Int J Adv Life Sci Res. Volume 8(4) 111-127
https://doi.org/10.31632/ijalsr.2025.v08i04.009

International Journal of Advancement in Life Sciences Research

Online ISSN: 2581-4877

Journal homepage http://ijalsr.org

Original Article

Evaluation of Potential Toxicity of *Calotropis gigantea* Leaf Extract on *Danio rerio*: Bioactive Compounds, Oxidative Stress and Histological Toxicity Analysis

Ganavi B. B., Kuppusamy Alagesan Paari*

Department of Life Sciences, CHRIST University, Hosur Road, 560029, Bengaluru, India

**Corresponding Author's Email: paari.ka@christuniversity.in*

Abstract

A greater understanding of the ecotoxicological effects of plant-based products is required due to their increasing use. Despite its long-recognised medicinal properties, *Calotropis gigantea* possesses toxic characteristics that necessitate further investigation into its chemical composition and biological effects. This study evaluates the toxicity of crude *C. gigantea* leaf extracts on the freshwater fish *Danio rerio* over a 30-day exposure period. Alongside GC–MS analysis, which identified 48 major constituents, the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity were assessed using the DPPH assay. The extracts exhibited a higher flavonoid content than phenolic content, indicating strong antioxidant potential. In contrast, biochemical analyses revealed that exposed fish showed significantly elevated levels of oxidative stress biomarkers, including succinate dehydrogenase (SDH), glutathione S-transferase (GST), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD), signifying cellular stress and disruption of redox homeostasis. Histological examinations of the liver, brain, muscle, and gills of treated fish demonstrated notable alterations. The gills exhibited lamellar fusion and epithelial lifting, impairing respiration. The brain showed neuronal vacuolation and degeneration, suggesting neurotoxicity, while muscle tissues displayed myofibrillar degeneration. Liver samples revealed hepatocyte necrosis and vacuolisation, indicative of metabolic dysfunction. Collectively, these enzymatic and systemic histopathological responses emphasise the toxicological impacts of *C. gigantea* extracts on aquatic organisms. The findings underscore the importance of ecotoxicological evaluations and the dual nature of medicinal plants such as *C. gigantea*, which can be both therapeutic and harmful. In addition to stressing the necessity for cautious application and further research to elucidate the mechanisms underlying their toxicity, this study provides valuable insights into the biological effects of plant-derived compounds.

Keywords: *Calotropis gigantea; Histology; Oxidative Stress; Phytochemical Analysis; Zebrafish*

Introduction

Medicinal plants are a double-edged sword, containing secondary metabolites of therapeutic significance that can serve as herbal medicines or, when administered in higher doses, act as poisonous substances for suicide or homicide (Subramanian *et al*., 2018). These plants encompass a wide range of applications, including osteopathy, naturopathy, chiropractic, and herbal therapies, with phytochemicals such as flavonoids, alkaloids, terpenoids, phenolics, and glycosides being predominantly utilised for therapeutic purposes (Agarwal *et al*., 2025; Miara *et al*., 2018). According to the World Health Organization (WHO), 70–80% of the global population depends on various herbs

Received on :15th April 2025; Revised version received on :10th July 2025; Accepted: 14th July 2025

for primary healthcare needs (Alotaibi *et al*., 2021). However, intentional or accidental use of poisonous plants and their derivatives can result in mortality or severe ailments (Azzalini *et al*., 2019). Toxins derived from plants have been employed for criminal purposes and as botanical weapons owing to their easy accessibility and lack of cost (Nithaniyal *et al*., 2021). Although plant toxicity is an emerging scientific discipline, the study of poisoning caused by plant materials remains insufficiently explored. A lack of botanical knowledge also hinders the use of botanical resources as forensic evidence. Conversely, there is very limited evidence available regarding the potential toxicity of such plant-based medicines and their adverse effects on human health. Therefore, assessing the potential toxicity of medicinal herbs prior to their use is crucial (Perumal *et al*., 2021).

Calotropis gigantea, commonly known as the gigantic milkweed or crown flower, belongs to the Apocynaceae family and has attracted significant research attention due to its potent toxicity. The plant is native to tropical and subtropical regions of Asia, Africa, and parts of the Middle East (Jayalekshmi *et al*., 2024). It contains a variety of toxic compounds, primarily cardiac glycosides, which have raised concerns regarding their effects on livestock and potential toxicity in humans (Mahale *et al*., 2023). Numerous studies have demonstrated that the leaves and latex of the plant possess several pharmacological properties, including anti-cancer, wound-healing, anti-asthmatic, hair-growth-promoting, antibacterial, antioxidant, anti-inflammatory, vasodilatory, and antivenom activities. The leaves and roots are often applied externally for wound cleansing and in the treatment of bronchitis and asthma, while the leaf juice is traditionally used to alleviate external swellings (Joshi *et al*., 2024).

Although the plant's therapeutic properties have been recognised for many decades, its toxic nature has prompted investigations into its chemical composition, mechanisms of toxicity, and detrimental effects on living organisms. Over time, numerous case reports have documented *Calotropis* poisoning, resulting in severe vision impairment due to corneal oedema and permanent blindness, diarrhoea, effects on lactation, and mouth ulcers. The plant has also been used for abortion, homicide, suicide, and as a cattle poison (Iyadurai *et al*., 2020; Kanchan & Atreya, 2016). Despite the awareness of its toxic properties, *C. gigantea* continues to be used in herbal medicine. Therefore, comprehensive toxicological studies are required to elucidate the dose-dependent toxicity of *Calotropis gigantea* leaf extracts. The present study aims to evaluate the phytochemical composition, oxidative stress enzyme responses, and histopathological alterations in various organs as biomarkers to investigate the toxic effects of *C. gigantea* leaf extracts in zebrafish (*Danio rerio*).

Materials and Methods

Plant Collection and Preparation of Crude Extract:

Calotropis gigantea plants were collected from the outskirts of Harohalli, Kaggalipura, Bengaluru, Karnataka, India. Healthy leaves were separated, washed twice with tap water, and shade-dried for 10–12 days at room temperature. The dried leaves were then ground into a coarse powder using a laboratory grinder, sieved, and stored under cold conditions (4 °C) until further use. The powdered leaf sample was subjected to crude extraction using the Soxhlet method, with methanol as the solvent. A total of 50 g of leaf powder was extracted with 500 ml of methanol in a 1:10 (w/v) ratio, with the extraction temperature maintained at 64 °C. The solvent was evaporated using a rotary evaporator, and the resulting crude leaf extract was stored in an airtight container at 4 °C (Alara *et al*., 2018).

Phytochemical Analysis

Determination of total Phenolic Content (TPC):

The total phenolic content (TPC) was determined using the Folin–Ciocalteu (FC) method, with gallic acid employed as the standard. The crude extract of *C. gigantea* was mixed with FC reagent and distilled water, followed by incubation for 5–10 minutes. Subsequently, 20% sodium carbonate solution was added, and the mixture was incubated in the dark for 2 hours. Absorbance readings

were recorded at 636 nm using a UV–Vis spectrophotometer (Shimadzu 1800). The TPC was calculated using a gallic acid standard calibration curve, and the results were expressed as gallic acid equivalents (GAE mg/g) of extract weight (Ismail *et al*., 2017).

Determination of Total Flavonoid Content (TFC):

The total flavonoid content (TFC) of the *C. gigantea* extract was estimated using the aluminium chloride method, with quercetin as the standard. Briefly, 1 mg of the crude extract was diluted with distilled water in a volumetric flask, after which 5% sodium nitrate solution was added to each flask. This was followed by the addition of 10% aluminium chloride solution and subsequently 1 M NaOH. Distilled water was then added to the flask, and the contents were mixed thoroughly. Absorbance readings were recorded at 533 nm. The TFC was expressed as quercetin equivalents (QE mg/g) of extract weight (Ismail *et al*., 2017).

GC-MS Analysis

Gas Chromatography–Mass Spectrometry (GC–MS) analysis of the methanolic extracts from *Calotropis gigantea* leaves was performed using a Thermo Scientific Trace 1300 GC system coupled with a TSQ 8000 triple quadrupole mass spectrometer. The system was equipped with a TG-5MS capillary column (30 m in length, 0.25 mm internal diameter, and 0.25 µm film thickness). Helium was used as the carrier gas and maintained at a constant flow rate of 1.0 mL per minute. A sample volume of 1.0 µL was injected, with the injector temperature set at 250 °C, while the ion source was maintained at 230 °C. The GC oven was programmed to start at 50 °C and increase gradually to a maximum of 280 °C. The transfer line connecting the GC to the MS was also kept at a constant temperature. The mass spectra were recorded, and the compounds present in the sample were identified by comparing their retention times and fragmentation patterns with those in spectral libraries for accurate characterisation.

