

Enzymatic responses of *Culex quinquefasciatus* Say larvae to *Clerodendrum infortunatum* Linn extract

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Abstract

Mosquitoes are of serious public health concern due to their vector competence. Among mosquitoes, *Culex quinquefasciatus*, commonly known as the Southern house mosquito, is of major importance due to its potency to spread Lymphatic filariasis. Due to the emergence of resistance to synthetic insecticides, more emphasis is now placed on plant-based compounds. In the present study, *Clerodendrum infortunatum*, a member of the Lamiaceae family, is investigated for its insecticidal potency against *Culex quinquefasciatus* larvae. Treatment using the acetone extract of *Clerodendrum infortunatum* against the fourth-instar larvae of *Culex quinquefasciatus* showed severe changes in the enzyme profile of the treated sample compared with the control. The activities of the antioxidant enzymes SOD, CAT, and GPx decreased, while GST levels increased compared with control levels. A significant decrease was observed in the neuroregulatory enzyme Acetylcholinesterase and the osmoregulatory enzyme Na⁺/K⁺-ATPase. Extract of *Clerodendrum infortunatum* thus adversely affects the growth and metabolism of *Culex quinquefasciatus*, leading to larval mortality.

Keywords: *Culex quinquefasciatus*, *Clerodendrum infortunatum*, antioxidant enzymes, Acetylcholinesterase, Na⁺/K⁺-ATPase

1. Introduction

Mosquitoes, particularly those within the genus *Culex*, are significant vectors of numerous diseases, including filariasis and various forms of encephalitis, thereby posing considerable threats to global public health (Pavunraj et al., 2017). The increasing resistance of *Culex quinquefasciatus* to traditional synthetic insecticides calls for the investigation of sustainable and environmentally acceptable vector control options (Oliveira et al., 2023).

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Natural products offer a promising avenue for developing novel larvicide agents due to their biodegradability and diverse bioactive constituents. Plant-based insecticides offer more advantages than synthetic chemical pesticides, as research shows that phytochemicals act through multiple modes of action (Sugumar et al., 2014).

Understanding the physiological and biochemical responses helps to formulate biopesticides that mitigate the environmental and health concerns associated with synthetic alternatives. Enzymatic modulation demonstrates potential synergistic interactions with existing control strategies, thereby improving overall efficacy and potentially mitigating the development of resistance (Aremu et al., 2024). Insecticide-based control of mosquitoes is a major method for preventing the spread of mosquito-borne diseases. These insecticides disrupt the mosquito's enzymatic system, reducing growth and development and leading to mortality. In mosquitoes, enzymes catalyse specific metabolic reactions essential for performing critical biological functions, including immune response, digestion and development. Some key enzymes in mosquitoes include antioxidant enzymes Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), and Glutathione-S-Transferase (GST), the neuroregulatory enzyme Acetylcholine esterase (AChE), and the osmoregulatory enzyme Na^+/K^+ -ATPase. Like synthetic insecticides, plant-derived larvicides induce physiological stress that modifies the normal enzyme profile as part of a defensive response. Disruption of these enzymatic activities can lead to severe oxidative stress, neurological dysfunction, and osmotic imbalance, thereby contributing to the overall larvicidal effect (Sofi et al., 2022).

Plants in the genus *Clerodendrum* have emerged as highly promising candidates for natural pest control. Crude extracts and isolated compounds from these plants have demonstrated diverse pharmacological and pesticidal properties (Darsana et al., 2019). The present study aims to elucidate the specific enzymatic alterations induced in *Culex quinquefasciatus* larvae upon exposure to *Clerodendrum infortunatum* extract, thereby contributing to the understanding of its larvicidal mechanism of action.

2. Materials and methods

2.1. Collection and rearing of *Culex quinquefasciatus*

The fourth-instar larvae of *Culex* were reared as described by Ayana Gayathri and Evans (2018). A 10 L bucket was filled with 5 L of dechlorinated tap water mixed with two beaten eggs and kept under laboratory conditions for 7 days to attract female *Culex* mosquitoes. Following oviposition, the egg rafts were carefully collected and transferred to small bottles containing an aliquot of egg solution and dechlorinated tap water. The reared larvae were fed with crushed dog biscuits. The eggs and larvae were identified using standard keys (Tyagi et al., 2015) and used for further experimental studies.

