Toxicological Assessment of a widely consumed Nigeria Seasoning powder (Benny Seasoning Powder) on *Drosophila Melanogaster* (Survival, Negative Geotaxis, and Neurotoxicity).

BY

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FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN

BENIN CITY

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AN UNDERGRADUATE DISSERTATION SUBMITTED TO THE DEPARTMENT OF ENVIRONMENTAL MANAGEMENT AND TOXICOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA; IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN ENVIRONMENTAL MANAGEMENT AND TOXICOLOGY.

November, 2024.

CERTIFICATION

This is to certify that this research titled **Toxicological Assessment of a widely consumed Nigeria Seasoning powder (Benny Seasoning Powder) on *Drosophila Melanogaster* (Survival, Negative Geotaxis, and Neurotoxicity),** was carried out by **ESTHER CHIOMA UGBO** and presented to the Department of Environmental Management and Toxicology, Faculty of Life Sciences, University of Benin, Benin City; in partial fulfilment of the requirement for award of Bachelor of Science (B.Sc.) in Environmental Management and Toxicology. It was conducted under stable conditions, was carefully supervised and subsequently approved as having met the requirements for the award of Bachelor of Science Degree in Environmental Management and Toxicology.

______________________________ ____________________

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(PROJECT SUPERVISOR)

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PROF. E. T. AISIEN **DATE**

(HEAD OF DEPARTMENT)

DECLARATION

I, ESTHER CHIOMA UGBO declare that Toxicological Assessment of widely consumed Nigeria Seasoning powder (Benny Seasoning Powder) in *Drosophila Melanogaster* (Survival, Negative Geotaxis ,and neurotoxicity) is my own work and that all sources that I have used or quoted have been acknowledged by means of complete references and that this work has not been submitted before for any other degree at any other University.

______________________________ ______________________________

Esther Chioma Ugbo Date

DEDICATION

I dedicate this project to my lovely God Jehovah, my parents, my lovely siblings and myself.

ACKNOWLEDGEMENT

I want to sincerely thank Jehovah God for the grace he has given me to complete my degree and this project, non of these would have been possible without Him.

I appreciate my lovely parents Mr. and Mrs. Ugbo and for their love, prayers and support all through my school days. I appreciate the best siblings I could ever ask for Mrs. Rita Eke, Mr. Davidson Ugbo, Mrs. Ogechi Ucheagwu and Mr. Onyeka Ugbo who love me unconditionally and supported me in every way possible. I thank Mr. Davidson Ugbo specially for being my biggest cheerleader, my ATM and my one call away sibling. This project would never be possible without you. Thank you so much.

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ABSTRACT

Benny seasoning is a cooking powder that is commonly used in Africa for improving the taste, aroma and color of food. The potential health risk from the use of Benny seasoning powder remains unclear. Oxidative stress has been implicated in disease onset. Hence in this study oxidative parameters were assessed in fruit flies (*Drosophila melanogaster*) exposed to Benny seasoning at various concentrations (Control, 0.025 g/mL, 0.05 g/mL and 0.1 g/mL) for seven (7) days. A survival study and climbing assay was conducted and the observations gotten from the study showed that with increasing concentration, mortality rate increased and climbing activity decreased. After homogenization, specific markers of oxidative stress response (Protein, Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA)) were assessed. The results showed that there was no significant difference ($p<0.05$) when control was compared with the treatment groups. However there were alterations (increase and decrease) in all of the markers indicating a response to oxidative stress. Some genes involved in neurotoxicity were observed (SPITZ, WINGLESS, EIGER, FOXO HEDGEHOG, KEAP1) after exposure to Benny seasoning. There were significant differences with increasing concentrations in the expression of these genes indicative of neurotoxicity. Further studies may be needed to fully assess oxidative effect at the genetic level in order to completely understand oxidative stress responses of *drosophila melanogaster* to Benny seasoning powder.

CHAPTER ONE

INTRODUCTION

1.1 Background study

Drosophila melanogaster belongs to the *Drosophilidae* family, a group often referred to as "small fruit flies," which are typically seen around rotting or overly ripe fruit (Atoki *et al.,* 2024). Their survival and locomotion are influenced by several physiological factors, with lifespan being a primary measure of survival. Under optimal conditions, *Drosophila melanogaster* generally lives for 40–50 days, although well-maintained populations may survive for about 70 days on average, with some reaching up to 90–100 days (Partridge *et al*., 2017).

These flies display a behavior known as negative geotaxis, which is their instinctive tendency to climb upward against gravity (Zhong *et al*., 2022). This climbing ability can be shaped by multiple factors:

Age – locomotor performance declines progressively with age (Tower, 2023).

Health and Disease – infections and oxidative stress contribute to reduced climbing activity (Gomes *et al*., 2023).

Genetics – the genetic background of the fly influences how rapidly motor decline occurs with aging (Bragina *et al*., 2025).

Environment – stressors such as low temperatures can impair climbing ability (Klesatel and Galikova, 2022).

Because of its well-studied genetics, *Drosophila melanogaster* has become one of the most important model organisms in biomedical and developmental biology research. While "fruit fly" and "Drosophila" are often used interchangeably in scientific literature, the genus itself contains more than 1,500 species with diverse traits and ecological preferences (Rohner *et al*., 2018). Importantly, over 65–70% of human disease-related genes have homologues in *Drosophila melanogaster*, underscoring its value in biochemistry, molecular biology, and genetics. Its short life cycle, rapid generational turnover, and ease of laboratory maintenance make it highly suitable for large-scale biological studies (Rocha, 2013). Although rodents such as mice and rats are also widely used, *Drosophila melanogaster* offers unique advantages, particularly its genetic accessibility and efficiency as a model system.

Drosophila melanogaster has a short generation cycle of about 10–12 days at room temperature, making it possible to observe multiple generations within a relatively short timeframe (Hales *et al*., 2015). This species is an excellent model for studying complex biological processes such as cancer, genetic mutations, and toxicological markers including oxidative stress and neurotoxicity (Atoki *et a*l., 2025).

Benny seasoning powder is a commonly used food additive in Nigeria and many other African countries, valued for enhancing the taste and aroma of traditional meals like stews, soups, rice, and vegetable dishes. Its main constituents include Monosodium Glutamate (MSG), Iodized Salt, Cornflour, Artificial Colourant and anti-caking Agents.

Excessive intake of these additives has been linked to adverse health outcomes through oxidative stress, with biomarkers indicating risks such as neurotoxicity, hypertension, and diabetes. Oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) in cells and tissues and the ability of the body to detoxify or neutralize these reactive products (Pizzino *et al.*, 2017). It is a critical factor in numerous pathological

conditions, including diabetic complications, neurological disorders, and cancer. Monitoring oxidative stress and inflammatory biomarkers may help detect early biochemical shifts toward disease, particularly during preclinical stages. This early detection can allow for more personalized and sensitive health profiling (Grant and Seyedsadjadi, 2020).

Neurotoxicity refers to any harmful effect on the structure or function of the central and peripheral nervous systems caused by biological, chemical, or physical agents (Soleimani *et al*., 2016). Neurotoxic agents can trigger a wide range of cellular and molecular disturbances, such as oxidative stress, inflammation, apoptosis, and impaired synaptic activity. Analyzing gene expression networks provides insight into these toxic responses by identifying critical genes and pathways involved (Kuwano, 2024).

1.2 Justification

Benny seasoning powder is a household staple across of Africa, used to improve the flavor and aroma of meals. However, its safety profile is of concern, as its major ingredients have been shown to induce oxidative stress and alter neurotoxic gene pathways, raising potential long-term health risks. For this reason, its use warrants careful consideration and continuous scientific evaluation.

The use of *Drosophila melanogaster* for this study is justified by several advantages:

1) It is a well-established model organism with close physiological, molecular, and genetic parallels to humans. It has a short life cycle, low maintenance cost, a fully sequenced genome, and is highly amenable to genetic manipulation.
2) *Drosophila melanogaster* possesses oxidative stress defense mechanisms (e.g., SOD, catalase, glutathione peroxidase, GSTs) that are highly conserved and comparable to those in humans (Wu, 2025).

1.3 Aim and Objectives of the Study

This study aims to evaluate the responses of *Drosophila melanogaster* to oxidative stress and neurological gene modifiers following exposure to Benny seasoning powder.

The specific objectives of this study are:

I. To determine the extent of survival in drosophila melanogaster on exposure to benny seasoning.
II. To assess behavioral responses in exposed drosophila melanogaster using the negative geotaxis (climbing assay).
III. To assess oxidative stress responses in drosophila melanogaster exposed to benny seasoning powder using specific markers of oxidation (SOD, CAT, GPX, GSH, Total Thiol and NO).
IV. To assess neurotoxic effects in exposred drosophila melanogaster using specific genetic markers of neurotoxicity (Wnt, Eiger, Foxo and KEAPI).

Specific objectives of the Study

1. The specific objective of this study are:
2. To observe behavioral changes using negative geotaxis as indicator
3. To carry out survival studies upon exposured to the toxicant
4. To identify oxidative and neurotoxic Responses in *Drosophila Melanogaster* upon Exposure to Benny seasoning powder.

5. To statistically determine differences in response to different concentrations of the benny seasoning powder exposed to the *Drosophila Melanogaster* for seven days in comparison with an unexposed group.

CHAPTER TWO

LITERATURE REFVIEW

2.1 *Drosophila melanogaster* as a biological model

Animal models serve as essential tools for exploring the mechanisms underlying the onset and progression of numerous human diseases. These models vary in complexity and, when applied through comparative medicine, have greatly contributed to biomedical research by enhancing understanding of both normal and abnormal biological processes (Domínguez-Oliva *et al.*, 2023). They have enabled the discovery of crucial insights into many diseases that would otherwise be difficult to obtain. Their usefulness often depends on the degree of genetic and physiological similarity they share with humans, allowing results to be applied to human health—though this extrapolation is not always accurate.

The fruit fly *Drosophila melanogaster*, a dipteran insect belonging to the Drosophilidae family, is one of the most extensively studied eukaryotic organisms and has made major contributions across several areas of biology (Chao *et al.*, 2017). It is increasingly recognized as a valuable model for investigating the biochemical basis of human diseases. Its short lifespan, high reproductive rate, ease of maintenance, simple genome with limited gene redundancy compared to vertebrate models, and the availability of advanced genetic tools for genome manipulation have all strengthened its position as a reliable animal model (Obafemi *et al.*, 2025). Research using *Drosophila melanogaster* has provided insights into a wide range of diseases, including neurodegenerative, cardiovascular, inflammatory, infectious, and metabolic disorders (Adesola et al., 2021). Comparative genomic studies further reveal that approximately 75% of human disease-related genes are conserved in *Drosophila melanogaster* (Calap-Quintana *et al.*, 2017).

2.1.1 History of drosophila melanogaster as biological model

Since its introduction into laboratories in the early 1900s, *Drosophila melanogaster* has played a pivotal role in many groundbreaking discoveries in genetics. Its use as a model organism was pioneered by Thomas Hunt Morgan, who received the 1933 Nobel Prize in Physiology or Medicine for demonstrating the role of chromosomes in heredity. His student, Hermann J. Muller, later earned the 1946 Nobel Prize for showing that X-rays can induce mutations. In 1995, Edward B. Lewis, Christiane Nüsslein-Volhard, and Eric F. Wieschaus were jointly awarded the Nobel Prize for uncovering the genetic mechanisms that control early embryonic development. More recently, Jules Hoffmann shared the 2011 Nobel Prize for identifying the activation of innate immunity in *Drosophila melanogaster*

The first person to rear *Drosophila melanogaster* was Harvard entomologist Charles Woodworth, shortly after the turn of the 20th century. While his reasons remain uncertain, the fly's short life cycle and ease of culture likely made it attractive for research. Woodworth introduced the species to his colleague William Castle, who, though initially focused on mammals, began using the flies to investigate inbreeding. Around the same time, Frank Lutz at the American Museum of Natural History also conducted extensive studies on the fly's basic biology, publishing numerous papers. Lutz later provided the flies to Thomas Hunt Morgan, who incorporated them into his research at Columbia University. At that time, the principles of heredity were still being debated, and Morgan's groundbreaking discoveries—along with the contributions of his talented graduate students—cemented *D. melanogaster* as a cornerstone model system in genetics.(Markow, 2015).

2.1.2 Biological makeup of drosophila melanogaster

The standard karyotype of *Drosophila melanogaster* consists of four chromosomes, visible in the dividing neuroblasts of larval brains. These include the X and Y sex chromosomes, two large autosomes (chromosomes 2 and 3), and a small fourth “dot” chromosome (Kaufman, 2017). The fruit fly’s genome has been fully sequenced and annotated, revealing over 14,000 genes distributed across the four chromosomes, with most genes concentrated on three of them. Remarkably, around 75% of human disease-associated genes have functional counterparts in *D. melanogaster* (Oyeniran, 2019).

Fruit flies reproduce rapidly, with females capable of laying up to 100 eggs daily for about 20 days. Development is also fast: at 25 °C, the full cycle from egg to adult takes around 10 days (Popis et al., 2018). Fertilized eggs develop into first-instar larvae within 24 hours, and the larvae progress through two further stages (second and third instars), each lasting roughly a day. During these larval phases, glycolysis is highly active, lactate production is elevated, and glycogen and triglycerides (TAGs) accumulate in preparation for metamorphosis. By 2–3 days, the larvae pupate, and over 3–5 days of pupation, adult tissues form. The fly then emerges (ecloses) from the pupal case and becomes sexually mature in about 24 hours. In laboratory settings, adults typically live 60–90 days depending on temperature and diet (Brischigliaro et al., 2023).

A key feature of *Drosophila melanogaster* development is the imaginal discs—clusters of embryonic cells that give rise to adult structures like wings, legs, and eyes. Unlike in humans, most adult tissues in flies cannot regenerate; for instance, a lost wing will not regrow. However, imaginal discs are capable of limited repair under certain conditions, making them valuable for studying tissue regeneration genetics (Bergantiños *et al*., 2010).

In the wild, *Drosophila melanogaster* primarily feeds on rotting and fermenting organic matter, particularly overripe fruits. They consume yeast, bacteria, and other microorganisms on fruit surfaces (Wong *et al.*, 2016). Yeasts such as *Saccharomyces cerevisiae* are especially important, providing essential nutrients like amino acids and sterols that the flies cannot synthesize (Colinet and Renault, 2020). In laboratories, fruit flies are usually kept on cornmeal–agar–sugar–yeast diets, which supply carbohydrates, proteins, and micronutrients. Some diets are supplemented with molasses or soy flour, while chemically defined "holidic" diets are used when researchers require precise nutrient control for metabolic or toxicological studies (Piper *et al.*, 2017).

Carbohydrates serve as the primary energy source, while yeast-derived proteins and sterols are vital for growth and reproduction. Flies also obtain water from their food medium. When deprived of protein sources such as yeast, they show reduced fertility and shorter lifespans (Piper and Partridge, 2016). The balance between carbohydrate and protein intake is critical, influencing longevity, fecundity, and stress resistance—making *Drosophila melanogaster* a useful model in nutrigenomics and dietary restriction studies (Lee *et al.*, 2017).

Their small size further adds to their convenience as laboratory organisms. On average, males weigh 0.8–1.0 mg, while females are slightly heavier (1.0–1.2 mg) due to egg production (Piper & Partridge, 2016). Larvae grow rapidly, increasing their mass more than 200-fold in just a few days before pupating and metamorphosing into adults (Colinet and Renault, 2020).

