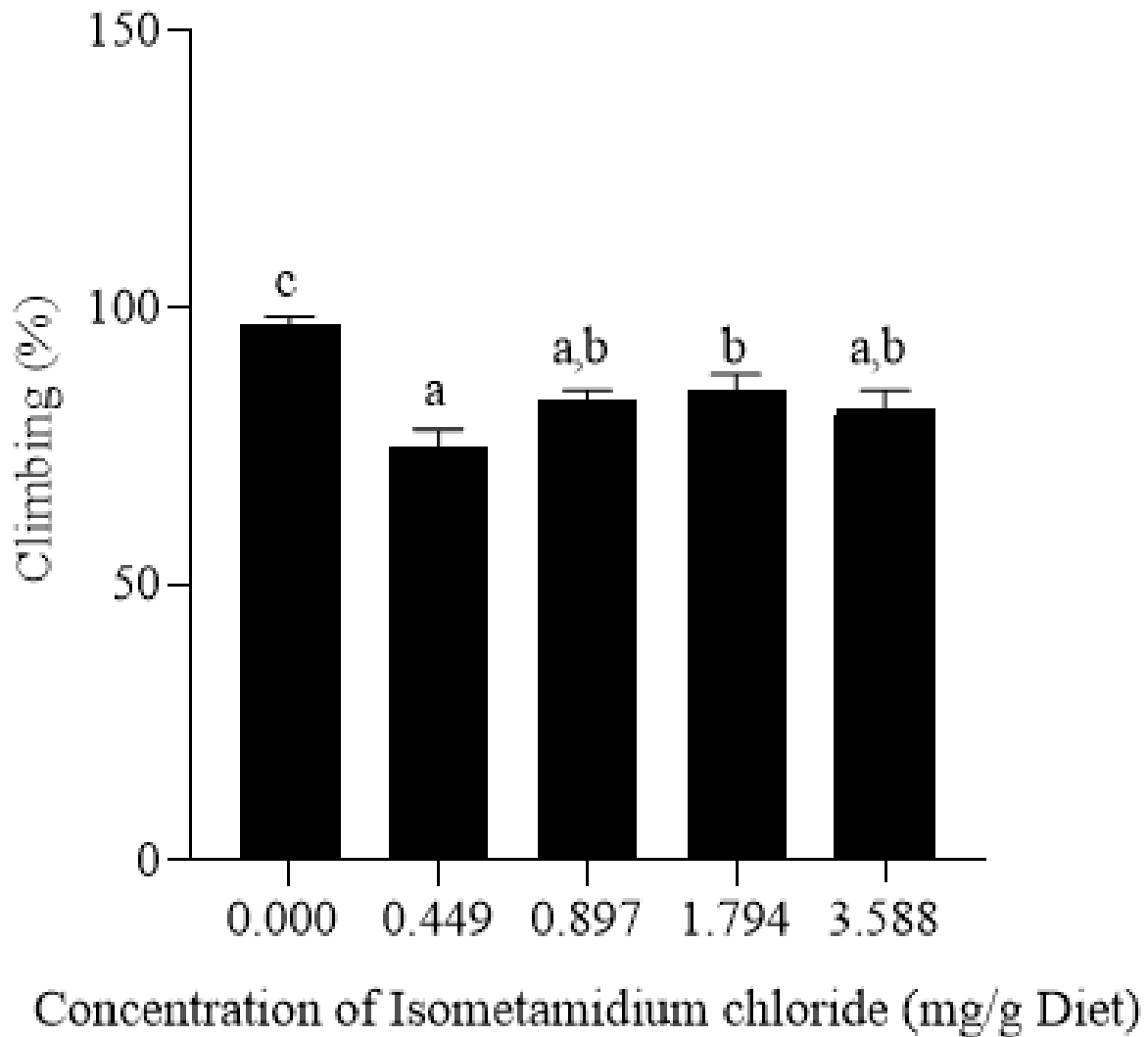


Figure 4. 4: Negative geotaxis (climbing performance) of *Drosophila melanogaster* exposed to Isometamidium chloride

Result represented as Mean±SEM for three independent treatments performed in duplicates. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are significantly different from each other

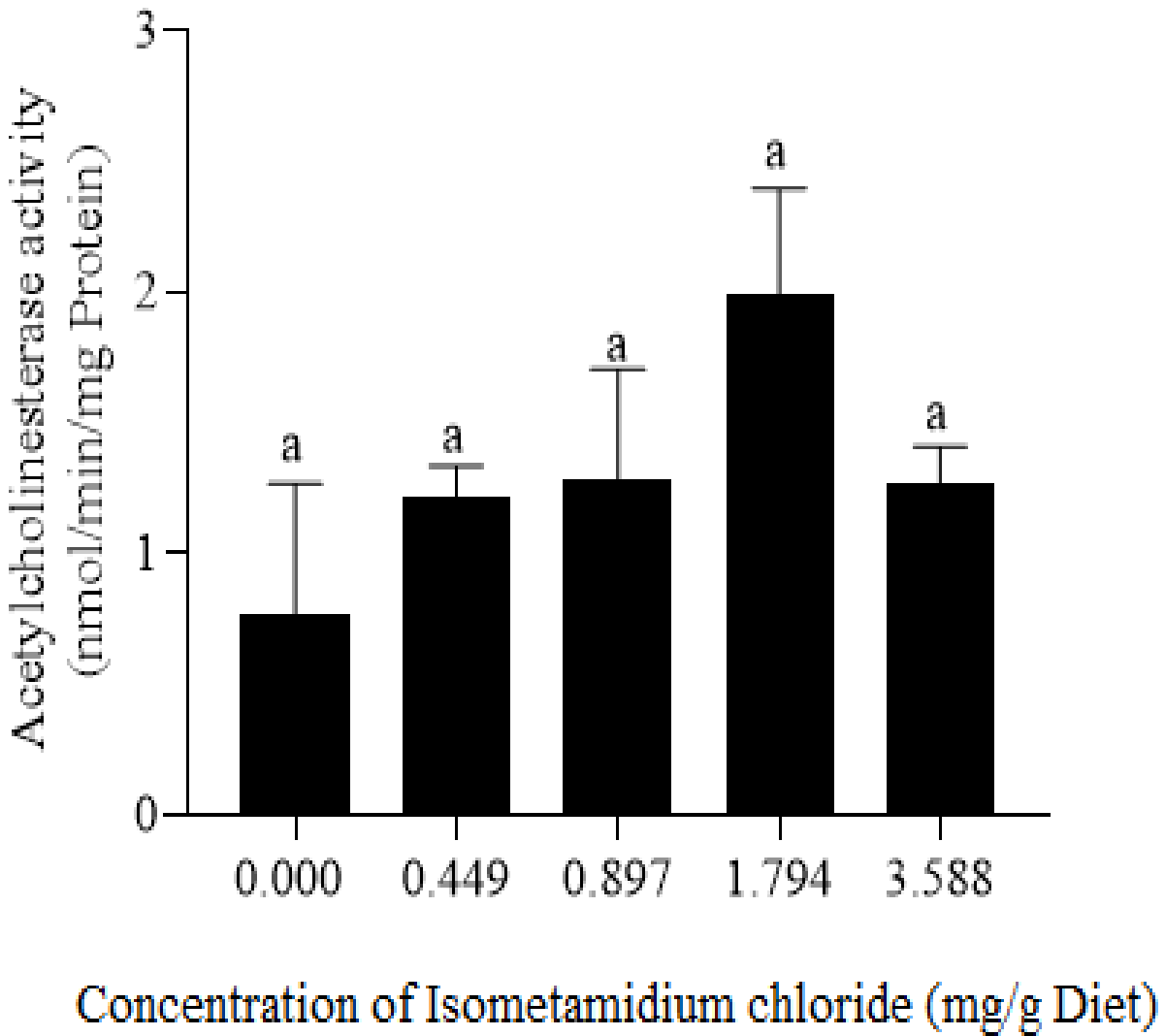


Figure 4. 5: Effect of Isometamidium chloride on Acetylcholinesterase (AChE) activity in *Drosophila melanogaster*

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at p<0.05. Bars with different alphabets are significantly different from each other

4.4 Effect of Isometamidium chloride Exposure on the Redox Status of *D. melanogaster*

There was significant ($P < 0.05$) increase in the level of H_2O_2 in groups exposed to 1.794 mg/g and 3.588 mg/g diet compared to the non-exposed flies (Figure 4.6). The level of H_2O_2 suddenly increased just after 0.897 mg/g, indicating a threshold concentration beyond which caused elevated levels of H_2O_2 . *D. melanogaster* was able to regulate the production of H_2O_2 but failed at concentration greater than 0.897 mg/g.

There was significant ($P < 0.05$) decrease in catalase (CAT) activity in groups exposed to 0.897, 1.794 and 3.588 mg of isometamidium chloride per g diet (Figure 4.7). Further indicating a threshold concentration of probable toxicity between 0.897 and 1.794 mg/g diet.

A significant ($P < 0.05$) decrease in the total thiol (TSH) level was observed in all the groups exposed to isometamidium chloride compared to the non-exposed group. The level of the TSH was significantly ($P < 0.05$) more reduced in the 3.588 mg/10g diet exposed group (Figure 4.8) compared to the other exposed groups as well as the non-exposed group.

The activity of glutathione-s-transferase (GST) was significantly ($P < 0.05$) reduced in the isometamidium chloride exposed groups compared to the non-exposed group (Figure 4.9). The reduction in GST activity was more significant ($P < 0.05$) in the 0.897 and 1.794 mg/g exposed groups compared to the groups exposed to 0.449mg/g and 3.588mg/g (Figure 4.9).

