Recuperative effect of the combined leaf extracts of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* in Tilapia (*Oreochromis mossambicus*) after sublethal exposure to Cadmium

by

PRARTHANA JAYESH NAIK
22P0440019

Under the supervision of

DR. AVELYNO HUBERT D'COSTA

School of Biological Sciences and Biotechnology
Zoology Discipline
GOA UNIVERSITY

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PRARTHANA JAYESH NAIK

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Under the Supervision of

DR. AVELYNO HUBERT D'COSTA

School of Biological Sciences and Biotechnology

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GOA UNIVERSITY

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Examined by:

SBSB, M.Sc., Zoology
Taleigao Plateau, Goa 403206
GOA UNIVERSITY

Seal of the School

PREFACE

Heavy metals like Cadmium have posed a persistent threat to life since years, their detrimental effects on humans can be tracked down to the late 19th century. However, it was only during mid to late 20th century as the industrialization surged, that their impact as some of the major toxicants began to be acknowledged. Several regulations were placed pertaining to health concern once Cd started getting recognised as a hazardous substance. However, despite of all the regulatory efforts since years, Cadmium toxicity still remains a concern today. One of the prominent avenues of Cadmium exposure to humans is the consumption of fish contaminated with this perilous metal. Fish being one of the cheapest and most accessible sources of protein has been consumed by humans since time immemorial. Recently fishes have also gained popularity in human diet because of their significant health benefits. However, the heavy metal content discovered in some of the fishes makes it detrimental to human health.

ABSTRACT

This study aimed at analyzing the potency of the combined leaf extracts (CLE) of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* in restoring the health of *O. mossambicus* affected by Cadmium toxicity. The study primarily focused on the synergistic potency of the CLE to act as a hepatoprotective agent. Two different concentrations of the CLE were utilized for better understanding of the dose response of the exposed fishes. The tilapias were divided into four groups: normal control, cadmium control, cadmium + extract 1 and cadmium + extract 2. Except for the normal control, all the other groups were subjected to cadmium chloride ($1/8^{th}$ of LC50) for a period of 7 days. Cadmium exposure was followed by treatment with the CLE for a duration of 15 days in the 3^{rd} and 4^{th} group. The cadmium control was however, left for natural detoxification for the same period. The results revealed that the CLE significantly restored the concentrations of catalase and TBARS. No significant results were observed pertaining to the protein concentration. While there was no significant change displayed in the carbohydrate concentration in the cadmium exposed fish. The study results indicate that the administration of the CLE can significantly improve the toxic effects of cadmium in *O. mossambicus.*

1. INTRODUCTION

1.1 BACKGROUND

1.1.1 METALS AS POLLUTANTS

A fraction of metals like iron, cobalt, zinc, copper, chromium, molybdenum, selenium and manganese play a pivotal role in various essential functions of the body (Mehrandish et al, 2019), while a plurality of heavy metals are evaluated as the major pollutants in the environment. Even though they occur naturally, their mounting demand in various sectors of industry has turned them into a threat.

These metals which do not find any significant role in the body, are known to invoke their influence even at scant concentrations. And since they have a predisposition to bioaccumulate in the tissues and later undergo biomagnification in the organisms at the successive trophic levels, their toxicity becomes of all the more concern in the biosphere. The toxicity of these dense metals depends on various aspects, including the route and dose of exposure, the species, age as well as the gender of the particular organism. Also, these trace metals are evident to cause multiple organ damage even at minimal levels.

1.1.2 CADMIUM

Cadmium (Cd) is one of the well-known heavy metal toxicants which is a threat to aquaculture and is a classified human carcinogen (group 1). Like all the other metals, Cadmium has its natural sources like volcanic eruptions, weathering of rocks and forest fires. However, it is the surging demand of such hazardous metals that has heightened its environmental measures to several notches, paving its path into the aquatic environment where they significantly affect the fauna as well as the flora in their vicinity. With such a ubiquitous distribution, fishes find no escape from the toxicity of these high-density elements.

Gills, digestive tract and body surface are the three ways by which the metals like Cadmium enter the body of the fish, with gills being the prime site for direct uptake of metals from the water (Afshan et al, 2013) They pose harm to the fishes and other aquatic life even in subdued amounts, causing tissue damage, biochemical changes, alterations in the levels of antioxidant enzymes as well as genotoxicity, ultimately deteriorating the quality of the fish. Since fishes are a part of the human diet, the toxic levels of these metals ultimately affect humans as well.

1.1.3 *Oreochromis mossambicus*

Several fish species have been utilised as bioindicators to ascertain aquatic pollution as well as for examining the methods for controlling these pollutants. One such fish, the Tilapia (*Oreochromis mossambicus*) has been used profoundly in various toxicological studies. Additionally, it has substantial economic importance and is comparatively easy to handle in laboratory conditions. (Naik et al., 2020) There are various studies concerned with the toxic effects of Cadmium in *O. mossambicus*, therefore in the present study it was chosen as the model organism to execute a new approach in ameliorating the oxidative stress as well as the genotoxicity caused by Cadmium chloride.

1.1.4 ROLE OF BODY'S ANTIOXIDANT DEFENCE

The toxicity of the harmful substances such as metals evokes the production of reactive oxygen species (ROS) in the body of the affected individual. These ROS causes a considerable damage to the tissues and organs where they are generated. They are even evident to cause DNA damage thus resulting in genotoxicity. These ROS are formed in the body even during the normal conditions in response to certain stress stimuli, however they are kept under check by the body's antioxidant defence. The antioxidant defence comprises

the antioxidant enzymes which contribute to the elimination of the ROS from the body. But in instances where the body is affected by toxicants, there is a rise in the levels of ROS that more than often even the antioxidant enzymes fail to keep the damage in control. The severity of the condition often ends up affecting these very enzymes, thereby decreasing their levels. In such conditions, antioxidants from external sources can provide relief by restoring the normal enzyme concentrations in the body.

The imbalance between reactive oxygen species (ROS) production and antioxidant defence system in fish can cause DNA hydroxylation, apoptosis, protein denaturation, lipid peroxidation and cell damage. Dietary approaches were recently proposed to reinforce antioxidant defence activity in different fish and shellfish species (Ghafarifarsani et al., 2022).

1.1.5 REMEDIATION OF CADMIUM TOXICITY

Many methods have been employed to detoxify the heavy metals from the fishes prior to their integration in the human diet. Most of these techniques rely on the use of chemicals to attain a toxicant free status. Chemicals like EDTA and a variety of other compounds which act as chelating agents are used for this purpose. However, the possible ill-effects of these chemicals on humans cannot be ignored. Thus, there is a need to switch to environment-friendly ways of remediation.

Recently, plant extracts and feeds have received great attention to solve this problem. Many plants with their various bioactive compounds and antioxidants serve as great alternatives to tackle the reactive oxygen species as well as their interaction with the toxicants enables the amelioration of these harmful substances.

1.1.6 USE OF PLANTS AS AMELIORATIVE AGENTS

India possesses a luxury of medicinal and aromatic plants, with more than 1,100 species of flowering plants listed in the Materia Medica of traditional ayurvedic and Unani medicines. Among these, over 46 species are in high demand and are regularly harvested from their natural habitats in the wild (Ayoub & Mehta, 2018).

The World Health Organization (WHO) describes traditional medicine as therapeutic approaches that have been employed for countless centuries before the ascent of modern medicine and are still in use today. Plants have played a paramount role in maintaining human health and refining the attribute of life for numerous generations throughout history. The practice of using plants for medicinal reasons can be pursued back to the dawn of human civilizations (Baral et al., 2022).

Since time immemorial, a variety of plants have been recognized as a source of antioxidants. These naturally occurring antioxidants have received great attention in recent years for their use in serving as a means to combat many toxicants. The phenolics, flavonoids and the condensed tannins present in the various plant parts are the major source of antioxidants in the plants. Thus, the plants having a significant content of these vital bioactive compounds can help to detoxify the effects of many toxicants which exert their fatal effects on the organisms. Scientists have utilised not just single plant extracts but even combinations of two or more plant extracts to ameliorate the toxicant's effects, obtaining great results. Researchers have exploited the synergistic effects wherein the sum of extracts have magnified the protective effect as compared to their individual effects. This has helped them to target multiple pathways and yield great results in the end. Additionally, the extracts in combinations, often referred to as the synergistic cocktails have shown to enhance each other's potency to provide a faster remedial effect.

