SUBLETHAL CONCENTRATIONS OF BISPHENOL A INDUCED TOTAL PROTEIN IN EXPOSED CATFISH.

BY

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DEPARTMENT OF ANIMAL AND ENVIRONMENTAL BIOLOGY

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UNIVERSITY OF BENIN

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF ANIMAL AND ENVIRONMENTAL BIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE IN ANIMAL AND ENVIRONMENTAL BIOLOGY(BSCAEB)

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CERTIFICATION

This is to certify that this project was carried out by **ANDREW EKHATOR** of the Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin, Benin City.

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DEDICATION

This work is dedicated to God Almighty.

AKNOWLEDGEMENT

My sincere gratitude goes to all who made this project a reality, first of all I acknowledge my parents, for the funding and encouragement and then to my project supervisor, Dr. Nosa, your guidance was invaluable. I also appreciate the dedication of my team mates and colleagues whose cooperative efforts brought our project to fruition and alo our institution for their crucial support in ensuring a smooth project.

TABLE OF CONTENT

LIST OF FIGURES

ABSTRACT

Bisphenol A (BPA), a pervasive endocrine-disrupting chemical, poses significant ecological risks to aquatic organisms due to its widespread environmental contamination. This study investigates the effects of sublethal BPA concentrations on total protein levels in *Clarias gariepinus* (African catfish), a bioindicator species, to evaluate its sublethal toxicity and physiological implications. Following the organization for economic cooperation and development **(**OECD) guidelines for sublethal testing, fish were exposed to four BPA concentrations (1.25, 2.50, 5.0, and 10.0 mg/L) alongside positive (0.0 mg/L BPA) and negative (1.25 mg/L DMSO) controls under static conditions for 28 days. Total protein levels were quantified spectrophotometrically using the Biuret method, with statistical analysis performed via one-way ANOVA and Dunnett's test ($p < 0.05$). Results revealed a significant dose-dependent reduction in total protein at higher concentrations (5.0 and 10.0 mg/L) after 7 days compared to controls ($p< 0.05$). However, these differences diminished by day 28, suggesting potential adaptive mechanisms or compensatory responses. The transient decline in protein levels at higher doses aligns with BPA-induced oxidative stress, which may impair hepatic protein synthesis or accelerate proteolysis. These findings underscore the time- and dose-dependent nature of BPA toxicity, highlighting its capacity to disrupt protein metabolism in aquatic organisms. The study emphasizes the need for stricter regulatory measures to mitigate BPA discharge into aquatic ecosystems, thereby reducing its sublethal impacts on fish physiology and broader ecological health.

CHAPTER ONE

1.0 Introduction

Water bodies serve as a primary sink for industrial, domestic, and other anthropogenic pollutants, leading to significant ecological consequences for aquatic organisms (Häder, *et al.*,, 2020). Among these pollutants, endocrine-disrupting chemicals (EDCs) have garnered increasing attention due to their pervasive nature and potential toxic effects on wildlife. Bisphenol A (BPA), a widely used EDC, is a synthetic compound extensively utilized in the production of polycarbonate plastics, epoxy resins, and other consumer goods (Vom Saal & Hughes, 2005). BPA contamination in aquatic systems occurs through industrial discharges, leaching, and runoff, exposing aquatic organisms to sublethal concentrations that can induce biochemical and physiological alterations. (Mishra *et al.*,, 2023)

Fish, as sentinel organisms in aquatic ecosystems, are highly susceptible to the detrimental effects of pollutants. Exposure to BPA can interfere with the metabolic and physiological processes of fish, often manifested through changes in biochemical parameters such as serum total protein and creatinine levels (Srivastava and Redd 2020) . Serum total protein is a key biomarker of hepatic function and overall protein metabolism, while serum creatinine levels serve as indicators of renal health and glomerular filtration rate (Pal & Reddy, 2018). Changes in these parameters can signal toxic effects, such as impaired liver function and nephrotoxicity, which are critical for understanding the sublethal impacts of BPA on fish physiology.

The toxic effects of BPA are primarily mediated through the generation of reactive oxygen species (ROS), leading to oxidative stress and cellular damage (Kabuto, *et al.*,, 2004). Oxidative stress disrupts the balance between ROS production and antioxidant defense mechanisms, resulting in lipid peroxidation, protein oxidation, and other cellular dysfunctions (Tabrez & Ahmad, 2009). Studies have demonstrated that BPA exposure induces significant oxidative damage, which can alter key biochemical pathways in aquatic organisms. For

instance, BPA has been linked to decreased serum total protein levels in stinging catfish (*Heteropneustes fossilis)*, suggesting hepatic impairment (Pal & Reddy, 2018). Similarly, elevated serum creatinine levels in African catfish (*Clarias gariepinus*) exposed to BPA indicate potential nephrotoxic effects (Oluah *et al.*,, 2019).

The ecological and physiological implications of these biochemical changes are profound. Reduced total protein levels can impair growth, reproduction, and immune responses, while elevated creatinine levels reflect renal dysfunction, potentially leading to the accumulation of toxic metabolites and increased physiological stress (Gounden *et al.*,, 2024). These alterations not only compromise the health and survival of individual fish but also have broader implications for aquatic ecosystems, given the role of fish as critical components of the food web. Therefore, the use of biomarkers such as serum total protein and creatinine levels is crucial for assessing the sublethal effects of BPA exposure in aquatic species. (Pal & Reddy, 2018; Kulkarni & Pruthviraj 2016)

1.1 Aim and Objectives

The aim of this study was to investigate the effects of sublethal concentrations of Bisphenol A (BPA) on total protein levels.

The objectives of this study were

i. To determine the impact of sublethal concentrations of Bisphenol A (BPA) on total protein levels in the test organism.

ii. To assess the dose-dependent variations in total protein concentration following BPA exposure.

iii. To evaluate the time-dependent effects of BPA on protein metabolism.

iv. To investigate the physiological implications of altered total protein levels due to BPA exposure.