Additionally, there was no significant ($P > 0.05$) difference in 8-HydroxyGuanosine (8-OHdG) level in both exposed and non-exposed groups (Figure 4.10).

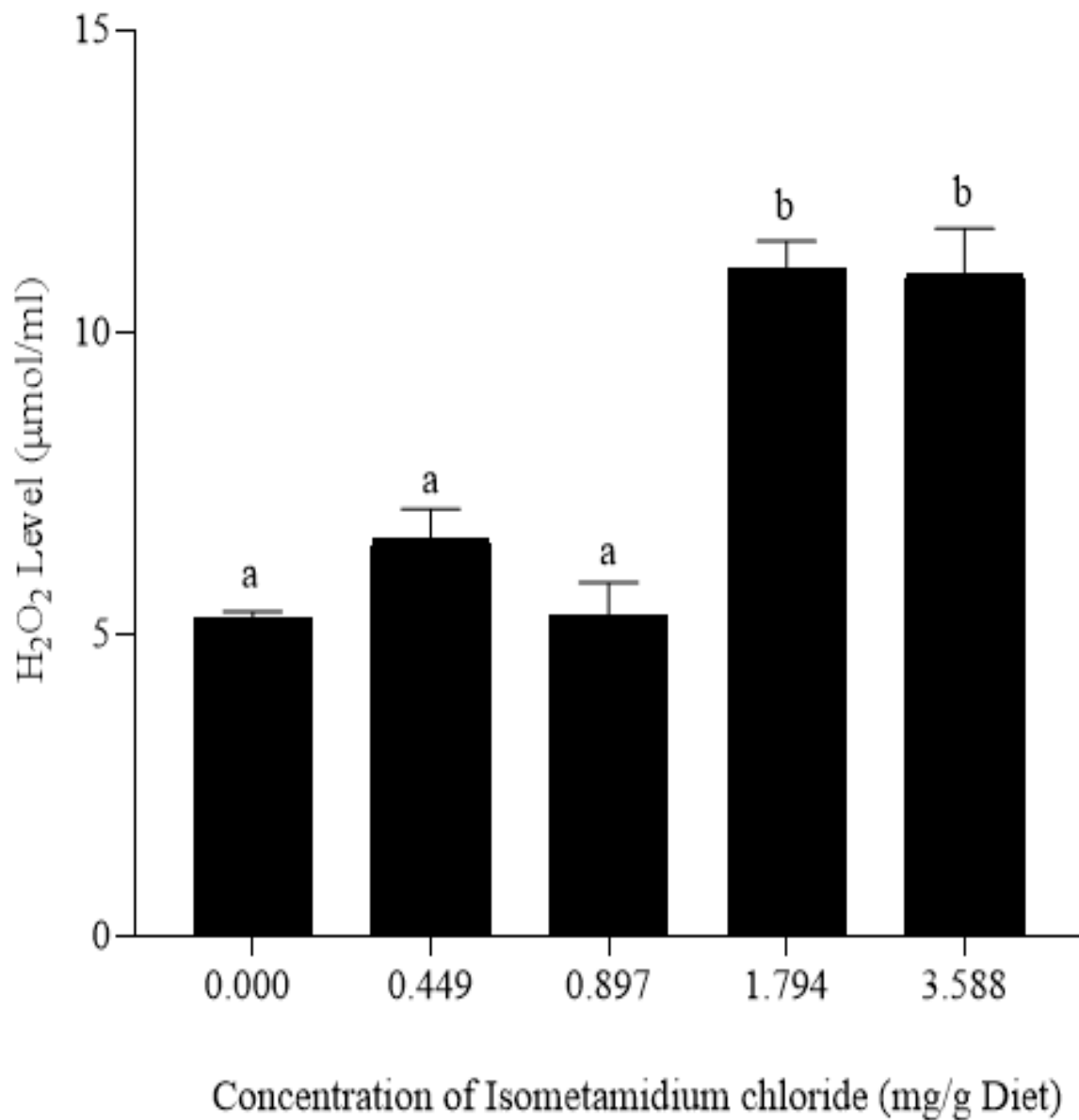


Figure 4. 6: Effect of Isometamidium chloride on the level of H₂O₂ in *Drosophila melanogaster*

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at p<0.05. Bars with different alphabets are significantly different from each other

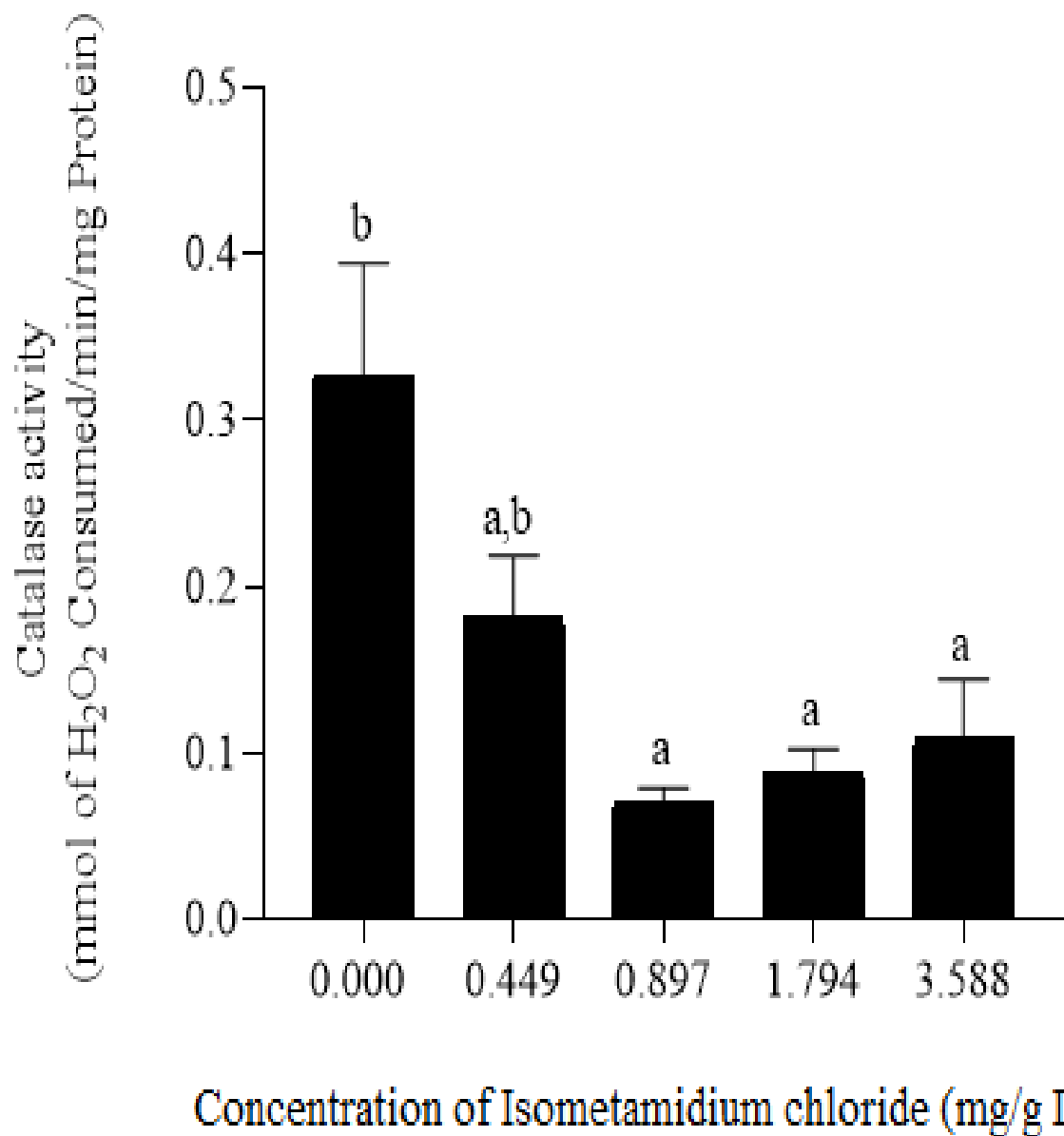


Figure 4. 7: Effect of Isometamidium chloride on Catalase (CAT) activity in *Drosophila melanogaster*

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are significantly different from each other