2.2. Collection and preparation of plant extract

The leaves of *Clerodendrum infortunatum* were collected from Thiruvananthapuram district. The plant material was taxonomically identified at the Department of Botany, University College, Thiruvananthapuram, utilising standard taxonomic keys (Bhattacharjee et al., 2011). Fresh leaves were thoroughly washed with water and air-dried in the shade for 1 week. The air-dried leaves were then pulverised using a domestic grinder. The powdered material was extracted in a Soxhlet apparatus with acetone. The dried extract was prepared as a 10% stock solution in distilled water (Indusree et al., 2019). A concentration of 300 ppm was used for further analysis, as the LC_{50} value obtained was 345 ppm (Sheenu et al., in press).

2.3. Experimental design

Laboratory-reared uniformly sized fourth-instar larvae of *Culex quinquefasciatus* were randomly selected from the stock culture. 20 numbers of fourth-instar larvae were placed in 250 ml paper cups containing plant extract at a concentration of 300 ppm and used for further studies. The control set was maintained simultaneously in distilled water without plant extract. For both the treated and control groups, six replicates were maintained.

100 mg of fourth-instar larvae of *Culex quinquefasciatus*, both treated and control, were thoroughly washed with 50 mM phosphate buffer solution (pH 7.4). Subsequently, the larval tissue was homogenised in 1 ml of Tris. HCl buffer, adjusted to a pH of 7.2, while maintaining a temperature of 4°C. The resulting homogenates were centrifuged at 10,000 rpm for a duration of 10 min at 4°C. The supernatant was collected and used for subsequent antioxidant enzyme assays.

2.4. Antioxidant enzyme assays

2.4.1. Superoxide dismutase (SOD)

The reaction mixture contained 1.2 mL of sodium pyrophosphate buffer, 100 µL of Phenazine methosulphate, 300 µL of Nitroblue tetrazolium, 200 µL of an appropriately diluted enzyme preparation and water to a final volume of 3 mL. The reaction was stopped by adding 1 mL of glacial acetic acid after incubation at 30°C for 90 seconds. Using 4 mL of n-butanol, the reaction mixture was stirred vigorously. After 10 minutes, the mixture was centrifuged at 2500 rpm for 10 minutes, and the butanol layer was pipetted out. The colour intensity was measured at 560 nm using a UV spectrophotometer (Perkin, Elmer). The enzyme activity was defined as the enzyme concentration required to reduce the chromogen-produced optical density at 560 nm by 50% in 1 minute under the assay conditions. The specific activity is expressed as IU/mg protein (Kakkar et al., 1984).

2.4.2. Catalase (CAT)

The test mixture was prepared by combining 3 mL of phosphate buffer, 20 mL of H_2O_2 , and 1 mL of approximately diluted extract. A mixture free of extract served as a blank. The decrease in optical absorbance was measured using spectrophotometry at a wavelength of 230 nm. The specific enzymatic activity was expressed as n moles of H_2O_2 decomposed/minute/mg of protein (Maehly & Chance, 1954).

2.4.3. Glutathione peroxidase (GPx)

The assay mixture comprised 2 mL of phosphate buffer, 300 μL of EDTA, 300 μL of sodium azide, 100 μL of reduced glutathione, 100 μL of NADPH, and 200 μL of tissue extract. Blank was prepared by mixing all the reagents except the tissue extract. The change in absorbance was measured at 340 nm at a 15-second interval for 1 minute. The enzyme activity was expressed as IU/mg protein (Lawrence & Burk, 1976).

2.4.4. Glutathione-S-Transferase (GST)

The assay mixture comprised 1 mL of phosphate buffer, 100 μL of CDNB, 0.1 mL of GSH, and 100 μL of tissue extract. The increase in optical density was measured at 340 nm for a period of 2 minutes against a blank. Enzyme activity was expressed as n moles of CDNB-GSH conjugate formed/minute/mg protein for tissue samples (Habig et al., 1974).

2.5. Neuroenzyme - Acetylcholinesterase (AChE)

The tissue samples from both treated and control larvae were weighed and homogenised in 0.1 mL of sodium phosphate buffer at pH 8 using a mortar and pestle. The homogenate was centrifuged at 5000 rpm for 30 minutes. An aliquot of 0.1 mL of the homogenate was added to a cuvette containing 2.6 mL of 0.1 M phosphate buffer, 0.1 mL of DTNB reagent, and 0.3 mL of distilled water. To this solution, 0.02 mL of acetylthiocholine iodide was added. The mixture was incubated at 25°C for 10 minutes in a water bath, and the absorbance was measured at 410 nm using a spectrophotometer. The absorbance change was recorded every 15 seconds, and the per-minute change was calculated. The specific activity of the enzyme was calculated as micromoles of substrate hydrolysed per minute per gram of enzyme (Ellman et al., 1961).