Justicia adhatoda, *Coleus amboinicus* and *Artemisia parviflora* have a history of use in traditional medicine, especially Ayurveda. These highly studied plants with assortments of bioactive compounds have shown immense potential as antioxidants. The leaves of each of these plants have revealed their efficacy against reactive oxygen species. A synergistic cocktail of the three of these plants' leaf extracts may as well show enhanced activity against the toxic effects of Cadmium in *Oreochromis mossambicus*.

1.1.7 *Justicia adhatoda*

Justicia adhatoda (L.) Nees, a shrub belonging to the family Acanthaceae is widespread throughout the tropical regions of Southeast Asia. It is a gregarious species of miscellaneous use. This shrub is highly revered in Unani and Ayurveda, owing to its healing properties against asthma, cold, cough and tuberculosis. The plant also exhibits antispasmodic and expectorant properties. The leaf, shoot and root of *Justicia adhatoda*, pervasively possess quinazoline alkaloids like vasicine and vasicinone, along with a non-crystalline steroid (vasakin) and various essential oils, sterols, fatty acids, glycosides and other phenolic components (Gantait & Panigrahi, 2018).

The leaves and roots contain numerous alkaloids, the principle alkaloids being quinazoline alkaloid, vasicine and vasicinone, vasicinolone and vasicol, which may exhibit a bronchodilator effect. These alkaloids are reported to exist in integration with an acid that has been entitled adhatoda acid. It acts as an anthelmintic, bronchial antiseptic, sedative, expectorant, antispasmodic and bronchodilator. The leaf extract has been harnessed for the remediation of bronchitis and asthma for ages (Singh et al., 2017).

1.1.8 *Coleus amboinicus*

Coleus amboinicus is a renowned plant belonging to the Lamiaceae family. It is found in many regions throughout India. It is a medicinal plant which has been used to cure many ailments in folkloric medicines. It also finds its use in diseases such as flu, bronchitis, and epilepsy. This plant is impactful in wound healing with very few side effects. The fatal toxic dose of this specific herb was assessed in laboratory mice which disclosed that the plant *C. amboinicus* is an impressive herb which has no side effects (Ce, 2013, Manjamalai & Grace, 2013). Photochemical study uncovered the presence of flavonoids such as luteolin, apigenin, and salvigenin in this plant (Janakiraman & Somasundaram, 2014).

The essential oil of *C. amboinicus* is rich in carvacrol, thymol, ethyl salicylate, eugenol and chavicol with thymol being the chief component in the oil. Additionally, rosmarinic acid was discovered to be the principal element for the radical scavenging activity of *C. amboinicus* (Rout et al., 2012).

1.1.9 *Artemisia parviflora*

Artemisia pertaining to the family Asteraceae is evident for their essential oils. More than 400 species of *Artemisia* have been delved into from different parts of the world. The terpenoids and sesquiterpene lactones constituting the essential oil, imparts the characteristic strong aroma and bitter taste concerned with this genus. Essential oils of *Artemisia* sp. have served numerous medicinal ailments such as antiviral, antimalarial, antibacterial, fungicidal and nematicidal for years.

Research studies highlight *Artemisia*'s role in addressing an entire spectrum of physiological imbalances through a rarefied combination of pharmacotherapeutic actions (Kshirsagar & Rao, 2021). *A. parviflora* yields a light yellow essential oil which is known to contain isobornyl acetate, ar-curcumene, limonene, propanol, B- caryophyllene, germacrene D,

camphor and artemisia ketone (Ahameethunisa & Hopper, 2012). Eminent among the phytochemicals is artemisinin and its derivatives (ARTs) that depict a new class of endorsed drugs due to the advent of bacteria and parasites that are resistant to quinoline drugs (Kshirsagar & Rao, 2021).

1.2.1 AIM

· To evaluate the effect of the combined leaf extracts of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* on the Cadmium induced toxicity in *Oreochromis mossambicus*.

1.2.2 OBJECTIVES

- To determine the ameliorative effect of the Combined Leaf Extract (CLE) on Cadmium induced hepatotoxicity in Tilapia.
- To analyze the effect of CLE on antioxidant enzymes affected by Cadmium toxicity in Tilapia.
- To assess the effect of CLE on the nutritional aspects of Tilapia subjected to Cadmium.

1.3 HYPOTHESIS

- The combined leaf extracts of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* will have a remedial effect on Cadmium toxicity in Tilapia (*Oreochromis mossambicus*).

1.4 SCOPE

1. Broadening the research to comprehend the mechanism of action of the extract and its effects on other aquatic species could lead to the advancement of more competent and targeted treatments.

2. Implementing the extract as a treatment or a preventative measure in aquaculture to attenuate the toxicity of Cadmium, thus improving the quality of the fish.

3. Venturing into the possible benefits or risks on human health concerned with consuming extract treated Tilapia fish.

4. Examining if the extract has the potency for bioremediation of water bodies contaminated with Cadmium.

2. LITERATURE REVIEW

2.1 Plant based remedies to combat toxicity in Tilapia

O. niloticus subjected to lead (II) nitrate were supplemented with *Thunbergia laurifolia* leaf extract along with normal fish supplementations to detect the ameliorative potential of the extract. *T. laurifolia* leaf extract protected the fish against toxic effects of lead pertaining to growth retardation, altering the blood parameters and organ histopathology (Palipoch et al., 2011).

Effect of Indian lotus leaf (ILL) powder was studied for its potential to alleviate the toxic effects of lead (Pb), cadmium (Cd), mercury (Hg), and zinc (Zn) on *O. niloticus*. Heavy metal exposure brought about elevated levels of serum enzymes as well as oxidative stress markers. In addition, there was an increase in metallothionein expression and high residues of heavy metals were found in the fish tissue. ILL supplementations however, lowered the HM concentrations in the fish, mitigating the toxic effects and enhancing the biochemical and antioxidant activities (Rahman et al., 2019).

Dietary *Coriandrum sativum* seed powder (CP) and extract (CE) were analysed for its remedial effect on the immunity of *O. niloticus* altered by lead. The CP and CE diets improved immune functions and showed a protective effect against Pb-induced immunosuppression while also decreasing the fish morality and lead residues in the fish muscles (Ahmed et al., 2019).

Chlorella vulgaris (Ch) was used as a dietary supplement to subdue the toxicity of Arsenic in *O. niloticus*. Severe histopathological changes, increased serum biomarkers and cytokine gene expression were displayed by the fish exposed to Arsenite. However, the fish fed with Ch exhibited decreased histopathological alterations, regulated biomarker levels, and lower cytokine gene expression (Zahran & Risha, 2014).

O. niloticus subjected to zinc toxicity were treated with *Salvadora persica* supplemented diets. The study revealed that the treatment elevated the antioxidant enzyme and histopathological changes caused by zinc as well as displayed protective effects against zinc-induced abnormalities (Abd El-Naby et al., 2020).

The study inspected the protective effect of *Chlorella vulgaris* (CV) and β-glucan supplements in remediating the toxicity of diazinon in *O. niloticus.* CV and β-glucan supplementations improved the hepatic damage, compromised immunity and retarded growth caused by diazinon. The study suggested that *Chlorella vulgaris* (CV) and β-glucan have a protective effect against the diazinon toxicity in Tillapia. (Abdelhamid et al., 2020)

The study accessed the protective effects of Guava leaves extract (GLE) on cypermethrin (CYP) induced alterations in *O. niloticus*. The GLE significantly enhanced the growth and immune variables affected by the toxicant. The study suggested the GLE has an antagonistic effect against the CYP toxicity in Tilapia (Abdel-Tawwab & Hamed, 2020).