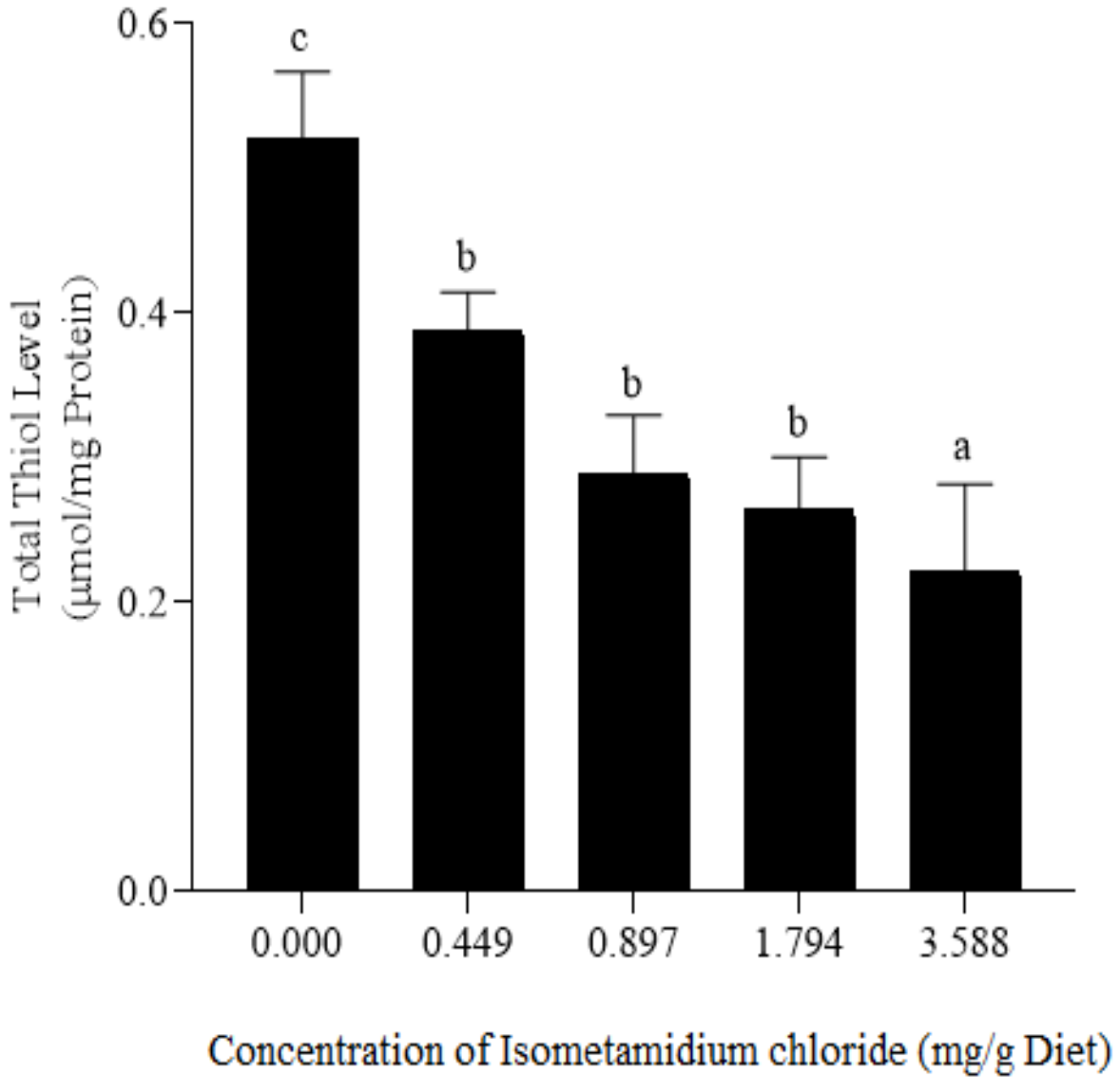


Figure 4. 8: Effect of Isometamidium chloride on the level of Total thiol (TSH) in *Drosophila melanogaster*

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are significantly different from each other

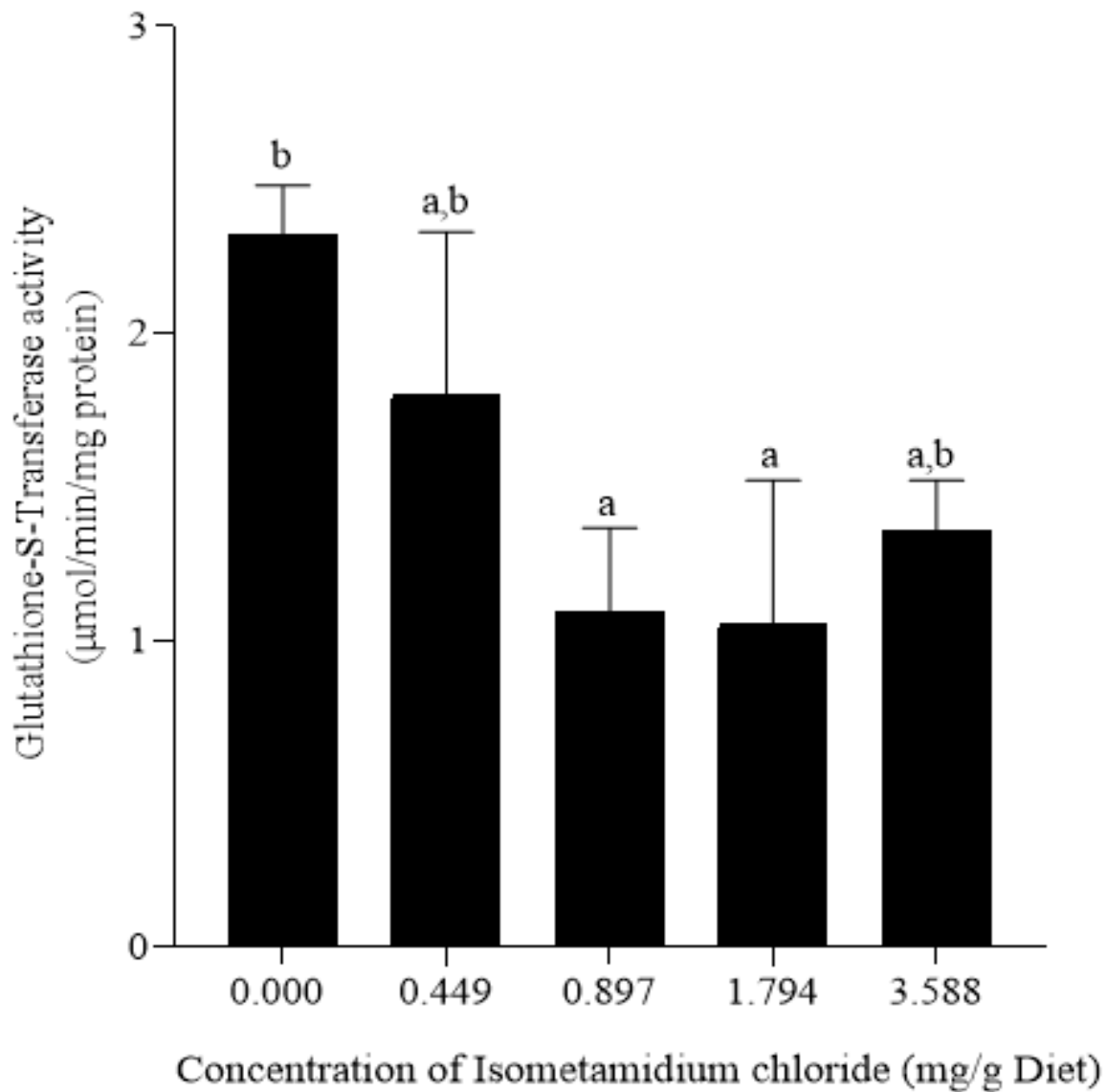


Figure 4. 9: Effect of Isometamidium chloride on Glutathione-S-Transferase (GST) activity in *Drosophila melanogaster*

Result presented as Mean \pm SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are statistically different from each other

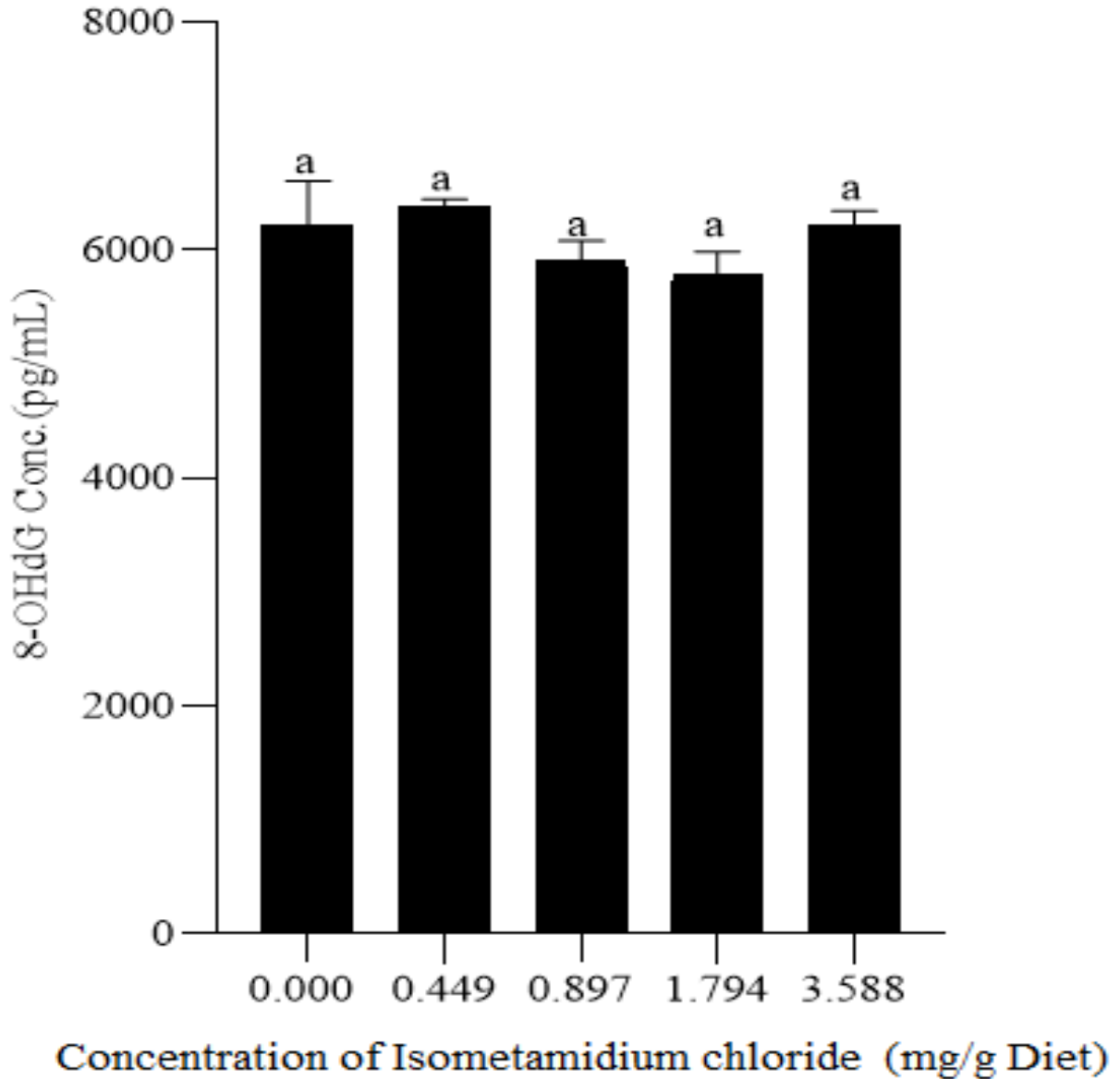


Figure 4. 10: Effect of Isometamidium chloride on 8-HydroxyGuanosine (8-OHdG) level in *Drosophila melanogaster*

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are significantly different from each other

4.5 Effect of Isometamidium chloride exposure on the mRNA Expression levels of *D. melanogaster* *p53* and *PARP1* Genes

There was significant ($P < 0.05$) down-regulation in the expression of *p53* gene in all the groups exposed to the compound compared to the non-exposed flies (Figure 4.11).