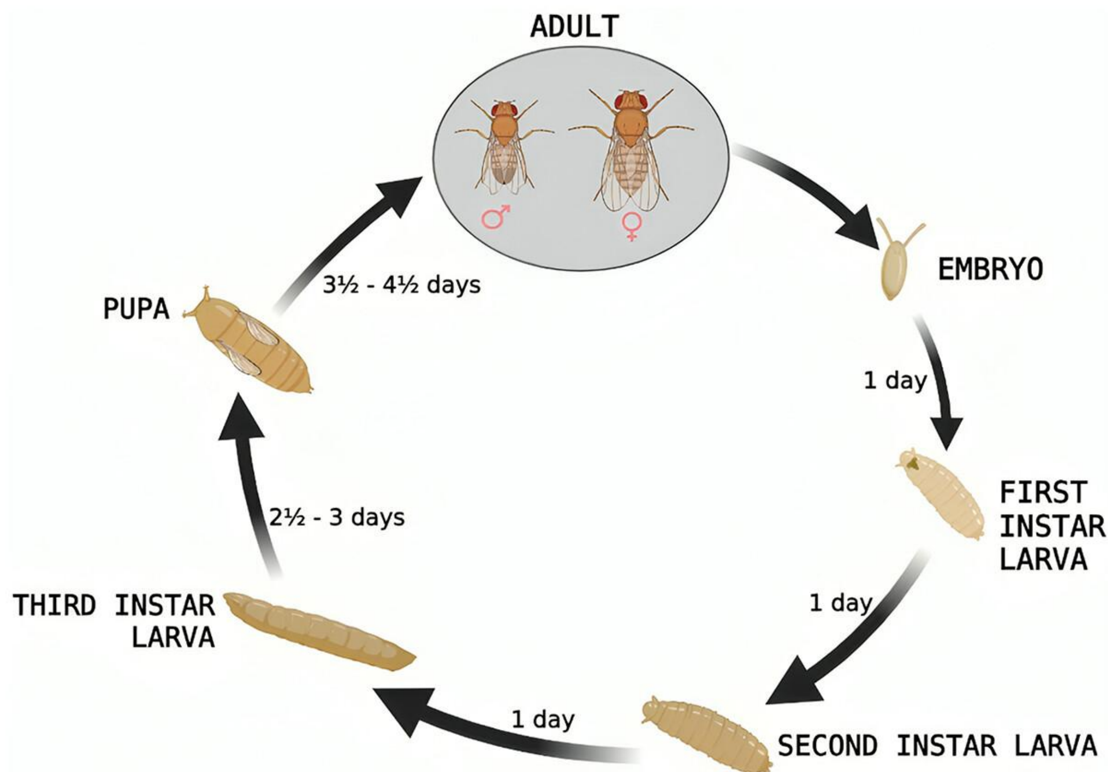


Figure 2.1: Life cycle of Drosophila Melanogaster

Source: (Atoki *et al.,* 2025).

2.1.3 Relevance of using D*rosophila melanogaster* as a model organism

Drosophila melanogaster (fruit fly) is an arthropod belonging to the *Drosophilidae* family of dipterans—an order of true, two-winged flies. It is one of the most extensively studied eukaryotic species and has contributed significantly to multiple areas of biology. In recent years, *Drosophila* has gained even greater importance as a model organism for investigating human diseases such as neurodegenerative, cardiovascular, inflammatory, infectious, and metabolic disorders (Chao *et al.,* 2017; Adesola *et al*., 2021).

Among the many *Drosophila* species, *Drosophila melanogaster* has been particularly valuable in genetic research for more than a century. It continues to serve as a leading model

in biomedical and developmental biology, especially in genetics and molecular biology (Yamaguchi and Yoshida, 2018). The genus itself includes over 1,500 species, displaying broad variation in traits, behaviours, and breeding habitats (Rohner *et al.*, 2018). Notably, over 65–70% of human disease-related genes have functional equivalents in *Drosophila melanogaster*, making it a powerful system for studies in biochemistry, genetics, molecular biology, and cell biology.

Its experimental advantages include rapid generation time, short lifespan, low maintenance costs, high fertility, and ease of handling and genetic manipulation. These qualities enable large-scale laboratory studies (Tolwinski, 2017). Consequently, *Drosophila melanogaster* has become a critical tool in uncovering fundamental mechanisms behind human conditions such as rare Mendelian disorders, cancer, and neurodegeneration, while also advancing understanding of key biological processes including development, nervous system function, and behaviour (Pratamo *et al.,* 2023).

Furthermore, *Drosophila* research has provided unparalleled insight into the molecular mechanisms of gene regulation, at a speed and resolution that few other model organisms can match. The genome of *drosophila melanogaster*, consisting of roughly 16,000 genes across four chromosome pairs, has been a cornerstone of biomedical and genetic studies. Despite its relative simplicity compared to the human genome, more than 60% of its genes have identifiable human homologs (Nguyen *et al*., 2021). This genetic conservation, along with anatomical similarities in certain tissues and organs, underscores the value of *Drosophila* in cross-species comparative research and its role in advancing our understanding of human biology and disease. (Figure 2.2).

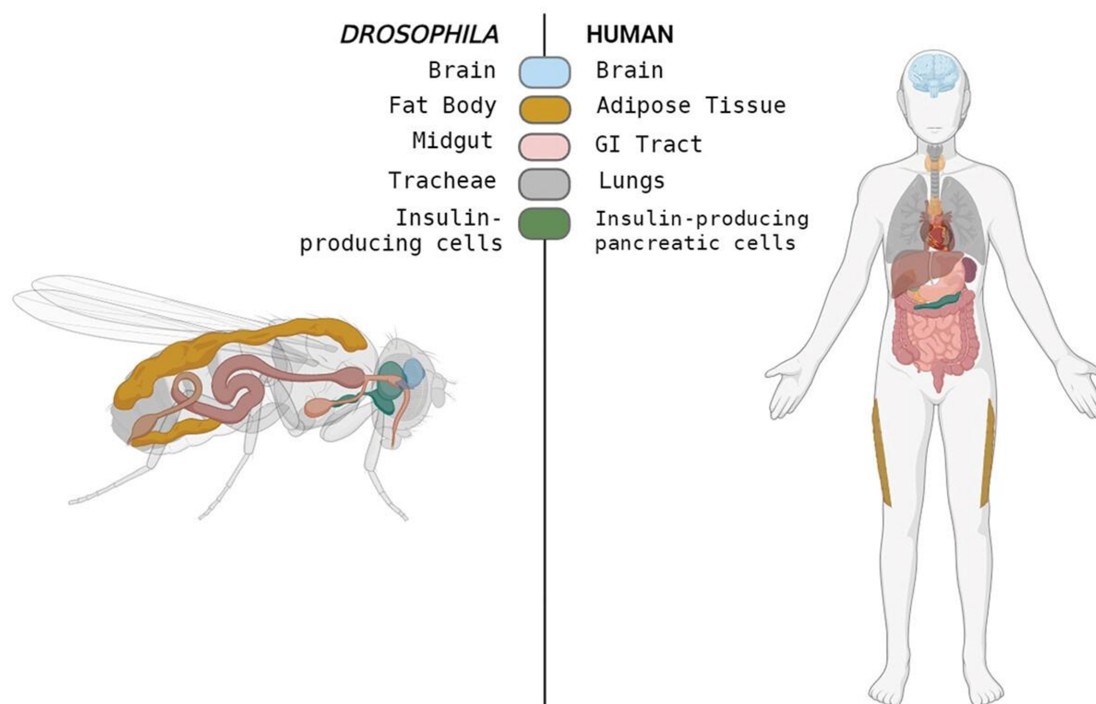


Figure 2.2: Tissue/Organ functional similarities shared by *Drosophila* and human.

Source: (Atoki *et al 2025)*

Drosophila melanogaster (fruit fly) has become a valuable model organism in toxicology, where it is used to study how environmental contaminants and toxic substances affect living systems. Its use is supported by regulatory guidelines, making it a suitable choice for experimental toxicological research (Alaraby *et al*., 2016). One of its greatest strengths is its short biological life cycle, which allows scientists to examine the effects of toxicants across all stages of development—from embryo to adult—in a relatively short period (Calap-Quintana *et al.*, 2017).

In addition, the fruit fly's adaptability and fully sequenced genome make it a powerful tool for pharmacological studies. Researchers can investigate how different chemicals interact with specific genes, leading to the discovery of potential new drugs and a deeper understanding of chemical–gene relationships (Ferrero, 2021).

Beyond toxicology, *Drosophila melanogaster* is highly valued because it is genetically amenable, meaning that its genes can be easily modified or studied. This feature makes it ideal for exploring gene functions and their roles in biological processes with both medical relevance, such as disease mechanisms, and economic importance, such as agricultural or environmental health.

2.1.4 Behavioral changes

Exposure to environmental toxicants often affects the negative geotaxis and survival rate of Drosophila melanogaster. Negative geotaxis — the innate tendency of flies to climb upward against gravity after being tapped to the bottom of a vial — is a standard indicator of locomotor and neuromuscular function. Toxicant exposure such as pesticides, heavy metals, and nanoparticles disrupts neuronal signaling and induces oxidative stress, which in turn reduces climbing ability. Toxicants often disrupt negative geotaxis by causing oxidative stress and neurodegeneration. When flies are exposed to harmful chemicals, reactive oxygen species (ROS) accumulate within nerve and muscle cells, damaging proteins, lipids, and DNA. This oxidative stress impairs the normal functioning of dopaminergic neurons that control movement, resulting in reduced climbing ability. In addition, many toxicants interfere with mitochondrial activity, leading to low cellular energy (ATP) levels, which further weakens muscle performance and slows locomotor responses (He *et al.*, 2020)

For example, Alghamdi *et al*. (2020) reported that *Drosophila melanogaster* exposed to cadmium oxide nanoparticles showed a marked decline in climbing performance due to oxidative stress and neurobehavioral changes. Similarly, chronic exposure to common insecticides such as imidacloprid or malathion caused flies to exhibit slower or uncoordinated climbing movements, reflecting damage to the nervous system (Wang *et al*., 2021). Even food additives like artificial sweeteners have been found to impair geotactic behavior (Ahmed

et al. 2023), who linked reduced climbing ability to disrupted energy metabolism and neuromuscular stress.

In terms of survival, *Drosophila melanogaster* exposed to increasing concentrations of toxicants often show dose-dependent mortality. Sublethal doses may allow survival but with behavioral impairments, while higher doses reduce lifespan through cumulative oxidative stress, apoptosis, and metabolic imbalance (Zhang *et al 2024).* Toxicants have a profound impact on the survival of *Drosophila melanogaster* by disrupting essential biological systems that sustain life. Survival, often measured through lifespan or mortality assays, reflects how exposure to harmful substances alters the fly's ability to maintain internal balance, repair cellular damage, and resist environmental stress.

When *Drosophila melanogaster* are exposed to toxicants such as heavy metals, pesticides, or nanoparticles, the most common effect is a dose-dependent decrease in lifespan. Toxicants impair survival by damaging cellular structures, interfering with detoxification enzymes, and overwhelming the fly's antioxidant defense system. This leads to excessive accumulation of reactive oxygen species (ROS), which cause oxidative stress and destroy macromolecules critical for normal cell function (He *et al.*, 2020). Over time, these cellular disruptions result in tissue degeneration, organ failure, and premature death.

Some toxicants also interfere with the fly's metabolic and reproductive systems, indirectly reducing survival. For example, chronic exposure to the pesticide imidacloprid reduces food intake, alters fat metabolism, and suppresses reproduction, which weakens the flies and shortens lifespan (Wang *et al.,* 2021). Similarly, exposure to cadmium oxide nanoparticles causes a significant reduction in adult survival by damaging the midgut epithelium and reproductive organs, leading to systemic toxicity (Alghamdi *et al.,* 2020).

Furthermore, toxicants can impair the expression of genes responsible for cell repair, stress resistance, and detoxification. When these genes are suppressed, the fly becomes less able to handle even mild chemical stress, accelerating mortality . artificial sweeteners like sucralose significantly shortened *Drosophila melanogaster* lifespan by disrupting normal carbohydrate metabolism, which resulted in energy imbalance and systemic failure

Together, impaired negative geotaxis and reduced survival serve as reliable biomarkers for assessing the neurotoxic and systemic effects of environmental pollutants in *Drosophila melanogaster* (Ali *et al.,* 2023).

2.2 Overview of Benny Seasoning Powder

Benny seasoning powders, commonly used as flavor enhancers or stock powders, are highly popular in Nigeria and across many African countries because they provide a cost-effective way to improve the taste of meals. These products typically contain a mixture of ingredients such as salt, starch or cornflour, monosodium glutamate (MSG) and related flavor enhancers, anti-caking agents, vegetable extracts, artificial colorants like tartrazine, and proprietary blends of seasonings.

While these additives serve specific purposes—such as enhancing taste, improving texture, and preventing clumping—there are growing concerns about their health implications when consumed in excess. Compounds like MSG, synthetic dyes, and anti-caking agents have been linked in some studies to potential metabolic disturbances, oxidative stress, and even neurotoxic effects, highlighting the need for moderation in their use.

The high sodium content in Benny seasoning is one of its major toxic components. (Aigberua *et al.*,2018) reported that common seasoning powders and condiments used in Nigeria contain sodium and heavy metals in concentrations capable of inducing adverse health effects when

consumed frequently. Excess sodium intake has been linked to hypertension, renal impairment, and oxidative stress due to disrupted osmotic balance and increased metabolic demand. Chronic ingestion of sodium-rich condiments has also been shown to alter ion homeostasis and enzyme function, predisposing organisms to oxidative damage and reduced longevity.

Another key ingredient, monosodium glutamate (MSG), has been widely studied for its potential neurotoxic and oxidative effects. (Kasozi *et al.,* 2018), prolonged dietary exposure to MSG in *Drosophila melanogaster* significantly reduced lifespan and altered antioxidant enzyme activity, suggesting induction of oxidative stress. MSG acts as an excitatory neurotransmitter mimic that can overstimulate neurons, leading to neurotoxicity, hepatic stress, and hormonal imbalance at high concentrations. Similar findings in mammalian studies have linked MSG consumption with oxidative damage and tissue degeneration.

In addition, vegetable fats and hydrogenated oils used in some seasoning powders contain trans fats, which promote lipid peroxidation and increase cardiovascular risk. Trans fats have been shown to elevate low-density lipoprotein (LDL) and reduce high-density lipoprotein (HDL) levels, predisposing consumers to atherosclerosis and metabolic disorders (Guardian, 2022). The anti-nutritional compounds such as phenols and alkaloids, detected in seasoning powders like Benny by Food Science Journal (2022), may further interfere with enzymatic activity and nutrient absorption.

Overall, the combination of high sodium, MSG, trans fats, anti-nutrients, and heavy metals makes Benny seasoning powder potentially toxic when consumed in large quantities or under prolonged exposure. These components can act synergistically to induce oxidative stress, impair neuromuscular function, and reduce organismal survival — effects that can be effectively modeled in *Drosophila melanogaster* and other experimental systems.

2.3 Oxidative Stress

Oxidative stress occurs when there is an imbalance between the excessive generation and buildup of reactive oxygen species (ROS) or free radicals in cells and tissues, and the ability of the body to neutralize or remove them. Although ROS are natural by-products of oxygen metabolism and play useful roles in processes such as cell signaling, their levels can be greatly increased by environmental factors such as ultraviolet radiation, ionizing radiation, pollutants, heavy metals, and certain drugs. This imbalance can damage cells and tissues (Pizzino *et al*., 2017).