The efficacy of dietary ginger and liquorice supplementation to mitigate the heavy metal induced alterations in *O. niloticus.* The combination of ginger and liquorice improved the growth and blood parameters as well as reduced the heavy metal accumulation in Tilapia. Additionally, histopathological analysis showed improved intestinal and gill morphology with liquorice supplementation. Overall, ginger and liquorice proved beneficial in counteracting the effects of heavy metals in Tilapia (Mohammed et al., 2020)

The study assessed the efficacy of *Trigonella foenum-graecum* extract to mitigate the toxicity induced by copper oxide nanoparticles (CuO-NPs) in *O. mossambicus.* The extract showed significant ameliorative effect on the enzymatic and histological alterations caused by CuO-NPs, indicating that the *Trigonella foenum-graecum* extract possesses potential intervention against CuO-NPs in Tilapia (Asad et al., 2021).

The study scrutinized the potential of *Moringa oleifera* flower in ameliorating the growth and antioxidant enzymes in *O. niloticus* affected by Lead toxicity. The study revealed that *Moringa oleifera* flower diets enhanced fish endurance to stress, restored the altered levels of antioxidant enzymes, lowered the histological damage while acting as a protective agent (Kumar et al., 2021).

The study aimed at investigating the protective effect of Spirulina and *Saccharomyces cerevisiae* against the toxicity of fipronil in *O. niloticus*. Fipronil displayed adverse effects on growth, hematology, biochemistry and gene expression (genotoxicity). However, the dietary supplementation with Spirulina and *Saccharomyces cerevisiae* remediated these alterations (Fald et al., 2022)

The study was concerned with the remedial effect of dietary cinnamon powder *(Cinnamomum zeylanicum)* against prolonged lead exposure in *O. niloticus*. Lead instigated adverse effects on growth, blood parameters, and immune and antioxidant indices. Supplementations of Cinnamon alone improved growth, hemato-biochemical, antioxidant, and immune indices. It also reduced the lead accumulation and provided protection against oxidative stress (Hamed et al., 2022).

The study determined the effect of *Asparagus racemosus* (ARE) extract in detoxifying the effects of deltamethrin (DM) in *O. niloticus*. ARE reversed the DM influenced immune alterations as well as liver and kidney damage. This suggested the potency of ARE to act as a protective agent against deltamethrin (DM) (Vineetha et al., 2022).

The study evaluated the waterborne lead toxicity in *O. niloticus* from a contaminated area and farmed fish exposed to lead acetate, besides also examining the efficacy of neem leaf powder (NLP). Lead prompted DNA damage, lipid peroxidation and decreased enzyme expression and pathological changes in gill and liver of wild as well as exposed tilapia. NLP ameliorated

the lead toxicity by reducing oxidative stress and pathological alterations (Abu-Elala et al., 2023).

2.2 Phytoremediation of cadmium in Tilapia

The study aimed at scrutinizing the mitigating effect of grape seed proanthocyanidis on Cd induced growth retardation and oxidative stress in hepatopancreas of juvenile tilapia (*Oreochromis niloticus).* There was a substantial decrease in the final body weight and weight gain rate of Cd exposed fishes as compared to GSP treated fishes. Levels of malondialdehyde, glutathione, and total antioxidant capacity, as well as the activities of SOD, CAT, GTP were significantly affected because of Cd while no prominent difference was observed between the GSP treated groups and control fishes. The results concluded that the GPS supplementation can combat the Cd induced growth retardation and oxidative stress in hepatopancreas of *O. niloticus* (Zhai et al., 2010).

The investigation sought to evaluate the antioxidant potential of *Lemna gibba* leaf extract, weed or weed plus extract to combat the toxic effect of Cd in *Oreochromis niloticus.* The effects of Cadmium were observed in the histopathological, haematological, and biochemical aspects of the fish. The Cd exposed fishes showed a noteworthy reduction in the erythrocyte count (RBCs), haemoglobin content (Hb) and haematocrit value (Hct) and a considerable increase in plasma aspartate aminotranseferase (AST) and alanine aminotransferase (ALT). However, the *Lemna gibba* leaf extract and weed presented the potential to scale down the levels of Cd in water as well as decreased the Cd uptake by the fish. The extract and weed proved to be efficient in ameliorating the effect of Cadmium by enhancing the blood parameters of the treated fish. The results suggested that the extract could not only chelate the Cd but also proved to render protection against the degenerative effect of Cd (Kaoud et al., 2011).

The research intended to examine the bioaccumulating potential of Cd in different muscles of *Oreochromis niloticus* and the changes in the oxidative stress with or without the treatment with waterborne vitamin C and Rosemary leaf extract. The findings revealed a notable reduction in the cadmium concentration in the treated fishes. Also, there was a decrease in lipid peroxides, catalase activity and an increase in superoxide dismutase activity in liver and kidney implying a minimised oxidative stress. The outcomes demonstrated the ameliorative potential of both the antioxidants used (Al-Anazi et al., 2015).

The study was concentrated on the toxic effects of Cd on protein, glucose and lipid peroxidation product malondialdehyde and the ameliorative potential of *Spirulina platensis* on *Oreochromis mossambicus*. The revelations implied that the *S. platensis* treatment improved the amount of protein, high blood glucose level and low level of malondialdehyde in the liver of the exposed fish. The study concluded that *spirulina* can detoxify the effects of Cadmium (Priya et al., 2018).

The study was concerned with the ameliorative ability of *Withnia somnifera* leaf extract on Cadmium exposed *Oreochromis niloticus*. The evaluations proposed that the high doses of *Withnia somnifera* leaf extract eminently improved upon the blood parameters and antioxidant status in the treated fish. Later, all groups were experimentally challenged with *Aeromonas hydrophila* and the relative protection survival (RPS) was estimated which was comparatively higher in *Withnia somnifera* treated fishes. Thus, *Withnia somnifera* leaf extract proved not only as an effective antioxidant against Cd but was also efficient in acting as an immunostimulant (El-Sabbagh et al., 2022).

Two concentrations (0.5% and 1%) of *Allium cepa* extract were studied for their restoring effect on *O. niloticus* against *Saprolegnia parasitica* as well as Cd induced immunosuppression. *A. cepa*-supplemented diets showed a significant improvement in the

growth performance and non-specific immune response, along with reduced oxidative stress and mortality. It not only lowered the Cd accumulation in fish tissues but also upregulated the *IL-1β* and *IFNγ* levels. The most preferred results were obtained with 0.5% *Allium cepa* extract. The study proved that using *Allium cepa* extracts can provide the *O. mossambicus* protection against *Saprolegnia parasitica* infections and the adverse effects of Cd (Elgendy et al., 2022).

3. METHODOLOGY

3.1 APPARATUS AND INSTRUMENTS

General laboratory wares like measuring cylinders, beakers, test tubes, glass rods, micropipettes, reagent bottles, mortar and pestle, vertical coupling jars, porcelain crucibles, volumetric flasks, Whatman No.1 filter papers, hemocytometer and microscopic slides were used. Instruments like pH meter (TMP 3), Hot air oven (MIC-165), Muffle furnace (i-therm Al-7981), Hot plate, weighing balance (PGB, 200), Centrifuge(R-24), UV-Visible spectrophotometer(BL 1073), and Agarose Gel Electrophoresis unit were also used. Olympus Fluorescence Microscope (BX53) were used for analysing comet assay slides.

3.2 COLLECTION AND IDENTIFICATION OF PLANT MATERIAL

Fresh leaves of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* were collected in good condition from numerous home gardens of Sanvordem, Goa and Colomb, Rivona, Goa. Identification and authentication of the plants was done by Dr. Annie Gomes, Assistant Professor in Botany, Government College of Arts, Science and Commerce, Quepem, Goa.

Fig. 3.1: Plants used in the study (A) Justicia adhatoda (B) Coleus amboinicus (C)Artemisia parviflora

3.3 PREPARATION OF DRIED LEAF POWDER

The leaves were first cleaned with water to remove the dirt and then kept on an aluminium foil to be dried in a hot air oven. *J. adhatoda* leaves were dried at 60°C (Asif et al., 2020). *C. amboinicus* leaves were dried at 70°C (Nurafifah et al., 2018). And the leaves of *A.*

parviflora were dried at 45°C (Ferreira & Luthria, 2010). The drying was continued until the moisture content was below 10 % after which they were ground into fine powder and stored in an airtight container away from the sunlight.