Similarly, there was significant ($P < 0.05$) down-regulation in the expression of *PARP1* gene in all the exposed groups compared to the non-exposed flies with 0.449 mg/g showing higher reduction (Figure 4.12).

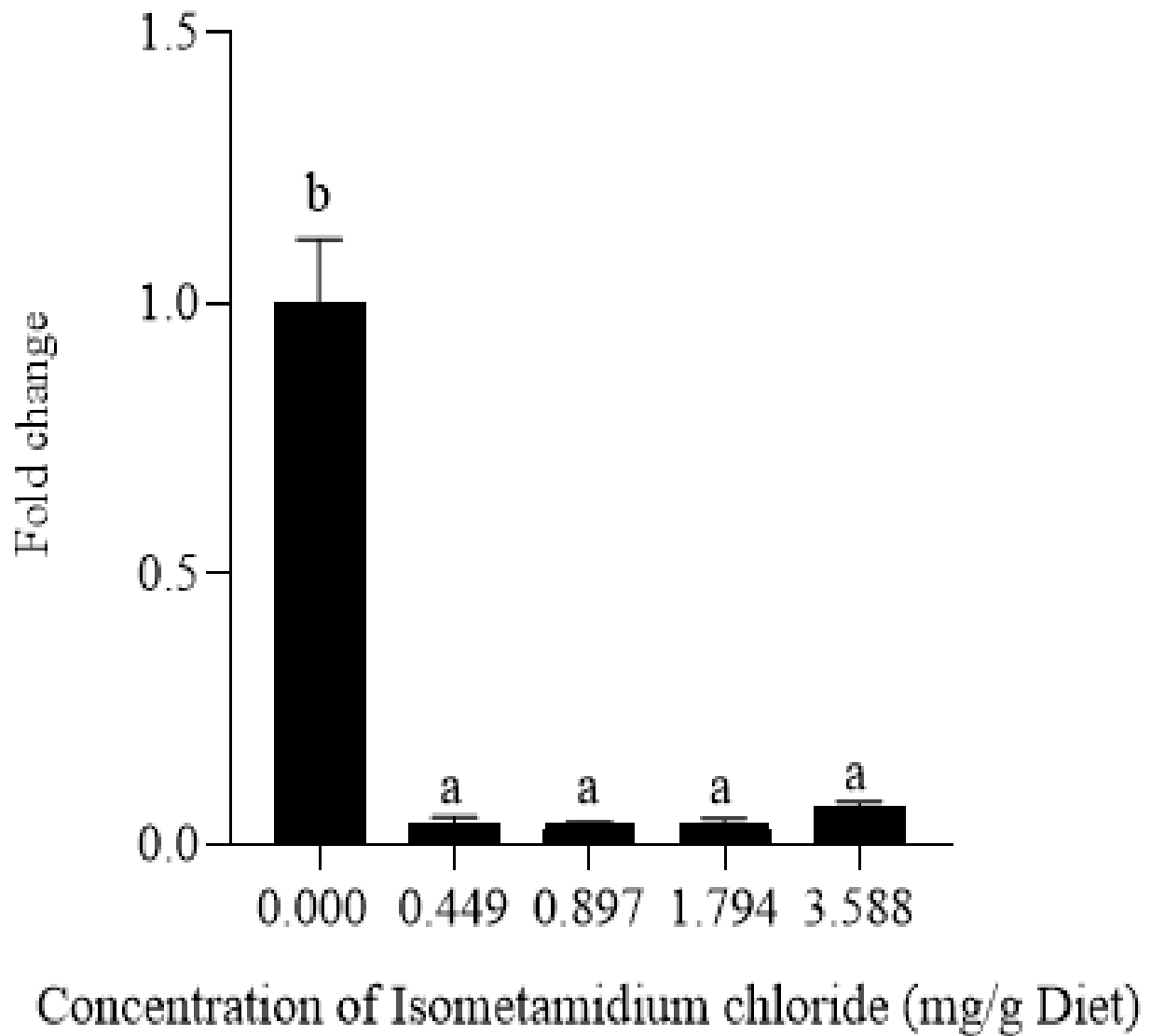


Figure 4. 11: Quantitative real time PCR (qPCR) mRNA expression analysis of *p53* gene in *Drosophila melanogaster* exposed to Isometamidium chloride

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are significantly different from each other

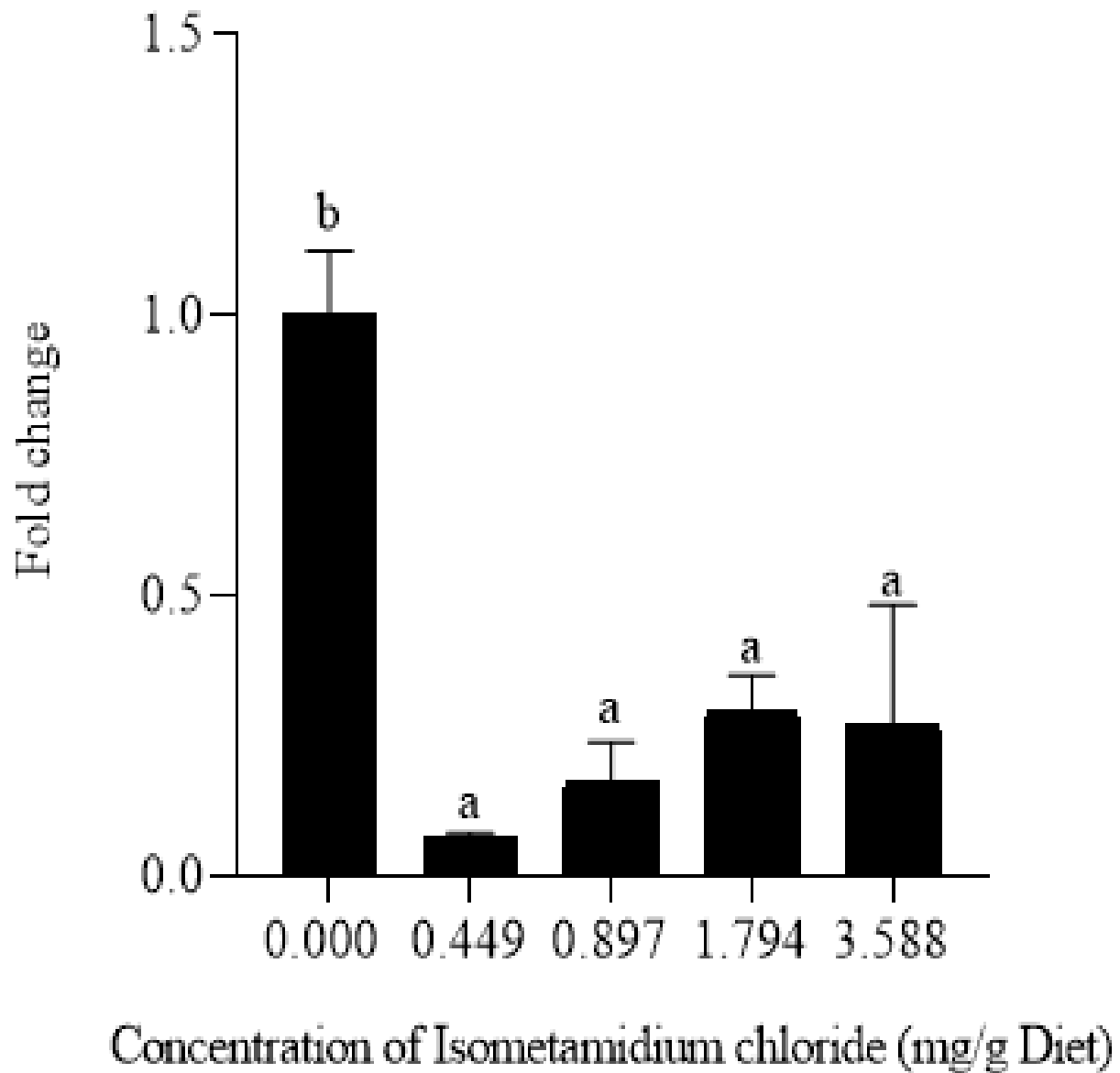


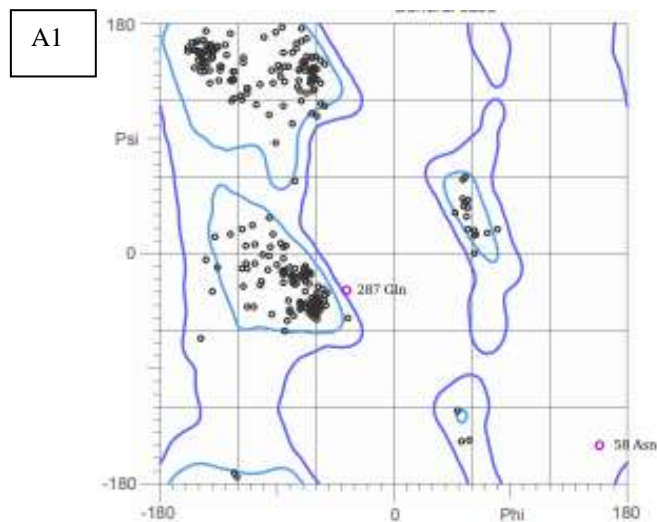
Figure 4. 12: Quantitative real time PCR (qPCR) mRNA expression analysis of *PARP1* gene in *Drosophila melanogaster* exposed to Isometamidium chloride