Under normal conditions, cells maintain a steady redox balance, often described as a basal redox state. Any disruption of this balance is considered stress and activates a stress response (Sies, 2020). ROS and reactive nitrogen species (RNS) are unavoidable products of normal metabolism, and while they can be toxic, they also act as important intracellular signaling molecules (Hales *et al.*, 2020). To prevent excessive oxidative damage, cells are equipped with antioxidant defenses, which include direct antioxidants that neutralize free radicals and indirect systems that either limit the formation of ROS/RNS or detoxify their by-products. When ROS/RNS production rises above the capacity of these defenses, oxidative stress develops.

At elevated levels, ROS can damage vital cellular components such as proteins, lipids, and nucleic acids, leading to functional decline and disease development (Wu *et al.,* 2013). At low concentrations, however, free radicals contribute to physiological regulation and signaling processes. In contrast, high concentrations are harmful and can disrupt normal cellular activity. Antioxidants protect cells by donating electrons to stabilize free radicals, thereby reducing their reactivity and minimizing cellular damage (Nahar *et al*., 2017).

Free radicals, particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS), are unstable molecules with unpaired electrons that participate in essential cellular activities but also contribute to disease development. While they are naturally produced during metabolic processes, enzymatic reactions, and through environmental exposure, excessive accumulation of these radicals can overwhelm the body's antioxidant defenses, leading to oxidative stress (Pooja *et al*., 2025).

Their formation arises from several sources, including normal metabolism, enzymes such as xanthine oxidase, immune responses like nitric oxide release from macrophages, and external factors such as ultraviolet radiation and drug metabolism (Singh *et al.*, 2019). Within cells, ROS and RNS are generated through both enzymatic and non-enzymatic pathways. A major route is the incomplete reduction of oxygen during mitochondrial metabolism. Key ROS include superoxide (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). Common RNS include nitric oxide (NO), peroxynitrite ($ONOO^-$), and nitrogen dioxide (NO_2), which often arise through interactions between nitric oxide and ROS or from the activity of nitric oxide synthases.

2.3.1 Oxidative stress biomarkers and oxidative stress parameters

Oxidative stress biomarkers represent measurable outcomes of oxidative damage. Lipid peroxidation products are some of the most investigated, with malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) formed during the oxidation of polyunsaturated fatty acids. F_2-isoprostanes are regarded as the most reliable indicators because of their specificity and chemical stability (Zhang *et al.,* 2020). Protein oxidation is also a major marker, where protein carbonyls denote irreversible amino acid modifications, and advanced oxidation protein products (AOPPs) are linked to chronic inflammation and kidney disorders (Vona et al., 2021). Nitrotyrosine levels further reflect protein modifications driven by reactive

nitrogen species. DNA oxidation is commonly monitored through 8-hydroxy-2′-deoxyguanosine (8-OHdG), which can be detected in blood or urine and is closely tied to cancer risk and mutagenesis (Gella and Durany, 2020).

Alongside biomarkers, oxidative stress parameters provide information about antioxidant defense mechanisms and the cellular redox environment. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) are frequently measured since they form the first defense line against ROS (Ziehr and MacDonald, 2024). In addition, levels of non-enzymatic antioxidants like reduced glutathione (GSH), vitamins C and E, and uric acid are used to assess both internal and dietary antioxidant capacities.

2.3.2 Superoxide Dismutase (SOD)

SOD facilitates the dismutation of superoxide radicals ($O_2\bullet^-$) into hydrogen peroxide (H_2O_2) and oxygen. Different isoforms exist, such as Cu/Zn-SOD in the cytoplasm and Mn-SOD in the mitochondria. Decreased SOD activity is typically associated with oxidative stress seen in cardiovascular and neurodegenerative diseases (Zhang *et al*., 2020).

2.3.3 Catalase (CAT)

CAT breaks down hydrogen peroxide into water and oxygen, preventing the Fenton reaction that produces hydroxyl radicals. If (CAT) levels decrease, hydrogen peroxide builds up and reacts to form hydroxyl radicals, which are very toxic to cells. This results in lipid peroxidation and cell membrane damage. Changes in CAT activity have been implicated in conditions such as diabetes, atherosclerosis, and cancer (Vona *et al.,* 2021).

2.3.4 Glutathione Peroxidase (GPx)

GPx reduces hydrogen peroxide and lipid hydroperoxides by using glutathione (GSH) as a cofactor. It is vital for maintaining membrane stability and is a sensitive marker of oxidative imbalance in both metabolic and neurological disorders (Gella and Durany, 2020). When (GPx) decreases, these peroxides accumulate, causing membrane injury and increased cell death. A rise in (GPx) activity indicates the organism is mounting a defense against oxidative stress by neutralizing peroxides. Persistent elevation may, however, reflect prolonged oxidative challenge (Rady *et al.,* 2022).

2.3.5 Glutathione Reductase (GR)

GR is responsible for converting oxidized glutathione (GSSG) back into its reduced form (GSH), thereby sustaining antioxidant defenses. GR activity reflects the ability of cells to restore redox balance after oxidative damage and is often studied alongside GPx (Ziehr and MacDonald, 2024). If GR activity decreases, the supply of GSH drops, weakening antioxidant protection and increasing susceptibility to oxidative damage. When GR increases, it suggests the cells are compensating to maintain redox balance and counteract toxicity (Aigberua *et al.*, 2018).

2.3.6 Hydrogen Peroxide (H_2O_2)

This relatively stable ROS is produced during superoxide dismutation. At low concentrations, it functions as a signaling molecule, but excessive H_2O_2 can trigger hydroxyl radical formation via the Fenton reaction, leading to oxidative injury. It is a widely used oxidative stress parameter in cardiovascular and metabolic studies (Sies and Jones, 2020).

2.3.7 Malondialdehyde (MDA)

MDA is a lipid peroxidation by-product, commonly applied as a biomarker of oxidative stress. Elevated levels signify membrane injury and are associated with metabolic syndrome, cancer, and neurodegenerative disorders. It is usually measured with the TBARS assay (Vona *et al.*, 2021).

2.3.8 Nitric Oxide (NO)

NO is a reactive nitrogen species generated by nitric oxide synthases. While it plays a physiological role in vascular relaxation and neurotransmission, excessive production leads to its reaction with superoxide, forming peroxynitrite and driving nitrosative stress. It is commonly measured indirectly through its stable end-products, nitrate and nitrite. (Miller *et al.*, 2019)

2.4 Biomarkers of Neurotoxicity in *Drosophila Melanogaster*

2.4.1 Neurotoxic Gene Modulation

Neurotoxic gene modulation describes the process by which toxic agents influence gene expression, resulting in neuronal impairment or death, while genetic predispositions and targeted gene therapies can in turn shape neurotoxic outcomes. Neurotoxins trigger diverse molecular and cellular disturbances, including oxidative stress, neuroinflammation, programmed cell death, and impairments in synaptic signaling. Mapping gene expression networks offers an integrated perspective of these responses, enabling the identification of pivotal genes and pathways implicated in toxicity. For example, exposure to heavy metals such as lead or mercury disrupts genes responsible for oxidative defense, often causing an increased expression of antioxidant enzymes like Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) (Kuwano, 2024).

2.4.2 Inflammatory pathways in neurotoxicity

Neuroinflammation represents a crucial component of neurotoxic processes. Persistent exposure to toxins activates microglia and astrocytes, the brain's main immune cells, producing a sustained inflammatory state. Gene network analyses can unravel the complex signaling cascades underlying these inflammatory reactions. A key mediator is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which governs many inflammation-related genes. Investigating NF-κB-regulated expression networks enables researchers to pinpoint strategies for suppressing excessive inflammatory activity in neurotoxicity (Jong Huat *et al.,* 2024).

2.4.3 Apoptosis and cell death pathways

A common outcome of neurotoxic exposure is apoptosis, or regulated cell death, which contributes significantly to the progression of neurodegenerative disorders. Apoptotic gene networks involve regulators of both the mitochondrial (intrinsic) and receptor-mediated (extrinsic) pathways. Critical players include BAX, Bcl-2, and various caspases. The interplay between genes that promote apoptosis and those that inhibit it determines neuronal survival under toxic stress. Therapeutic modulation of these networks may help limit or prevent neuron loss caused by neurotoxic insults (Kebieche *et al.*, 2025).

2.4.4 Disruptions in synaptic function

Synaptic integrity is another major target of neurotoxins, as effective neurotransmission is vital for brain function. Gene expression networks governing synaptic plasticity and neurotransmitter release are often perturbed by toxic exposure. Changes in the transcription of synaptic proteins such as synapsins and neuroligins interfere with vesicle recycling and neurotransmitter signaling. Analysis of these networks offers valuable insight into how toxins

impair cognition and behavior (Adesola *et al.,* 2021). The following neurotoxic genes can give us insight to our studies

1. Spitz (Spi) — EGFR pathway activator

Spitz is the principal activating ligand for the Drosophila EGF receptor (EGFR) pathway and controls many developmental processes (cell proliferation, differentiation, and patterning) via paracrine signalling. Regulation of Spitz (and its antagonists like Argos) determines EGFR signalling amplitude and tissue outcomes. (Pasnurii *et al.,* 2023)

EGFR signalling (Spitz→EGFR) shapes neural precursor specification and glial/neuronal behaviours in the developing CNS. Disruption of EGFR signalling can alter neural cell fate and survival, making perturbations of Spitz–EGFR relevant when studying developmental neurotoxicity. Recent Drosophila studies show that altering EGFR signalling affects apoptosis patterns and tissue homeostasis — mechanisms that are often engaged during chemical or physical neurotoxic insults (e.g., increased cell death propagation). Thus Spitz expression changes or EGFR dysregulation can be mechanistically linked to neurotoxic outcomes that involve aberrant apoptosis or impaired tissue repair. (Yuswan *et al.,* 2024).

2) Wingless (Wg) / Wnt pathway

Wingless (Wg) is the founding Drosophila Wnt ligand and drives canonical Wnt/β-catenin signalling in many patterning, proliferation and synaptogenesis contexts. Wg signalling is evolutionarily conserved and regulates neuronal connectivity, synapse formation and stem cell behaviours (Bejsovec, 2013).

Proper Wg/Wnt signalling is required for neural patterning and synaptic maintenance. Both loss- and gain-of-function can impair synapse development and function, which makes this

pathway sensitive to neurodevelopmental toxicants. Emerging literature implicates aberrant Wnt signalling in neurological disease and aging; excessive or dysregulated Wnt activity has been associated with neurodevelopmental defects and contributes to disease-relevant phenotypes in model organisms. Consequently, Wg perturbat on in Drosophila is a useful readout for compounds that disturb pathways critical for neuronal survival and connectivity. Recent reviews emphasize how altered Wnt signalling contributes to neural dysfunction

Eiger (Egr) — the Drosophila TNF homolog

Eiger is the single TNF-family ligand in Drosophila that activates JNK signalling through TNF receptors (Grindelwald, Wengen). It is a potent inducer of apoptosis and a mediator of stress responses, immunity and cell competition (Palmerini *et al.,* 2021)

Eiger-triggered JNK activation propagates apoptosis and inflammatory-like responses; in the nervous system, such activation can cause neuronal death or degeneration if uncontrolled. Thus Eiger is a key mediator when neurotoxic exposures trigger stress- or apoptosis-related cascades. Drosophila studies show Eiger participates in apoptosis-induced apoptosis (AiA) and in eliminating damaged or oncogenic cells; similar JNK/TNF-driven mechanisms are central to secondary injury cascades after traumatic or toxic insults to the nervous system, making Eiger a logical marker and mechanistic actor in neurotoxicity models (Buhlman *et al.,* 2021).

4) FOXO (forkhead box O) transcription factors

Drosophila Foxo is the insulin/IGF signalling (IIS)–responsive transcription factor that controls stress responses, autophagy, metabolism, and longevity. Neuron-specific FOXO activity regulates neuronal homeostasis and synaptic function (Birnbaum, 2021)>

FOXO mediates transcriptional programmes in response to oxidative and metabolic stress. In neurons, FOXO activity influences synaptic maintenance, neuromuscular junction stability, and responses to proteotoxic/oxidative insults — processes central to neurotoxicity and neurodegeneration. For example, FOXO upregulation is observed after traumatic brain injury and in models of age-related cognitive decline, suggesting it's a stress-responsive node in neuronal injury.

Studies in Drosophila show FOXO helps maintain neuromuscular junction (NMJ) integrity and regulates MAPK signalling; manipulating FOXO modifies vulnerability to stressors, making it an attractive target/marker in neurotoxicity assays. (Asadi *et al.,* 2021).

5) Hedgehog (Hh) pathway

Hedgehog (Hh) is a secreted morphogen that patterns the nervous system during development, regulates neural progenitor fate, and influences glial behaviour. Canonical Hh signalling is conserved and crucial for correct neurogenesis (Kaushal *et al.,* 2022).

Dysregulation of Hh signalling affects neuronal survival and proteostasis in glia; recent studies highlight Hh's role beyond development — adult Hh signalling in Drosophila glia contributes to proteostasis and dopaminergic neuron integrity (Yang *et al.,* 2021). Thus Hh perturbation can sensitize neurons to toxic insults or accelerate degeneration. In vertebrate models, SHH pathway dysregulation has been linked to neurodegenerative diseases (Alzheimer's, Parkinson's), suggesting conserved mechanisms by which Hh pathway disruption contributes to neuronal vulnerability under stress or toxic exposure.

6) Nrf2 / Keap1 (dNrf2 / dKeap1 in Drosophila) — oxidative stress master regulator

Nrf2 (CncC in flies) with its regulator Keap1 is the canonical oxidative stress response pathway. Activation of Nrf2 drives expression of antioxidant and cytoprotective genes; in Drosophila, this module controls stress tolerance, lifespan, and cellular redox homeostasis (Yuh Chew *et al.,* 2021).

Oxidative stress is a common mediator of chemical neurotoxicity. Activation of the Keap1–Nrf2 axis provides neuronal protection by inducing antioxidant defenses; conversely, Keap1 loss or Nrf2 dysfunction alters neuronal resilience. Multiple Drosophila studies use Keap1/Nrf2 manipulation to modulate oxidative vulnerability and model neurodegenerative phenotypes (Chatterjee *et al.,* 2022).

Recent translational studies link Nrf2 activation to protection in ALS/FTD models and show pharmacological modulation of Keap1/Nrf2 can suppress neuronal pathology, highlighting the pathway's relevance to both neurodegenerative disease and neurotoxic stress responses. (Au *et al.,* 2024).

CHAPTER THREE

METHODOLOGY

3.1 Materials Used

A sachet of benny seasoning pwder, Falcon tube, 1000 microlitre micropipitte, 100 microlitre micropipitte, centifuge, cotten wool, tissue paper, glass jar, foil papper, paint brush, homogenizing stick, eppendorf tube, polyurethane foam, phosphate buffer, distilled water (H_2O), corn meal, agar agar, glucose, yeast, nipargin, ethanol, test tubes, beakers and UV-spectrophotometers.

3.1.1 Model Organism

The organism selected for this study is *drosophila melanogaster.* It was provided and breeded at Biomedical Toxicology and Chemistry Research (Biotoxs) Laboratory. Located at University of Benin Central research laboratory (CRL), Benin City, Edo State. Nigeria.

3.2 Methods

3.2.1 Experimentation design, grouping and treatment of experimentation animal

The treatment conditions was prepared using three (3) prepared concentrations of 0.025 g/mL, 0.05 g/mL and 0.1 g/mL of benny seasoning powder in distilled water (H_2O) with three replicate each and three control groups with only distilled water (H_2O), the concentrations were then added to 9.8g of meal in a falcon tube.