2.6. Osmoregulatory enzyme - Na^+/K^+ -ATPase

The Zuagg (1982) protocol was used to estimate the Na^+/K^+ activity, and the Guernsey and Edelman (1986) procedure was used to estimate the liberated inorganic phosphorus. The samples were prepared at 4°C . 100 mg of tissue from both treated and control larvae was homogenised in 2 mL of SEI buffer

(75 mM sucrose, 20 mM EDTA, 10 mM imidazole) and centrifuged at 1000 rpm for 10 minutes. The supernatant was then used to determine the specific activity of ATPase. The experimental and control cocktails for Na⁺/K⁺-ATPase2 contained 30 mM Tris, 10 mM NaN₃, 1 mM EDTA, 130 mM NaCl, 10 mM KCl, and 3 mM ATP. The ouabain-containing cocktail also included 3 mM ouabain along with all these reagents.

The pH of the cocktails was adjusted to 7.4. The assay medium was shaken and incubated at room temperature for 15 minutes. After incubation, the reaction was terminated by adding 1 mL of 10% TCA. The microfuge tubes were kept on ice for 30 minutes, then centrifuged at 2000 rpm for 10 minutes. The clear supernatant was collected to determine the inorganic phosphate. The absorbance was measured at 640 nm using a UV-visible spectrophotometer. Adenosine triphosphate activity was calculated from the inorganic phosphate liberated and expressed as nanomoles of inorganic phosphate liberated per minute per milligram of protein.

Statistics

Data assessment involved the utilisation of the Student's t-test for comparison. All statistical calculations and analyses were performed using SPSS version 20.0 on the Windows Operating System.

3. Results

3.1. Effect of *Clerodendrum infortunatum* on the activity of antioxidant enzymes

To evaluate the biochemical impact of the plant extract, the activities of key antioxidant and detoxification enzymes were quantified in whole-body homogenates of fourth-instar *Culex quinquefasciatus* larvae following a 24-hour exposure period. As illustrated in Figure 1, exposure to the extract significantly altered the larval enzymatic profile compared to the untreated control group.

A marked reduction was observed across all measured antioxidant enzymes; specifically, superoxide dismutase (Figure 1(A)), catalase (Figure 1(B)), and glutathione peroxidase (Figure 1(C)) activities decreased post-treatment. Conversely, the activity of glutathione S-transferase, a primary detoxification enzyme, exhibited a statistically significant increase in the treated larvae relative to the control (Figure 1(D)).

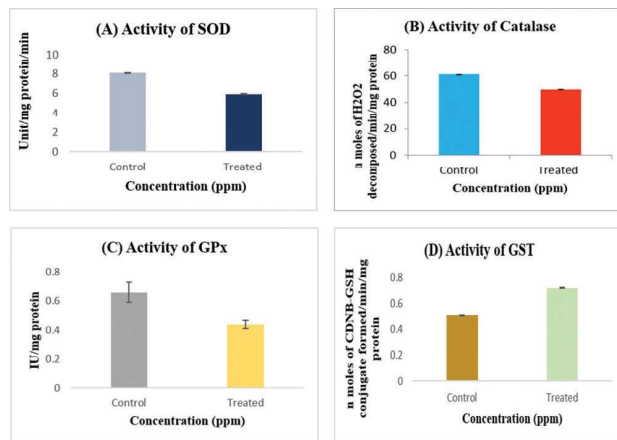


Figure 1: Effect of acetone extract of *Clerodendrum infortunatum* (300 ppm) on the activity of (A) SOD, (B) CAT, (C) GPx and (D) GST in the whole body of fourth- instar larvae of *Culex quinquefasciatus*. Each bar represents mean $\pm$ SEM six replicates ($p < 0.001$)

3.2. Effect of *Clerodendrum infortunatum* on the activity of non-antioxidant enzymes

To evaluate the neurotoxic and physiological disruptions induced by the plant extract, the activities of acetylcholinesterase (AChE) and Na⁺/K⁺-ATPase were quantified in the whole bodies of fourth-instar *Culex quinquefasciatus* larvae after 24 hours of exposure. Both essential enzymes exhibited a marked decline in activity post-treatment relative to the control groups.