Result presented as Mean±SEM for three independent treatments. One-Way ANOVA Duncan's Post-hoc Test was used to determine the statistical differences across all groups at $p < 0.05$. Bars with different alphabets are significantly different from each other

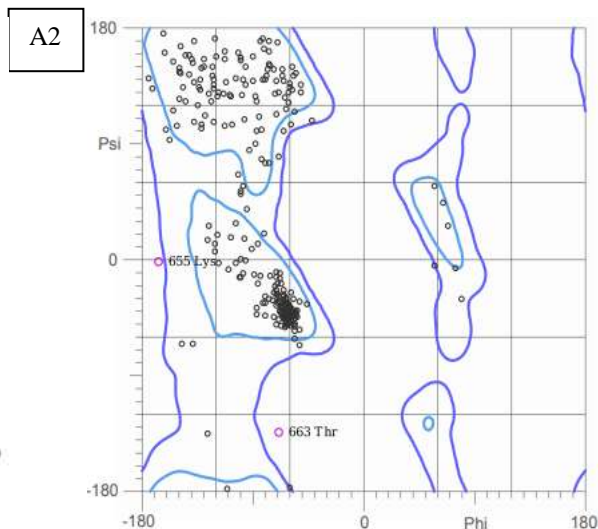
4.6 Molecular Docking of p53 and PARP1 proteins with Isometamidium chloride

The Ramachandran plot (Figure 4.13), showed that the modelled p53 protein of *B. taurus* had 90.6% of all its amino acid residues in the favoured (98%) regions and 97.7% in the allowed (>99.8%) regions (Figure 4.13 A1) while the modelled p53 protein of *D. melanogaster* had 87.4% of its total amino acid residues in the favoured (98%) regions and 96.1% in the allowed (>99.8%) regions (Figure 4.13 B1). Similarly, the modelled PARP1 protein of *B. taurus* had 91.2% of all its amino acid residues in the favoured (98%) regions and 99.5% in the allowed (>99.8%) regions (Figure 4.13 A2) while the modelled PARP1 protein of *D. melanogaster* had 91.2% of its total amino acid residues in the favoured (98%) regions and 98.5% in the allowed (>99.8%) regions (Figure 4.13 B2). From the molecular docking (Figure 4.14), isometamidium was found to bind to p53 protein of *B. taurus* with binding energy of -8.1Kcal/mol (Table 4.1) and formed hydrogen bond with Asp179 as well as other forms interactions like Attractive Charges, Pi-Alkyl, Pi-anion, Pi-Donor hydrogen bond, Pi-sulfur and Van der Waals (Figure 4.14 A1 and Table 4.1) while p53 protein of *D. melanogaster* interacted with isometamidium with binding energy of -9.4 Kcal/mol (Table 4.1) and formed about four hydrogen bonds with Pro220, Leu221, Asp400 and Lys404 residues as well as other forms of interaction like attractive charges, Pi-Alkyl, Unfavourable positive-positive and Van der Waals interactions (Figure 4.14 B1 and Table 4.1). Similarly, the molecular docking results showed that Isometamidium interacted with PARP1 protein of *B.taurus* with binding energy of -10.3Kcal/mol forming a hydrogen bond with Ser906 and as well as other forms of bonds that include; Covalent bond and Van der Waals bonds (Figure 4.14 A2 and Table 4.1) while PARP1 protein of *D. melanogaster* interacted with isometamidium with binding energy of -9.2Kcal/mol and formed four hydrogen bond with Asn534, Glu626, Tyr629 and Asp630 (Figure 4.14 B2 and

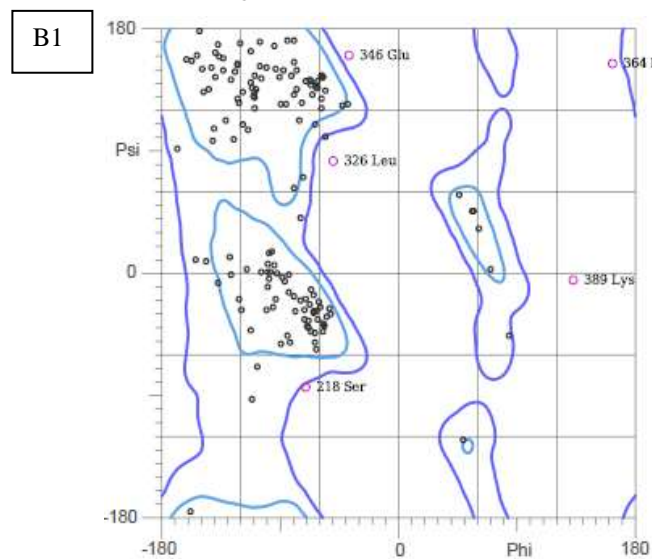
Table 4.1). In addition to the Hydrogen bonds formed, other forms of attraction like Amide Pi Stacked, Attractive charges, Pi-Alkyl, Pi-Sigma, Pi-Pi T-Shaped and Van der Waals interactions were also formed (Figure 4.14 B2 and Table 4.1).



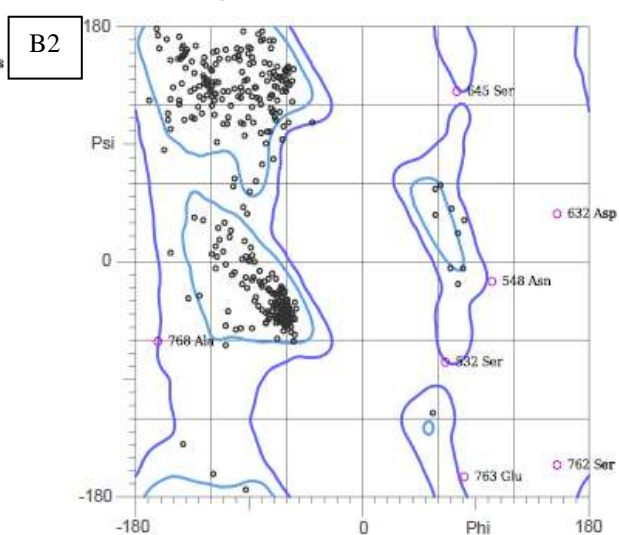
90.6% (348/384) of all residues were in favored (98%) regions. 97.7% (375/384) of all residues were in allowed (>99.8%) regions



91.2% (343/376) of all residues were in favored (98%) regions. 99.5% (374/376) of all residues were in allowed (>99.8%) regions



87.4% (181/207) of all residues were in favored (98%) regions. 96.1% (199/207) of all residues were in allowed (>99.8%) regions



91.2% (436/478) of all residues were in favored (98%) regions. 98.5% (471/478) of all residues were in allowed (>99.8%) regions

Figure 4. 13: Ramachandran plots of modelled proteins of *Bos Taurus* (A) and *Drosophila melanogaster* (B); A1 and B1 are Ramachandran plots for p53 while A2 and B2 are Ramachandran plots for PARP1