CHAPTER TWO

2.0 Literature Review

Bisphenol A has been widely used in the production of polycarbonate plastics, electronic equipment, food storage, and sports health products (Bousoumah *et al.*,, 2021; Catenza et al., 2021; Das *et al.*,, 2023). In 2023, the global market value for bisphenol A (BPA) is estimated to be USD 20.95 billion. The BPA market is projected to reach USD 35.59 billion with a compound annual growth rate (CAGR) of 8.9% from 2023 to 2028, driven by the increasing demand for polycarbonate plastics and epoxy resins across various industries (Research and Markets, 2023). Concerns about bisphenol A leaching from plastics have been significant since 2007, as it is a hazardous and enduring compound that interferes with the endocrine system (Xie *et al.*,, 2022; Xu *et al.*,, 2011). Its structural resemblance to hormones produced in the human body makes it a notable contaminant (Almeida et al., 2018; Matuszczak et al., 2019). Bisphenol A has been identified in rivers, groundwater, surface waters, and oceans globally (Sangeetha et al., 2021; Akram et al., 2021; Huang *et al.*,, 2020). It enters aquatic environments through inadequate wastewater treatment and leaching from BPA-containing plastics or microplastics (Akbari *et al.*,, 2016; Almeida *et al.*,, 2018). Leachates from landfills and the discharge of municipal wastewater also significantly contribute to bisphenol A pollution in water bodies and groundwater (Akbari *et al.*,, 2016; Catenza *et al.*,, 2021; Kusvuran & Yildirim, 2013). Bisphenol A disrupts endocrine functions, leading to issues such as infertility, reproductive disorders, precocious puberty, and obesity. It may also negatively impact human health and the ecosystem (Abraham & Chakraborty, 2020; Almeida et al., 2018; Valentino et al., 2016; Yang et al., 2021). The contamination of aquatic environments with BPA presents a major environmental challenge and a pressing issue. Several methods, such as ozonation, ultrasound irradiation, and biodegradation, can effectively remove BPA from water (AnakJuan *et al.,* 2021; Mohapatra *et al.*, 2011; Villegas et al., 2016; Zielińska et al., 2016). This review provides comprehensive information about the sources, environmental impacts, and

sustainable remediation techniques using microbes and enzyme systems for the removal of bisphenol A from aquatic ecosystems.

2.1 Physico-chemical properties of bisphenol A

To produce BPA, phenol and acetone must undergo condensation. This reaction is catalyzed by an acidic ion-exchange resin. The central carbon atom of BPA's molecular structure takes on a tetrahedral configuration, with two phenolic groups and two methyl groups surrounding it. Bisphenol A dissociates in alkaline environments and exhibits significant water solubility (120–300 mg/L) at ambient temperature (pKa 10.29–0.69). The log Kow for BPA is reported as 3.64 + 0.32, based on the Hazardous Substances Data Bank from the US National Institutes of Health. Traditionally, BPA was thought to exhibit minimal bioaccumulation (Heisterkamp *et al.*, 2004), although recent studies have highlighted the importance of understanding bioaccumulation and the relevance of moderately lipophilic substances concerning toxicity (Salapasidou *et al.*, 2011; Nichols et al., 2015; Valentino *et al.*, 2016). BPA's elevated melting points, moderate solubility, and low vapor pressure result in its low volatility. Due to its rapid atmospheric degradation and photo-oxidation, BPA has a short half-life in the environment (0.2 days). It has been found in various environmental matrices, including wildlife, humans, air, water, and soil.

2.2 Sources of Bisphenol A in environment

Due to its extensive production, usage, and resulting environmental pollution, BPA has disseminated throughout the ecosystem. The primary sources of BPA in the environment are categorized as pre-consumer and post-consumer items. Before reaching consumers, BPA is released into the environment from various pre-consumer sources, with the effluent discharge from manufacturing plants being the leading contributor. Other channels for its pre-consumer release include transportation, processing, and goods containing BPA (Flint *et al.*,., 2012).

BPA is commonly found in both marine sand and seawater globally. Researchers examined sand and saltwater from over 200 locations across 20 countries, mainly in Southeast Asia and North America. Each site exhibited BPA levels ranging from 0.01 parts per million (ppm) to 50 ppm, which Saido classified as "significant" levels (Laskawy & Laskawy, 2021).

Sources of BPA related to post-consumer activities include waste disposal mechanisms, such as leaching from landfills, incineration of domestic waste, and degradation of plastics in the environment (Czarny-Krzymińska et al., 2022; Flint et al., 2012). The bisphenol analogs triclosan and triclocarban were found to have different dispersal patterns in seawater and sediment samples taken from the East China Sea. In seawater, the most prevalent bisphenols were bisphenol A (BPA; average 23 ng/L), bisphenol S (BPS; 2.2 ng/L), and tetrabromobisphenol A (TBBPA; 2.3 ng/L) (Xie *et al.,* 2022). In the Bouregreg River, Chafi et al. (2022) studied the distribution of pharmaceuticals and endocrine-disrupting substances (Rabat, Morocco). He searched for 27 active pharmaceutical ingredients and endocrine disruptors in Moroccan surface waters. His findings revealed that bisphenol A was present at the highest concentrations, ranging from 142 to 368 ng/L in all samples, with detection frequencies surpassing 68% (Chafi *et al.,* 2022). Reports indicate that BPA is emerging as an organic pollutant in the lower stretches of the Ganga River in India and along the northeastern Bay of Bengal coast. Plastic waste in tourist areas may contribute to BPA levels in the Ganga River. BPA concentrations in surface and stormwater from this region have been found to be between 117.9 and 2147 ng/L (Mukhopadhyay & Chakraborty, 2021). In Africa, an increase in the use of products containing BPA has led to higher levels of environmental bioaccumulation and human exposure due to heightened industrial production and food packaging. To assess the extent of this bioaccumulation, Rotimi et al., 2021 investigated BPA levels across Africa (Rotimi *et al.,* 2021).

According to a published study on Africa, the highest BPA concentrations observed were in wastewater and water, recorded at 251 ng/mL and 384.8 ng/mL, respectively. To explore the seasonal variations, spatial distribution, source analysis, and ecological issues concerning endocrine-disrupting chemicals (EDCs) in the surface water of the Xiangjiang River in south China, Luo et al. (2019) conducted seasonal studies (Luo *et al.,* 2019).

The insufficient degradation of microplastics containing various bisphenol derivatives may be responsible for the ineffective removal of BPA by wastewater treatment plants (WWTP) (Luo et al., 2019; Montagner et al., 2018). Santhi et al. found that surface water samples collected in Malaysia near areas where sewage and industrial treatment facilities discharge contained significant levels of BPA ranging from 1.3 to 215 ng/L (Santhi et al., 2012). Additionally, a study led by Santhi et al. indicated that the concentrations of BPA in tap water and drinking water were 11.3 ng/L and between 3.5 to 59.8 ng/L, respectively. These findings imply that BPA infiltrates drinking water from plastic bottles and pipelines (Santhi *et al.,* 2012).