Table 3.1: Shows the experimental set-up for exposure of drosophila melanogaster to benny seasoniing powder.

Control (A)	200µl of distilled water + 9.8g of meal
Control (B)	200µl of distilled water + 9.8g of meal
Control (C)	200µl of distilled water + 9.8g of meal
Treatment 1(A)	200µl of 0.025 g/mL (Beenny Seasoning powder) + 9.8g of meal
Treatment 1(B)	200µl of 0.025 g/mL (Benny Seasoning powder) + 9.8g of meal
Treatment 1(C)	200µl of 0.025 g/mL (Benny Seasoning Powder)) + 9.8g of meal
Treatment 2 (A)	200µl of 0.05 g/mL (Benny seasoning powder) + 9.8g of meal
Treatment 2 (B)	200µl of 0.05 g/mL (Benny seasoning powder)+ 9.8g of meal
Treatment 2 (C)	200µl of 0.05 g/mL (Benny Seasoning powder)+ 9.8g of meal
Treatment 3 (A)	200µl of 0.1 g/mL (Benny Seasoning powder)+ 9.8g of meal
Treatment 3 (B)	200µl of 0.1 g/mL (Benny seasoning powder))+ 9.8g of meal
Treatment 3 (C)	200µl of 0.1 g/mL (Benny Seasoning powder)+ 9.8g of meal

Thirty (30) flies were transferred into each of the twelve (12) covered with cotton wool, This setup was left for seven (7) days, before homogenization.

Plate 3.1: Shows the experimental set-up for exposure of drosophila melanogaster to benny seasoning powder

3.2.2 Meal preparation

The meal was composed of corn meal, agar, glucose, nipargine, ethanol, yeast, and distilled water at quantities depending on the volume of meal needed. Cornmeal was mixed with one quarter (⅓) the quantity of water needed for preparation, while the rest of the water is allowed to boil, then yeast, agar, glucose, ethanol, nipargin and the mixed cornmeal were added in the boiling water with continuous stirring to obtain a creamy consistency. Table 3.2 shows the measurement of various ingredients needed for meal preparation.

Table 3.2: Measurement of various ingredients needed to prepare drosohilia meal

INGREDIENTS	STANDARD MEAL (g)	HALF STANDARD MEAL (g)	QUARTER STANDARD MEAL (g)
Corn meal	52 g	26 (g)	13 (g)
Agar	7.9 (g)	3.95 (g)	1.975 (g)
Glucose	3.5 (g)	1.75 (g)	0.875 (g)
Yeast	5 (g)	2.5 (g)	1.25 (g)
Nipargin	1 (g)	0.5 (g)	0. 25 (g)
Ethanol	1-2 (ml)	0.5 (ml)	0.25 (ml)
Water	850 (ml)	425 (ml)	212.5 (ml)

3.2.3 Breeding of flies

The prepared meal was poured into glass jars to cool down and solidified. Then flies were then transferred into the glass jar and covered with polyurethane foam that was pre-cut to fit the jar opening perfectly. Polyurethane form is used in order to allow aeration in the jar, and after few day (8-10) the flies should have reproduced.

3.2.4 Preparation of treatment concentration

Three (3) treatment concentrations were prepared 0.025 g/mL, 0.05 g/mL and 0.1 g/mL. This is achieved by measuring 0.2g of Benny seasoning sample with a weighing balance and adding 2ml of water using a micro-pipette in a plastic tube to make 0.1 g/mL of Benny seasoning in water. Then 1ml (1000µl) was taken from the prepared 0.1 g/mL using a micro-pipette and mixed with another 1ml of water to make 0.05 g/mL, lastly 1ml was taken from the prepared 0.05 g/mL and mixed with another 1ml of water to make 0.025 g/mL.

3.2.5 Preparation of treatment set-up

Two hundred (200) micro-litre of prepared concentration (0.025 g/mL, 0.05 g/mL or 0.1 g/mL) was taken using a micro-pipette and added to 9.8 grams of solidified prepared meal (standard, half standard or quarter standard) in a falcon tube, this mixture was then crushed into the bottom of the tube, and the side of the tube is cleaned with issue paper, thirty flies were added to the tube and covered with cotton wool.

3.2.6 Homogenization and extraction of supernatant

After seven (7) days of exposure the flies were transferred into empty falcon tubes with appropriate labels corresponding to the experimental group and immobilized by freezing for about four (4) minutes. After that empty eppendorf tubes were labeled and weighed using a weighing balance, then the immobilized files from the freezer were added to each tube with appropriate labels, in order to calculate the exact weight of flies in each group. After calculating the exact weight of flies, the flies were crushed inside the eppendorf tubes, then phosphate buffer (PO_4) in micro-liter were added at a proportion of ten (10) times the calculated weight of flies in milligram in the eppendorf tube. The eppendorf tubes were placed inside a centrifuge and set to run at 4000rpm for seven (7) minutes. Then the supernantant were collected from the samples using micro-pipette into labeled eppendorf tubes and stored in a freezer before analysis.

3.2.7 Survival Assessment

Survival assessment is a technique used to determine the percentage of *Drosophila Melanogaster* that remains alive or active over a given period of time after expsure to toxicants.

In this study survival test was conducted on experimental flies using different treatment concentrations, a Control, 0.025 g/mL, 0.05 g/mL, and 0.1 g/mL. Each concentration had three replicates making a total of twelve tubes for the analysis. Daily mortality of flies was recorded for the purpose of the study. A fly was confirmed dead if failed to move upon gently hitting of tubes.

The objective of this study was to determine how long fruit flies could survive at different concentrations of the toxicant (Benny seasoning powder).

3.2.8 Negative Geotaxis (Climbing Assay)

The experiment was designed to determine the number of flies capable of moving against gravity.

A 6cm mark was drawn on the body of a falcon tube, ten immobilized flies were then placed into the marked tubes following the standard fly transfer proccedure. The tube was gently tapped to ensure all the flies settled at the bottom before starting the 6-second timer. The number of flies that crossed the marked line within the limit was recorded. Afterwards. The flies were returned to their respective tubes, and the proceedure was repeated for the other treatment groups.

3.3 Gene Expression Study

3.3.1 Isolation of Total DNA

Total RNA was isolated from the tissue samples with Quick-RNA MiniPrep™ Kit (Zymo Research). The DNA contaminant was removed following DNAse I (NEB, Cat: M0303S) treatment. The RNA was quantified at 260 nm and the purity confirmed at 260 nm and 280 nm using A&E Spectrophotometer (A&E Lab. UK).

3.3.2 cDNA conversion

One (1 µg) of DNA-free RNA was converted to cDNA by reverse transcriptase reaction with the aid of cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs) in a condition of 3-step reaction: 65 °C for 5 min, 42 °C for 1 h, and 80 °C for 5 min (Elekofehinti et al., 2020).

3.3.3 PCR amplification and agarose gel electrophoresis

Polymerase chain reaction (PCR) for the amplification of gene of interest was carried out with OneTaqR2X Master Mix (NEB) using the following primers (Inqaba Biotec, Hatfield, South Africa). PCR amplification was performed in a total of 25 µl volume reaction mixture containing cDNA, primer (forward and reverse) and Ready Mix Taq PCR master mix. Under the following condition: Initial denaturation at 95 ◦C for 5 min, followed by 30 cycles of amplification (denaturation at 95 ◦C for 30 s, annealing for 30 s and extension at 72 ◦C for 60 s) and ending with final extension at 72 ◦C for 10 min. The amplicons were resolved on 1.0% agarose gel. The GAPDH gene was used to normalize the relative level of expression of each gene, and quantification of band intensity was done using "image J" software (Olumegbon et al., 2022).

Primer sequences

CNC

Forward GCGTCCCGTTAACTGTCTTTA

Reverse CGTATCTGTAGCTGTGGCTTAG

Wingless

Forward: CAGTTAGTCCGAATGCAGCC

Reverse: GTTCGGGTGATGGATCTTGC

Eiger

Forward: TCGATAATCTCCAGCAGCGT

Reverse: CGCCAACATCATCCACAGAG

Hh droso

Forward CCAGTTGCTATGCGGTGATC

Reverse: GACCTTGTAGAGCGCATTGG

KEAP1

Forward: ATTTATCAGCCGGCGTGTTC

Reverse: CCGTACGTGTTTTGTGGTGT

FOXO

Forward: CAACACAAACGGCTACCACA

Reverse: CACACACAACAACGACGACT

3.4 Statistical Analysis

One way ANOVA analysis of variance was used to check the significant differences between treatment and control group. I used Normal (Gaussian) to assume sampling from normal distribution and to compare means. The Resullts of the statistical analysis was represented graphically using a graphpad prism.

3.5 Oxidative Stress Analysis

3.5.1 DETERMINATION OF CATALASE (CAT)

Catalase (CAT) activity was estimated by the method described by Cohen et al., (1970).

Reagents

Hydrogen peroxidase (H_2O_2)

Suphuric acid (6M) H_2SO_4

Preparation of reagents

0.01M $KMnO_4$ was prepared by distilling 0.158g of $KMnO_4$ in 100ml of distilled water. Phosphate buffer (pH 7.4) 0.426g of $NaHPO_4$ and 0.240g of NaH_2PO_4 was weighed and dissolved in 100ml of distilled water. 6M H_2SO_4 and 32.3ml of conc. H_2SO_4 was added to 66.7ml of distilled water.

Procedure

To an unknown volume of plasma (0.5ml), 5.0ml of H_2O_2 was added. This was mixed by inversion and allowed to stand for 30min. The reaction was stopped by adding 1.5ml of 6M H_2SO_4 and 7ml of 0.01M $KMnO_4$. These were mixed by inversion and allowed to stand for 10min. The absorbance was read at 480nm within 30-60 seconds against distilled water. The enzyme blank was run simultaneously with 1.0ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as µmoles of H_2O_2 decomposed/min/mg/protein.

Calculation

$$\text{Activity} = \frac{\text{OD/min} \times V}{M \times V \times L \times Y}$$

Where OD = Absorbance

L= Light path

V= Total volume of reaction sample

M= Molar coefficient of H_2O_2 (40/m/cm)

V= Volume of sample

Y= mg protein in the sample

3.5.2 ESTIMATION OF SUPEROXIDE DISMUTASE ACTVITY (SOD)

This was determined according to teh methods of Masra and Fridorich (1972)

Principle

Adrenaline undergoes auto oxidation rapidly to adrenochrome whose concentration can be determined at 420nm with the aid of a spectrophotometer. The auto oxidation of adrenaline depends on the presence of superanions.

Superoxide dismutase inhibits the auto-oxdiation of adrenaline by catalysing the breakdown of superoxide anion. The degree of inhibition reflects the activity of SOD which is determined at 420nm.

Reagent and preparation

Carbonate buffer (0.05M) pH 10.2: This was prepared by dissolving 0.2014g of Na_2CO_3, 0.2604g $NaHCO_3$ and 0.0372g of EDTA in 100ml of distilled water. The pH was adjusted to 10.2 using Sodium hydroxide.

Hydrochloric acid (0.005M): This was prepared by adding 0.044 concentration of HCL to 99.96mls of distilled water.

Adrenaline solution (0.3mM): This was prepared by dissolving 0.01098g of adrenaline in 100mls of 0.005M HCL solution.

Plasma volume of 100ml was mixed with 125ml of carbonate buffer and 150ml of adrenaline solution. 100ml of distilled water was mixed with 1.25ml of carbonate buffer as reference sample. These were mixed and absorbance read at 420nm.

These were mixed and read at 420nm

$$\%\text{inhibition} = \frac{(\text{O.D test} - \text{ODref}) \times 100}{\text{OD test}}$$

Enzyme concentration can thus be calculated

$$\text{unit/mg protein} = \frac{\%\ \text{inhibition}}{50 \times Y}$$

Where Y = mg of protein in the volume of sample used

3.5.3 ESTIMATION OF GLUTHATHIONE PEROXIDASE (GPx)

This was determined according to Nyman (1959)

Principle

This is based on the oxidation of pyrogallol to purpurogallin by peroxidase activity, resulting to a deep brown color disposition, read at 420nm.

Reagent and preparation

Pyrogallol (20mM): 0.2552g of pyrogallol was dissolved in 100mls of distilled water.

Procedures

To an aliquot of plasma (0.2ml), 2.5ml of phosphate buffer, 2.5ml of H_2O_2, 1.5ml of distilled water and 2.5ml of pyrogallol was added.

The reaction was allowed to stand for 30mins at room temperature. A deep brown color was formed which was read at 480nm.

Calculations

$$\text{Activity} = \frac{\text{OD/min x vt x Df}}{\text{E x Vs x Y}}$$

OD= Absorbance of test

Vt= Total volume of reaction mixture

Df= Diution factor = 1

E= Molar extinction co-efficient (12/m/cm)

Vs= Volume of sample

Y= mg of protein used

3.5.4 DETERMINATION OF MALONDIALDEHYDE (MDA)

Malonaldehyde was determined using the thiobarbituric acid assay (Buege and Aust, 1978)

Principle

Malonaldehyde which is a product of lipid peroxidation react with thiobabituric acid (TBA) to give a red species.

Procedure

A volume of plasma (1.0ml) was added to 2.0ml of TCA-TBA-HCL and mixed thoroughly. The solution was heated for 15mins in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifuged at 1000g for 10min. The absorbance was determined using the formula;

$$\text{MDA (mol/mg protein)} = \frac{A \times V \times 100}{M \times V \times Y}$$

A= Absorbance

V= Total volume of reaction mixture

M= Molar extinction coefficient

V= volume of the sample

Y= mg protein

3.5.5 DETERMINATION OF HYDROGEN CONCENTRATION

Hydrogen peroxide levels measured as described by Wolff (1994)

PRINCIPLE

In dilute acid hydrogen peroxide oxidizes Fe (II) to Fe (III) which then selectively forms a blue purple complex with xylenol orange with an absorption maximum at 560nm. The addition of sorbitol initiates a chain reaction with produced hydroxyl radical that increases the yield of Fe (III) and therefore greatly amplifies the response per H_2O_2 molecule present, thereby increasing the sensitivity of the method.

REAGENTS

1. Xylenol orange, 100 uM (MW760.0)

   7.6 mg of xylenol orange was dissolved in 10mL of distilled water.

2. Sorbitol, 100 mM

   1.822 g of sorbitol was dissolved in 10mL distilled water.

3. Sulphuric acid, 25 mM

   140 uL of concentrated H_2SO_4 was added to distilled water and made up to 50mL

4. Ammonium ferrous sulfate (AFS), 250 uM (MW 392.14)

   9.8mg of ammonium ferrous sulfate was dissolved in 50mL of 25 mM H_2SO_4.

5. Hydrogen peroxide stock, 100 uM

   57 uL of 30% H_2O_2 was added to distilled water and the volume made up to 100 mL.

   1mL of the resulting solution was taken and made up to 50mL.

   Fox 1 reagent (100 mL) = 10 mL xylenol orange + 10 mL sorbitol + 50 mL AFS + 30 mL distilled water

Test tube	**Blank**	**1**	**2**	**3**	**4**	**5**
H_2O_2 stock (uL)	-	20	40	60	80	100
Distilled water (uL)	100	80	60	40	20	-
FOX1 reagent (mL)	1.9	1.9	1.9	1.9	1.9	1.9
H_2O_2 conc (uM)	0	1	2	3	4	5

The total reaction mixture was vortexed and incubated at room temperature for 30 min before being read against the reagent blank at 560nm.