Specifically, target-site acetylcholinesterase activity was distinctly suppressed following the 24-hour exposure period (Figure 2). Concurrently, a significant inhibition of the critical ion-transporting enzyme Na⁺/K⁺-ATPase was observed in the treated larvae compared to the controls (Figure 3)

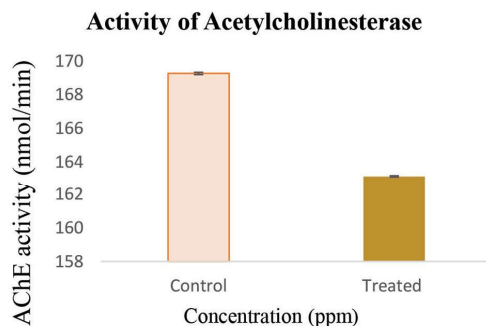


Figure 2: Effect of acetone extract of *Clerodendrum infortunatum* (300 ppm) on the activity of Acetylcholinesterase in the whole body of fourth- instar larvae of *Culex quinquefasciatus*. Each bar represents mean $\pm$ SEM of six replicates ($p < 0.001$)

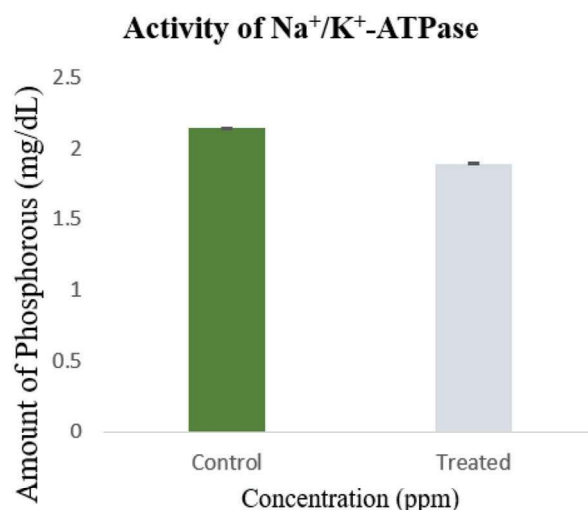


Figure 4: Effect of acetone extract of *Clerodendrum infortunatum* (300 ppm) on the activity of Na⁺/K⁺-ATPase in the whole body of fourth-instar larvae of *Culex quinquefasciatus*. Each bar represents the mean $\pm$ SEM of six replicates ($p < 0.001$)

4. Discussion

The *Culex quinquefasciatus* mosquito transmits a wide range of vector-borne diseases. To control mosquito-borne diseases, larval control with larvicidal agents is necessary. Plant-derived larvicides are preferred as a viable alternative for larval control. Phytochemical compounds act as toxicants against both adult and larval stages of mosquitoes; they also function as growth inhibitors, chemosterilants, repellents, or attractants. Natural products from different parts of plants are more effective at reducing mosquito populations (Elumalai et al., 2017). Diverse organisms have evolved a range of protective mechanisms to mitigate the effects of these natural products. Living organisms can be protected from various toxicants by robust antioxidant defence mechanisms. Insects, especially mosquitoes, have an efficient detoxification system to guard against reactive oxygen species (ROS). The enzymatic and non-enzymatic antioxidant activities present are sufficient to eliminate various kinds of ROS generated (Datkhile et al., 2009). In the present study, the major antioxidant enzyme GST increased relative to the control, whereas SOD, CAT, and GPx decreased in response to treatment with *Clerodendrum* extract, indicating oxidative stress. Due to oxidative stress, the production of free radicals increases when exposed to insecticides, inducing an imbalance in the antioxidant system (Sharifi-Rigi & Heidarian, 2019).

SOD protects mosquitoes from oxidative damage caused by ROS. Reduced SOD activity increases oxidative damage, compromising mosquito cellular

integrity and making them more susceptible to stressors, thereby decreasing survival. All eukaryotic cells, including yeast, plants and animals, possess superoxide dismutase. SOD activity decreased in the haemolymph of *Galleria mellonella* larvae exhibiting oxidative stress when treated with *Vairimorpha ephestiae* (Lozinskaya, 2004). When treated with alpha-terthienyl, SOD levels were markedly inhibited in the anal gills of *Aedes aegypti* larvae, indicating a buildup of ROS, as phytochemicals disrupt antioxidant enzymes, leading to cellular damage (Nivsarkar et al., 1991). Catalase, an important antioxidant enzyme, breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water. It is also linked to the suppression of apoptosis associated with oxidative stress (Nandi et al., 2019). Decreased CAT in the current study indicates oxidative damage induced by the plant extract. RNAi-mediated knockdown of Catalase resulted in reduced oviposition and lifespan in *Aedes aegypti* (Oliveira et al., 2017).