Antioxidant Activity Evaluation

DPPH free Radical Scavenging Assay

The standard protocol was followed as described by Yeganegi *et al*. (2018) to determine the antioxidant activity of the *C. gigantea* extract. 1ml of plant extract and 2ml of DPPH was added in the test tube and kept for incubation for 30min in the dark at ambient temperature, and the readings were noted at 517 nm (Sherikar & Mahanthesh, 2015). The percentage inhibition of the antioxidant activity was calculated using the formula:

$$\%of\ inhibition = \frac{A\ blank - A\ sample}{A\ blank}$$

Collection and Maintenance of Zebrafish

Healthy adult male and female fish (in equal ratio), aged 6–7 months, with an average weight of 0.50 ± 0.08 g and length of 3.50 ± 0.06 cm, were obtained from Aqua-Pets Aquarium, Bengaluru. The fish were acclimatised to laboratory conditions and transferred to a pre-aerated glass aquarium for a 7-day acclimation period. They were fed twice daily at a rate of 5% of their body weight, and the tanks were cleaned on alternate days to remove debris and uneaten food. The fish were maintained under standard conditions with an appropriate environment provided, while any dead zebrafish were promptly removed and monitored (Ni *et al*., 2019).

Acute Toxicity Test and LC_{50} Determination

The 96-hour acute toxicity of *D. rerio* exposed to *C. gigantea* leaf extract was assessed using a semi-static test method in accordance with OECD Guideline 203 (1992). Five chemical-free tubs, each containing 4 litres of tap water, were aerated one day prior to the experiment to ensure a suitable environment. Feeding was discontinued 24 hours before exposure to prevent faecal contamination. Based on preliminary tests, 10 zebrafish of mixed sexes were exposed to *C. gigantea* extract at concentrations of 0.5 g/L, 0.25 g/L, 0.125 g/L, 0.075 g/L, and 0.050 g/L for 96 hours, along with a

control group. Mortality was recorded every 12 hours up to 96 hours, and the LC_{50} value was calculated using Probit analysis (Ni *et al*., 2019).

Chronic Toxicity Test

The toxic effects of *C. gigantea* leaf extracts on zebrafish were evaluated over a 30-day period. Based on the LC_{50} value, one-tenth of the LC_{50} concentration was selected for the chronic toxicity study. The experimental groups of zebrafish were exposed to this concentration for 30 days. Pre-acclimatised zebrafish were divided into three groups of 20 fish each, maintained in 8 litres of tap water with constant aeration, along with a control group. The exposure followed a static renewal method, in which the water was regularly replaced with fresh water containing *C. gigantea* extract. The fish were fed twice daily with commercial feed, and faecal matter was removed from the tanks to prevent contamination. Mortalities were monitored throughout the study, and any dead zebrafish were immediately removed (Ni *et al*., 2019).

Sampling And Sample Storage

From each tank, five fish were collected from the triplicate setups at intervals of 0, 10, 20, and 30 days of the exposure period. The fish were carefully removed without disturbing the others to minimise handling stress and experimental error. After collection, the fish were anaesthetised and dissected using a dissection kit to obtain the gill, liver, muscle, and brain tissues. The dissected tissues were temporarily stored in PBS buffer at –20 °C for subsequent biochemical and enzyme assays. A separate set of tissues was fixed in 10% buffered formalin for histological analysis (Ni *et al*., 2019).

Stress Physiology

Homogenisation

Dissected tissues (gill, liver, brain and muscle) were brought on ice and homogenised individually in a cold PBS in 1:10, w/v. The tissues were thoroughly homogenised using pestle and mortar, the homogenate samples were then centrifuged at 3000 rpm at 4 ℃ for 15 min and the supernatants were kept separately at 4 ℃ for further analysis. Enzymatic activities like SOD, CAT, GST, GR and SDH induced by *C. gigantea* extract on tissues were analysed using standard protocols (Ni *et al.,* 2019).

Analysis of Stress Enzyme

Catalase (CAT) activity was measured following the protocol of Bergmeyer (2012). Briefly, CAT activity was analysed based on the decomposition of hydrogen peroxide, which was monitored as a decrease in absorbance at 240 nm over 3 minutes, with readings taken at 30-second intervals. CAT activity was calculated as the total amount of enzyme required to decompose 1 µM of H_2O_2 per minute per gram of tissue and expressed as U mg^{-1} protein.

Superoxide dismutase (SOD) activity was estimated following the method of Marklund and Marklund (1974). The assay measured the inhibition of pyrogallol autoxidation, observed as an increase in absorbance at 540 nm over 3 minutes, with readings taken every 30 seconds. One unit of SOD activity was defined as the amount of enzyme required to inhibit autoxidation by 50%. The results were expressed as U/min/mg protein.

Glutathione reductase (GR) activity was determined according to the method of Carlberg and Mannervik (1975). The assay measured the oxidation of NADPH in the presence of oxidised glutathione (GSSG), indicated by a decrease in absorbance at 340 nm over 3 minutes, with measurements taken at 30-second intervals. One unit of GR activity was defined as the amount of enzyme required to oxidise 1 µM of NADPH per minute per gram of tissue, and the results were expressed as µM/min/mg protein.

Glutathione S-transferase (GST) activity was assessed following the method of Chein and Dauterman (1991). GST catalyses the conjugation of electrophilic compounds with reduced

glutathione (GSH), thereby increasing the water solubility of the product. The absorbance of the reaction was measured at 340 nm over 3 minutes. One unit of GST activity was defined as the amount of enzyme required to catalyse the formation of 1 µM of the conjugated CDNB product per minute per gram of tissue at 25 °C, and the results were expressed as µM/min/mg protein.

Succinate dehydrogenase (SDH) activity was assayed according to the method of King (1967). SDH catalyses the oxidation of succinate to fumarate using electron acceptors such as ferricyanide. The reduction of ferricyanide was measured as a decrease in absorbance at 420 nm over 3 minutes. A standard curve was prepared using potassium ferricyanide (200–1000 µM) at 420 nm. SDH activity was defined as the amount of enzyme required to reduce potassium ferricyanide per minute per gram of tissue at 30 °C and expressed as U/mg protein.

Histopathological Observations

The fish were freshly dissected, and the collected organs were fixed in 10% buffered formaldehyde for further processing. The pre-fixed tissue samples were then dehydrated through a graded ethanol series (50%, 60%, 70%, 80%, 90%, and twice in 100%) and subsequently immersed in 50% paraffin before being embedded in 100% paraffin wax to prepare tissue blocks. The blocks were trimmed and sectioned at a thickness of 5 µm using a microtome, and the resulting tissue sections were temporarily mounted onto glass slides. The wax was then removed by gentle heating, and the slides were stained with haematoxylin and eosin (H&E) following standard hydration and dehydration procedures. The prepared tissue sections were examined under a Leica microscope equipped with an integrated camera, and photomicrographs were captured for further analysis.

Statistical Analysis

The data were collected in triplicates, the mean and Standard Deviation (SD) were calculated using MS Excel (Mean, Average, SD and probit analysis).

Results

Chemical Profile

TPC and TFC Content

The total phenolic contents (TPCs) in the *C. gigantea* extracts were determined using the standard gallic acid linear equation ($y = 0.0154x – 0.3285$; $R^2 = 0.989$; $p = 0.005$). The TPC in the methanolic extract was calculated as 35.76 ± 0.166 µg gallic acid equivalents (GAE)/mg of extract. The total flavonoid contents (TFCs) were estimated using the standard quercetin hydrate calibration curve ($y = 0.0242x – 0.1845$; $R^2 = 0.976$; $p = 0.012$). The total flavonoid content of the extract was recorded as 60.73 ± 0.181 µg quercetin equivalents (QE)/mg of extract. These results indicate that *C. gigantea* possesses considerable antioxidant potential, capable of neutralising free radicals and aiding in metal chelation.

DPPH Radical Scavenging Assay

In this current study, the antioxidant activity of the methanolic leaf extract of the *Calotropis gigantea* was evaluated using DPPH assay, reducing power of the *C. gigantea* extract and by measuring the total antioxidant capacity. The leaf extract of *C. gigantea* demonstrated a concentration-dependent inhibition of DPPH radicals, with 58.96% inhibition observed at 100 µg/ml, suggesting potent radical scavenging activity. This activity was comparable to that of the reference antioxidant, ascorbic acid, implying that *C. gigantea* possesses significant natural antioxidant potential.