PROCEDURE FOR SAMPLES

Samples were treated similarly as standard (100 uL 0f sample added to 1.9 mL FOX1 reagent) except that the mixtures were centrifuged at 3000rpm for for 5 min before incubation.

3.5.6 NITRIC OXIDE DETERMINATION

Nitric oxide was assayed by the method of Marcocci et al., (1994) PROTOCOL From the reaction mixture containing 500 uL of sample dissolved in dimethyl sulfoxide (DMSO) saline mixed with 5 mM sodium nitropusside prepared in 10 mM potassium buffer (PH 7.4), then incubated at 25 oC for 15min. At the end of incubation, the samples were allowed to react with 1 ml of Greiss reagent containing equal volume of solution A (2 % sulfanilamide and 5 % H3PO4) and B (0.2 % naphthyethylenediamine dihydrochloride). The absorbance of the chloromphore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyethylenediamine was read at 540nm.

CHAPTER FOUR

RESULTS

4.1 Survival Study

The survival studies showed that with increasing concentration *Drosophila melanogaster* showed extreme mortality rate. The highest mortality rate was recorded in the 0.1 g/mL concentrations.

Table 4.1: Mean of mortality values of drosophila on exposure to different concentration of Benny seasoning powder.

Days	Control	0.025 g/mL	O.05g/ml	0.1 g/mL
1	0	0	0	0
2	0	1	0	1
3	4	4	3	4
4	4	6	4	9
5	4	5	4	11
6	5	6	4	15
7	6	8	5	18

4.2 Negative Geotaxic Response

During the negative geotaxis assay, flies exhibited varied response to the stimulus. While some flies crawled upwards along the walls of the tube, others immediately took flight within the 6-second observation period. Some of the *drosophila melanogaster* showed contradictory behavior, initially moving upwards but quickly reversing and descending the tube. The observations was carried out in triplicate for each concentration. The mean differences in levels of response is seen in Figure 4.2

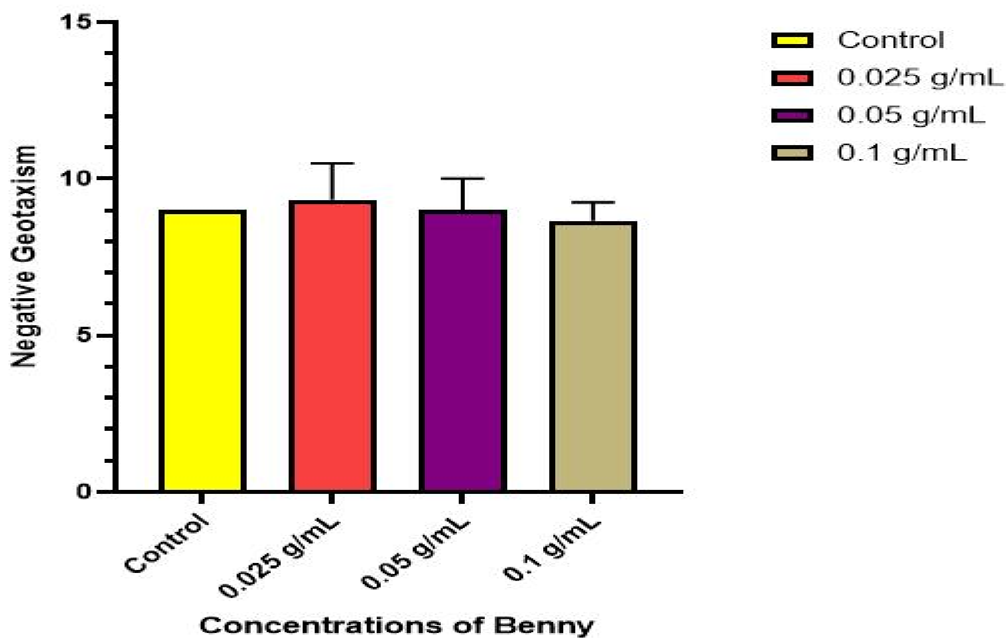


Figure 4.1: Negative geotaxis responses of *Drosophila Melanogaster* on exposure to varying concentrations of Benny Seasoning powder

4.3 Oxidative Stress Responses

4.3.1 Protein

A non significant difference in protein levels was observed between the control group and the treatment groups (F=1.200, P< 0.05). Protein levels decreased across the treatment group in a dose dependent manner when compared with the control.

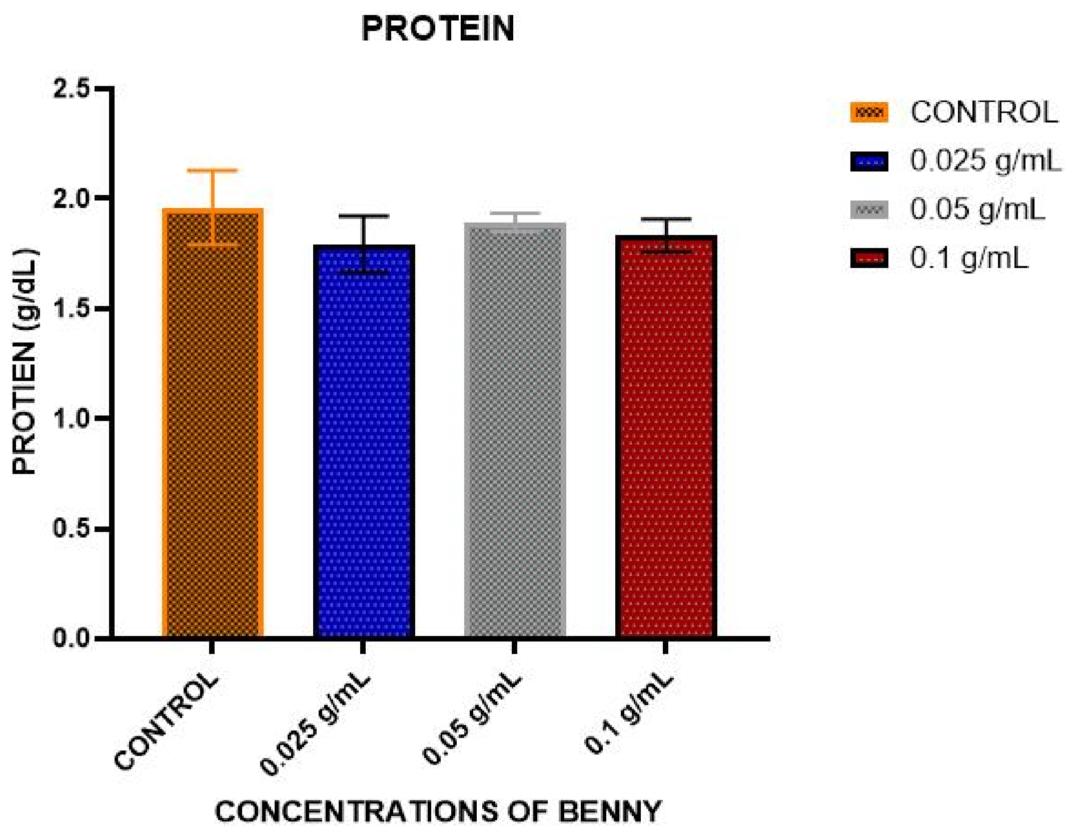


Figure 4.2: Protein activity in *drosophila melanogaster* when exposed to different concentrations of Benny seasoning powder. Each bar represents the Mean ± SEM (F=1.200, P< 0.05).

4.3.2 Superoxide Dismutase (SOD)

There a non significant difference in SOD levels between the control and treatment groups ($f = 2.33$ $P < 0.05$). There was a significant increase in SOD values across the different treatment groups in a dose dependent manner when compared with control.

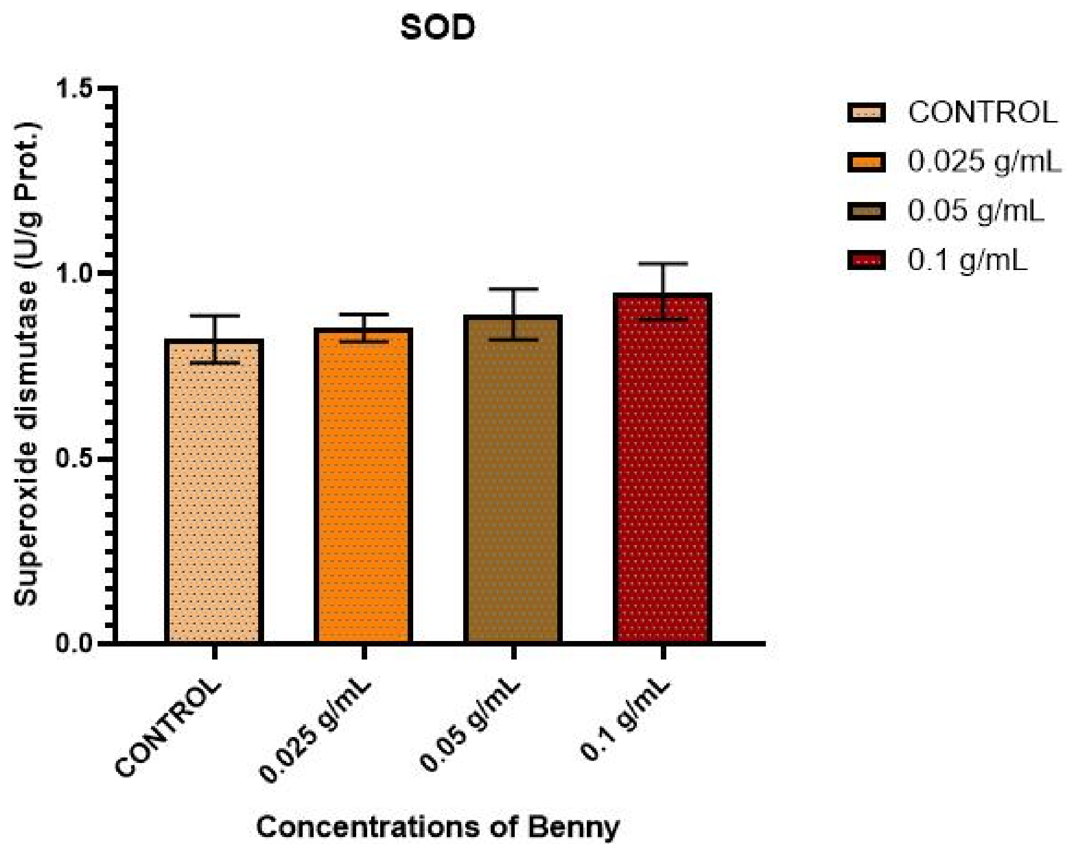


Figure 4.3: Superoxide dismutase activity in drosophila melanogaster when exposed to different concentrations of Benny seasoning powder. Each bar represents the Mean ± SEM where ($f = 2.33$ $P < 0.05$).

4.3.3 Catalase (CAT)

A non significant difference in CAT levels was observed between the control and treatment groups (f = 1.200 P < 0.05). There was a significant increase in CAT levels across the various treatment groups in a dose dependent manner when compared with control.

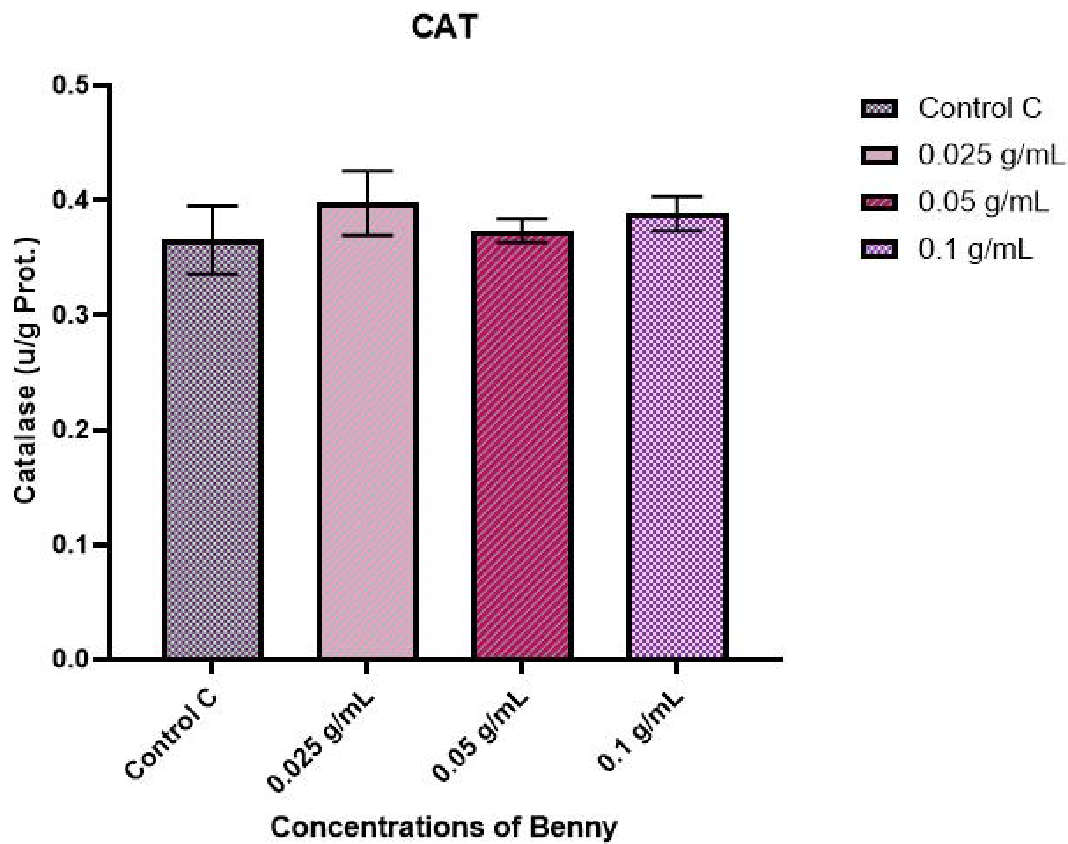


Figure 4.4 CAT activity in *drosophila melanogaster* exposed to different concentrations of Benny Seasoning Powder. Each bar represents the Mean ± SEM where (f = 1.200 P < 0.05).

4.3.4 Gluthathione Peroxidase (GPx).

There was a non significant difference in GPx levels between the control and treatment groups (F= 0.2547 P > 0.05). There was a significant increase in GPx levels across the various treatment groups in a dose dependent manner when compared with control.