3.4 PROXIMATE ANALYSIS OF DRIED LEAF POWDER

Proximate Analysis is performed to inspect the composition of the biomass. The storage capacity and energy contents of the biomass is evaluated based on this analysis. (Kamran, 2023) It involves the quantification of major components: moisture content, ash content, total proteins, total carbohydrates and total lipids. These nutrients were analyzed by following the methods given in the AOAC 2010.

3.4.1 Moisture Content (Ahn et al., 2014)

Determining the moisture content in dried leaf powder meant for extraction is fundamental for ensuring accurate dosing, stability, and alignment with standards. It directly governs the quality, potency, and longevity of the extract, influencing extraction efficacy and enabling persistent production across various iterations.

Apparatus required:

Crucibles, dessicator, hot air oven, weighing balance

Procedure:

The crucible was dried at 110°C for 15 minutes and then cooled in a dessicator. The dried crucible was weighed (Wi) and 2-5g powdered sample (Ws) was added to it. The crucible was then kept in the hot air oven at 135°C for 20 minutes to remove the moisture. The crucible was then kept in the dessicator until cooled prior to taking its final weight (Wf). The moisture content was calculated by the formula:

Moisture % = {[Ws-(Wf-Wi)]×100}/Ws

3.4.2 Ash Content (Marshall, 2010)

Estimation of ash content is necessary for ascertaining the consistency, purity, and compliance with standards for extract production. It analyses impurities, sets quality standards, and optimizes extraction precision. By enabling accurate yield, ash content analysis maintains extract's integrity and efficacy.

Apparatus required:

Crucibles, dessicator, hot air oven, muffle furnace, weighing balance

Procedure:

The crucible was dried at 110°C for 15 minutes and then cooled in a dessicator. The dried crucible was weighed (Wi) and 2-5g powdered sample (Ws) was added to it. The crucible was then kept in the muffle furnace at 550°C for 2 hours to obtain white ash. The crucible was then kept in the dessicator until cooled prior to taking its final weight (Wf). The ash content was calculated by the formula:

Ash % = [(Wf-Wi)/Ws]×100

3.4.3 Total Proteins (Lowry et al., 1951)

The total proteins content was evaluated by Lowry's method. Approximation of protein content in dried leaf powder prior to extraction is critical for extraction efficiency, quality control, regulatory compliance and extract standardization. It guarantees purity, optimizes parameters of extraction, maintains consistency along with meeting regulatory guidelines, thus, confirming efficacy and safety of the resultant extract.

Apparatus required:

Mortar and Pestle, centrifuge tubes, centrifuge, test tubes, test tube stand, reagent bottles, micropipettes, glass cuvettes, spectrophotometer

Chemical Preparations:

- Lowry's reagent: 98.0 ml of a 4% solution of sodium carbonate were combined with 1 ml each of a 2% solution of copper sulfate and a 4% solution of sodium-potassium tartrate, resulting in a total volume of 100 ml.
- Follins reagent (1:1): Dissolve equal volume of Folin-Ciocalteu and d/w. The reagent should be freshly prepared.
- Protein Standard: BSA 5 mg/ml.

Procedure:

0.5% of 10ml homogenate of the dried leaf powder was prepared in 0.01M Phosphate buffer. The homogenate was centrifuged at 5000 rpm for 15 minutes. To 1ml of supernatant, 5 ml of Lowry's reagent was added and allowed to undergo incubation for a duration of 15 minutes at room temperature. Following this step, 0.5 ml of Folin-Ciocalteu reagent was introduced and allowed to incubate for an additional 15 minutes. The intensity of the resultant blue-colored complex was assessed at 690 nanometers relative to a suitable blank, as outlined by Lowry et al. (1951). The protein concentration within the samples was determined utilizing a standard curve established with bovine serum albumin (BSA) at a concentration of 100 mg per ml in 1N sodium hydroxide solution.

The standard graph of BSA was used to compute the protein content and the results were represented as BSA equivalent (mg /mL).

3.4.4 Carbohydrates (Ludwig & Goldberg, 1956)

The carbohydrates were estimated by the Anthrone method. Analysing the carbohydrate content in dried leaf powder meant for extraction is vital to preserve extract quality, enable accurate dosing, and standardize the extracts. This guarantees consistency in product quality, helps determine suitable dosage and adherence to regulatory requirements.

Apparatus required:

Mortar and pestle, centrifuge tubes, centrifuge, test tubes, test tube stand, reagent bottles, micropipettes, glass cuvettes, spectrophotometer

Chemical Preparations:

- Anthrone Reagent: 2g Anthrone was dissolved in 1L conc. Sulphuric acid.
- Standard solution: D-glucose 1000 mg/mL

Procedure:

0.5% of 10ml homogenate of the dried leaf powder was prepared in 0.01M Phosphate buffer. The homogenate was centrifuged at 5000 rpm for 15 minutes. To 1mL of supernatant, 4mL of Anthrone reagent was added followed by incubation in a boiling water bath for 15 minutes. The absorbance was measured at 650nm. The total carbohydrate concentration within the samples was evaluated by employing a standard curve established with D-glucose at a concentration of 1 mg per ml.

The standard graph of D-glucose was used to compute the carbohydrate content and the results were expressed as D-glucose equivalent (mg /mL).

3.4.5 Total Lipids (Lee et al, 1995)

Evaluating the lipid content in dried leaf powder prior to preparation of leaf extract is vital for quality, compliance, efficiency and standardization. This is done to guarantee purity, optimization, ensure consistency, and meet regulatory standards so as to ensure the extract's effectiveness and safety.

Apparatus required:

Reagent bottles, measuring cylinders, funnel, separating funnel, petri plates, hot air oven

Chemical Preparations:

- Chloroform-Methanol solvent: 20mL Chloroform was mixed with 10 mL Methanol

Procedure:

3g of powder was homogenized in chloroform-methanol solvent (2:1). The homogenate was filtered using Whatmann filter No. 1. The filtrate was then transferred to a separating funnel, followed by addition of 10 mL 5% NaCl. The funnel was corked and the solutions were mixed by gently tilting the funnel 5 times. The mixture was then allowed to stand until distinct separation of layers was achieved. The lower chloroform layer was removed carefully and measured. It was then separated in 3 pre-weighed petri plates (3 mL each), followed by oven drying at 110°C for 30 minutes. The dried plates were then cooled and weighed. The total lipid content was measured as

Lipid content (%) = (Lipid extraction (g))/(Sample weight (g))×(chloroform layer+amount lost)(mL)/(3 mL)˙×100

3.5 PREPARATION OF COMBINED LEAF EXTRACT

The dried leaf extract was prepared by maceration technique. The aqueous leaf extract was formulated by using the method described by Mitra et al., (2013) with some modifications.

Appararatus required:

Conical flasks, funnel, muslin cloth, centrifuge tubes, cold centrifuge, sterile polypropylene tubes

Procedure:

Dried leaf powder of the 3 samples were soaked in double distilled water overnight and then filtered by using a muslin cloth. The filtrate was transferred in a centrifuge tube and centrifuged for 10 minutes in a Remi cold centrifuge. The supernatant obtained was referred to as the aqueous leaf extract. Two concentrations were prepared; 50mg/ml and 100mg/ml. The extract was freshly prepared for every subsequent use.

3.6 IN VIVO STUDY

3.6.1 MODEL ORGANISM

After attaining the ethical clearance (IAEC Approval Reference No : GUZ/IAEC/23-24/N21 Dated: 13/09/23) *Oreochromis mossambicus* of 10cm ± 2cm were procured from ICAR, Old Goa, Goa. The fishes were acclimatised in the animal house of Goa University (Reg. No: 2104/G0/Re/S/20/CPCSEA dt. 10/08/2020) for a period of 15 days in large aerated tanks. Only chlorine free water was used and the fishes were fed with nutritious commercial feed. The water conditions were maintained as follows: temperature 25 °C ± 2°C, 12 h light/dark cycle and pH 7.0 ± 0.15.