GC-MS Profile of C. gigantea Leaf Extract

The GC–MS analysis of the *C. gigantea* leaf extract revealed the presence of several major bioactive compounds. These compounds were identified based on their retention time (RT), peak area percentage, and comparison with the NIST spectral library. The detected compounds included various alkaloids, terpenoids, phenolics, fatty acids, esters, and phytosterols, many of which are well-

documented for their antimicrobial, antioxidant, and anti-inflammatory properties. The presence of these constituents provides a chemical basis for the observed antioxidant activity of the extract and highlights the potential pharmacological applications of *C. gigantea* in herbal medicine and drug development.

Table 1: *Bioactive Compounds Identified in Leaf Extract of Calotropis gigantea*

Peak No.	Retention Time (RT) (min)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Peak Area (%)
1	5.751	Nonanal	$C_9H_{18}O$	142	0.22
2	6.196	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	0.89
3	7.662	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0.43
4	8.146	L-Proline, 5-oxo-, methyl ester	$C_6H_9NO_3$	143	1.37
5	8.764	1-Hexadecanol	$C_{16}H_{34}O$	242	0.23
6	9.125	2-Nonanol, acetate	$C_{11}H_{22}O_2$	186	0.34
7	9.37	Phenol, 4-ethenyl-2,6-dimethoxy-	$C_{10}H_{12}O_3$	180	0.35
8	10.454	Tetradecanoic acid (Myristic acid)	$C_{14}H_{28}O_2$	228	0.61
9	10.878	Neophytadiene	$C_{20}H_{38}$	278	1.53
10	11.329	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	0.92
11	12.016	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	294	1.1
12	12.197	9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z)-	$C_{19}H_{32}O_2$	292	2.05
13	12.247	Phytol	$C_{20}H_{40}O$	296	2.89
14	12.408	9,12,15-Octadecatrienoic acid (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	15.14
15	15.306	Squalene	$C_{30}H_{50}$	410	1.77
16	18.727	Campesterol	$C_{28}H_{48}O$	400	5.22
17	18.989	Stigmasterol	$C_{29}H_{48}O$	412	4.37
18	19.718	γ-Sitosterol	$C_{29}H_{50}O$	414	4.66
19	21.086	α-Amyrin	$C_{30}H_{50}O$	426	5.78
20	24.135	Triterpenoid Derivative	-	-	8.44

Acute Toxicity Test

In this study, 96-hour acute toxicity was performed to determine the LC_{50} concentration by keeping five different range of concentration (500, 250, 125, 75 and 50mg/L) for leaf extract and the LC_{50} value of 96h was found to be 244mg/L from probit analysis. After obtaining LC_{50}, $1/10^{th}$ of obtained concentration was selected for chronic exposure of 30days to analyse the effect of these extracts on Zebrafish (*D. rerio*). The healthy fishes were segregated and exposed to the LC_{50}concentration of leaf extract for 30 days to observe biochemical and histological changes in the *Danio rerio*.

Chronic Toxicity Test

In chronic exposure study carried out for 30 days, no mortality was observed in the control and in the treated groups. Fishes were active and healthy swimming throughout the exposure period. The sampling was done on every 15-day interval. Dissected organs were homogenised and stored for further biochemical analysis. The organs for histology were stored in buffered formalin and was fixed for further histological process.

Effect of Leaf Extract on Oxidative Stress Enzymes in Zebrafish

The variation in the stress enzymes like SOD, CAT, GST, GR & SDH in zebrafish organs due to chronic leaf exposure is depicted in Fig 1,2,3,4 and 5.

Catalase (CAT):

In the current study, the CAT activity was significantly increased in all organs on10thand 20th day of exposure showing the antioxidant defence reaction to the stress caused by toxins in a long-term exposure and markedly declined on 30th day of exposure. Liver and gills showed highest activity among all the organs on 20th day of exposure compare to control group. Catalase activity significantly reduced on the30thday of the exposure period in all the selected organs.

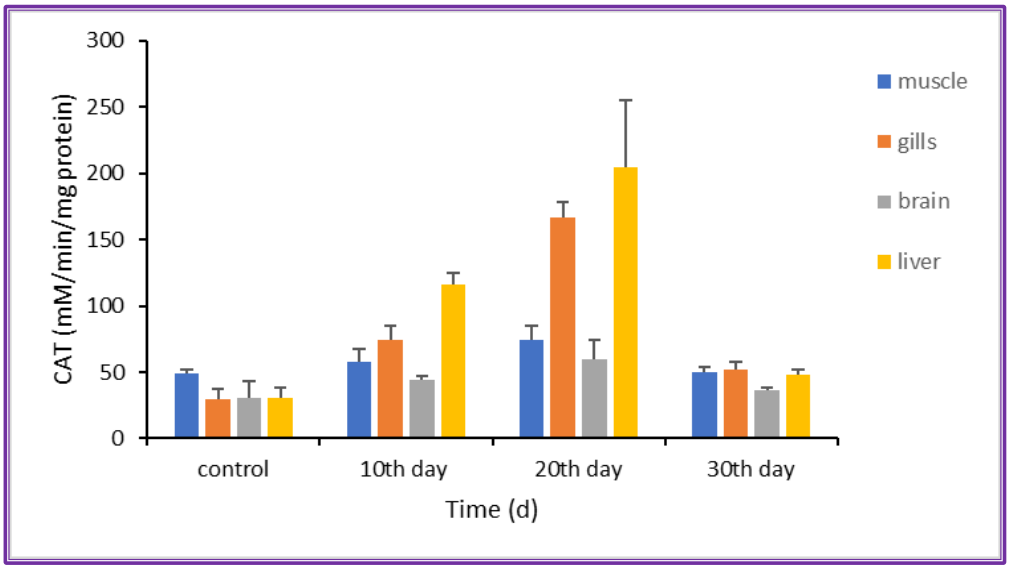

Figure 1: *Activity of Catalase (CAT) In Control and Experimental Setup Exposed to Calotropis gigantea Leaf Extract on Danio Rerio. Data Are Presented as Mean ± SD.*

SOD (Superoxide Dismutase)

A significant variation was seen in the SOD activity but not highly differing with the control among all organs. MusclehasshownagradualincreaseintheSODactivityovertheperiodcomparedto control and gills showed the least SOD activity on 30th day.

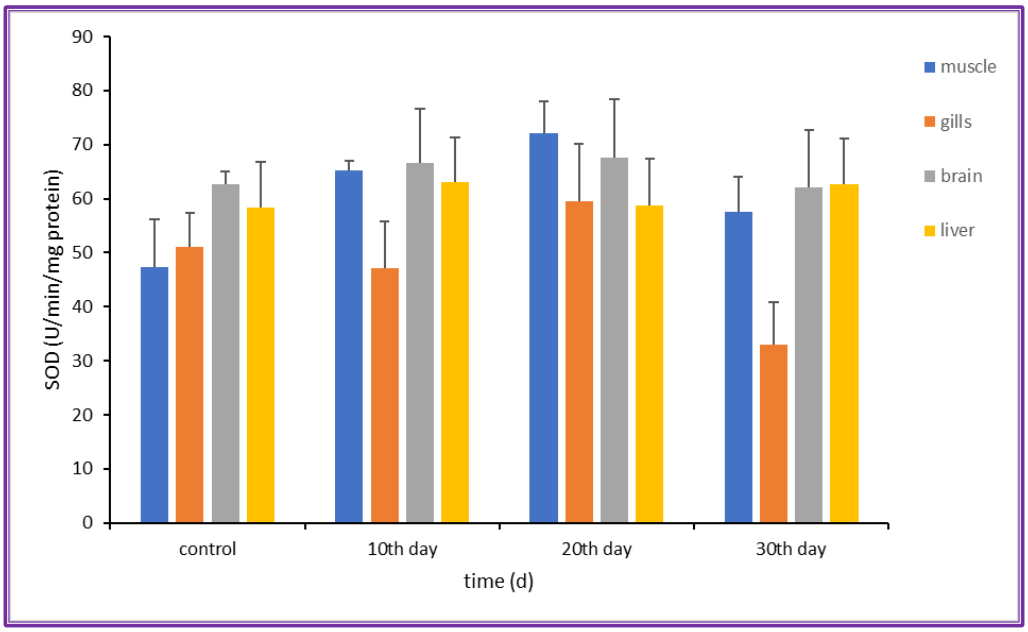

Figure 2: *Activity of Superoxide Dismutase (SOD) in Control and Experimental Setup Exposed to Calotropis gigantea Leaf Extract on Danio Rerio. Data Are Presented As mean ± SD.*

GST(Glutathione S-Transferase):

A prominent increase in the GST activity was recorded in all the organs over the exposure period (10^{th}, 20^{th}, 30^{th}). Among all the organs, liver expressed its highest activity on both 20^{th} and 30^{th} day of exposure. A slight increase in the GST activity was registered in brain and gills on 30^{th} day of exposure. However, compared to control group, muscle showed least activity during the exposure period.