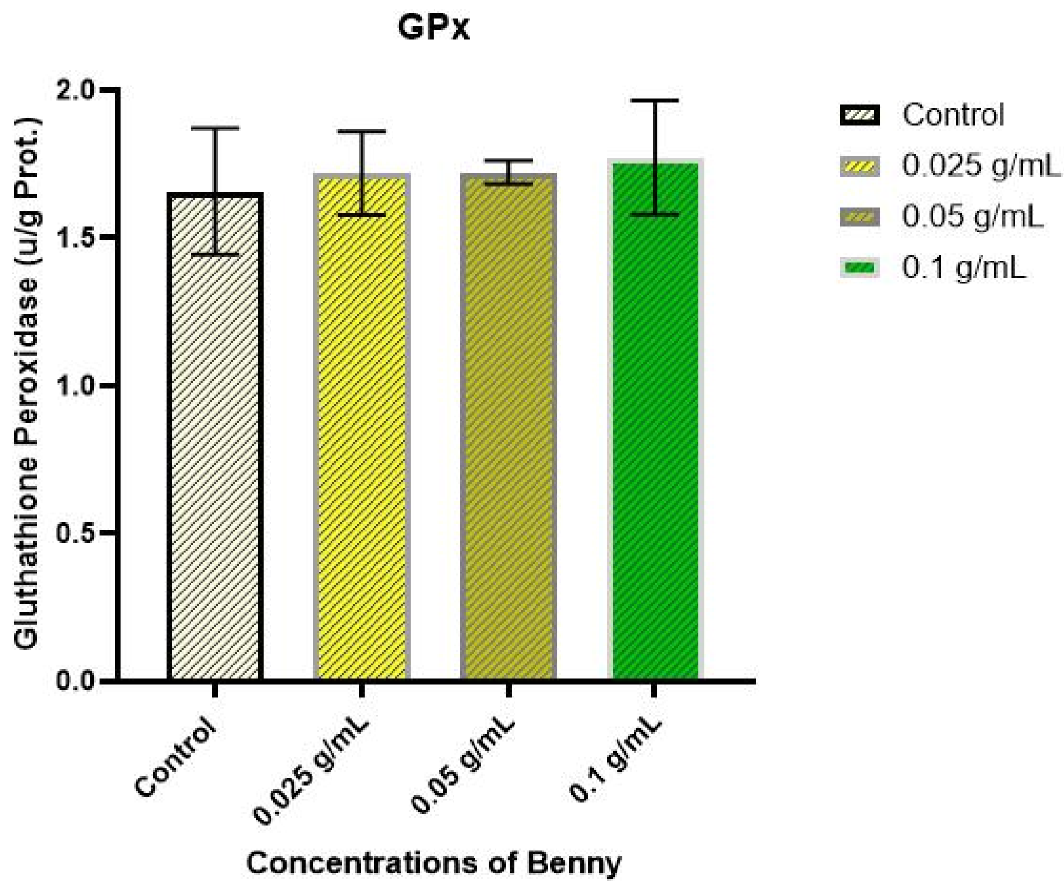


Figure 4.5: GPx activity in *Drosophila melanogaster* exposed to different concentrations of Benny Seasoning powder. Each bar represents the Mean ± SEM where (F= 0.2547 P > 0.05).

4.3.5 Total Thiol (Gluthathione S- transferase (GST)

There was a non significant difference in the activity of GST and GSH when control was compared with treatment groups ($f=0.3672$ $P>0.05$). However, There was significant increase in GST and GSH activity with increasing concentration.

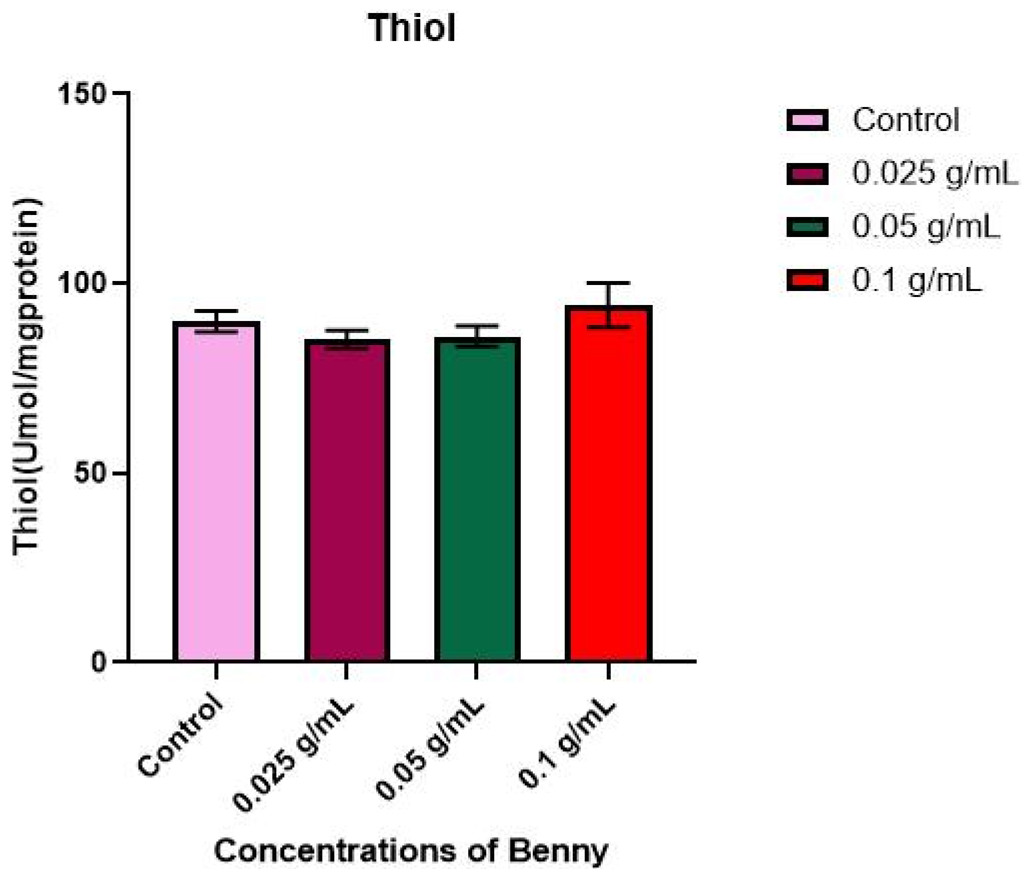


Figure 4.6: Thiol activity in *Drosophila melanogaster* exposed to different concentrations of Benny Seasoning powder. Each bar represents the Mean ± SEM where ($f=0.3672$ $P>0.05$).

4.6. Hydrogen Peroxide (H_2O_2)

There was a non significant difference in GPx levels between the control and treatment groups ($F = 1.317$ $P < 0.05$). There was a significant increase in H_2O_2 levels across the various treatment groups in a dose dependent manner when compared with control.

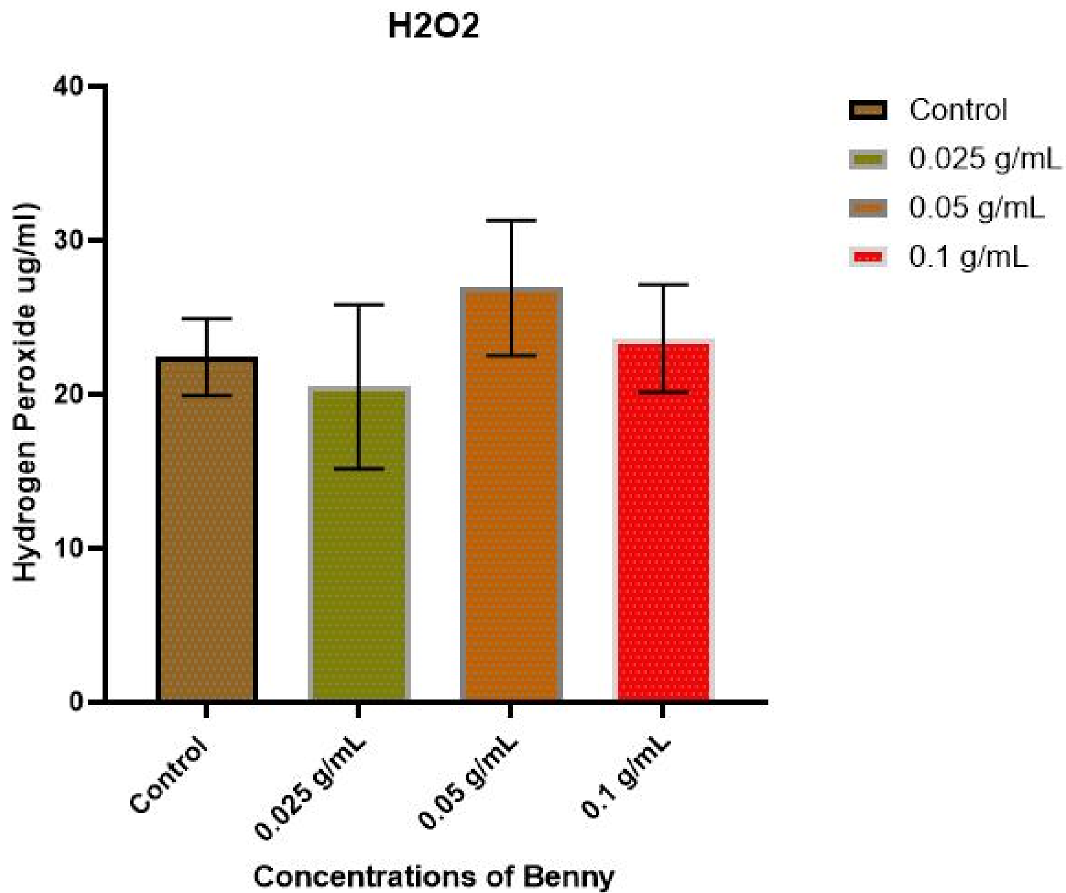


Figure 4.7: H_2O_2 activity in *drosophila melanogaster* exposed to different concentration of Benny Seasoning powder. Each bar represents the Mean ± SEM where ($F = 1.317$ $P < 0.05$).

4.3.6 Malondialdehyde (MDA)

There a non significant difference in MDA between the control and treatment groups (F =0.8841 P< 0.05). a significant increase in MDA was observed across the various treatment groups in dose dependent manner when compared with control.

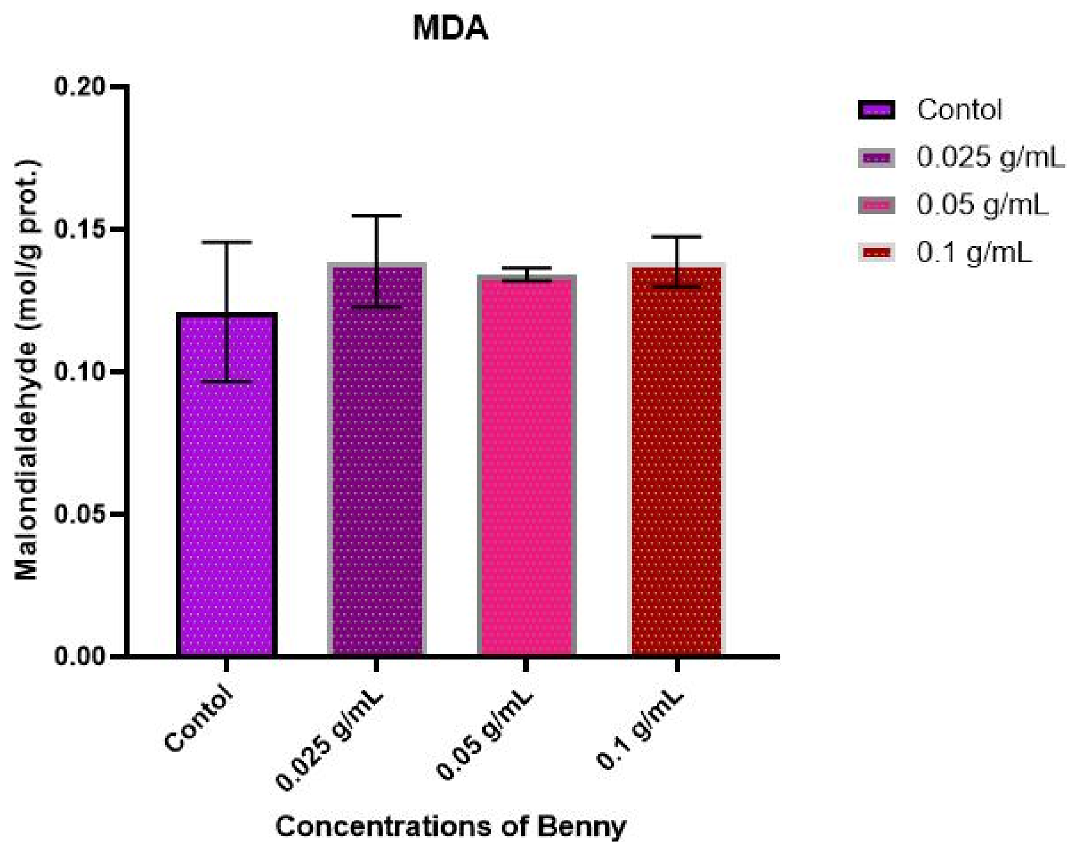


Figure 4.8: MDA activity in *drosophila melanogaster* when exposed to different concentrations of Benny Seasoning powder. Each bar represents the Mean ± SEM where (F =0.8841 P< 0.05).

4.3.7 Nitric Oxide (NO_2)

There was a non significant difference in NO_2 between the control and treatment group ($F=1.897$ $P<0.05$). There was a significant decrease in $N0_2$ across the various treatment groups in a dose dependent manner when compared with control.

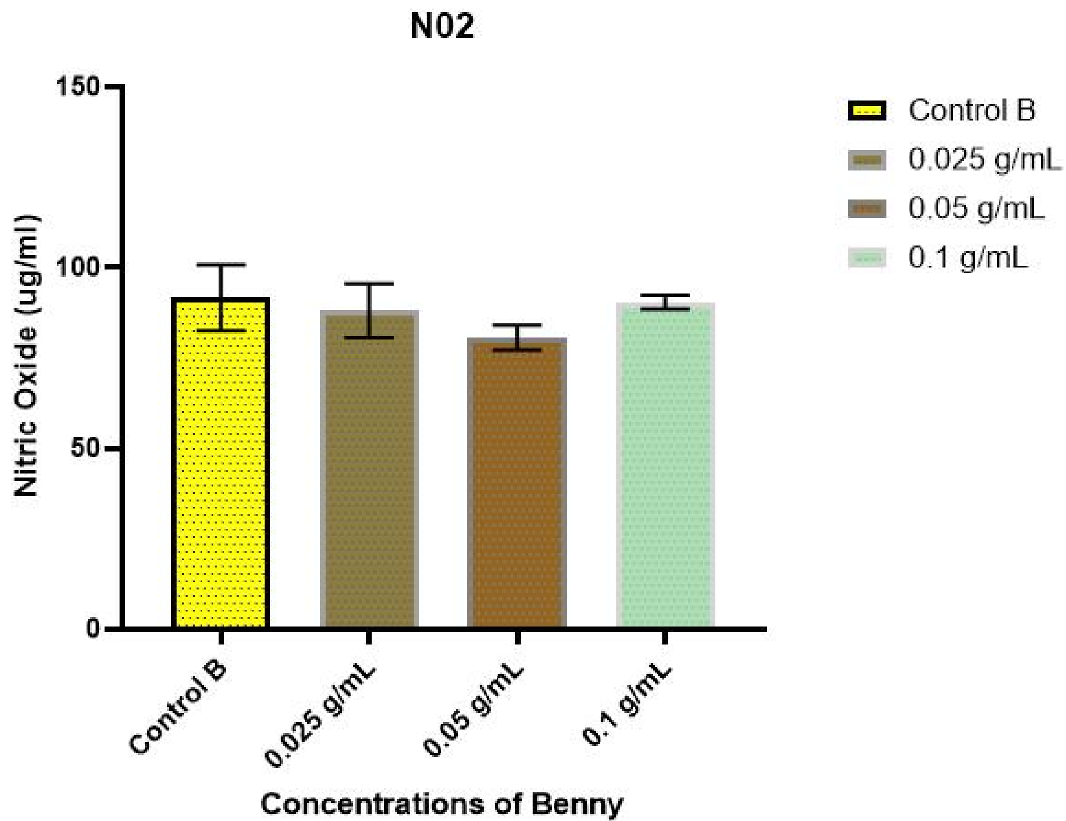


Figure 4.9: NO2 activities in *drosophila melanogaster* when exposed to different treatments of Benny Seasoning powder. Each bar represents the Mean ± SEM where (F=1.897 P<0.05)

4.4 Gene Modulation

4.4.1 Spitz (Spi) - EGFR pathway activator

There was a significant difference in the spitz gene between the control and various treatment groups (F=181.9 P<0.05). There was a significant increase in spitz gene levels across the various treatment groups when compared with the control group in a dose dependent manner.