In Sao Paulo, Brazil, research analyzing drinking water, surface water, groundwater, and wastewater for BPA content was conducted from 2006 to 2015. The highest BPA concentrations were identified in surface water samples, with values spanning from 2 to 13,016 ng/L (Montagner et al., 2018). The levels of BPA in surface water rise due to the discharge of industrial wastewater and effluent from wastewater treatment facilities. Researchers concluded that regions close to industrial sites and densely populated areas exhibited elevated BPA concentrations (Montagner *et al.,* 2018). In a study from Lower Austria, it was found that BPA concentrations were particularly high in industrial effluents from the paper sector, with the wastewater showing the greatest BPA levels at 2500 ng/L (Fürhacker et al., 2000). Between 2013 and 2016, the concentration of BPA in Taihu Lake, China, increased dramatically from 8.5 to 97 ng/L (Rodriguez-Mozaz et al., 2004). At the Spanish water treatment facility, BPA was detected in every water sample taken throughout the purification process (Rodriguez-

Mozaz et al., 2004). Research indicated that the highest BPA concentrations were recorded in Japan, China, Korea, and India, with levels in the rivers of Chennai, India, ranging from 54 to 1950 ng/L (Yamazaki *et al.*, 2015). Due to the interactions between the polar characteristics of polylactic acid (PLA) and the hydroxyl groups in BPA, it has been demonstrated that PLA-based plastics release BPA into surface water less frequently than polypropylene (PP)-based plastics (dos Santos Rosa, 2018).

2.3 Toxicity of Bisphenol A

BPA can potentially influence the endocrine system in both direct and indirect ways. It interferes with hormonal balance by acting as an antagonist on nuclear hormone receptors, including the estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR), thyroid receptor (TR), insulin receptor (IR), and retinoid receptor (R) (Diamanti-Kandarakis et al., 2009). Golub and Doherty (2004) note that BPA's binding to hormone receptors leads to either the activation or inhibition of the downstream biological pathways in target cells. By attaching to or stimulating hormone receptors, altering hormone levels, or modifying the turnover of hormone-binding receptors, BPA may adversely affect the regulation of natural hormones (Mazur *et al.*, 2015; Zoeller *et al.*, 2014).

2.4 BPA-induced reproductive system toxicity

BPA leads to infertility in both genders by disrupting the function of sex hormones (Li *et al.*, 2010; You *et al.*, 2021; Shamhari *et al.*, 2021). Exposure to BPA has been associated with increased blood levels of hormones like luteinizing hormone (LH), progesterone, and estradiol, while cortisol levels were found to decrease. Research indicated a connection between BPA levels in the body and varying thicknesses of the uterine wall (Mínguez-Alarcón & Gaskins, 2017). In women under 37, a strong correlation was observed between BPA levels in urine and the thickness of the endometrial wall. However, this correlation was not significant among women older than 37. It has been revealed that women suffering from polycystic ovarian

syndrome (PCOS) tend to have elevated serum BPA levels (Vahedi et al., 2016). Various pregnancy complications, such as miscarriages and premature births, were often associated with different levels of BPA (Li *et al.* 2023, b, c; Namat *et al.*, 2021). Men with higher bisphenol exposure exhibited issues like lateral head displacement, reduced sperm counts, decreased motility, and angular displacement, all of which negatively impacted their reproductive capabilities (Ji *et al.*, 2018). Additionally, BPA exposure has been linked to problems with ejaculation, erectile dysfunction, and diminished sexual desire.

2.5 Developmental toxicity

According to various studies, exposure to BPA during pregnancy has been linked to inhibited growth in children, especially regarding weight, height, and neurological development. The anogenital distances of girls are shorter compared to boys (Sun *et al.*, 2018; Yu *et al.*, 2023). There is also a significant correlation between BPA concentrations and the timing of puberty. Both boys and girls who experience delayed puberty often have higher levels of BPA (Watkins et al., 2017). Increased exposure to BPA during pregnancy adversely affects children's neurological and mental development. Elevated environmental BPA levels have led to unusual reactions and behaviors among boys in school (Chen *et al.*,. 2017, b). The similar consequences of BPA exposure during and following pregnancy were investigated, revealing that anxiety, depression, restlessness, and hyperactivity were among the most common behavioral issues.

2.6 Metabolic disorders

When neuroendocrine function is impaired, metabolic irregularities and other complications start to emerge. Studies involving a sample of potential participants have revealed a significant association between plasma glucose levels and BPA levels. Research indicates that BPA may elevate the risk of developing type 2 diabetes (T2D) (Wang *et al.*, 2022). Although age has a minor impact on the incidence of diabetes mellitus, there is a strong link between BPA concentrations and the occurrence of the disease (Shankar & Teppala, 2011). The interference

of BPA with calcium metabolism has been linked to a reduction in bone density (Adoamnei *et al.,* 2018). Elevated BPA levels have been associated with cardiovascular issues, including pre-eclampsia, coronary heart disease, and peripheral artery disease (Shankar & Teppala, 2011; Ye *et al.,* 2017). Additionally, higher BPA levels may impair liver enzyme activity and potentially lead to renal failure, which could cause fat accumulation (Abdulhameed *et al.,* 2022; Li *et al.,* 2022). BPA exposure is often associated with the development of type I diabetes, bronchial dysfunction, and asthma (Zhou et al., 2017). A substantial number of studies have identified increased BPA levels as a primary factor in various cancers and neurological disorders, including autism spectrum disorder (occurring in tissues like the breast, ovary, and lung). A similar impact was observed when BPA in trace amounts was present in cancer cells such as HeLa and OVCAR-3 (Pugsley et al., 2022; Welch & Mulligan, 2022). The neuroendocrine dysfunction resulting from BPA interference permanently disrupts transporter proteins, which subsequently influences the production, action, and effectiveness of endogenous hormones. Research has shown a relationship between fluctuations in BPA levels and decreased testosterone levels in men, along with increased estradiol levels in women (Wisniewski *et al.,* 2015). Rat pups exposed to BPA orally exhibited excessive insulin production, which contributed to type 2 diabetes (Manukyan *et al.,* 2019). BPA exposure has also been linked to cognitive and behavioral issues, as well as alterations in neurosteroid levels.

2.8 Malfunctioning of hypothalamus

The brain produces gonadotropin-releasing hormone, which serves as the primary regulator of sexual behavior. The pituitary gland also secretes two additional hormones. Children exposed to BPA displayed irregular estrus cycles due to dysfunction in the hypothalamus, pituitary, and gonadal glands. The malfunction of the hypothalamus, pituitary, and gonadal glands in BPA-exposed children led to abnormal estrus cycles. Research has indicated that elevated BPA levels disrupt transcription of kisspeptin and gonadotropin-releasing hormone (Kurian *et al.,*

2015; Xi *et al.*, 2011). The signaling pathway that is regulated by inositol triphosphate and extracellular protein kinases has been shown to be obstructed by gonadotropin-releasing hormone. In an attempt to restore hormonal balance, the pituitary gland increased the levels of LH, FSH, and Er. The ability of BPA to mimic glucocorticoid activity caused interference with pituitary-adrenal function. Thanks to BPA's attraction to thyroid hormone receptors, thyroid homeostasis is prone to disruption. Evidence suggests that BPA affects thyroid hormone production in conjunction with the pituitary gland in rats. There is a noted association between chronic BPA exposure and its effects on memory and emotional behavior (Huang *et al.*, 2022).