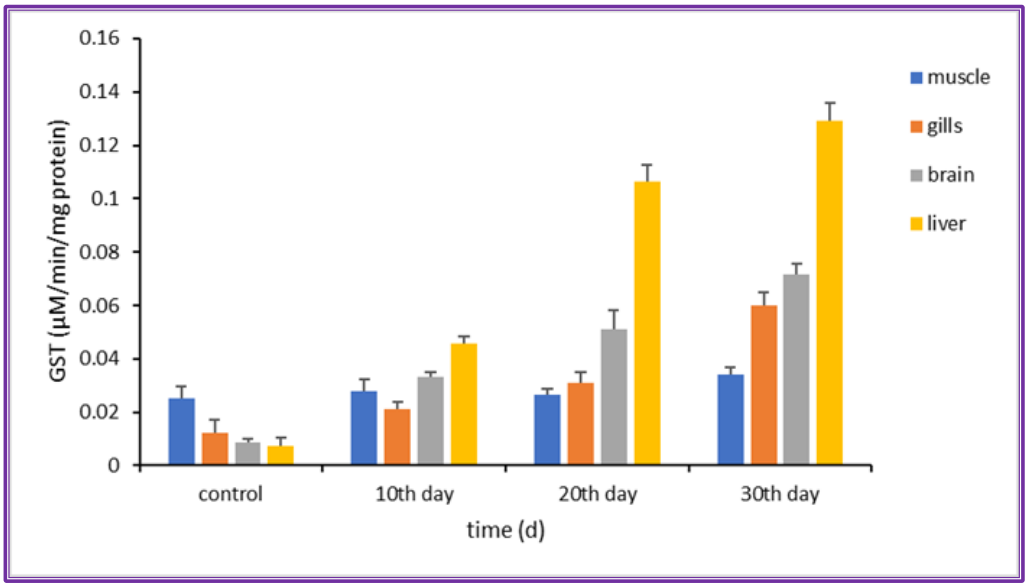

Figure 3: *Activity of Glutathione S-Transferase (GST) in Control and Experimental Setup Exposed to Calotropis gigantea Leaf Extract on Danio rerio. Data are presented as mean ± SD.*

GR(GlutathioneReductase):

GR activity raised initially (on 10^{th} day) in liver, muscle whereas brain showed decreased activity on 20^{th} day compared to control. The variation in gill found was found to be similar on 10^{th} and 20^{th} day of exposure but a slight increase was observed on 30^{th} day of the exposure. All the organs showed increase in activity in all sampling days compared to the control group.

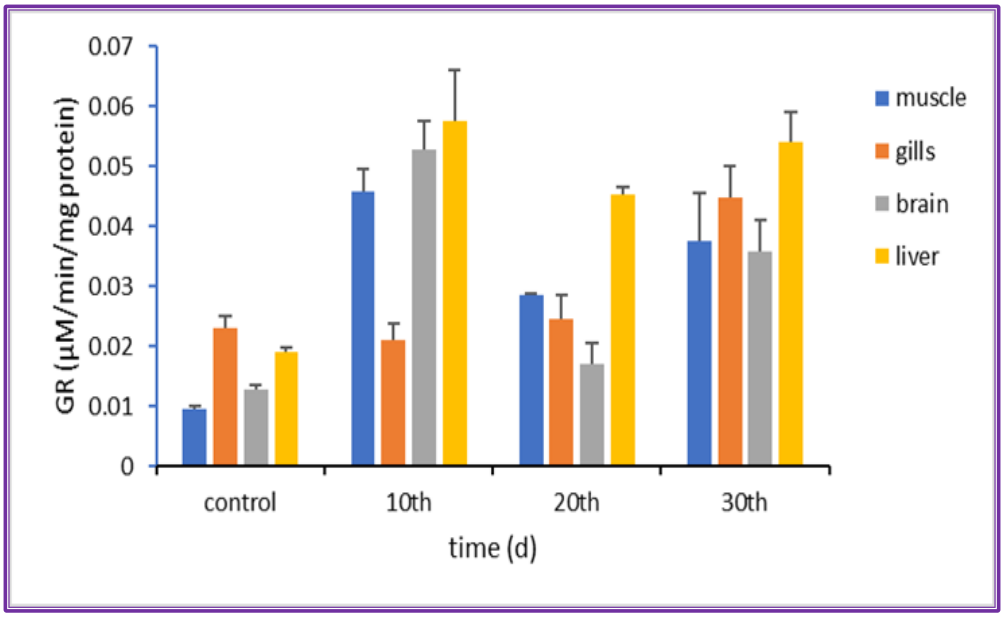

Figure 4: *Activity of GlutathioneReductase (GR) in Control and Experimental Setup Exposed to Calotropis gigantea Leaf Extract on Danio Rerio. Data Are Presented as mean ± SD.*

SDH (Succinate Dehydrogenase)

AprominentincreaseintheSDHactivitywasseeninalltheorganson10thdaycomparedto control group and a gradual decline was observed on the 20th and 30th days in all the organs. Liver showed an increased SDH activity on initial exposure (10thday) but declined as exposure days increased. All the exposed organs showed decreased SDH activity on the 30th day of sampling.

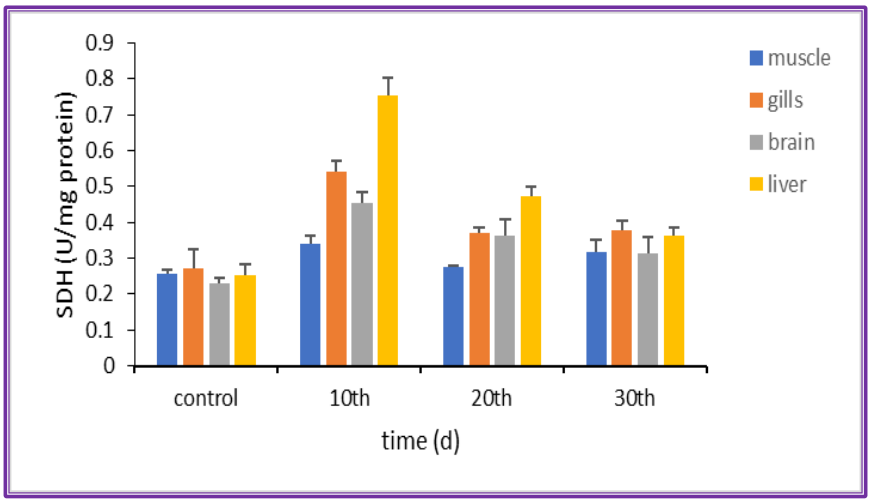

Figure 5: *Activity of Succinate Dehydrogenase (SDH) In Control and Experimental Setup Exposed to Calotropis gigantea Leaf Extract on Danio Rerio. Data Are Presented as Mean ± SD.*

Analysis of Histopathology

Effect on Muscle

Histological alteration in muscle like Necrosis (N), Haemorrhage (Hr), Degradation (D), Hyperplastic Muscle Fibre (HMF), Edema (E), Separation of Dermal Layer (S) in zebrafish exposed to *C. gigantea* leaf extract depicted in fig (6). Muscle of the control group exhibited the normal cell structure, with well-organized muscle fibres and showed no sign of cellular damage. However, separation of dermal layer from underlying tissue and disruption of dermal tissue was majorly observed in the treatment group. However, findings including haemorrhage, cellular degradation, hyperplastic muscle fibre edema was also seen prominent in the all-sampling days. More effect and aberrations were observed on 30th day sampling.

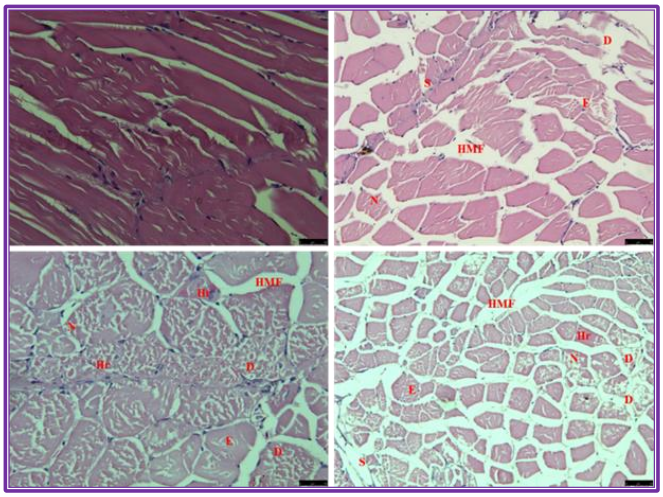

Figure 6: *Photomicrograph Showing Change in Histological Structures in Danio Rerio Exposed to C. gigantea. Control Muscle Showing Normal Structure, Exposed Showing Necrosis (N), Haemorrhage (Hr), Degradation (D), Hyperplastic Muscle Fibre (HMF), Degradation (D), Edema (E), Separation of Dermal Layer (S).*

Effects on Gill

Histological alterations in gill like lifting of respiratory epithelium (LRE); fusion of lamellae (FL); epithelial lifting (EL); vasodilation (VD); disorganisation of cartilaginous core (DCC); haemosiderin (HM); necrosis (NC); curling of lamellae (CL) in zebrafish exposed to *C. gigantea* leaf extract is depicted in fig (7). The structure of gill in the control group was normal with the gill arch with prominent filaments and secondary lamellae and minimal effect was seen on the 10th day sample. However, severe effect was observed on 20th and 30th day samples with severe epithelial lifting, fusion and curling of lamellae, vasodilation and cellular necrosis along with disorganisation of the cartilaginous core.