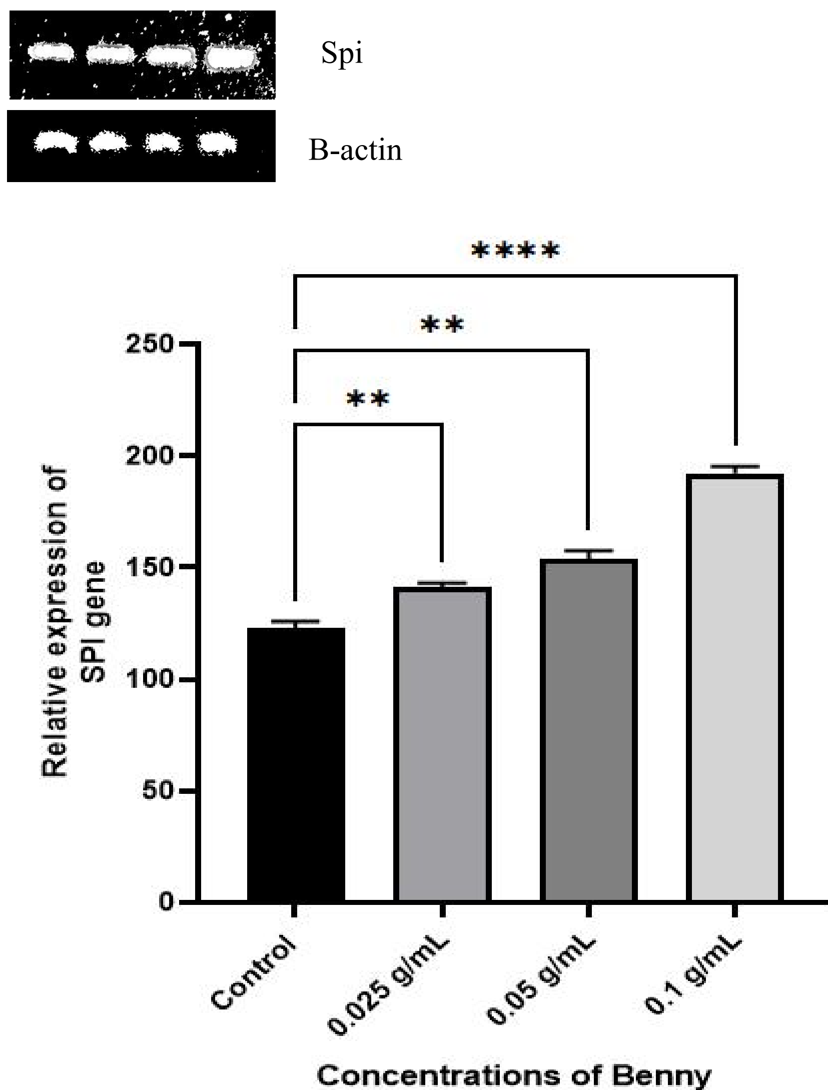


Figure 4.10: Activities of spitz gene in *Drosophila melanogaster* when exposed to diferent concentration of Benny Seasoning powder. Each bar represents the Mean± SEM where (F=181.9 P<0.05).

4.4.2 Wingless (Wg)- Wnt pathway

There was a significant difference in Wingless gene between the control and treatment groups (F=40.67 P<0.05). There was a significant increase in Wingless gene levels in the 0.025 and 0.05 treatment groups in a dose dependent manner when compared to the control groups.

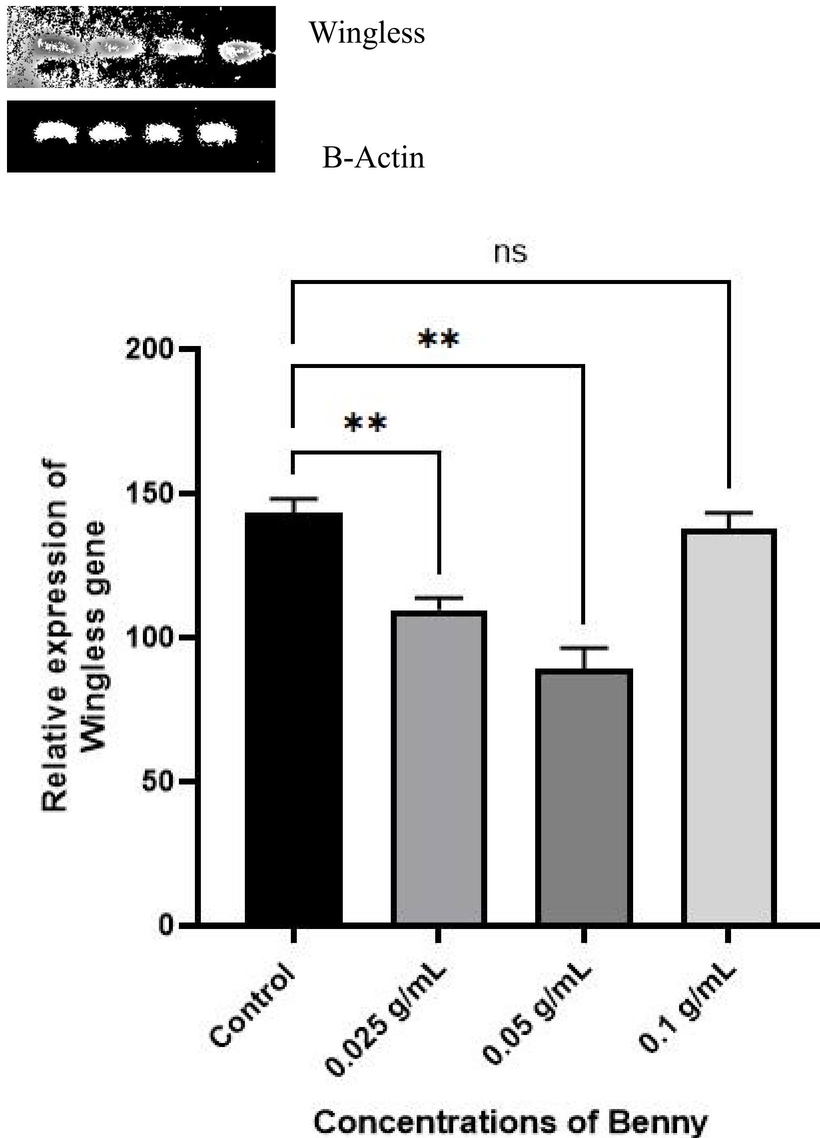


Figure 4.11: Actvity of Wingless gene in *drosophila melanogaster* when exposed to different concentrations of Benny seasoning powder. Each bar represents the Mean± SEM where (F=40.67 P<0.05).

4.4.3 Eiger (Egr)- the drosophila homolog

There was a significant difference in Eiger gene between the control and different treatment groups (F=114.3 P< 0.05). There was a significant increase in Eiger gene in the 0.1 treatment group in a dose dependent manner when compared with the control

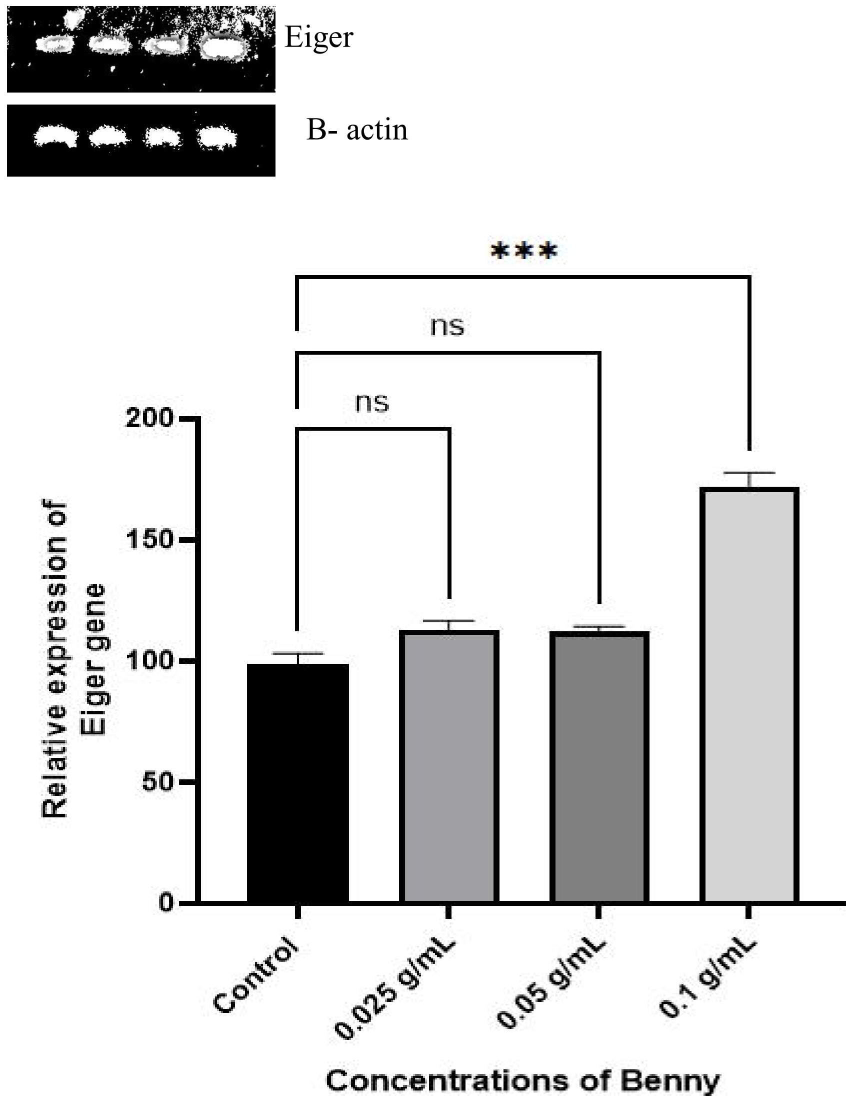
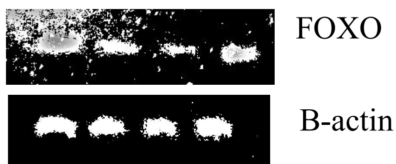


Figure 4.12: Activity of Eiger gene in *drosophila melanogaster* when exposed to different concentration of Benny Seasoning powder. Each bar represents the Mean ± SEM where (f=114.3 P< 0.05).

4.4.4: FOXO (Forkhead box O) transcription factors

There was a significant difference in the FOXO gene between the control and treatment groups.(F=127.8 P<0.05). There was a significant decrease of FOXO in the 0.025 and 0.05 groups and a significant increase in the 0.1 treatment groups when compared with the control group



FOXO

B-actin

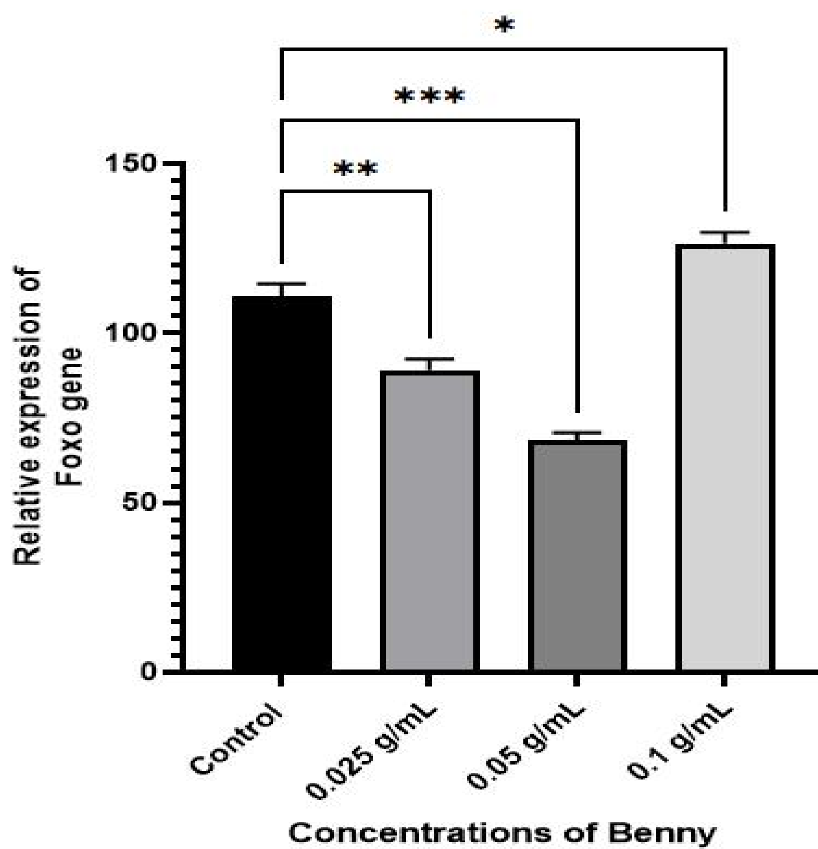


Figure 4.13 Activity of FOXO gene in *drosophila melanogaster* when exposed to various treatment groups. Each bar represents the Mean ± SEM where (F=127.8 P<0.05).

4.4.5: Hedgehog (Hh) pathway

There was a significant difference in Hedgehog gene between the control and treatment groups (F=22.48 P<0.05). A significant increase in Hedgehog was observed in the 0.05 and 0.1 treatment group when compared to the control groups.

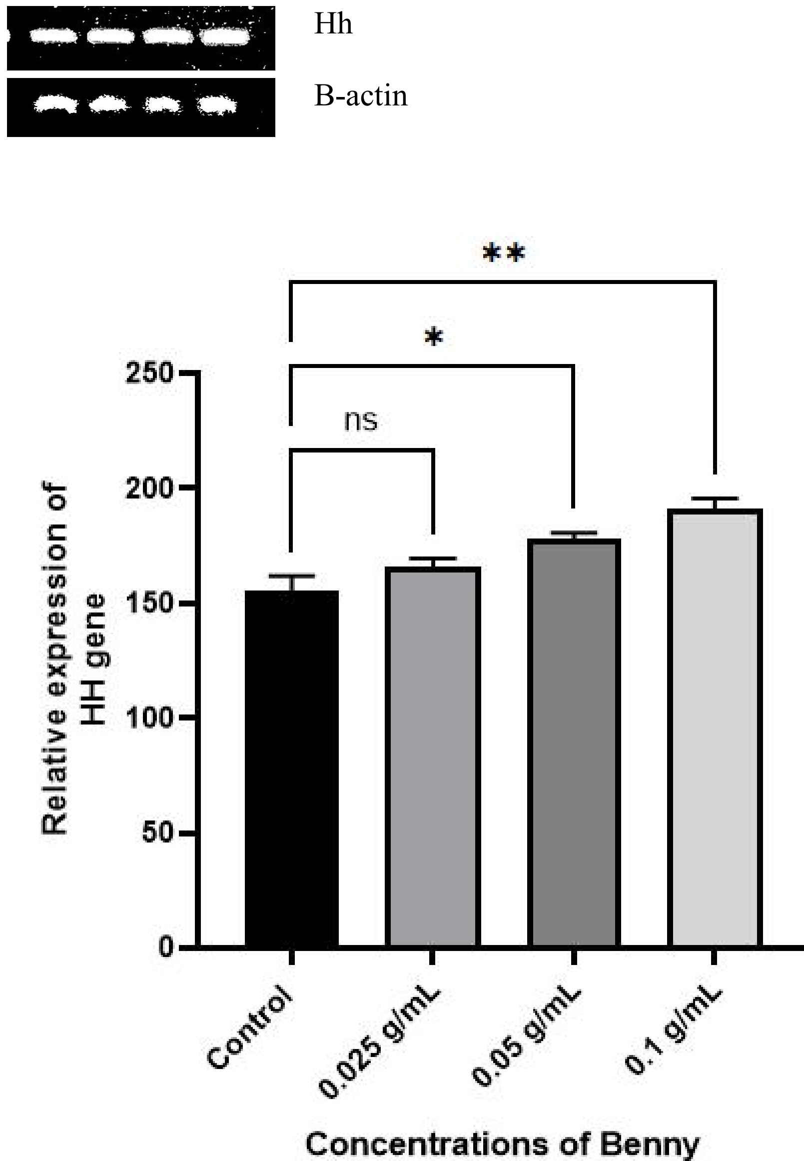


Figure 4.14: Activity of FOXO gene in *drosophila melanogaster* when exposed to different concentrations of Benny seasoning powder. Each bar represents the Mean ± SEM where (f=22.48 P<0.05).