2.9 Malfunctioning of estrogen receptor pathways

The interaction of BPA with the estrogen receptor is believed to act as a potent antagonist to estradiol (ER). The binding of BPA to the ER disrupts normal functions even at extremely low concentrations (Alonso-Magdalena *et al.*, 2012; Yuan *et al.*, 2023). The BPA-ER complex's preference for estrogen-sensitive regions influences gene expression patterns at estrogen response elements (ERE). Furthermore, studies have examined how BPA exposure impacts HepG2 cells. There is a strong correlation between an ERE site on the CYP2C9 gene promoter and the BPA-ER complex, which enhances the transcription of CYP2C9 (Xu et al., 2016). BPA also influences the uncontrolled differentiation of blood cells, known as hemangiomas. Through the ER pathway, BPA modifies epithelial-mesenchymal cells (EMC). In hemangiomas, the transcription factor Snai1 drives epithelial cells to undergo transformation into mesenchymal cells. BPA regulates the expression or silencing of Snai1. Following BPA exposure, cancer cells from the ovarian and breast tissues exhibited changes in expression. The binding of BPA-ER enhanced the motility and invasion of MCF-7 breast cancer cells, which also increased CXCR4 expression (Lee *et al.* 2021, b). BPA stimulated the expression of the CXCL12 gene, thereby boosting the proliferation capacity of ovarian cells. Regarding the effects on non-estrogen receptors, research indicates that BPA has a substantial impact on

androgens, akin to its effects on estrogens. BPA competes with androgens by binding to the androgen receptor (AR). These interactions proceed out of sequence, hindering the initiation of gene transcription and disrupting reproductive mechanisms. BPA precisely modulates the confirmation of the AR protein, allowing effective communication between the thyroid hormone receptor silencing mediator, nuclear receptor co-suppressor, and AR. In MDA-MB 231 cells, downregulation of the estrogen-related receptor (ERR) pathway resulted in decreased ERK, AKT, and MMP activities. The aryl hydrocarbon receptor (AHR), which has functionalities similar to the ERR, regulates growth and metabolism. Analysis of AHR responses in mice revealed that overexpression of AHR negatively affected sperm quality and motility. Infertile individuals commonly exhibited elevated BPA levels, corresponding with increased expression of ER, AR, and HR, which led to a reduction in sex hormones and sperm quality (La Rocca *et al.,* 2018). There exists a connection between peroxisome proliferator-activated receptor (PPAR) and type 2 diabetes. Male mice exposed to BPA developed fat accumulation due to upregulation of the Ap2 gene and activation of the PPAR pathway (Long *et al.,* 2021). Levels of PPAR were quadrupled, accompanied by increased inflammatory factors in adipocytes. Additional research indicated a downregulation of both insulin production and fat storage. BPA's effects hindered a membrane receptor- or integrin-dependent pathway. In mice, beta 1, alpha 5, and MMP exhibited higher expression levels in the placenta despite relatively low BPA doses. The inhibition of MAPK and the phosphatidylinositol pathway enhanced trophoblast migration by reversing the expression trend. Integrin beta 1 was directly activated by BPA, promoting migration in cancer cell lines (Huang *et al.*, 2019).

2.10 Impacts on enzyme activity

The impact of BPA has enabled the enhancement of several enzyme activities. The interaction between BPA and xanthine oxidase increased the liver's ability to produce uric acid (hyperuricemia) (Ma *et al.,* 2018). BPA influences three main enzymes associated with lipid

metabolism: a carboxylase, fatty acid synthase, and lipoprotein lipase. A genomic study confirmed the effects of BPA on these enzymes in 3T3-L1 cells (Park *et al.,* 2019). BPA also inhibits fatty acid amide hydrolase, the enzyme responsible for breaking down endocannabinoids (Padmanabhan *et al.*, 2021). Additionally, aromatase, an enzyme found in both humans and fish that plays a vital role in converting testosterone to estrogen, is affected by BPA. The CYP19b aromatase gene was examined to determine how BPA influenced its expression, revealing an irregular pattern in the findings. Alterations in aromatase gene expression that impacted gonadotropic cell function ultimately led to anovulation and a lasting dysfunction of the HPG axis, resulting in infertility (Molina-Molina *et al.,* 2019).

Exposure to Bisphenol A (BPA) has been associated with disturbances in protein metabolism and renal function, with creatinine levels being an important marker of kidney health. BPA is one of the most widely produced chemicals globally and is commonly utilized in dental sealants, water bottles, and the packaging of food and beverages. As a result of its extensive use, BPA poses a potential risk to various organisms and public health. In this investigation, a total of 80 bighead carps were randomly assigned to four different groups (A–D). Fish in groups B, C, and D were exposed to BPA concentrations of 500, 1000, and 1500 μg/L, respectively, for a duration of 60 days, while group A served as a control without treatment. There was a notable decrease in body weight, while both the absolute and relative weights of various internal organs significantly increased ($p < 0.05$) among fish exposed to the highest concentration (1500 μg/L) of BPA. The proximate analysis results indicated significantly lower levels of crude proteins, lipids, and moisture, along with an increase in ash content in the muscles of treated fish. Erythrocyte counts, hemoglobin levels, lymphocytes, and monocytes significantly decreased, while total leukocyte and neutrophil counts showed a significant increase in the exposed fish. The evaluation of serum biochemistry parameters revealed significant reductions ($p < 0.05$) in serum albumin and total protein levels, while increases were

observed in alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine, glucose, cholesterol, and lactate dehydrogenase (LDH) ($p < 0.05$) in treated fish. Histopathological examinations revealed issues such as pyknosis, degeneration of glomeruli, increased Bowman's space, and ceroid formation in the kidneys, alongside ceroid formation, hemorrhage, pyknosis, karyorrhexis, karyolysis, nuclear hypertrophy, and eccentric nuclei in the liver of the treated fish. Histological analysis of various brain sections from the treated fish showed neuron degeneration in the cerebellum, lipofuscin deposition, microgliosis, necrotic neurons, inflammatory cell presence, and hemorrhages. Light microscopic investigations of different heart sections of the bighead carp revealed necrosis, inflammatory responses, neutrophilic myocarditis, and hemorrhages. In conclusion, the findings suggest that BPA causes negative effects on physical conditions, blood biochemistry parameters, and histopathological changes in various internal tissues of the exposed fish.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3. 1 Chemicals

Bisphenol A (BPA) was purchased from a certified supplier. The BPA (4,4'-isopropylidenediphenol; CAS No. 80-05-7) is an industrial chemical primarily used in the production of polycarbonate plastics and epoxy resins. BPA is an endocrine-disrupting compound (EDC) known to mimic estrogen and has been associated with various adverse developmental and reproductive effects. It is sparingly soluble in water but highly soluble in organic solvents.