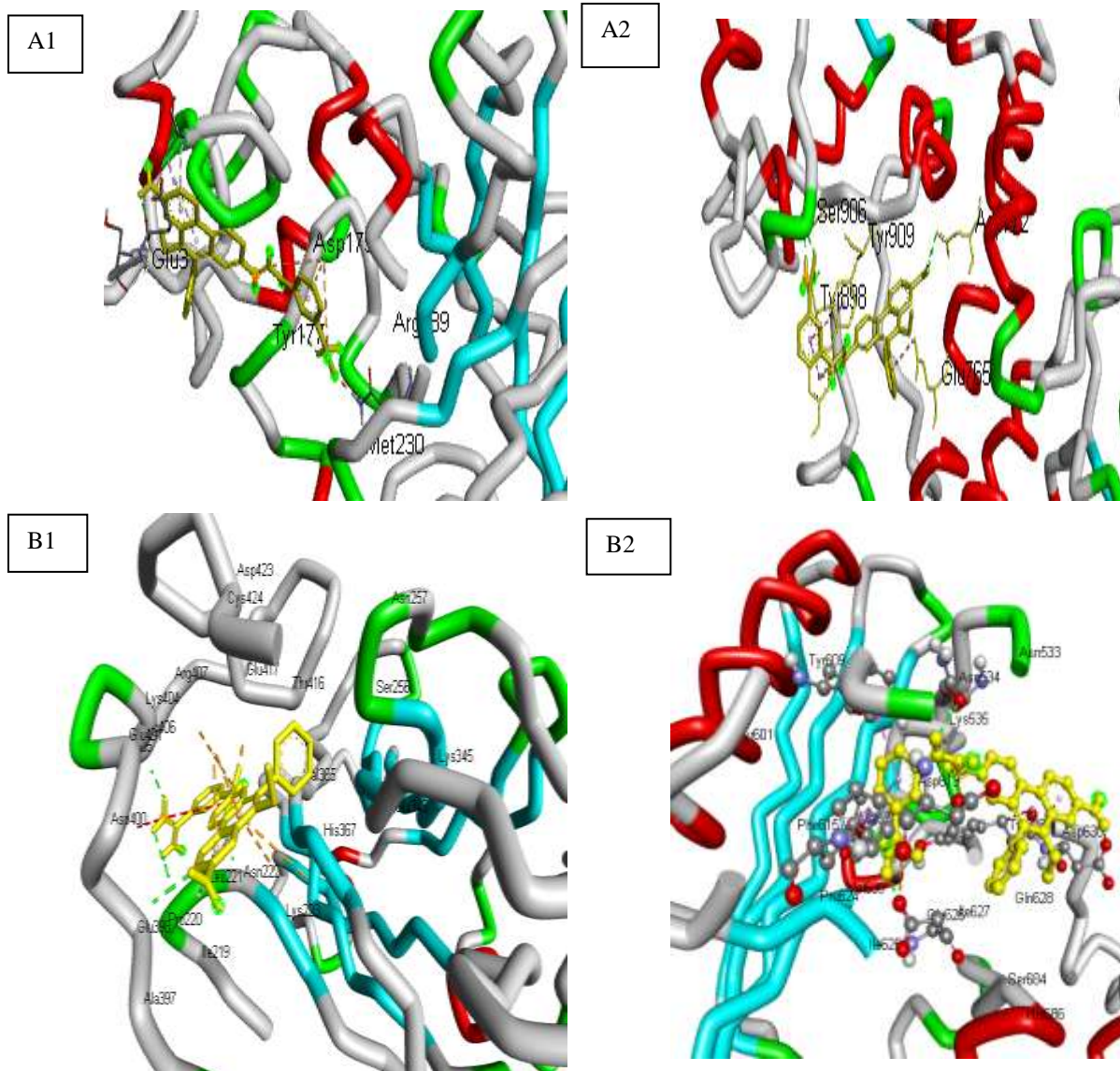


Figure 4. 14: 3D Molecular docking interactions of Isometamidium chloride with Modelled proteins of *Bos taurus*(A) and *Drosophila melanogaster* (B); A1 and B1 are for p53 while A2 and B2 are for PARP1

Table 4. 1Summary of *in silico* interaction of Isometamidium chloride with Modelled p53 and PARP1 proteins of *D. melanogaster* and *Bos taurus*

<i>Drosophila melanogaster</i>			<i>Bos taurus</i>		
Protein(s)	Binding Energy	Interaction(s)	Protein(s)	Binding Energy	Interaction(s)
p53	-9.4 Kcal/mol	Attractive Charges (Lys223, Glu346, Glu401 and Glu417) Hydrogen bonds (Pro220, Leu221, Asp400 and Lys404) Pi-Alkyl (Leu221) Unfavourable positive-positive (Arg406). Van der Waals (Ile219, Asn222, Ser256, His367, Val 405 and Cys424)	p53	-8.1 Kcal/mol	Attractive Charges (Glu3 and Asp179) Hydrogen bonds (Asp179) Pi-Alkyl (Arg189) Pi-anion (Asp179). Pi-Donor hydrogen bond (Tyr177) Pi-sulfur (Met230) Van der Waals (Glu2, Ser4, Leu32 Ser34, Glu35, Leu36, Ser37, Ala38, Ala130, Lys131, Asp176, His226 and Asn228)
PARP1	-9.2 Kcal/mol	Attractive Charges (Asp613 and Glu626) Amide-Pi Stacked (Tyr609 and Tyr629) Pi-Alkyl (Val538) Pi-Pi T-Shape (Phe615) Pi-Sigma (Tyr629) Hydrogen bonds (Asn534, Glu626, Tyr629, Asp630) Van der Waals (Asn533, Lys535, Val539, Tyr601, Pro624, Ile625, Ile627, Gln628, Ser684 and His686)	PARP1	-10.3 Kcal/mol	Covalent bond (Asn908 and Cys910) Hydrogen bonds (Ser906) Van der Waals (Glu865, Ser866, Arg867, Lys905, Ala907, Asn908 and Cys910)

CHAPTER FIVE

5.0 DISCUSSION

Isometamidium chloride, Diminazene and Homidium are the main compounds used in the treatment of AAT (Bengaly *et al.*, 2018). Isometamidium chloride has been shown to be effective both in the prevention and treatment of this disease but with adverse side effects reported in its use (Ali and Haroun, 1984; Ali and Hassan, 1986; Ardelli and Woo, 2001; Raftery *et al.*, 2019). For the first time, we evaluated if isometamidium chloride could cause oxidative stress as well as DNA damage in *Drosophila melanogaster*. A study revealed that trypanocidal drug (feximidazole) can cause DNA damage (Tweats *et al.*, 2012). Likewise, another study also showed that diminazene aceturate caused cytotoxic and genotoxic effects in the liver and kidney cells of rats (Baldissera *et al.*, 2017).

The acute toxicity study indicated the LC₅₀ of Isometamidium chloride in *D. melanogaster* to be 3.588 mg/g diet, suggesting the drug is toxic to the flies. Longevity or the ability to continue living and reproduce are indicators of good health in any organism and different ranges of factors can affect the survival of an organism (Chattopadhyay *et al.*, 2015). By treating or exposing an organism to different concentrations of a particular compound, its effect on the survival of the organism can be studied. Evidence from the sub-chronic exposure (28 days) of *D. melanogaster* to isometamidium, demonstrated a reduction in the percentage survival of the flies in a dose and time dependent manner. Above 0.897 mg/g, a drastic mortality was observed. After five (5) days exposure, at 3.588 mg/g mortality was significant. Suggesting that at concentration above 1.794 mg/g, isometamidium chloride is more toxic. The observation is similar to the study conducted

on rabbits where the drug caused rapid death of the experimental animals and as well caused some cytotoxic effects (Ali and Haroun, 1984). Reactive oxygen species (ROS) are constantly being generated in the cells during cellular respiration (Henle and Linn, 1997). At lower levels ROS serve some beneficial biological functions but at higher levels are deleterious to the cells (Chetterjee and Walker, 2017). To moderate the negative effects caused by higher levels of ROS, antioxidant enzyme activities serve as the main mechanism via which the levels of ROS is regulated in the cell (Mates and Sanchez-Jimenez, 1999). The results of this study indicated higher levels of H₂O₂ in the groups where flies were exposed to isometamidium chloride especially in 1.794 and 3.588 mg/g diet groups. The elevated levels of ROS in this study is similar to a study that showed increased ROS levels in rats exposed to diminazene (Baldissera *et al.*, 2017). From literature, the reaction of H₂O₂ with Fe²⁺ produce ⁻OH which is one of the most reactive ROS known to cause numerous deleterious effects such as the degradation of DNA, proteins and lipids (Imlay *et al.*, 1988; Dizdaroglu *et al.*, 1991). Catalase is a key enzyme in the neutralization of free radicals, where it decomposes H₂O₂ into water and oxygen (Nandi *et al.*, 2019). It was observed in this study that catalase activity was greatly reduced in all the groups exposed to isometamidium chloride for the period of 5 days. This suggests that the level of H₂O₂ observed could be as a result of the inability of the enzyme to decompose it optimally. Thiols are compounds with carbon-bound sulfhydryl groups (Garcia-Garcia *et al.*, 2012) and are important biochemical markers of oxidative stress (LoPachin and Barber, 2006). The total thiol (protein and non-protein thiols) level was found to be reduced in all the groups exposed to isometamidium chloride with the 3.588 mg/g diet group showing the lowest level of total thiol.

Glutathion S- transferases (GSTs) as family of phase II detoxification enzymes serve an important function in protecting macromolecules by catalyzing the conjugation of glutathione

(GSH) to reactive electrophiles (Townsend and Tew, 2003). The result of this study showed, a reduction in GST activity in the groups exposed to isometamidium chloride for 5 days. This outcome could be attributed to the elevated level of ROS such as H₂O₂ in this study as excess ROS may inhibit the activities of antioxidant enzymes (Kim and Hyun, 2006).

To further ascertain if the disruption of the redox status of *Drosophila melanogaster* as observed in this study caused DNA damage, 8-OHdG was assayed for. 8-OHdG is one of the prominent oxidative DNA lesions formed when hydroxyl radical attack guanine and as a result, 8-OHdG has been widely used as a biomarker for oxidative stress and carcinogenesis (Valavanidas *et al.*, 2009; Fenga *et al.*, 2017). Contrary to the report of Nie *et al.* (2012) which showed increase in the level of 8-OHdG and ROS in rats exposed to radon gas, the result in this study showed no difference in the level of 8-OHdG between the control and the isometamidium exposed groups. This suggests that the oxidative stress observed does not show clear involvement in DNA damage.

The central nervous system is key in motor control. Different sensory inputs are conveyed via neurons and muscles and any perturbation within the central nervous system can result in motor defects (Manjila *et al.*, 2018). In an attempt to know if isometamidium chloride could affect the behavior of the flies, the climbing performance and acetylcholinesterase (AChE) activity in the flies were examined. A significant reduction in the climbing performance of the flies exposed to isometamidium chloride was observed. In contrast, no significant difference was observed in the activity of AChE in both control and isometamidium chloride-exposed flies. This observation is not similar to the positive correlation between inhibition of AChE activity and reduction of climbing activity reported by Abolaji *et al.* (2015). The result here suggested that isometamidium chloride impaired climbing behavior. However, this effect may not necessarily involve AChE

activity but may be attributed to the effect of the compound on other neurotransmitters not assessed in this study.