Figure 3.4: *Oreochromis mossambicus*

3.6.2 CLASSIFICATION

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cichliformes

Family: Cichlidae

Genus: *Oreochromis*

Species: *O. mossambicus*

3.6.3 EXPERIMENTAL SETUP

The Tilapia were divided into 4 groups, each consisting of 7 fishes for the study duration of 22 days. The fishes were maintained in 20L aquaria containing dechlorinated tap water. Water was changed every day and the respective concentration of the toxicant or the CLE was added. The fishes were fed once a day with high quality commercial aquarium fish feed. The fishes were exposed to Cd based on LC50 data of Goswami et al., (2016) as follows:

Group 1: Negative Control

Group 2: Cadmium Control; here the fishes were given 4.606mg/L of Cadmium Chloride for 7 days.

Group 3: Concentration I; here the fishes were first given 4.606mg/L of Cadmium Chloride for 7 days and later treated with 50mg/ml CLE for 15 days.

Group 4: Concentration II; here the fishes were first given 4.606mg/L of Cadmium Chloride for 7 days and later treated with 100mg/ml CLE for 15 days.

3.7 ESTIMATION OF BIOMOLECULES IN THE LIVER

3.7.1 Protein Estimation (Lowry et al., 1951)

The Lowry method implements the reaction of peptide nitrogen with copper (II) ions under alkaline conditions to determine the protein concentration. The reaction results in the reduction of Folin-Ciocalteu reagent and the creation of heteropolymolybdenum blue due to copper-catalyzed oxidation of aromatic acids.

Apparatus Required:

Homogenizer, centrifuge tubes, centrifuge, test tubes, test tube stand, micropipettes, glass cuvettes, spectrophotometer

Chemical Preparations:

- Lowry's reagent: 98 ml of a 4% sodium carbonate solution was combined with 1 ml each of 2% copper sulfate and 4% sodium-potassium tartrate.
- Follins reagent (1:1): Dissolve equal volume of Folin-Ciocalteu and d/w. It should be freshly prepared.

Procedure:

10% liver homogenate was prepared with 0.01M phosphate buffer (pH 7). 200µl of the supernatant obtained after centrifugation at 4500 rpm in a Remi cold centrifuge, was combined with distilled water until the total volume reached 500 µl. Rest of the steps were performed as detailed in the estimation of proteins of plant powder.

3.7.2 Carbohydrate Estimation (Ludwig & Goldberg, 1956)

The carbohydrates were estimated by the Anthrone method. Here, carbohydrates are quantitatively measured via a chemical reaction with anthrone reagent under acidic conditions. The carbohydrates go through acid hydrolysis, forming a blue-green chromophore whose intensity correlates with carbohydrate concentration in the sample.

Apparatus required:

Mortar and pestle, centrifuge tubes, centrifuge, test tubes, test tube stand, reagent bottles, micropipettes, glass cuvettes, spectrophotometer

Chemical Preparations:

- Anthrone Reagent: 2g Anthrone was dissolved in 1L concentrated Sulphuric acid.
- Standard solution: 1g/ml D-glucose

Procedure:

10% liver tissue homogenate was prepared with 0.01M phosphate buffer. The homogenate was centrifuged at 5000 rpm in a Remi cold centrifuge for 10 minutes. To 200µl of supernatant, 4mL of Anthrone reagent was added followed by incubation in a boiling water bath for 15 minutes. The absorbance was measured at 650nm. The total carbohydrate concentration within the samples was evaluated by employing a standard curve established with D-glucose at a concentration of 1 mg/ ml.

The standard graph of D-glucose was used to compute the carbohydrate content and the results were expressed as D-glucose equivalent (mg /mL).

3.8 ESTIMATION OF ANTIOXIDANT ENZYMES IN THE LIVER

3.8.1 Catalase (CAT) Assay (Sinha, 1972)

A Catalase assay was performed based on the modified method of Sinha 1972. The assay ascertains the enzyme's ability to break down hydrogen peroxide. When heated in the presence of H_2O_2, the chromate in acetic acid undergoes reduction to form chromate acetate and an unstable intermediate called perchromic acid. By using a spectrophotometer, the chromate acetate formed can be measured at 620nm.

Apparatus Required:

Homogenizer, centrifuge tubes, centrifuge, test tubes, test tube stand, micropipettes, glass cuvettes, spectrophotometer

Chemical Preparations:

- Dichromate Acetic Acid Reagent: 5% potassium dichromate and glacial acetic acid were taken in the ratio 1:3.
- Standard solution: 50mg/ml Catalase

Procedure:

The tissue homogenate was prepared in cold conditions to minimize enzyme activity. To 200µl of 10% homogenised tissue in a final volume of 1000 µl in 0.01M phosphate buffer (pH 7), 500 µl of 0.2M H_2O_2 was added. After addition of 2mL Dichromate Acetic Acid reagent, the reaction mixture was incubated in a boiling water bath for 10 minutes. The

absorbance was measured at 620nm. 2ml Dichromate Acetic acid reagent and reaction mixture without homogenate was taken as blank. Protein content was determined by Lowry method described earlier. The Catalase was calculated with the help of a standard curve of 50mg/ml Catalase and was measured as µg/ mg protein.

3.9 DETERMINATION OF HEPATOTOXICITY BIOMARKERS

3.9.1 Estimation of Lipid Peroxidation (LPO) (Buege & Aust, 1978)

Lipid peroxidation (LPO) was determined through a modified version of the technique described by Ohkawa, et al 1979. During the process of polyunsaturated fatty acid degradation, malondialdehyde (MDA) is formed, working as an indicator of lipid peroxidation. It forms a characteristic chromogenic adduct with two molecules of TBA. This pink-hued compound termed as thiobarbituric acid reactive substances (TBARS), shows its peak absorption at 532 nm.

Apparatus Required:

Homogenizer, centrifuge tubes, centrifuge, test tubes, test tube stand, micropipettes, glass cuvettes, spectrophotometer

Chemical Preparations:

- TBA reagent: 0.67% in glacial acetic acid
- Standard solution: 0.6mg/ml MDA

Procedure:

A 10mL of 10% liver homogenate was prepared with Tris HCl. To 200 µl of the supernatant, 1 mL 5% TCA and 1 mL TBA were added. The reaction mixture was incubated in a boiling

water bath for 10 minutes. After cooling the test tubes, the optical density was measured at 535nm using a spectrophotometer. A solution that contained all the reagents except the sample was taken as a blank. The determination of TBARS content was enabled through a standard graph, with the concentration of MDA serving as the standard ranging from 20 to 100µg/ml. Subsequently, the TBARS content was quantified and expressed as µg /mg protein.

3.10 STATISTICAL ANALYSIS

The data collected from the present study was subjected to statistical analysis using Graphpad Prism 10 software. The data was represented as Mean ± Standard deviation. It was subjected to test of normality using Kolmogorov-Smirnov test and Shapiro-Wilk test prior to performing Levene test of homogeneity for checking the equal variance among the samples. Once the normality and homogeneity assumptions were satisfied, parametric test called one-way ANOVA was utilized. Significant F values for the one-way ANOVA ($p<0.05$), were further compared by post-hoc Dunnettes Multiple Comparison test for comparing the treatment groups against the Cd control group.

4. ANALYSIS AND CONCLUSIONS

4.1 RESULTS

In the present chapter, a comprehensive account of the results is entailed that were obtained during the research study. The experimental study was carried out to evaluate the efficacy of combined leaf extract of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* against the Cadmium chloride induced toxicity in *Oreochromis mossambicus*. The extract was predominantly evaluated for its potential as a hepatoprotective agent. The genotoxic status of the extract was also examined towards the end of the exposure.

4.1.1 Proximate analysis of the dried leaf powder

The results of the proximate analysis of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* are given in figures 4.1- 4.5. The data revealed that the moisture content (%) in the dried leaf powder of the three plants was 8.18 ± 0.55, 9.33 ± 0.58 and 7.65 ± 0.52 respectively. The ash content (%) was found to be 12.15 ± 3.69, 16.17 ± 4.25 and 13.67 ± 2.00 respectively. The protein content (mg/g powder) of the dried leaf powder was 6.18 ± 0.57, 7.78 ± 0.51 and 3.99 ± 0.48 respectively. The carbohydrate (µg/g powder) content was found to be 26.44 ± 0.37, 15.41 ± 0.37 and 18.91 ± 2.62 respectively. The lipid content (%) of the respective dried leaf powder was 3.13 ± 0.32, 2.11 ± 0.30 and 2.12 ± 0.25.