Dimethyl Sulfoxide (DMSO) was obtained from a commercial vendor. The DMSO (Dimethyl sulfoxide; CAS No. 67-68-5) is an organosulfur compound widely used as a solvent in biological and chemical research due to its ability to penetrate biological membranes. It is a polar aprotic solvent, fully miscible with water and many organic solvents. DMSO is known for its anti-inflammatory properties and is used as a vehicle for drug delivery in experimental studies.

3.2 Test organism

Clarias gariepinus (African catfish) is widely distributed in tropical freshwater ecosystems and relished by the tropical region populace (Ogueji *et al.*, 2018). The fish is omnivorous, feeding on invertebrates, fish, small mammals, seeds and fruit (Hastuti *et al*., 2019). According to Ogueji *et al.* (2018), it is also rugged, adaptive to laboratory conditions produces reasonable quantities of blood for haematological parameter estimation. It is among the most widespread of the African freshwater fish (Nguyen and Janssen, 2002).

3.3 Source of experimented fish and acclimatization

The experimental fish, *Clarias gariepinus* juveniles were purchased at a fish farm in Benin city, Edo state and transported to the ecotoxicology laboratory of Animal and Environmental Biology, University of Benin, Benin City, Edo state where the experiment was conducted. The

average weight and average length of the fish was 4.18±1.67g, 9.73±1.34cm and 7.87±0.98cm SD respectively. The juveniles were acclimatized in a 160L cylindrical plastic tank for two days and fed on a pelleted commercial feed daily. They were however left unfed for the first 24 hours to adapt to a change in the environment before feeding commenced with the fish diet. Approximately, 80% of the water was replaced every two days. They were kept under natural photoperiod.. There was no mortality during the acclimatization period.

3.4 Experiment design

A total of 126 *Clarias gariepinus* juveniles used were divided into six treatments which included a positive control (+control), negative control (-control), and four different concentrations of the toxicant) with two replicates. Seven juveniles were stocked into each tank with two replicates treatment. The water used was tap water left to stand for 24 hours.

3.5 Experimental protocol

The experiment was conducted following the OECD guidelines for fish acute toxicity testing (Test, 1997). A stock solution was prepared by dissolving 2 g of bisphenol A in 100 mL of DMSO. This stock solution was then used to prepare four different concentrations of the toxicant: 1.25 mg/L, 2.50 mg/L, 5.0 mg/L, and 10.0 mg/L. Each concentration was introduced into a 20-liter experimental tank. The positive control (+control) contained 0.0 mg/L of the toxicant, while the negative control (-control) contained 1.25 mg/L of DMSO. Each treatment, including the controls, was conducted in duplicate. The test was performed under static conditions and lasted for 28 days. During this period, the organisms were fed once daily, and mortality was recorded daily

3.6 Determination of Concentration of Plasma Total Protein

3.6.1 Principle

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex.

3.6.2 Assay Procedure

Biuret reagent (2.5 mL) was added to 0.05 mL of sample and 0.05 mL of standard. The blank contained 2.5 mL of Biuret and 0.05 mL of distilled water. The solution in each tube was incubated for 10 min at 37 ^{o}C, and the absorbance was read at 546 nm against the reagent blank.

3.6.3 Calculations

- When measurements are taken at 546 nm, total protein concentration may be calculated as follows:

$$Total\ Protein\ (g/L) = 190 \times A_{Sample}$$

$$Total\ Protein\ (g/dL) = 19 \times A_{Sample}$$

- When using a standard

$$Total\ Protein\ Concentration = \frac{A_{Sample}}{A_{Standard}} \times Standard\ Conc.$$

3.7 Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM). The difference between groups was analyzed with one-way ANOVA followed by Dunnett's multiple comparison test. The data were analyzed using GraphPad Prism version 5.01. The level of significance was set at $p < 0.05$. Graph analyses were plotted using the GraphPad Prism software as well.

CHAPTER FOUR

4.0 Results

4.1 Total Protein

On day 7 of exposure, the mean value of total protein in the +ve Control group was 53.65 ± 0.2355 g/dL, whereas in the -ve Control group, it was 52.55 ± 0.6675 g/dL. Additionally, at a concentration of 1.25 mg/L, the mean value was 51.70 ± 0.009192 g/dL, while at 2.50 mg/L, it was 51.61 ± 1.363 g/dL. Furthermore, at 5.0 mg/L and 10 mg/L, the mean values were 49.81 ± 0.5862 g/dL and 50.24 ± 0.5989 g/dL, respectively. Notably, only the concentrations of 5.0 mg/L and 10 mg/L were significantly different from the +ve Control group.

On day 14 of exposure, the mean value of total protein in the +ve Control group was 49.65 ± 0.2008 g/dL, while in the -ve Control group, it was 50.26 ± 0.2263 g/dL. At 1.25 mg/L, the mean value was 49.78 ± 0.5134 g/dL, whereas at 2.50 mg/L, it was 49.73 ± 0.7608 g/dL. Additionally, at 5.0 mg/L and 10 mg/L, the mean values were 50.03 ± 0.1810 g/dL and 49.82 ± 0.4483 g/dL, respectively. None of the concentrations showed significant differences when compared to the +ve Control group.

On day 21 of exposure, the mean value of total protein in the +ve Control group was 50.11 ± 0.5911 g/dL, while in the -ve Control group, it was 50.06 ± 0.04808 g/dL. Further analysis revealed that the mean value was 49.73 ± 0.04384 g/dL at 1.25 mg/L and 50.85 ± 0.3274 g/dL at 2.50 mg/L. Similarly, at 5.0 mg/L and 10 mg/L, the mean values were 50.96 ± 2.066 g/dL and 53.15 ± 0.8888 g/dL, respectively. None of the concentrations differed significantly from the +ve Control group.