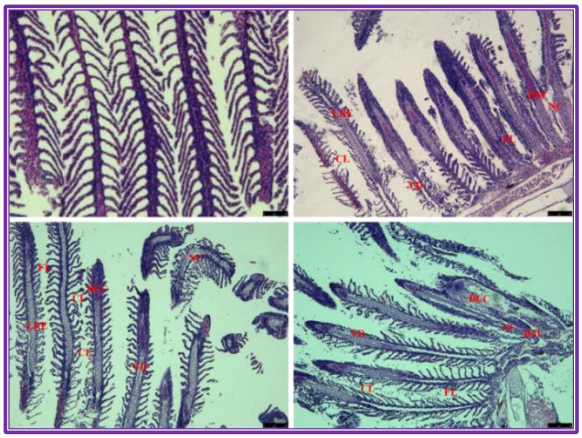

Figure 7: *Photomicrograph Showing Change in Histological Structures in Danio Rerio Exposed to C. gigantea. Control Gill Showing Normal Structure, Exposed Showing Lifting of Respiratory Epithelium* (LRE); Fusion of lamellae (FL); Epithelial lifting (EL); Vasodilation (VD); Disorganisation of cartilaginous core (DCC); Haemosiderin (HM); Necrosis (NC); Curling of lamellae (CL).

Effect on Liver

Histological alteration in liver like Haemorrhage (Hr), Haemosiderin (Hn), Fatty degeneration (Fd), Degeneration (D), Necrosis (N), Congestion in blood sinusoid (Cn), Hypertrophy (H), Vacuolation (V), Hepatic cell dealation (Di) in zebrafish exposed to *C. gigantea* leaf extract is depicted in fig (8). Liver in the control group exhibited no aberrations in the structure. Apparently, excessive bleeding within the liver causing maximum haemorrhage was observed in treatment liver on 10th day sampling. Hepatic cell dilation, fatty degradation, congestion in blood sinusoid and necrosis was prominently seen in the treatment group in all sampling days.

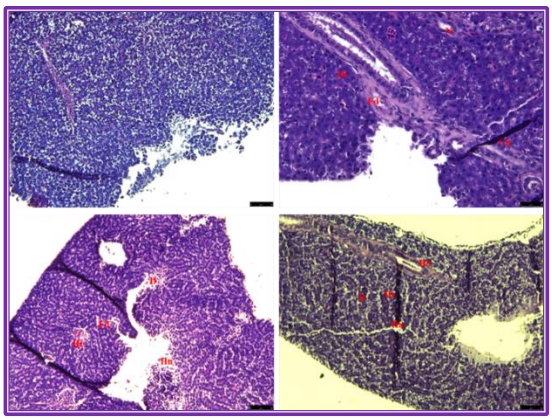

Figure 8: *Photomicrograph Showing Change in Histological Structures in Danio Rerio Exposed to C. gigantea. Control Liver Showing Normal Structure, Haemorrhage (Hr), Haemosiderin (Hn), Fatty Degeneration (Fd), Degeneration (D), Necrosis (N), Congestion in Blood Sinusoid (Cn), Hypertrophy (H), Vacuolation (V), Hepatic Cell Dealation (Di).*

Effect on Brain

Histological alterations in the brain, such as melano-macrophage centre (MMC) accumulation, neuronal cell degeneration (Nd), vacuolar degeneration (Vd), optic lobe atrophy (OPA), and neuronal degeneration in the stratum album (Sa) and indusium griseum (Sg) of zebrafish exposed to *C. gigantea* leaf extract, are illustrated in Fig. 9. Histological examination of brain damage revealed cellular and tissue-level changes associated with exposure to toxic agents. The observations included neuronal necrosis, characterised by shrunken cell bodies, pyknotic nuclei, and eosinophilic cytoplasm. Inflammatory responses were evident, marked by microglial activation and astrocytosis, as indicated by hypertrophied astrocytes and gliosis in the treatment group, whereas the control fish displayed normal brain architecture. Evidence of vascular damage, including oedema, haemorrhage, and disruption of the blood–brain barrier, was also noted. Furthermore, chronic exposure over 30 days resulted in demyelination, loss of neural connections, and glial scar formation in the exposed fish tissue.

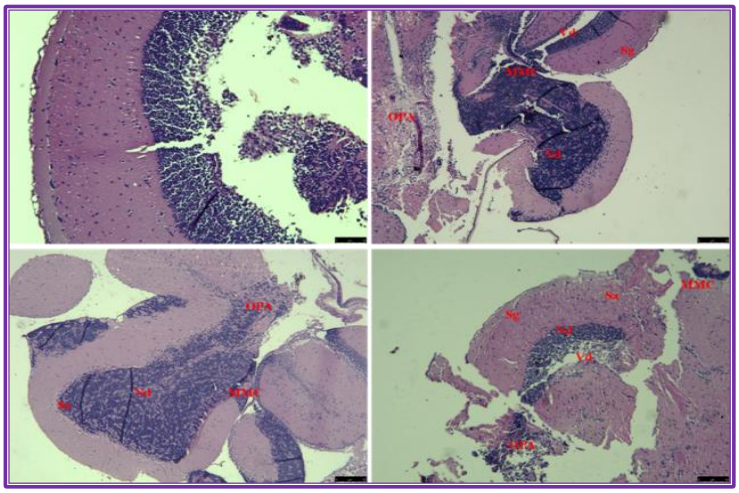

Figure 9: *Photomicrograph Showing Change in Histological Structures in Danio Rerio Exposed to C. gigantea. Control Brain Showing Normal Structure, Exposed Showing Melano Macrophage Centres Accumulation (MMC), Neuronal Cell Degeneration (Nd), Vacuolar Degeneration (Vd), Optic Lobe Atrophy (OPA) Neuronal Degeneration in the Stratum album (Sa) And Indusium griseum (Sg)*

Discussion

Plant Extraction and Yield of The Sample

In this study, methanol was chosen as the effective solvent to extract the major phytochemical and bioactive compounds from leaf sample. The yield of methanolic crude extract of the leaf was found to be 12.5%. Similarly, a comparative study between different solvents for phytochemical extraction from the leaves of *Datura metel* identified methanol as an appropriate solvent for maximum extraction of flavonoids and phenols from the leaf sample (Dhawan & Gupta, 2017).

Acute Toxic and Chronic Toxic Effects of C. gigantea Leaf Extract on Zebrafish

Acute oral toxicity testing is a preliminary and crucial step in assessing the toxicity of any compound before undertaking detailed toxicological evaluations. In the present study, the LC_{50} value of the *Calotropis gigantea* leaf extract was determined to be 244 mg/L using Probit analysis. A similar study by Nur Khasanah investigated the effects of *C. gigantea* leaf extract on *P. xylostella* larvae (Khasanah *et al*., 2021), where tested concentrations of 15, 7.5, 3.75, 1.875, and 0.937 µg/L yielded an LC_{50} value of 2.958 µg/L. In another study, the LC_{50} of green-synthesised CuO nanoparticles derived from *C. gigantea* on embryonic zebrafish was found to be 175 ± 10 mg/L, while that of green-synthesised MgO nanoparticles was 520 µg/mL (Kumari *et al*., 2017; Verma *et al*., 2020). The

variation in LC_{50} values observed across studies may be attributed to differences in the susceptibility of test species to the concentrations of the samples used.