4.1.2 Estimation of biomolecules in the liver

4.1.2.a Protein Content in fish liver samples

The protein content of the liver samples was found to be significantly decreased when exposed to cadmium chloride ($p < 0.001$). However, both the concentrations of the combined leaf extracts (CLE) failed to bring back the protein content to normal in cadmium-exposed Tilapia, evident by a statistically non-significant p-value ($p > 0.99$ and $p = 0.77$) (Figure 4.8).

4.1.2.b Carbohydrate content in fish liver

With regard to the carbohydrate content, the results reveal that the alterations in the carbohydrate content among the groups was not statistically significant, suggesting that cadmium did not induce any apparent alterations to the carbohydrate concentration in the liver. Also, the treatment with both the extracts didn't display any significant findings (F= 1.480) (Figure 4.9).

4.1.3 Estimation of antioxidant enzymes in the liver

4.1.3.a Catalase concentration in liver

The data regarding the level of the catalase in the liver of the exposed fishes is given in figure 4.10. The catalase concentration was observed to increase significantly in the group exposed to cadmium only ($p < 0.001$). However, the study concluded that the cadmium affected catalase levels decreased significantly after treatment with combined leaf extract (CLE). Both the concentrations of CLE revealed the potency to decrease the level of the affected enzyme

4.1.4 Determination of hepatotoxicity biomarker

4.1.4.a Estimation of lipid peroxidation (LPO)

The outcomes of the level of TBARS in the liver of the exposed fishes are shown in figure 7. A significant increase in TBARS was found in the group subjected to cadmium only ($p < 0.001$). The concentration of TBARS decreased significantly after treatment with the lower concentration of the combined leaf extract (CLE) ($p< 0.001$). The higher concentration of CLE showed no significant results against reducing the lipid peroxidation caused by cadmium ($p = 0.8746$).

LIST OF GRAPHS

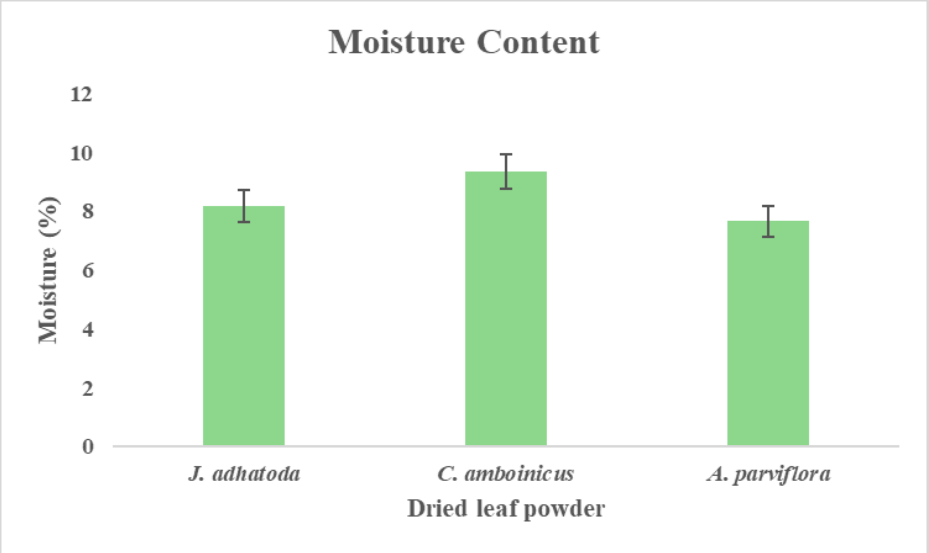

Figure 4.1: Moisture content of the dried leaf powder.

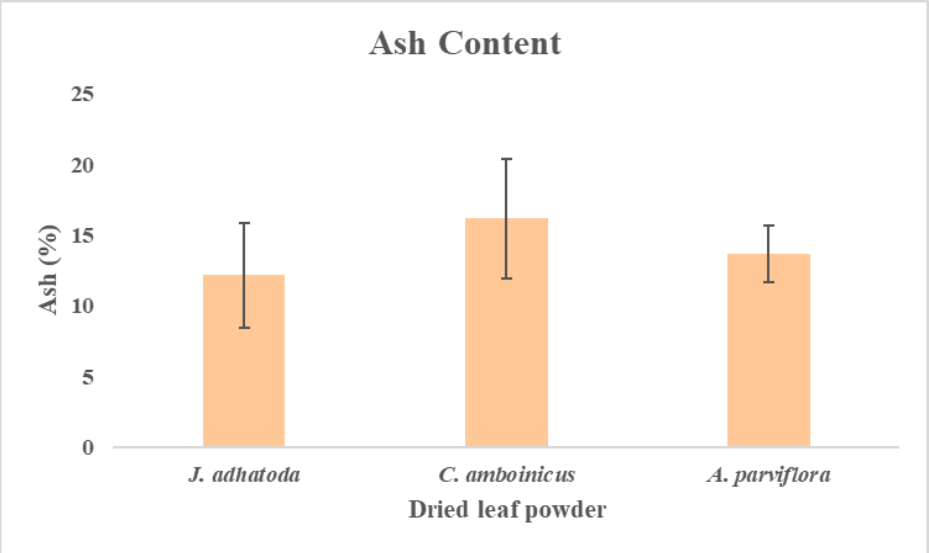

Figure 4.2: Ash content of the dried leaf powder.

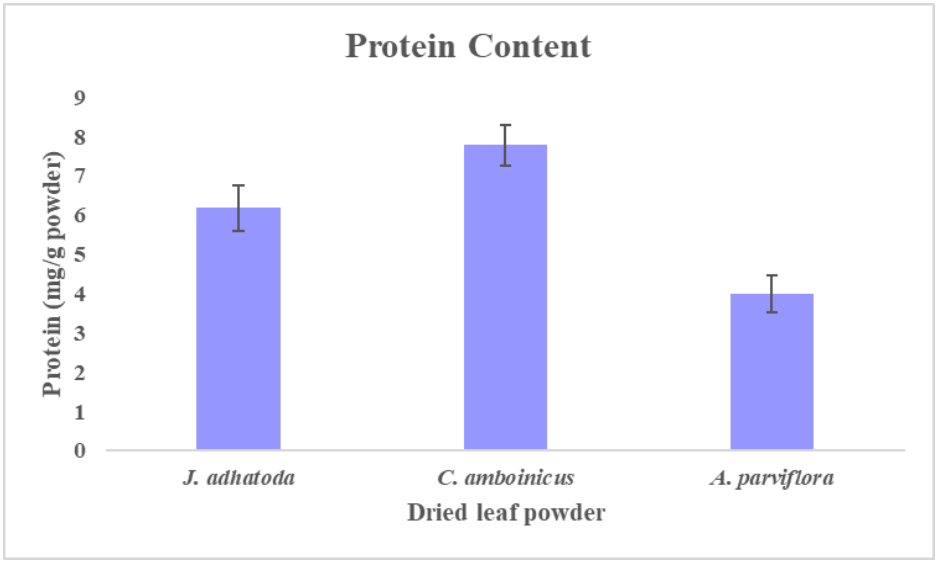

Figure 4.3: Protein content of the dried leaf powder.