The mammalian cells are constantly harmed by genotoxic insults. In order to maintain genome stability, two important proteins namely p53 and PARP1 are involved in DNA damage response and repair mechanisms (Fischbach *et al.*, 2018). The interplay between p53 and PARP1 is not completely understood, though thoughts that PARP1 may be involved in the activation of p53 through its poly (ADP-ribosyl)-ation activity exists (Süsse *et al.*, 2004). Both p53 and PARP1 mRNA levels were shown to be higher in tumor cells (Zerdoumi *et al.*, 2015; Barton *et al.*, 2009), suggesting the up-regulation of either of these two genes as an indicator of genotoxic stress. The result of this study showed that both p53 and PARP1 relative mRNA levels were reduced after five (5) days exposure to isometamidium chloride. This observation suggests that isometamidium chloride did not cause genotoxic stress, further validating the result obtained when 8-OHdG level was determined where we observed no difference in the level of 8-OHdG in both the flies exposed to isometamidium chloride and the control.

Molecular docking is an important tool in structural biology used in studying how a ligand interacts with a known protein and in the same manner used to predict how structurally the ligand inhibits the target protein (Morris and Lim-Wilby, 2008). In this study, the Ramachandran plot showed that a significant percentage of the amino acid residues of both proteins were found in the favoured (98%) regions and allowed (>99.8%) regions. This suggests that the modelled p53 and PARP1 proteins of both *D. melanogaster* and *B. taurus* were good models for docking. The DNA-binding domain of *Drosophila* p53 protein lies within residues 90-275 (Ollmann *et al.*, 2000). Generally, the activation domain of p53 protein lies at the N-terminal region (amino acid residues 1-64), while the DNA binding domain lies between the C and N-terminals (amino acid

residues 101-292) (Mishra *et al.*, 2013). Here, the result showed that isometamidium interacted with p53 modelled protein of *B. taurus* with binding energy of -8.1Kcal/mol and formed hydrogen bond with Asp179 and Van der Waals bonds with Glu2, Ser4, Leu32, Ser34, Leu36, Ser37 and Ala38. Likewise, Isometamidium bind to modelled p53 protein of *D. melanogaster* with binding energy of -9.4Kcal/mol and formed strong hydrogen bonds with Pro220 and Leu221 as well as Van der Waals bonds with Ile219, Asn222 and Ser256. *Drosophila* PARP is identical to the mammalian PARP1 (which is conserved across all mammals) and includes all the conserved domains except for the canonical caspase cleavage site (Tulin *et al.*, 2002). The catalytic domain of PARP1 generally found at the C-terminal of the protein is known to be formed by HYE (His-Tyr-Glu) motive (Hassa and Hottiger, 2008). As observed from the molecular docking of isometamidium with the modelled PARP1 proteins of *B. taurus* and *D. melanogaster*, most of the interactions were formed with the amino acid residues at the C-terminal regions. Of note is the hydrogen bonds formed with Glu626, Tyr629 as well as the Van der Waals interactions with Tyr601 and His686 amino acid residues of modelled PARP1 protein of *D. melanogaster*. Similarly, isometamidium interacted with modelled PARP1 protein of *B. taurus* where three important bonds were formed; covalent, hydrogen and van der waals bonds and all the bonds formed were with the C-terminal amino acid residues. These interactions formed together with the high binding affinity (-9.2 Kcal/mol and -10.3 Kcal/mol) of isometamidium to PARP1 proteins of *D. melanogaster* and *B. taurus* respectively, showed that the compound will readily bind to the proteins thereby making it a potential inhibitor of the modelled proteins. Similar to this result, a study showed that diminazene inhibited PARP1 in *D. melanogaster* (Kirsanov *et al.*, 2014).

CHAPTER SIX

6.0 Summary, Conclusion and Recommendation

6.1 Summary

The LC₅₀ of isometamidium chloride for 7 days exposure in *D. melanogaster* was 35.88 mg/10 g diet. At the fifth day of the sub-chronic exposure of *D. melanogaster* to isometamidium chloride, the earliest effect was observed where there was at least 80% survival across all the groups and the percentage survival of the flies at the end of the sub-chronic (28 days) exposure was reduced in a time and concentration-dependent manner. The climbing performance of flies exposed to isometamidium chloride for 5 days was significantly ($P < 0.05$) reduced. Whereas, the redox status of flies was disrupted after 5 days exposure to isometamidium chloride, H₂O₂ level was significantly ($P < 0.05$) increased and TSH levels was significantly ($P < 0.05$) reduced. While GST and CAT activities were significantly ($P < 0.05$) reduced. Both p53 and PARP1 mRNA Levels were significantly ($P < 0.05$) reduced in all isometamidium chloride-exposed groups. Isometamidium bind to p53 and PARP1 modelled proteins of both *B. taurus* and *D. melanogaster* with great binding affinity of -8.1 Kcal/mol, -9.4 Kcal/mol (p53) and -10.3 Kcal/mol, -9.2 Kcal/mol (PARP1) respectively and interacted with key amino acids important in the DNA binding domain (DBD) of p53 and C-terminal (Catalytic domain) of PARP1.

6.2 Conclusion

Isometamidium chloride is toxic to *D. melanogaster* as it impedes the fly's survival and disrupts the redox status indicating that it is potentially cytotoxic. However, based on the insignificant levels of 8-OHdG observed, there was no clear role of isometamidium chloride being genotoxic per se to *D. melanogaster* in this study, but the down-regulation of *p53* and *PARP1* genes and the inhibition of p53 and PARP proteins could make the DNA prone to damage.

6.3 Recommendations

- i. To further ascertain if isometamidium chloride is genotoxic or not, other cytogenetic analyses should be carried out. This is so because the analyses in this study focused on the quantification of oxidative DNA lesions (8-OHdG) and the expression levels of *p53* and *PARP* genes which are not necessarily the only indicators of genotoxic stress.
- ii. Other model organisms can also be employed to further study the mechanism of toxicity in the use of isometamidium chloride.

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APPENDICES

Appendix I: RNA Concentration and Purity

Groups Conc.(mg/g diet)	Conc. (ng/ μ L)	A (260/280)	A (260/230)
0.000 A	2556.0	1.99	0.89
0.000 B	2409.4	2.05	1.28
0.000 C	1080.4	1.82	0.51
0.000 D	1992.6	1.93	0.74
0.000 E	1141.3	1.97	0.85
0.449 A	1354.5	2.02	1.06
0.449 B	1835.5	1.93	1.00
0.449 C	1117.2	1.80	0.53
0.449 D	972.3	1.93	0.79
0.449 E	1864.7	2.06	1.08
0.897 A	2035.9	2.08	1.27
0.897 B	2732.7	2.12	1.67
0.897 C	959.4	1.88	0.74
0.897 D	4722.9	2.17	1.71
0.897 E	1054.1	2.01	0.98
1.794 A	806.3	1.74	0.41
1.794 B	2452.4	2.04	1.48
1.794 C	923.9	1.92	0.83
1.794 D	1068.9	1.77	0.49
1.794 E	3264.7	2.14	1.58
3.588 A	2009.4	2.12	1.29
3.588 B	620.1	1.80	0.44
3.588 C	1463.8	2.04	1.16

3.588 D	576.5	2.00	0.92
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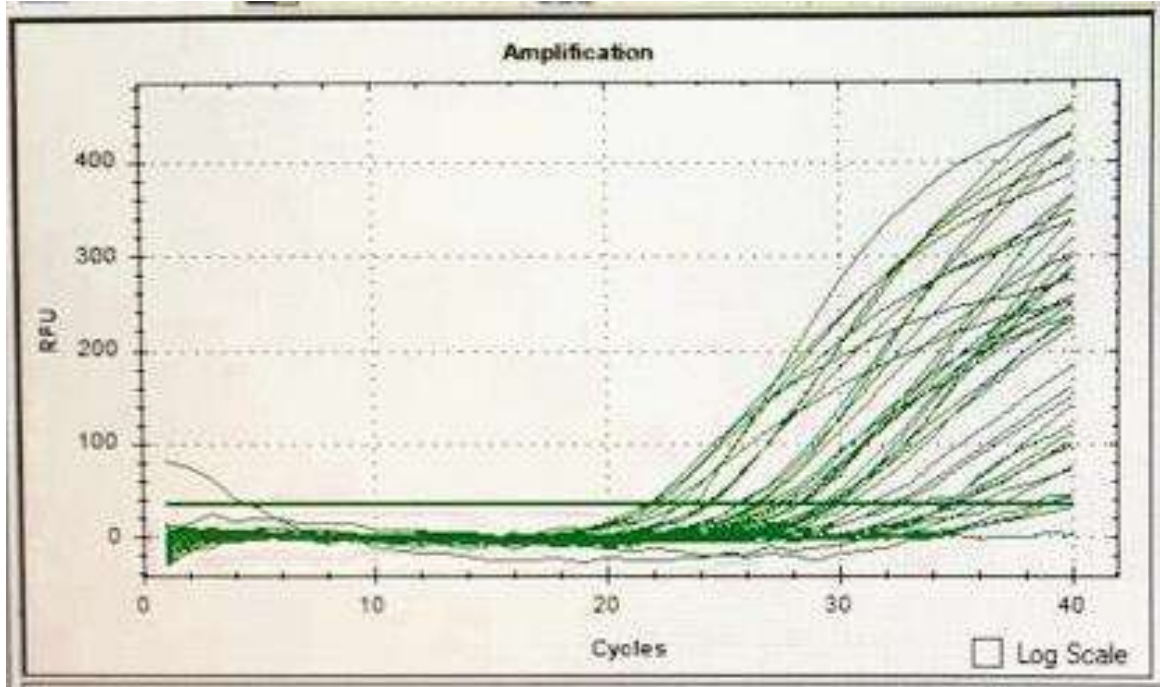
Appendix II: cDNA Quantification