On day 28 of exposure, the mean value of total protein in the +ve Control group was 52.21 ± 2.214 g/dL, whereas in the -ve Control group, it was 50.42 ± 0.7941 g/dL. At 1.25 mg/L, the mean value was 50.69 ± 0.9504 g/dL, while at 2.50 mg/L, it was 52.98 ± 0.6406 g/dL. Moreover, at 5.0 mg/L and 10 mg/L, the mean values were 54.31 ± 4.321 g/dL and 52.60 ±

2.973 g/dL, respectively. None of the concentrations exhibited significant differences compared to the +ve Control group

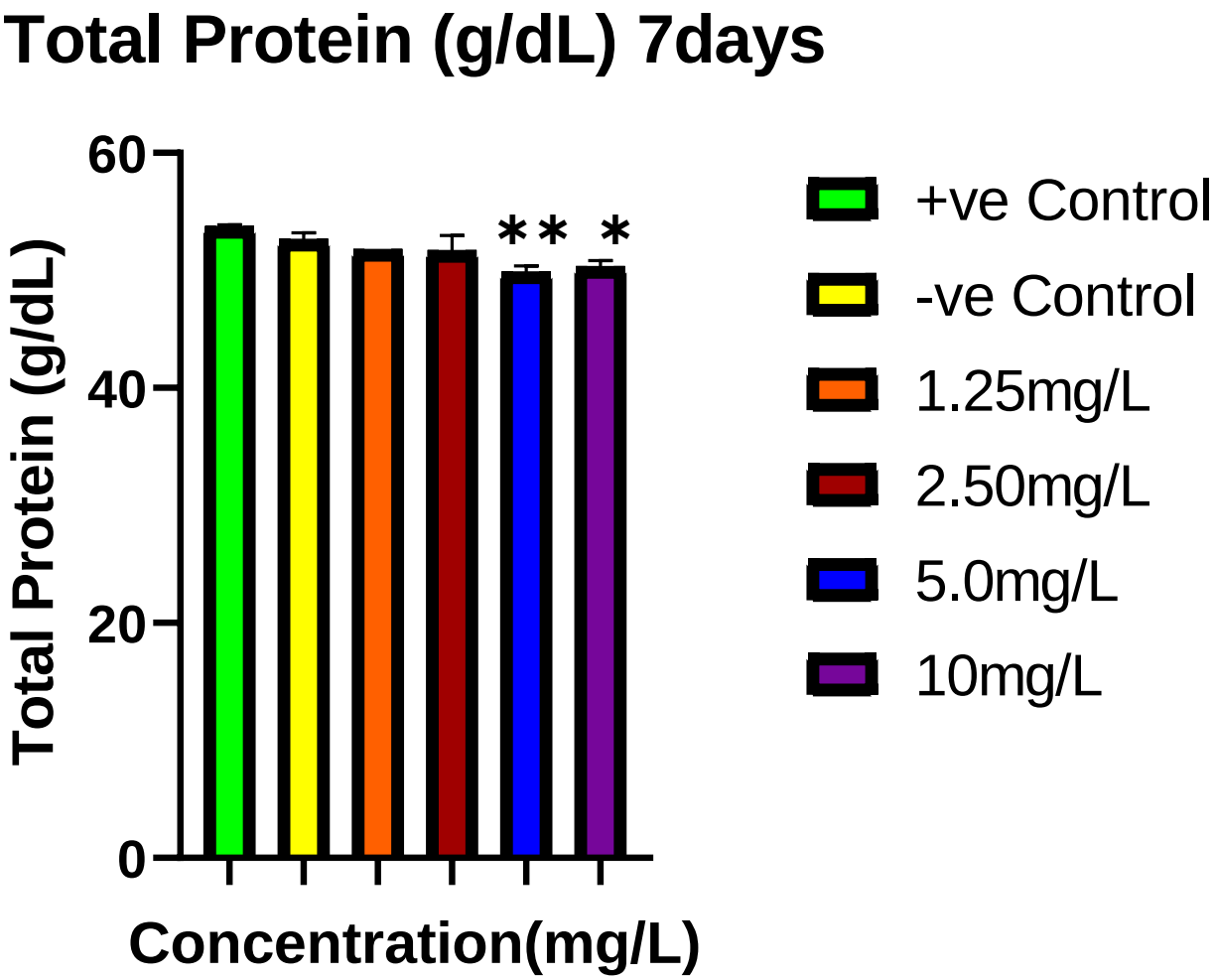

Fig. 4.1.1Total Protein (g/dL) levels in homogenate of juvenile catfish exposed to varying concentrations of Bisphenol A on day 7. Data are expressed as mean± SD(n=7)