Oxidative Stress Enzyme Studies

Many organisms possess unique defence mechanisms to protect themselves from the harmful effects of reactive oxygen species (ROS). Variations in enzymatic activity are therefore regarded as early indicators of toxic effects, occurring before the manifestation of severe pathological damage in exposed organisms (Yousefi *et al*., 2025). In the present study, exposure to crude *C. gigantea* leaf extract induced oxidative damage in the liver, gills, muscle, and brain of zebrafish. The activities of CAT, SOD, GR, GST, and SDH enzymes were assessed to evaluate the biochemical alterations caused by the induced toxicity of *C. gigantea*. SOD and CAT are considered the primary first-line defence enzymes against ROS generated during the bioactivation of foreign compounds within tissues (da Silva *et al*., 2024). CAT, an enzyme found in nearly all aerobically respiring organisms, plays a crucial role in protecting cells from oxidative stress caused by hydrogen peroxide (H_2O_2) (Tehrani & Moosavi-Movahedi, 2018). Chronic, time-dependent exposure to the leaf extract resulted in significant alterations in both CAT and SOD activity in the treated fish. Over the 30-day exposure period, samples collected on the 10th and 20th days showed a marked increase in CAT activity across all four organs, indicating antioxidant responses against ROS, followed by a notable decline by the 30th day in all tissues. Similarly, a previous study reported a significant reduction in CAT activity after 21 days of exposure to dimethoate and alphamethrin, with levels markedly lower than the control (Ansari & Ansari, 2012). In another study, acetaminophen-induced liver damage in mice, caused by *Calotropis procera*, led to reduced CAT activity, reflecting high levels of hepatic toxicity (Olaleye & Rocha, 2008). The time-dependent decline in CAT activity observed in the present study is consistent with these reports (Ali *et al*., 2025; Aziz & Abdullah, 2023; Lee *et al*., 2023).

By catalysing the conversion of superoxide anions (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), SOD plays a crucial role in protecting cells from oxidative damage. In this study, SOD activity was comparatively lower than that of CAT. Slight variations in SOD activity were observed between different organs and sampling days. Initially, an elevation in SOD levels was recorded across all exposure groups, which may be attributed to the stress induced by the *C. gigantea* leaf extract on the enzymatic activity of the fish. However, a significant increase in ROS generation was also detected within the tissues. As the experiment progressed, SOD activity in the gills showed a noticeable decline, likely due to the consumption of antioxidant enzymes in scavenging free radicals. Changes—either increases or decreases—in enzyme activity are indicative of the intensity of cellular damage. Similarly, the activities of GR, GST, and SDH enzymes showed a marked increase in all organs of the treatment groups compared to the control. This elevation reflects the oxidative stress imposed by the leaf extract on fish tissues and the excessive production of ROS within the antioxidant system. The induction of GR activity is regarded as a potential biomarker of oxidative stress in living organisms (Jomova *et al*., 2024). The observed time-dependent increase in GR activity across all tissues suggests that prolonged exposure to *C. gigantea* leaf extract may induce oxidative stress, triggering compensatory responses in the antioxidant defence system through the accumulation and recycling of glutathione (GSH). Consistent with this finding, enhanced GR activity has been reported in the liver of rats exposed to azo dyes (Oh *et al*., 1997). Likewise, Visweswaran and Krishnamoorthy (2012) observed fluctuations in GR activity in the testes of tartrazine-treated rats (Visweswaran & Krishnamoorthy, 2012). GST, a well-established phase II detoxification enzyme, facilitates the conjugation of glutathione with various xenobiotic compounds and their metabolites (Vašková *et al*., 2023). In fish, the increased GST activity across all organs may reflect an adaptive response aimed at detoxifying and eliminating toxicants (Ji *et al*., 2024). SDH, an exclusively mitochondrial marker enzyme, is located within the inner mitochondrial membrane and functions as part of both the tricarboxylic acid (TCA) cycle and the respiratory electron transport chain (Yang *et al*., 2020). The observed increase in SDH activity provides further insight into the potential toxic effects of the *C. gigantea* leaf extract on the antioxidant defence mechanisms within fish tissues.

Histopathology Analysis

Histopathological analysis is a sensitive and effective method for assessing cellular alterations in vital organs caused by toxicants (Brum *et al*., 2018). Numerous studies have demonstrated the adverse effects of certain medicinal plants, even when administered at low doses over extended periods. In the present study, prolonged exposure to a low concentration of *C. gigantea* extract resulted in notable changes and variations across all organs of the treated fish. As the liver is the principal site of various metabolic processes, any structural alterations in its cellular components are of great significance when evaluating the toxicity of a given substance (Zhong *et al*., 2022). In this study, significant histopathological changes were observed in the liver of fish exposed to the leaf extract, including haemorrhage (Hr), haemosiderin deposition (Hn), fatty degeneration (Fd), general degeneration (D), necrosis (N), congestion in blood sinusoids (Cn), hypertrophy (H), and hepatic cell dilation (Di). Supporting this observation, a chronic toxicological evaluation of Curcuma longa exposure in fish revealed several hepatic alterations such as liver degeneration, hepatic regeneration, inflammation, and necrosis (Amer *et al*., 2022). Similarly, a study examining histopathological changes in the liver and kidney tissues of male Sprague–Dawley rats treated with Rhaphidophora decursiva extracts demonstrated acute toxicity at doses of 2100 and 3500 mg/kg, with the presence of activated Kupffer cells, sinusoidal dilatation, and cytoplasmic vacuolation. Comparable abnormalities were also observed in rats treated with 140 and 210 mg/kg extracts during both sub-acute and sub-chronic exposure periods, indicating significant alterations in liver histology (Arsad *et al*., 2014). In agreement with these findings, the present results suggest that prolonged exposure to the medicinal plant *C. gigantea*, even at lower dosages, can cause severe hepatic damage.

The gill is a multifunctional organ in fish, playing essential roles in respiration, osmoregulation, excretion of nitrogenous wastes, pH regulation, and hormone production (Kaur & Dua, 2015). It serves as a semipermeable barrier between the organism and its external environment. In the present study, the gills of fish from the experimental group exhibited distinct structural alterations and tissue abnormalities when exposed to *C. gigantea* leaf extracts, compared with the control group. The control gills displayed normal architecture without any visible damage, whereas the treated gills showed lifting of the respiratory epithelium (LRE), fusion of lamellae (FL), epithelial lifting (EL), vasodilation (VD), haemosiderin accumulation (HM), necrosis (NC), and curling of lamellae (CL). Additional observations included mild to severe lamellar fusion and inflammation. Several studies have reported that such structural changes represent defensive responses of fish against toxicants. These include epithelial lifting, partial fusion of secondary lamellae, and hyperplasia and hypertrophy of epithelial cells, which collectively increase the distance between the blood and the external environment, thereby reducing toxicant uptake (Panchamoorthy *et al*., 2022; Shobana *et al*., 2018; Ale *et al*., 2018).

Muscle tissue in fish is typically influenced by the composition of fatty acids, texture, fibre characteristics, and endogenous cross-linking of connective tissue (Cheng *et al*., 2016). Prolonged exposure of fish to plant-derived toxins can lead to several detrimental effects on muscle tissues (Eula *et al*., 2025). However, there are relatively few studies reporting the impact of toxicants or harmful plant compounds on muscle fibre integrity and structure. In the present study, the control muscle tissue displayed normal architecture, with well-organised muscle fibres and intact muscle bundles. In contrast, the exposed fish showed signs of necrosis (N), haemorrhage (Hr), degradation (D), oedema (E), separation of the dermal layer (S), and general cellular disintegration, indicating the toxic effects of *C. gigantea* leaf extract on the dermal and muscular tissues, even at low oral doses. Furthermore, the brain exhibited notable structural and metabolic alterations. In this study, sections of control brain tissue showed normal architecture with tightly packed and intact cells. However, the treatment groups displayed distinct abnormalities in both muscle and brain tissues compared with the control fish, providing strong evidence of the toxicological impact of *C. gigantea* leaf extract on histological structure and the disruption of biological pathways in the organism (Kapoor *et al*., 2025; Gulzar *et al*., 2023).

Limitations and Future Prospects

The present study was limited to *in vitro* antioxidant assays and GC–MS profiling. *In vivo* validation of the signalling pathways, along with the assessment of antioxidant-related genes, stress proteins, and molecular docking studies, would provide further insight into the current findings and support future pharmacological applications.

Conclusion

The findings of the present investigation highlight the significant impact of *Calotropis gigantea* leaf extracts on the morphological integrity of adult *Danio rerio*, particularly following prolonged exposure to low dosage levels. Notably, after a 30-day exposure period, pronounced organ deformities and morphological abnormalities were observed across various anatomical structures, as evidenced by detailed histological analyses. The enzymatic profiles and antioxidant capacities of key zebrafish organs showed marked deviations from the control group, even when exposed to a highly diluted concentration (one-tenth of the LC_{50}) of the leaf extract. Overall, this study clearly demonstrates that even minimal exposure to *Calotropis gigantea* can induce substantial toxicological effects on vital organs in zebrafish, underscoring the plant's inherent toxicity, even at seemingly low concentrations. Although *C. gigantea* is widely recognised for its medicinal properties, the present findings advocate for a cautious approach regarding its therapeutic application. Prior to any medicinal use, it is essential to conduct comprehensive evaluations of the plant's toxicological effects and to understand its potential health implications, particularly in the context of long-term exposure. This research reinforces the importance of prudent and systematic assessment of the safety profiles of medicinal plants to ensure that their beneficial properties do not inadvertently result in adverse health outcomes.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

The authors sincerely thank and gratefully acknowledge the MRP and CIF of CHRIST University Bengaluru for providing the necessary facilities and resources to carry out this research work.