4.4.6 Nrf2/ Keap1 - Oxidative stress master regulator

There was an observed significant difference in the Keap1 gene between the control and treatment groups. (F=7.261 P<0.05) There was a significant decrease in the 0.05 and 0.1 treatment groups when compared to the control.

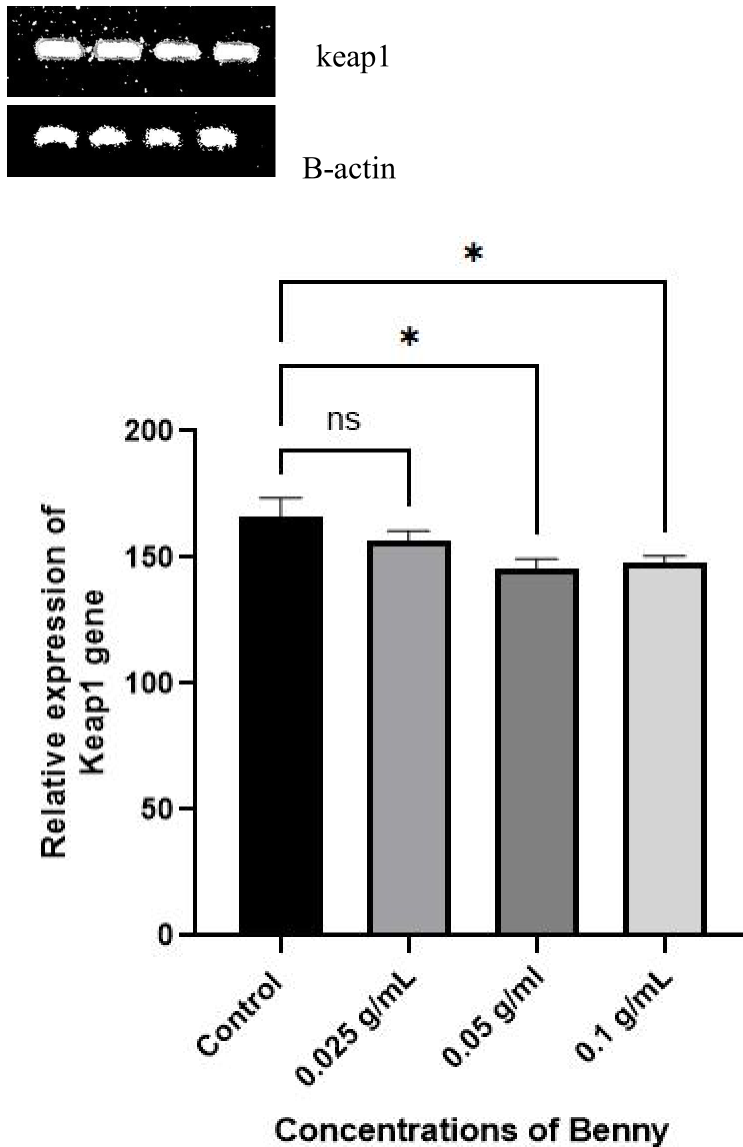


Figure 4.15: Activity of Keap1 gene in *drosophila melanogater* when exposed to different concentrations of Benny Seasoning powder. Each bar represents the Mean ± SEM where (F=7.261 P<0.05)

CHAPTER FIVE

5.0 DISCUSSION OF RESULTS

The potential health risks associated with *Benny* seasoning powder are of increasing concern due to its widespread consumer use. This study investigated the effects of *Benny* seasoning powder on locomotor behavior (negative geotaxis), oxidative stress biomarkers, and neurotoxic gene modulation in *Drosophila melanogaster*.

The survival assay revealed that flies exposed to higher concentrations of *Benny* seasoning powder exhibited markedly elevated mortality rates, particularly at 0.1 g/mL (Table 4.1). This observation aligns with previous findings reported by Alghamdi *et al.* (2020).

Negative geotaxis analysis demonstrated a concentration-dependent decline in climbing ability among exposed flies (Figure 4.1), indicating neuromuscular impairment. These findings are consistent with those of He *et al.* (2020), who also reported reduced locomotor performance in *Drosophila* following exposure to similar food additives.

Regarding oxidative stress biomarkers, there was no statistically significant difference in total protein activity between the control and treatment groups (Figure 4.2). Nonetheless, a downward trend in protein activity was observed, suggesting potential oxidative damage and metabolic disruption, as decreased protein activity is indicative of cellular stress and impaired metabolic homeostasis (Arsac *et al.*, 2021).

Superoxide dismutase (SOD) activity showed no significant difference between control and exposed groups (Figure 4.3); however, an increase in SOD activity was observed, suggesting activation of antioxidant defense in response to oxidative stress. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide, and its elevation indicates the presence of reactive oxygen species (ROS). A similar trend has been documented in studies

involving monosodium glutamate (MSG), a common flavor enhancer, where elevated SOD activity was observed as a compensatory response to ROS generation (Onyema *et al.*, 2006).

Catalase (CAT) activity also showed no significant difference between groups (Figure 4.4), though a slight increase was detected, implying an adaptive antioxidant response to oxidative stress induced by *Benny* seasoning powder constituents (Vona *et al.*, 2021). Comparable findings have been reported with tartrazine exposure, where increased CAT activity served as a compensatory mechanism to neutralize hydrogen peroxide accumulation (Mehedi *et al.*, 2013).

Similarly, glutathione peroxidase (GPx) activity did not differ significantly between the control and treated groups (Figure 4.5), though elevated GPx levels were observed. As GPx reduces hydrogen peroxide and lipid peroxides, its increase indicates an antioxidant response to elevated oxidative stress (Rady *et al.*, 2022). This aligns with oxidative adaptations previously observed in MSG-exposed models (Onyema *et al.*, 2006).

Total thiol content, including glutathione (GSH) and glutathione-S-transferase (GST) activities, showed no statistically significant difference (Figure 4.6). However, both GSH and GST levels were elevated at higher concentrations, indicating increased ROS production and the activation of phase II detoxification pathways (Aigberua *et al.*, 2018). Similar GST elevation has been noted with tartrazine exposure, representing an enzymatic defense mechanism against oxidative stress (Mehedi *et al.*, 2013).

Malondialdehyde (MDA) levels were not significantly altered (Figure 4.7), but a progressive increase was noted with rising *Benny* seasoning concentrations. Elevated MDA levels signify lipid peroxidation and membrane damage, consistent with findings from sodium benzoate toxicity studies (Saha and Verma, 2015).

Hydrogen peroxide (H_2O_2) measurements showed no significant difference between groups (Figure 4.8); however, an upward trend was evident, indicating excessive ROS accumulation beyond enzymatic detoxification capacity. This pattern parallels findings from butylated hydroxytoluene (BHT) exposure, which disrupts redox balance and promotes oxidative stress (Madi *et al.*, 2018).

Nitric oxide (NO_2^-) levels displayed no significant variation between control and treated groups (Figure 4.9), though a noticeable decrease was recorded. This reduction suggests that *Benny* seasoning powder may inhibit nitric oxide synthase activity or enhance NO scavenging, disrupting nitric oxide signaling and redox balance (Radi, 2018).

Gene expression analysis revealed significant alterations in several neurotoxic markers. The *Spitz* (Spi) gene, an activator of the epidermal growth factor receptor (EGFR) pathway, showed elevated expression, particularly at 0.1 g/mL (Figure 4.10). This upregulation may indicate compensatory activation of cellular proliferation or repair mechanisms following tissue injury, consistent with stress-induced modulation of EGFR signaling reported in toxicant-exposed *Drosophila* (Shilo, 2003).

Conversely, *Wingless* (Wnt) expression decreased significantly at 0.025 g/mL and 0.05 g/mL (Figure 4.11), implying potential neurodevelopmental perturbation, as dysregulated Wnt signaling has been linked to neurodegenerative processes (Ranjan *et al.*, 2019).

Eiger (Egr), the *Drosophila* homolog of tumor necrosis factor (TNF), showed increased expression at 0.1 g/mL (Figure 4.12). Elevated *Eiger* expression reflects activation of apoptotic and immune pathways, commonly observed following oxidative stress and paraquat exposure (Moreno *et al.*, 2002; Palmerini *et al.*, 2021).

Forkhead box O (FOXO) transcription factor expression was significantly downregulated at 0.025 g/mL and 0.05 g/mL (Figure 4.13). Since FOXO plays a critical role in oxidative stress response, its suppression suggests impaired cellular defense and increased neurotoxic vulnerability (Asadi *et al.*, 2021).

Hedgehog (Hh) pathway components were significantly upregulated at 0.05 g/mL and 0.1 g/mL (Figure 4.14). Such increases indicate altered signaling dynamics that could compromise neuronal stability and proteostasis, consistent with reports linking Hh dysregulation to dopaminergic neuron vulnerability (Yang *et al.*, 2021).

Finally, a significant downregulation of *Keap1/Nrf2* (dKeap1/dNrf2) pathway expression was observed (Figure 4.15). Given the pivotal role of this pathway in regulating oxidative defense, its suppression suggests increased susceptibility to oxidative damage and potential neurodegenerative risk (Au *et al.*, 2024).

5.1 Conclusion

From this study we can conclude that Benny seasoning powder has a significant negative impact on *Drosphila melanagaster.* Exposure to the seasoning resulted in increased mortality, impaired climbing activity , alteration in oxidative stress markers and significant alterations in expressions of key genes involved in neuromuscular integrity and development processes. Specifically, higher concentrations of Benny seasoning powder led to extreme mortality and reduced climbing abilities. Although certain seasoning may offer beneficial properties at moderate concentrations, excessive exposure can lead to negative effects on survival, climbing activities and neuromuscular function. Some additives show increased toxicity with prolonged consumption and higher dose. These finding suggest that moderate consumption is preferable to higher concentrations as excessive Intake poses potential health. The

upregulation of Eiger, Hedgehog and Spitz genes suggest oxidative stress, while the downregulation of Wingless (Wnt), FOXO and Keap1 a compromised neural patterning. These findings collectively demonstrate that Benny seasoning powder particularly at high concentration poses significant threat to neural, cellular functions and survival due to induction oxidative stress and impairment of crucial defense mechanism. This raises concern about the potential health risk associated with its consumption.

5.2 Recommendations

Further studies is needed to determine the precise molecular mechanism in which Benny season exerts its toxicity. This could involve studying its interaction with specific cellular targets, oxidative stress markers, signaling pathways and metabolic processes. Focus should be placed on reasons why oxidative stress biomarkers were altered although proven not significantly different. Also investigating the toxicity of Benny seasoning in other insect species and mammals. This would provide a broader understanding of its potential health risk. It would also indicate if the observed effects in *Drosophila* is similar to those gotten from other species.This would serve as a useful investigation in the identifying the potential health risk associated with high consumption of Benny seasoning.

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APPENDIX

Survival test (Benny Seasoning)

Condition	D0	D1	D2	D3	D4	D5	D6	D7	Mean	Standard Deviation
Control A	0	0	4	4	4	4	5	6	3.38	2.20
Control B	0	0	5	5	5	5	5	6	3.88	2.42
Control C	0	0	4	4	4	4	5	6	3.38	2.20
0.025 A	0	0	6	6	6	6	6	8	4.75	3.01
0.025 B	0	2	3	4	6	5	6	8	4.25	2.55
0.025 C	0	1	2	3	5	5	6	7	3.63	2.50
0.05 A	0	0	2	3	4	4	4	5	2.75	1.91
0.05 B	0	1	5	5	5	5	5	5	3.88	2.10
0.05 C	0	0	2	3	3	3	3	4	2.25	1.49
0.1 A	0	1	4	6	10	12	15	18	8.25	6.56
0.1 B	0	3	5	7	10	12	15	17	8.63	5.93
0.1 C	0	0	2	3	6	10	15	20	7.00	7.39

Climbing Assay (Benny seasoning)

Condition	T1	T2	T3	Mean	Standard Deviation
Control A	9	10	9	9.33	0.58
Control B	8	9	9	8.67	0.58
Control C	9	9	8	8.67	0.58
0.025 A	9	8	8	8.33	0.58
0.025 B	10	10	10	10.00	0.00
0.025 C	10	9	10	9.67	0.58
0.05 A	8	9	9	8.67	0.58

0.05 B	8	9	8	8.33	0.58
0.05 C	10	9	10	9.67	0.58
0.1 A	9	9	10	9.33	0.58
0.1 B	9	9	10	9.33	0.58
0.1 C	8	8	9	8.33	0.58

OXIDATIVE STRESS BIOMARKERS (Benny seasoning)

Marker	Control	0.025 g/mL	0.05 g/mL	0.1gml
Protein	I.959±0.297	1.793±0.742	1.893±0.0240	1.832±0.043
SOD	0.822±0.0395	0.8526±0.0213	0.890.±0.0300	0.9514±0.436
CAT	0.3655±0.0172	0.395±0.0161	0.3757±0.0060	0.3882±0.0087
GPx	1.6858±1.2321	1.719±0.0815	1.722±0.0023	1.773±0.1111
Thiol	90.00±15998	85.18±1.389	86.09±1.575	94.27±3.442
H2O2	22.41±1448	20.49±3.080	26.91±2.538	94.27±3.442
MDA	0.211±0.0141	0.1387±0.0009	0.1342±0.0013	0.1387±0.0050
NO	91.61±5.2781	88.63±4.284	80.63±1.951	90.43±1.106

Neurotoxic Gene Modulation (Benny seasoning Powder)

Gene	Control	0.025 g/mL	0.05 g/mL	O.1g/ml
Spi	122.7±2.210	141.0±1.452	153.7±2.6735	192.1±2.1989
Wingless	143.4±3.319	109.6±2.869	89.04±5.2423	137.5±4.0288
Eiger	98.54±3.156	112.9±2.509	111.8±1.7754	171.7±4.228
FOXO	110.9±2,626	89.13±2.259	66.46±1.6066	126.5±2.2343
HH	155.5±4.418	165.7±2.7046	177.7±20066	191.0±3.2531
Keap1	166.0±5.404	156.7±2.6671	145.7±2.7123	148.1±17.97