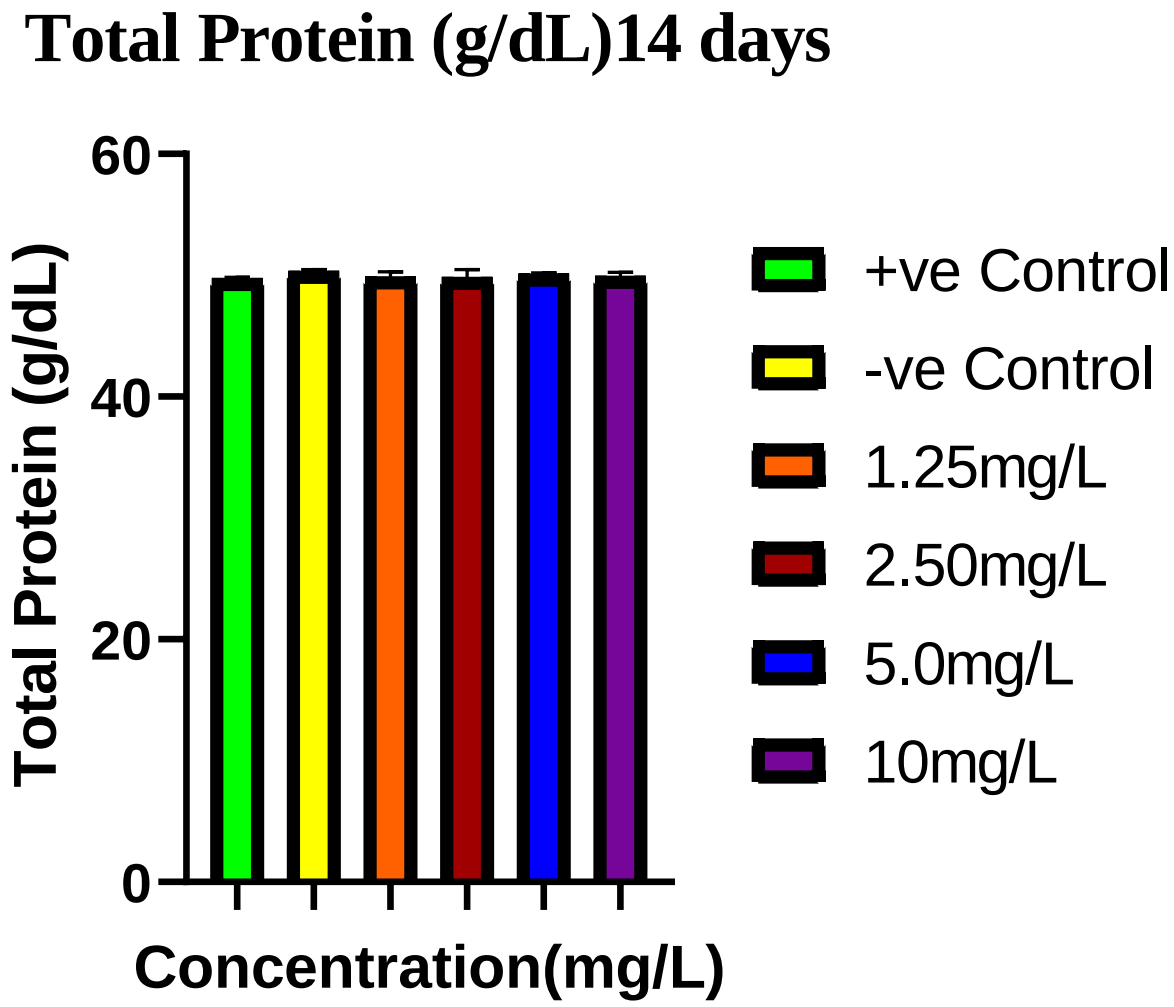

Fig. 4.1.2 Total Protein (g/dL) levels in homogenate of juvenile catfish exposed to varying concentrations of Bisphenol A on day 14. Data are expressed as mean± SD(n=7)

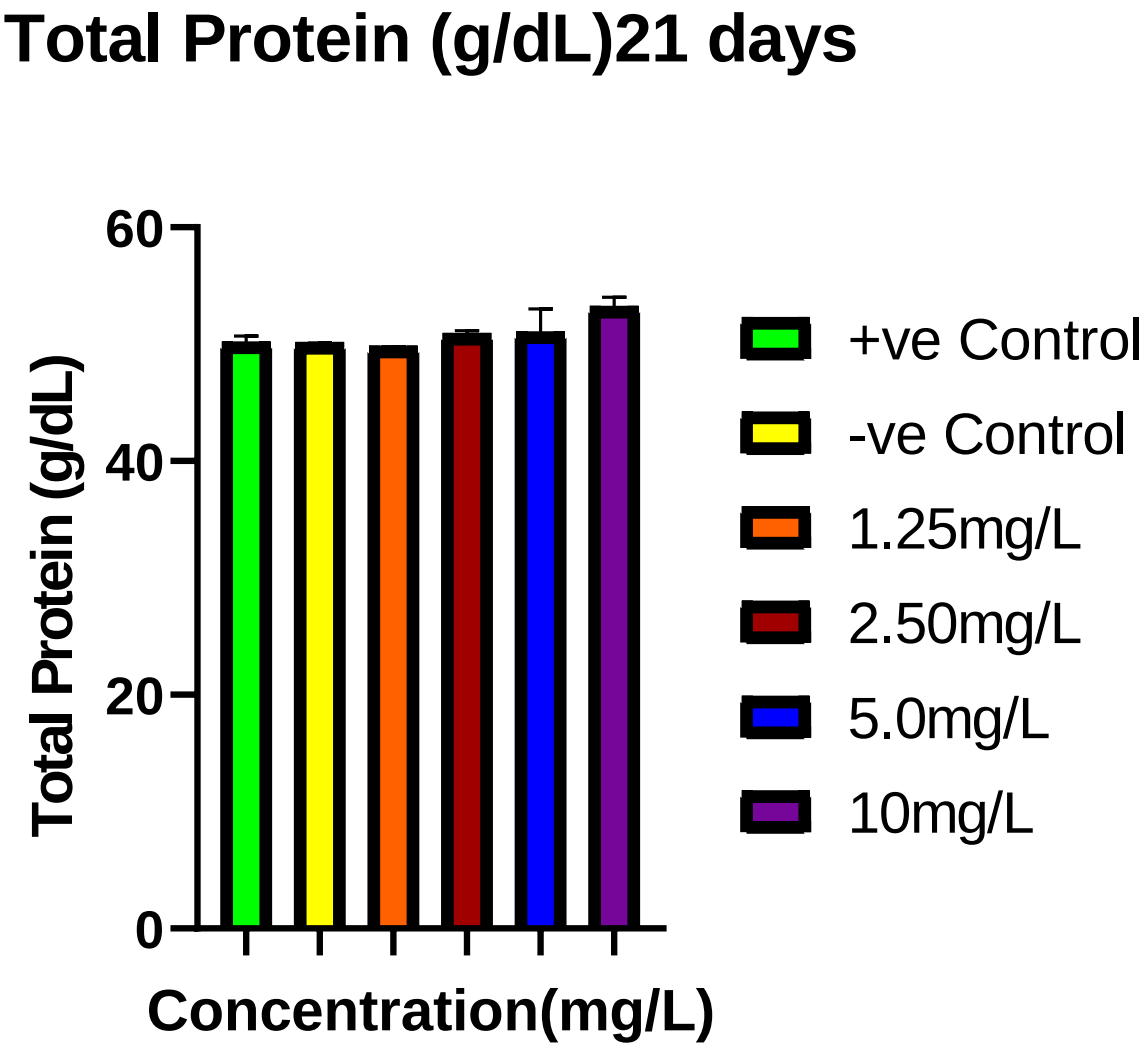

Fig. 4.1.3 Total Protein (g/dL) levels in homogenate of juvenile catfish exposed to varying concentrations of Bisphenol A on day 21. Data are expressed as mean± SD(n=7)