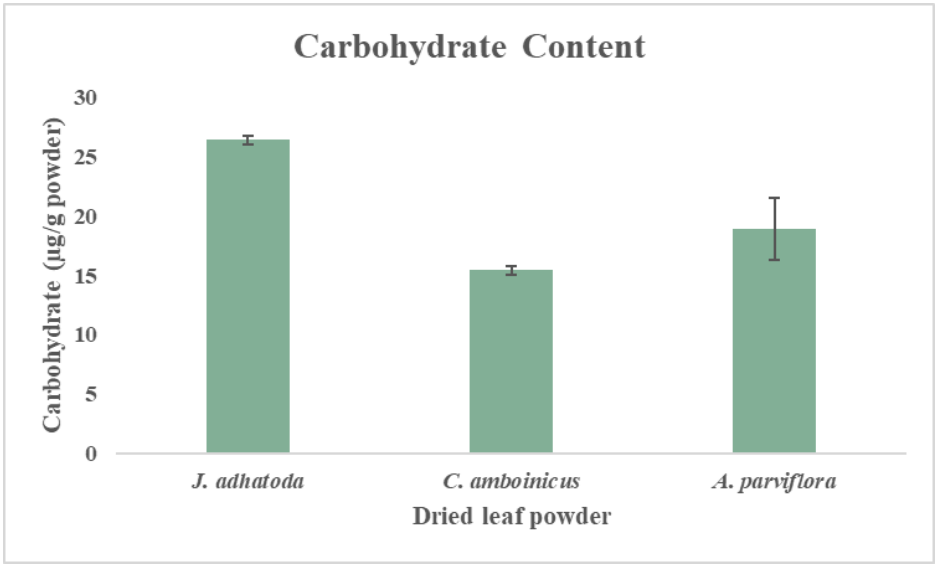

Figure 4.4: Carbohydrate content of the dried leaf powder.

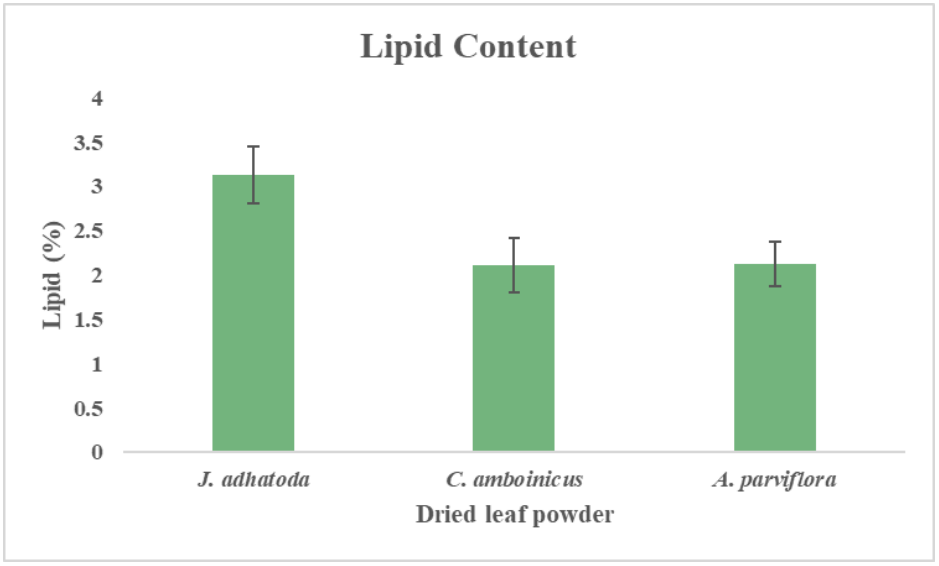

Figure 4. 5: Lipid content of the dried leaf powder.

Different values indicate significant differences at p <0.05 as per Dunnett's test. Values are significant at *p ≤0.05, **p ≤0.01 and ***p ≤0.001. NS represents non significant values.

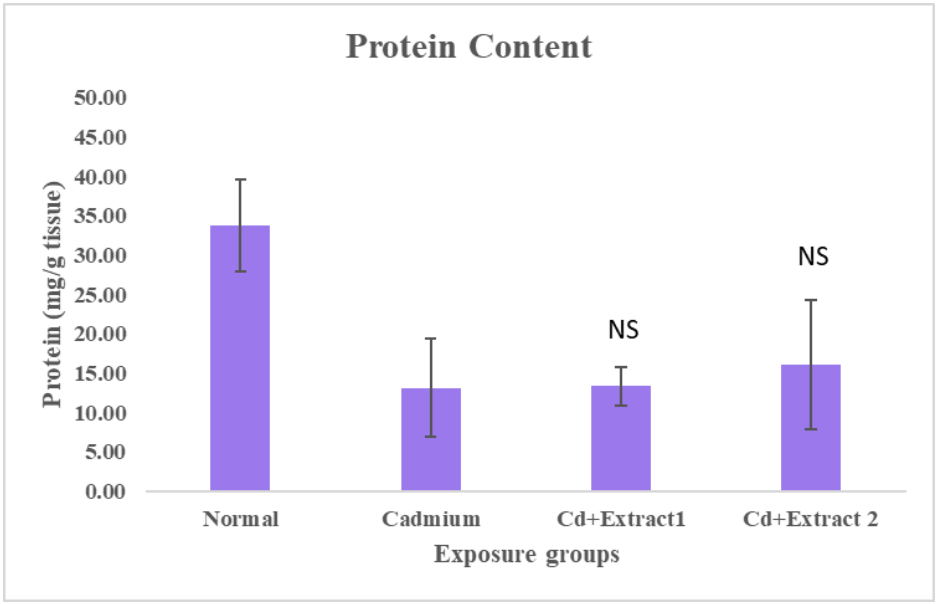

Figure 4.8: Protein concentration in liver of cadmium-exposed fish treated with the combined leaf extract (CLE).

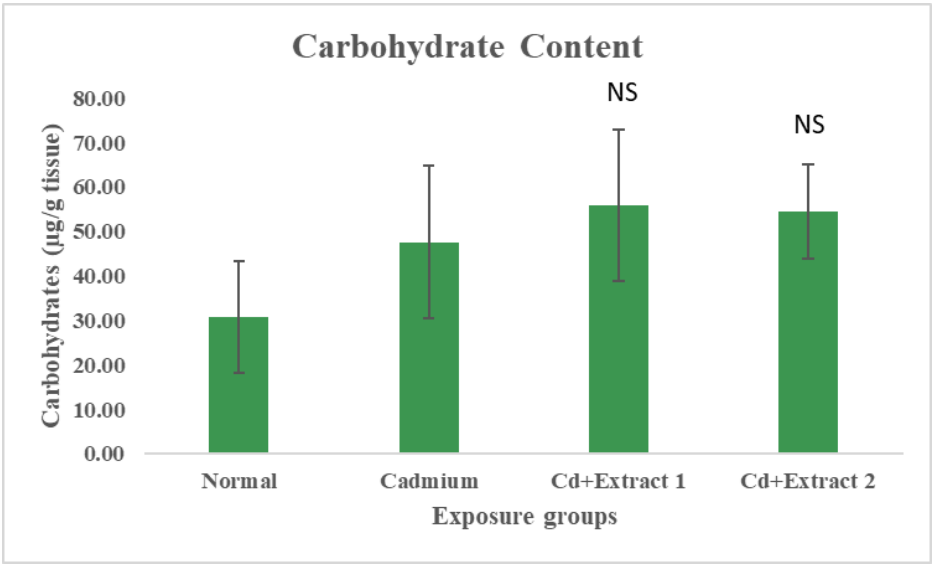

Figure 4.9: Carbohydrate concentration in liver of cadmium-exposed fish treated with the combined leaf extract (CLE).

Different values indicate significant differences at p <0.05 as per Dunnett's test. Values are significant at *p ≤0.05, **p ≤0.01 and ***p ≤0.001. NS represents non significant values.

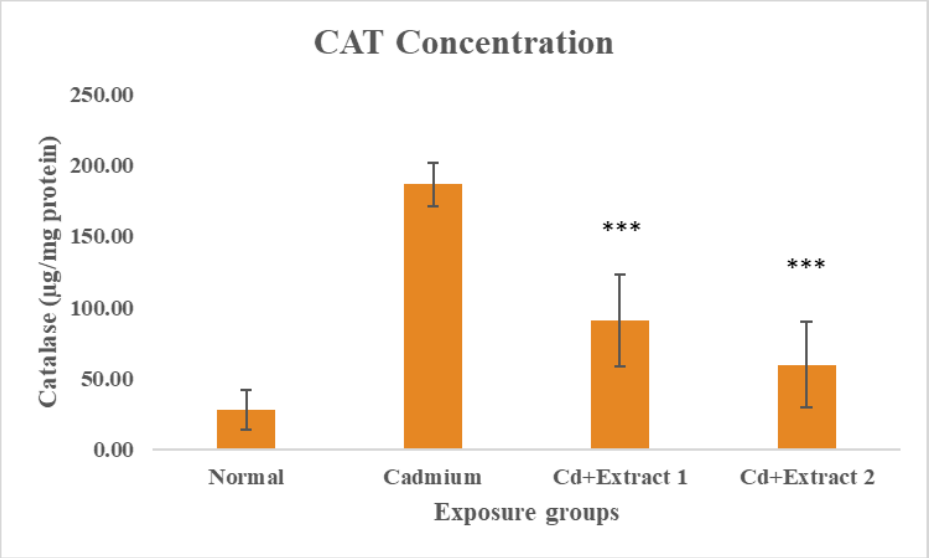

Figure 4.10: Catalase concentration in liver of cadmium-exposed fish treated with the combined leaf extract (CLE).