References

Agarwal, S., Kaushik, S., Saha, H., Paramanick, D., Mazhar, M., Basist, P., ... & Alhalmi, A. (2025). Therapeutic potential of traditional herbal plants and their polyphenols in alleviation of mercury toxicity. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 1-27. https://doi.org/10.1007/s00210-025-03807-7

Alara, O. R., Abdurahman, N. H., & Ukaegbu, C. I. (2018). Soxhlet extraction of phenolic compounds from Vernonia cinerea leaves and its antioxidant activity. *Journal of Applied Research on Medicinal and Aromatic Plants*, *11*, 12-17. https://doi.org/10.1016/j.jarmap.2018.07.003

Ale, A., Bacchetta, C., Rossi, A. S., Galdopórpora, J., Desimone, M. F., de la Torre, F. R., ... & Cazenave, J. (2018). Nanosilver toxicity in gills of a neotropical fish: metal accumulation, oxidative stress, histopathology and other physiological effects. *Ecotoxicology and Environmental Safety*, *148*, 976-984. https://doi.org/10.1016/j.ecoenv.2017.11.072

Ali, Z., Sher, N., Muhammad, I., Nayab, G. E., Alouffi, A., Almutairi, M. M., ... & Ali, A. (2025). The combined effect of cadmium and copper induces bioaccumulation, and toxicity and disrupts the antioxidant enzymatic activities of goldfish (Carassius auratus). *Toxicology Reports*, *14*, 101972. https://doi.org/10.1016/j.toxrep.2025.101972

Alotaibi, S. S., Alshoaibi, D., Alamari, H., Albogami, S., Khan, E., Alshanbari, A., ... & Almalki, W. (2021). Potential significance of medicinal plants in forensic analysis: A review. *Saudi Journal of Biological Sciences*, *28*(7), 3929-3935. https://doi.org/10.1016/j.sjbs.2021.03.071

Amer, S. A., El-Araby, D. A., Tartor, H., Farahat, M., Goda, N. I., Farag, M. F., ... & Osman, A. (2022). Long-term feeding with curcumin affects the growth, antioxidant capacity, immune status, tissue histoarchitecture, immune

expression of proinflammatory cytokines, and apoptosis indicators in Nile tilapia, Oreochromis niloticus. *Antioxidants*, *11*(5), 937. https://doi.org/10.3390/antiox11050937

Ansari, S. & Ansari, B. A. (2012). Alphamethrin toxicity: effect on the reproductive ability and the activities of phosphatases in the tissues of zebrafish, Danio rerio. *International Journal of Life Science& Pharma Res*earch *2*, 89-100. https://ijlpr.com/index.php/journal/article/view/70/45

Arsad, S. S., Esa, N. M., & Hamzah, H. (2014). Histopathologic changes in liver and kidney tissues from male Sprague Dawley rats treated with Rhaphidophoradecursiva (Roxb.) Schott extract. *J Cytol Histol S*, *4*(1), 1-6. https://doi.org/10.4172/2157-7099.S4-001

Aziz, S., Abdullah, S. (2023). Evaluation of Toxicity Induced by Engineered CuO Nanoparticles in Freshwater Fish, Labeorohita. *Turkish Journal of Fisheries and Aquatic Sciences, 23*(8), TRJFAS18762. https://doi.org/10.4194/TRJFAS18762.

Azzalini, E., Bernini, M., Vezzoli, S., Antonietti, A., &Verzeletti, A. (2019). A fatal case of self-poisoning through the ingestion of oleander leaves. *Journal of Forensic and Legal Medicine*, *65*, 133-136. https://doi.org/10.1016/j.jflm.2019.05.016

Bergmeyer, H. U. (Ed.). (2012). *Methods of enzymatic analysis*. Elsevier. https://books.google.co.in/books?hl=en&lr=&id=GDd2zYuLpRwC&oi=fnd&pg=PP1&dq=Bergmeyer,+H.+U.+(Ed.).+(2012).+Methods+of+enzymatic+analysis.+Elsevier.+&ots=z5TFdpz3im&sig=6-l_t9ozEXEN9q3bI5dAwejJWc4&redir_esc=y#v=onepage&q&f=false

Brum, A., Cardoso, L., Chagas, E. C., Chaves, F. C. M., Mouriño, J. L. P., & Martins, M. L. (2018). Histological changes in Nile tilapia fed essential oils of clove basil and ginger after challenge with Streptococcus agalactiae. *Aquaculture*, *490*, 98-107. https://doi.org/10.1016/j.aquaculture.2018.02.040

Carlberg, I. N. C. E. R., &Mannervik, B. E. N. G. T. (1975). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *Journal of biological chemistry*, *250*(14), 5475-5480. https://www.jbc.org/article/S0021-9258(19)41206-4/pdf

Cheng, A. J., Yamada, T., Rassier, D. E., Andersson, D. C., Westerblad, H., & Lanner, J. T. (2016). Reactive oxygen/nitrogen species and contractile function in skeletal muscle during fatigue and recovery. *The Journal of Physiology*, *594*(18), 5149-5160. https://doi.org/10.1113/jp270650

Chien, C., & Dauterman, W. C. (1991). Studies on glutathione S-transferase in Helicoverpa (= Heliothis) zea. *Insect Biochemistry*, *21*(8), 857-864. https://doi.org/10.1016/0020-1790(91)90092-S

da Silva, A. P., Hernández, H. V. P., Comelli, C. L., Portugal, M. A. G., Delavy, F. M., de Souza, T. L., ... & de Castilhos Ghisi, N. (2024). Meta-analytical review of antioxidant mechanisms responses in animals exposed to herbicide 2, 4-D herbicide. *Science of The Total Environment*, *924*, 171680. https://doi.org/10.1016/j.scitotenv.2024.171680

Dhawan, D., & Gupta, J. (2017). Research article comparison of different solvents for phytochemical extraction potential from datura metel plant leaves. *International Journal of Biological Chemistry*, *11*(1), 17-22. https://doi.org/10.3923/ijbc.2017.17.22

Eula, M. A. C., Bayona-Serrano, J. D., Nishiyama-Jr, M. Y., Squaiella-Baptistão, C. C., Santoro, M. L., & Junqueira-de, I. D. L. M. (2025). The underestimated local effects of Micrurus corallinus venom revealed by gene expression and histopathological alterations. *Toxicon*, 108368. https://doi.org/10.1016/j.toxicon.2025.108368

Gulzar, R., Riaz, Z., Gillani, Q. U. A., Mehreen, A., Jameel, F., & Nawaz, R. (2023). Bioaccumulation and influence of cadmium chloride on histology of muscles and gills in Nile tilapia (Oreochromis niloticus). *Journal of Survey in Fisheries Sciences*, *10*(2), 992-1000. https://doi.org/10.53555/sfs.v10i2.1509

Ismail, H. F., Hashim, Z., Soon, W. T., Ab Rahman, N. S., Zainudin, A. N., & Majid, F. A. A. (2017). Comparative study of herbal plants on the phenolic and flavonoid content, antioxidant activities and toxicity on cells and zebrafish embryo. *Journal of Traditional and Complementary Medicine*, *7*(4), 452-465.https://doi.org/10.1016/j.jtcme.2016.12.006

Iyadurai, R., Gunasekaran, K., Jose, A., &Pitchaimuthu, K. (2020). Calotropis poisoning with severe cardiac toxicity A case report. *Journal of Family Medicine and Primary Care*, *9*(8), 4444-4447. https://doi.org/10.4103/jfmpc.jfmpc_783_20

Jayalekshmi, C., Das, N. M., &Periakaruppan, R. (2024). Bioactive compounds of *Calotropis gigantea* for cancer treatment. *Oral Oncology Reports*, *10*, 100336. https://doi.org/10.1016/j.oor.2024.100336

Ji, F., Zhang, J., Ding, X., Rong, L., Liu, X., Yan, T., & Li, J. (2024). Associations of GST gene polymorphisms and GST enzyme activity with the development of noise-induced hearing loss in Chinese han males. *Public Health Genomics*, *27*(1), 168-176. https://doi.org/10.1159/000541618