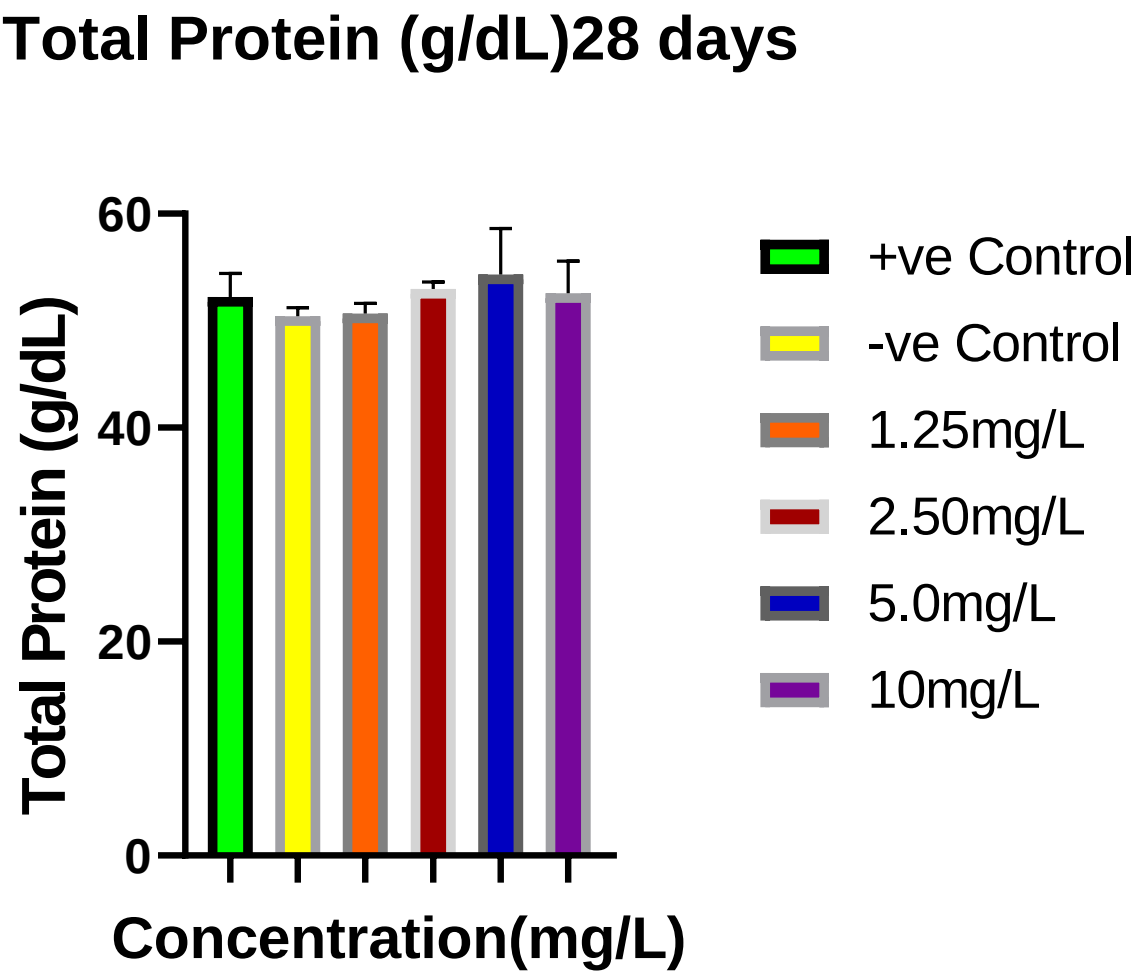

Fig. 4.1.4 Total Protein (g/dL) levels in homogenate of juvenile catfish exposed to varying concentrations of Bisphenol A on day 28. Data are expressed as mean± SD(n=7)

CHAPTER FIVE

5.0 DISCUSSION

Fish species are very sensitive to aquatic pollution and specific haematological and biochemical biomarkers of fish were demonstrated as direct indicators for assessing the ecological status of an aquatic environment. (Srivastava Reddy 2020). The findings of this study demonstrate that sublethal concentrations of Bisphenol A (BPA) influenced total protein levels in test subjects over the study period. While variations were observed across different concentrations and exposure durations, significant differences were limited primarily to higher concentrations (5.0 mg/L and 10 mg/L) during the initial days. Over time, the changes in total protein levels generally appeared less pronounced, with no significant differences observed between the experimental groups and the positive control (+ve Control) by the 28th day of exposure. These results align with and extend the findings of similar studies, shedding light on BPA's complex impact on protein metabolism.

Protein metabolism is a sensitive indicator of physiological stress and toxicant exposure, as proteins play crucial roles in cellular function, immune responses, and overall organismal health. Previous studies have documented the effects of BPA on protein metabolism, noting alterations in total protein levels as a potential biomarker of sublethal toxicity. For instance, Abdel-Tawwab, & Hamed, (2018) observed significant reductions in total protein levels in fish exposed to sublethal concentrations of BPA, particularly at higher concentrations and shorter exposure periods. Their results suggest that BPA-induced oxidative stress and disruptions in hepatic function may impair protein synthesis or accelerate protein degradation. These observations align with the significant reductions in total protein observed in this study at 5.0 mg/L and 10 mg/L after 7 days.

Similarly, a study by Martínez *et al.,*. (2020) reported that BPA exposure in zebrafish caused disturbances in metabolic pathways, including protein metabolism, especially under chronic

exposure conditions. They attributed these effects to BPA's endocrine-disrupting properties, which interfere with hormonal regulation of metabolic processes. The results of the current study, particularly the lack of significant differences in total protein levels by the 28th day, are consistent with these findings, suggesting a potential compensatory mechanism or adaptive response to prolonged BPA exposure.

Interestingly, certain studies, such as those by Adamovsky, *et al.*, (2024), have reported no significant changes in total protein levels at low BPA concentrations, emphasizing the dose-dependent nature of BPA's toxic effects. This aligns with the current findings, where lower concentrations (1.25 mg/L and 2.50 mg/L) consistently showed no significant differences compared to the +ve Control group across all time points.

The observed variations in total protein levels at higher concentrations and shorter exposure periods could be attributed to BPA's known ability to induce oxidative stress, leading to damage to proteins and other macromolecules. Oxidative stress disrupts hepatic function, which is critical for protein synthesis, and may also accelerate proteolysis (Poli, 2000).). Additionally, BPA's interaction with endocrine pathways, particularly its estrogenic effects, may disrupt hormonal regulation of protein metabolism, further contributing to the observed changes (Chen *et al.*, 2009).

The absence of significant differences at lower concentrations suggests that the toxic effects of BPA on protein metabolism are threshold-dependent and may not manifest below a certain dose. Furthermore, the lack of significant changes at higher concentrations by the 28th day suggests the activation of compensatory mechanisms, such as upregulation of antioxidant pathways or repair processes, which may mitigate the initial effects of BPA exposure.

5.1 Conclusion

This study adds to the growing body of evidence that BPA exerts dose-dependent and time-dependent effects on protein metabolism. While higher concentrations (5.0 mg/L and 10 mg/L) initially caused significant reductions in total protein levels, prolonged exposure led to an apparent normalization, potentially due to adaptive responses. These findings underscore the importance of considering both dose and exposure duration when evaluating the toxicological effects of BPA and highlight the need for further research to elucidate the underlying mechanisms of these adaptive responses.

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