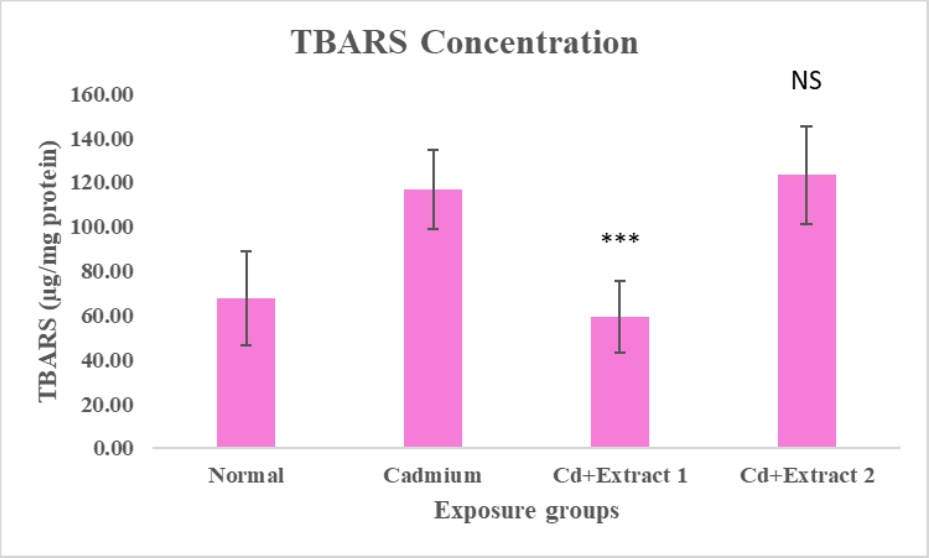

Figure 4.11: TBARS concentration in liver of cadmium-exposed fish treated with the combined leaf extract (CLE).

4.2 DISCUSSION

Proximate Analysis

Prior to preparation of the extract, the proximate analysis of the three dried leaf powders was conducted so as to gain the valuable information with regard to the nutrients, bioactive components, safety and the purity of the dried leaf powder. This analysis is a crucial step preceding any extract or feed preparation (Kamath & Dawda, 2023).

Biomolecules in the liver

Biomolecules in the fish liver perform diverse functions which contribute to the growth, development and health of the fish. Two of these biomolecules include the proteins and carbohydrates. The functions performed by the proteins in the liver are critical for the fish's overall well-being nonetheless also providing metabolic stability. The enzymes in the liver catalyse the metabolic reactions guaranteeing effective nutrient processing along with energy production. Furthermore, the proteins in the liver play a pivotal role in detoxification mechanisms, helping the body to eliminate the harmful substances. They are also a part of the immune system of the fish while also playing a pivotal role in the digestive processes. In the present study the protein content of the liver was significantly altered by cadmium exposure. Similar results were also encountered by Jose et al. (2013) in their study on *O. mossambicus* wherein they concluded that this change was on account of Cadmium induced catabolism of proteins to meet the immediate energy demands of the fish body. In their study this was displayed by increased levels of free amino acids and enzyme activities. Several ameliorative studies concerned with the use of plant extracts such as *Lemna gibba* and *Withania somnifera* have shown remedial effects on the protein content affected by Cadmium toxicity in Nile Tilapia (Hussein et al., 2011 and Nasser et al., 2021). On the other hand, the presently studied

combined leaf extracts failed to bring back the affected protein content to normal. In various studies conducted by researchers like Zhang et al., (2008) and Adetutu et al., (2020) they obtained similar results, concluding that time played a crucial role in the recovery of the liver affected by any toxicant. This might be a reason for the CLE's failure to restore the protein concentration back to normal.

Carbohydrates serve as an important source of digestible energy for fish, regardless of not being essential. The level of carbohydrates however, also regulates glucose metabolism, lipogenesis as well as inflammatory response. In the research conducted by Jadhav et al. (2022), they discovered that sublethal exposure to cadmium decreased the glycogen levels in the liver of *Channa punctatus* and *Cyprinus carpio*, indicating the influence of cadmium in altering the carbohydrate content in the fish. However, the current study did not exhibit any significant alterations in the carbohydrate content of the liver of *O. mossambicus* after cadmium exposure.

Antioxidant enzymes in liver

The antioxidant enzymes in the liver of the fish, such as catalase (CAT) play a vital role in mitigating the oxidative stress induced by a wide array of pollutants. The function is concerned with the elimination of the harmful reactive oxygen species like superoxide anions, hydroxyl radicals, etc. produced as a result of oxidative stress. The antioxidants scavenge these free radicals, inhibit lipid peroxidation and thus, prevent cellular damage (Jaydeokar et al., 2012).

As per the numerous studies carried out previously concerned with the amelioration of Cadmium toxicity such as the use of *Hibiscus sabdariffa* calyx extract in African catfish (Obi et al., 2022) as well as the supplements of trace elements like calcium and zinc utilized in study concerned with *O. mossambicus* (Obaiah & Rani, 2012), decreased the catalase level

altered by cadmium. Similar results were obtained with both the extracts in the present study. This signified that the extracts counteracted the oxidative stress and restored the enzyme balance.

Indicator of Hepatotoxicity

During the process of lipid peroxidation in the liver, malondialdehyde (MDA) is generated as a peroxidation product of polyunsaturated fatty acids (PUFA). This MDA in turn damages the cell membranes, leading to cellular dysfunction. They can also form covalent adducts with biomolecules thus affecting the fish well- being. Thus, MDA levels can also serve as a vital biomarker for liver damage in the fish (Shen et al., 2010).

Jamakala & Rani (2017) and Rani (2012) in each of their studies on *O. mossambicus* have reported significant increase in lipid peroxidation (LPO) induced by Cadmium. Similar outcomes have also been reported in the present research, indicating liver damage in the fish.

The lower concentration of the combined leaf extract has revealed significant results in reducing the lipid peroxidation caused by Cadmium in *O. mossambicus*. Several other studies have also utilized plant extracts to ameliorate the hepatotoxic effects of Cadmium such as *Withnia somnifera* leaf extracts used in *O. niloticus* (El-Sabbagh et al., 2021), *Lemna gibba* leaf extract in *O. niloticus* as well as the rosmary leaf extract and vitamin C utilized in *O. niloticus* (Al-Anazi et al., 2015). The plant extracts have been studied to exert ROS scavenging activity, thus preventing the lipid peroxidation and restoring the damage caused by the toxicant (Hassim et al., 2022).

4.3 CONCLUSIONS

The findings of the present study revealed the antioxidant potential of the combined leaf extracts (CLE) of *Justicia adhatoda*, *Coleus amboinicus* and *Artemisia parviflora* to combat the toxic effects of Cadmium in the liver of *Oreochromis mossambicus*. The study presented the synergistic activity of the CLE to remediate the various biochemical as well as the enzymatic alterations caused by Cd except for protein concentration. It was noted in the previous studies that these changes in response to Cadmium were a result of the body's first line of defences. Furthermore, in the present study the addition of the CLE removed the burden from the body's defences' and significantly restored their concentrations.

4.4 LIMITATIONS

The findings of the present study need to be substantiated with AAS as well as histopathological analysis. Also, the period of treatment needs to be increased to confirm that extended therapy period can show significant results pertaining to restoring the protein concentration altered by Cd.

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