Jomova, K., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., & Valko, M. (2024). Several lines of antioxidant defense against oxidative stress: antioxidant enzymes, nanomaterials with multiple enzyme-

mimicking activities, and low-molecular-weight antioxidants. *Archives of Toxicology*, *98*(5), 1323-1367. https://doi.org/10.1007/s00204-024-03696-4

Joshi, S. V., Gupta, S., Tripathi, K., Mishra, S., & Kumar, S. (2024). Antiviral plants of India. *Ambika Prasad Research Foundation, Odisha, India.*

Kanchan, T., & Atreya, A. (2016). *Calotropis gigantea*. *Wilderness & Environmental Medicine*, *27*(2), 350-351. https://doi.org/10.1016/j.wem.2015.12.011

Kapoor, V. K., Kaur, N., & Rana, S. (2025). Safety concern of drugs of herbal origin. *Phytochemistry Reviews*, 1-16. https://doi.org/10.1007/s11101-025-10070-4

Kaur, R., & Dua, A. (2015). 96 h LC50, behavioural alterations and histopathological effects due to wastewater toxicity in a freshwater fish Channa punctatus. *Environmental Science and Pollution Research*, *22*(7), 5100-5110. https://doi.org/10.1007/s11356-014-3710-1

Khasanah, N., Martono, E., Trisyono, Y. A., &Wijonarko, A. (2021). Toxicity and antifeedant activity of *Calotropis gigantea* L. leaf extract against PlutellaxylostellaL.(Lepidoptera: Plutellidae). *International Journal of Design & Nature and Ecodynamics*, *16*(6), 677-682. https://doi.org/10.18280/ijdne.16060

King, T. E. (1967). [58] Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. In *Methods in enzymology* (Vol. 10, pp. 322-331). Academic Press. https://doi.org/10.1016/0076-6879(67)10061-X

Kumari, P., Panda, P. K., Jha, E., Kumari, K., Nisha, K., Mallick, M. A., & Verma, S. K. (2017). Mechanistic insight to ROS and apoptosis regulated cytotoxicity inferred by green synthesized CuO nanoparticles from *Calotropis gigantea* to embryonic zebrafish. *Scientific Reports*, *7*(1), 16284. https://doi.org/10.1038/s41598-017-16581-1

Lee, J. H., Kang, J. C., & Kim, J. H. (2023). Toxic effects of microplastic (Polyethylene) on fish: Accumulation, hematological parameters and antioxidant responses in Korean Bullhead, Pseudobagrusfulvidraco. *Science of The Total Environment*, *877*, 162874. https://doi.org/10.1016/j.scitotenv.2023.162874

Mahale, D. S., Phulapagar, M., Gosavi, T., & Bharti, V. (2023). Review on *Calotropis gigantea* use on Diabetic. *Research Journal of Pharmacology and Pharmacodynamics*, *15*(1), 36-41. https://doi.org/10.52711/2321-5836.2023.00008

Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European journal of biochemistry*, *47*(3), 469-474. https://doi.org/10.1111/j.1432-1033.1974.tb03714.x

Miara, M. D., Bendif, H., Ait Hammou, M., & Teixidor-Toneu, I. (2018). Ethnobotanical survey of medicinal plants used by nomadic peoples in the Algerian steppe. *Journal of ethnopharmacology*, *219*, 248-256. https://doi.org/10.1016/j.jep.2018.03.011

Tehrani H. S., & Moosavi-Movahedi A. A. (2018). Catalase and its mysteries. *Progress in Biophysics and Molecular Biology*, *140*, 5-12. https://doi.org/10.1016/j.pbiomolbio.2018.03.001

Ni, H., Peng, L., Gao, X., Ji, H., Ma, J., Li, Y., & Jiang, S. (2019). Effects of maduramicin on adult zebrafish (Danio rerio): acute toxicity, tissue damage and oxidative stress. *Ecotoxicology and environmental safety*, *168*, 249-259. https://doi.org/10.1016/j.ecoenv.2018.10.040

Nithaniyal, S., Majumder, S., Umapathy, S., & Parani, M. (2021). Forensic application of DNA barcoding in the identification of commonly occurring poisonous plants. *Journal of forensic and legal medicine*, *78*, 102126. https://doi.org/10.1016/j.jflm.2021.102126

Oh, S. W., Kang, M. N., Cho, C. W., & Lee, M. W. (1997). Detection of carcinogenic amines from dyestuffs or dyed substrates. *Dyes and Pigments*, *33*(2), 119-135. https://doi.org/10.1016/S0143-7208(96)00038-1

Olaleye, M. T., & Rocha, B. J. (2008). Acetaminophen-induced liver damage in mice: effects of some medicinal plants on the oxidative defense system. *Experimental and Toxicologic Pathology*, *59*(5), 319-327. https://doi.org/10.1016/j.etp.2007.10.003

Panchamoorthy, R., Thada, R., & Chockalingam, S. (2022). Short-term Co-exposure of celery leaf powder exerts detoxifying action against acetaminophen-induced toxicity in fish gills. *Journal of Hazardous Materials Advances*, *8*, 100148. https://doi.org/10.1016/j.hazadv.2022.100148

Perumal, S., Gopal Samy, M. V., & Subramanian, D. (2021). Developmental toxicity, antioxidant, and marker enzyme assessment of swertiamarin in zebrafish (Danio rerio). *Journal of Biochemical and Molecular Toxicology*, *35*(9), e22843.https://doi.org/10.1002/jbt.22843

Sherikar, A. S., &Mahanthesh, M. C. (2015). Evaluation of aqueous and methanolic extract of leaves of Epipremnumaureum for radical scavenging activity by DPPH Method, total phenolic content, reducing capacity assay and FRAP assay. *Journal of Pharmacognosy and Phytochemistry*, *4*(4), 36-40. https://www.phytojournal.com/archives/view-pdf/678/4-3-40

Shobana, C., Rangasamy, B., Poopal, R. K., Renuka, S., & Ramesh, M. (2018). Green synthesis of silver nanoparticles using Piper nigrum: tissue-specific bioaccumulation, histopathology, and oxidative stress responses in Indian major carp Labeorohita. *Environmental Science and Pollution Research*, *25*(12), 11812-11832. https://doi.org/10.1007/s11356-018-1454-z

Subramanian, K., Sankaramourthy, D., & Gunasekaran, M. (2018). Toxicity studies related to medicinal plants. *In Natural products and drug discovery* (pp. 491-505). Elsevier. https://doi.org/10.1016/B978-0-08-102081-4.00018-6

Vašková, J., Kočan, L., Vaško, L., &Perjési, P. (2023). Glutathione-related enzymes and proteins: A review. *Molecules*, *28*(3), 1447. https://doi.org/10.3390/molecules28031447

Verma, S. K., Nisha, K., Panda, P. K., Patel, P., Kumari, P., Mallick, M. A., ... & Das, B. (2020). Green synthesized MgO nanoparticles infer biocompatibility by reducing in vivo molecular nanotoxicity in embryonic zebrafish through arginine interaction elicited apoptosis. *Science of The Total Environment*, *713*, 136521. https://doi.org/10.1016/j.scitotenv.2020.136521

Visweswaran, B., & Krishnamoorthy, G. (2012). Oxidative stress by tartrazine in the testis of Wistar rats. *Journal of Pharmacy and Biological Sciences*, *2*(3), 44-49. https://www.iosrjournals.org/iosr-jpbs/papers/vol2-issue3/J0234447.pdf

Yang, Y., Dong, F., Liu, X., Xu, J., Wu, X., & Zheng, Y. (2020). Thifluzamide induces the toxic effects on zebrafish (Danio rerio) via inhibition of succinate dehydrogenase (SDH). *Environmental Pollution*, *265*, 115031. https://doi.org/10.1016/j.envpol.2020.115031

Yousefi, M., Adineh, H., Al Sulivany, B. S., Gholamalipour Alamdari, E., Yilmaz, S., Mahboub, H. H., & Hoseini, S. M. (2025). The potential of the inclusion of Prosopis farcta extract in the diet on the growth performance, immunity, digestive enzyme activity, and oxidative status of the common carp, Cyprinus carpio, in response to Ammonia stress. *Animals*, *15*(6), 895. https://doi.org/10.3390/ani15060895

Zhong, L., Liu, H., Zhang, H., Zhang, W., Li, M., Huang, Y., ... & Yin, L. (2022). High starch in diet leads to disruption of hepatic glycogen metabolism and liver fibrosis in largemouth bass (Micropterus salmoides), which is mediated by the PI3K/Akt signaling pathway. *Frontiers in Physiology*, *13*, 880513. https://doi.org/10.3389/fphys.2022.880513