Group Conc. (mg/g diet)	Conc. (ng/ μ L)	A (260/280)	A (260/230)
0.000 A	1312.8	1.75	2.24
0.000 B	1268.6	1.78	1.96
0.000 C	1644.5	1.80	1.73
0.000 D	1357.2	1.77	1.78
0.000 E	1530.4	1.84	1.92
0.449 A	1338.1	1.78	1.98
0.449 B	1501.2	1.83	2.03
0.449 C	1710.7	1.84	1.72
0.449 D	1544.5	1.81	1.79
0.449 E	1639.0	1.83	1.86
0.897 A	1410.4	1.76	2.24
0.897 B	1504.6	1.78	2.0
0.897 C	1364.6	1.78	1.82
0.897 D	1356.1	1.78	2.10
0.897 E	1466.8	1.81	1.99
1.794 A	1759.8	1.83	1.69
1.794 B	1327.3	1.76	2.14
1.794 C	1397.1	1.78	1.89
1.794 D	1412.8	1.77	1.51
1.794 E	1359.4	1.77	2.15
3.588 A	1503.1	1.79	2.12
3.588 B	1299.2	1.77	1.59
3.588 C	1282.3	1.78	2.00
3.588 D	1337.6	1.78	2.02

Appendix III: cDNA Reconstitution

Group Conc.(mg/g diet)	Initial Conc. C₁ (ng/μL)	Initial Volume V₁ (μL)	Nuclease-free water Volume (μL)
0.000 A	1312.8	7.6	92.4
0.000 B	1268.6	7.8	92.2
0.000 C	1644.5	6.0	94.0
0.000 D	1357.2	7.4	92.6
0.000 E	1530.4	6.6	93.4
0.449 A	1338.1	7.4	92.6
0.449 B	1501.2	6.6	93.4
0.449 C	1710.7	5.8	94.2
0.449 D	1544.5	6.4	93.6
0.449 E	1639.0	6.0	94.0
0.897 A	1410.4	7.0	93.0
0.897 B	1504.6	6.6	93.4
0.897 C	1364.6	7.4	92.6
0.897 D	1356.1	7.4	92.6
0.897 E	1466.8	6.8	93.2
1.794 A	1759.8	5.6	94.4
1.794 B	1327.3	7.6	92.4
1.794 C	1397.1	7.2	92.8
1.794 D	1412.8	7.0	93.0
17.94 E	1359.4	7.4	92.6
3.588 A	1503.1	6.6	93.4
3.588 B	1299.2	7.6	92.4
3.588 C	1282.3	7.8	92.2
3.588 D	1337.6	7.4	92.6
3.588 E			

Final Concentration (C₂) = 100ng, Final Volume (V₂) = 100 μL



Appendix III: qPCR Amplification Curve

RESEARCH ARTICLE



Isometamidium chloride alters redox status, down-regulates *p53* and *PARP1* genes while modulating at proteomic level in *Drosophila melanogaster*

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ABSTRACT

As trypanocide, several side effects have been reported in the use of Isometamidium chloride. This study was therefore, designed to evaluate its ability to induce oxidative stress and DNA damage using *D. melanogaster* as a model organism. The LC_{50} of the drug was determined by exposing the flies (1–3 days old of both genders) to six different concentrations (1 mg, 10 mg, 20 mg, 40 mg, 50 mg and 100 mg per 10 g of diet) of the drug for a period of seven days. The effect of the drug on survival (28 days), climbing behavior, redox status, oxidative DNA lesion, expression of *p53* and *PARP1* (Poly-ADP-Ribose Polymerase-1) genes after five days exposure of flies to 4.49 mg, 8.97 mg, 17.94 mg and 35.88 mg per 10 g diet was evaluated. The interaction of the drug *in silico* with *p53* and *PARP1* proteins was also evaluated. The result showed the LC_{50} of Isometamidium chloride to be 35.88 mg per 10 g diet for seven days. Twenty-eight (28) days of exposure to Isometamidium chloride showed a decreased percentage survival in a time and concentration-dependent manner. Isometamidium chloride significantly ($p < 0.05$) reduced climbing ability, total thiol level, Glutathione-S-transferase, and Catalase activity. The level of H_2O_2 was significantly ($p < 0.05$) increased. The result also showed significant ($p < 0.05$) reduction in the relative mRNA levels of *p53* and *PARP1* genes. The *in silico* molecular docking of Isometamidium with *p53* and *PARP1* proteins showed high binding energy of -9.4 Kcal/mol and -9.2 Kcal/mol respectively. The results suggest that Isometamidium chloride could be cytotoxic and a potential inhibitor of *p53* and *PARP1* proteins.

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KEYWORDS

Isometamidium chloride;
oxidative stress; *p53*; Poly-
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Drosophila melanogaster

Introduction

Animal trypanosomiasis encompasses different wasting diseases caused by numerous species of unicellular flagellated protozoan parasites belonging to the genus *Trypanosoma*. The parasite is transmitted actively by blood-sucking insects from infected to uninfected hosts (Pan et al. 2006). There are three important pathogenic trypanosomes *T. vivax*, *T. congolense*, and *T. brucei* spp belonging to the Salivaria group, so called because their transmission to the vertebrate host is mainly through the infected saliva of the blood-sucking insects (Giordani et al. 2016). There are several clinical signs shown by the disease, ranging from fever to neuropathology which may lead to death if left untreated (Rodrigues et al. 2009). The disease has a high economic and social impact in many areas where transmission occurs as it affects animal production and husbandry with Africa suffering the highest burden historically (Stevenson 2008). Absolute drug specificity with no or relatively side effects are key to the management and treatment of this unfortunate disease.

Isometamidium, Diminazene, and homidium are the main compounds used in the treatment of African Trypanosomiasis

(Bengaly et al. 2018). Isometamidium chloride is a cationic phenanthridine, synthesized by linking homidium with *p*-aminobenzamide which is a moiety of diminazene (Giordani et al. 2016). Although the drug is used mainly in the treatment of *T. congolense* and *T. vivax*, it can also be used against *T. evansi* but not when the infection has reached the central nervous system as the drug does not cross the blood-brain barrier (Giordani et al. 2016). Ever since the withdrawal of Quinapyramine due to high cases of resistance, Isometamidium became the only compound used for chemoprophylaxis purposes (Uilenberg 1998). The drug has proven to be effective in the treatment of this disease with mild side effects (Raftery et al. 2019). However, adverse effects like cell damage in rabbits and the death of small fish have been reported (Ali and Haroun 1984, Ardell and Woo 2001). Also, there was a report of some neurological reactions when the drug was used in the treatment of *T. evansi* in camels (Ali and Hassan 1988). Oxidative stress has been implicated in anaphylaxis (Mohamed and Hussein 2019), itches in mice (Liu and Ji 2012), and neurodegenerative diseases (Uttara et al. 2009) characterized by accumulation senile plaques and neurofibrillary changes in the brains of affected organisms

Appendix IV: Paper Publication

NIGERIAN SOCIETY OF BIOCHEMISTRY AND MOLECULAR BIOLOGY (NSBMB)
DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF MAIDUGURI, MAIDUGURI BORNO STATE

39th

ANNUAL SCIENTIFIC CONFERENCE
MAIDUGURI 2023

THEME:
BIOCHEMISTRY AND MOLECULAR BIOLOGY-POTENTIAL TOOLS FOR ALLEVIATING ECONOMIC AND SECURITY CHALLENGES IN NIGERIA

DATE: Sunday 11th - Friday 15th June, 2023
VENUE: Muhammadu Indimi International Learning Centre, University of Maiduguri

BOOK OF ABSTRACTS

SPECIAL GUEST OF HONOUR
His Excellency,
Professor Babagana Umara Zulum, PhD, CON, FRSC
The Executive Governor of Borno State

Chief Guest:
Professor Aliyu Shugaba
The Vice-Chancellor, University of Maiduguri

Chair:
Prof. D.A. Saifudeen, *Executive Director, NISBMB*
President 7-65316

NIGERIAN SOCIETY OF BIOCHEMISTRY AND MOLECULAR BIOLOGY (NSBMB)

EAT 011

ISOMETAMIDIUM CHLORIDE IMPEDES SURVIVAL, CLIMBING PERFORMANCE AND ALTERS THE REDOX STATUS OF DROSOPHILA MELANOGASTER

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ABSTRACT
Isometamidium chloride is a drug used in the prevention and treatment of Animal African Trypanosomiasis (AAT). However, several side effects have been reported in the use of this drug. This study was, therefore, designed to evaluate its ability to induce oxidative stress using *D. melanogaster* as a model organism. The LC₅₀ of the drug was determined by exposing the flies (1-3 days old of both genders) to six different concentrations (1mg, 10mg, 20mg, 40mg, 50mg and 100mg per 10g of diet) of the drug for a period of seven days. The effect of the drug on survival (28 days), climbing behavior and redox status after five days exposure of flies to 4.49mg, 8.97mg, 17.94mg and 35.88mg per 10g diet was evaluated. The result showed the LC₅₀ of isometamidium chloride to be 35.88mg per 10g diet for seven days. Twenty eight (28) days exposure to isometamidium chloride showed a decreased percentage survival in a time and concentration-dependent manner. Isometamidium chloride significantly ($p < 0.05$) reduced climbing ability, total thiol level, Glutathione-S-transferase and Catalase activity. The level of H₂O₂ was significantly ($p < 0.05$) increased. The results suggest that isometamidium chloride could be cytotoxic as it impedes survival and disrupts the redox status of *D. melanogaster*.

Appendix V: Conference Presentation