The current investigation demonstrated reduced GPx and increased GST in the treated larvae compared with the control after 24 hrs. Insects attempt to counteract the negative effects of toxic substances by expressing and activating detoxifying enzymes. When zinc oxide nanoparticles were used against *Culex pipiens* larvae, GPx levels decreased (Ibrahim et al., 2023). GPx reduces oxidative damage by neutralising hydrogen peroxide, enabling overall detoxification, and protecting the insect from insecticide exposure. A reduction in GPx activity causes ROS accumulation, resulting in oxidative tissue damage and impaired cellular functions, ultimately leading to cell death. Bioactive components in *Psidium guajava* extract modulate GPx levels against selected mosquito species (Aremu et al., 2024). GSTs sequester insecticides and reduce their effectiveness in almost all organisms. GSTs catalyse the reaction of the sulphhydryl group of the tripeptide glutathione with various xenobiotics. This sulphhydryl group reacts with electrophilic sites on xenobiotics, forming conjugates that are more readily excreted and typically less toxic than the parent insecticide (Labbe et al., 2011).

The increased GST activity amid declining antioxidant enzyme function suggests a compensatory shift towards xenobiotic conjugation and detoxification pathways, potentially overwhelming the larvae's intrinsic antioxidant defence system (Aly et al., 2023; Rajan & Puthur, 2021). In the present study, the increased GST levels may represent a defence mechanism against stress induced by the plant extract, thereby enhancing detoxification. This result is in agreement with studies conducted using methanol, ethyl acetate, chlorobenzene and hexane extracts of leaves of *Origanum syriacum*, *Pergularia tomentosa*, *Senna italica* and *Otostegia fruticosa* against third-instar larvae of *Culex pipiens*. When treated with these extracts at different time intervals, the GST activity increased in treated larvae, whereas the activity of AChE decreased compared to the control. Increased GST activity indicates activation of the detoxification system. Usually, GSTs and AChE act as

biomarkers of detoxification and neurotoxicity (Shahat et al., 2020). GST-based resistance appears to be associated with increased enzyme levels resulting from gene duplication or increased transcription rates (Labbe et al., 2011).

A reduction in AChE levels was observed in *Clerodendrum* extract-treated *Culex* larvae compared with the control in this study. Insecticides inhibit AChE in insect nervous tissue, resulting in excess acetylcholine at the synapse and leading to paralysis (Kim et al., 2008). Previous studies have shown that when different plant extracts were tested against *Culex pipiens*, AChE and GST activities decreased in larvae treated with *Lantana camara*, whereas other plant extracts showed elevated AChE and GST levels. AChE and GST play a significant role in stress physiology in plant extract-treated insects (Al-Solami, 2021). Studies on *Annona muricata* against *Aedes aegypti* exhibited decreased levels of Acetylcholinesterase and GST (Parthiban et al., 2020).

ATPases are a category of enzymes located on cellular membranes. Their primary function is to regulate ionic equilibrium. The Na⁺/K⁺-ATPase, also known as the sodium pump, couples the hydrolysis of ATP to the movement of 2 K⁺ into the cell and the expulsion of 3 Na⁺ out of the cell. This results in the development of an electrochemical gradient, which plays a fundamental role in maintaining cellular osmotic equilibrium (Pederson, 2007).

The genus *Clerodendrum* is widely recognised in traditional and modern pharmacognosy for its diverse phytochemical constituents. Phytochemical screening of *Clerodendrum infortunatum* revealed a distinct, tissue-specific distribution of secondary metabolites across different plant parts; notably, the leaves and flowers are highly enriched with polyphenols like Scutellarin, Apigenin, and Quercetin, alongside unique clerodane diterpenoids such as Clerodinin A and key fatty acid derivatives including linoleic acid (Mahedi et al., 2026). Furthermore, various phytochemicals act synergistically to disrupt insect metabolism, highlighting the plant's viability as a botanical pesticide.

In the present study, fourth-instar larvae of *Culex quinquefasciatus* treated with the acetone extract of *Clerodendrum infortunatum* showed reduced Na⁺/K⁺-ATPase activity compared with the control, indicating disruption of the electrochemical gradient and the onset of oxidative stress. Toxic secondary metabolites in the plant extract target a wide range of biomolecules, including enzymes, ion channels, nucleic acids, biomembranes, and other cellular components, thereby affecting insect metabolism (Ghosh et al., 2012). Changes in sodium and potassium ion fluxes affect the mosquito's physiological system (Ramsay, 1953). Comprehensive analysis of enzymatic alterations in *Culex quinquefasciatus* exposed to *Clerodendrum infortunatum* extract reveals significant metabolic disruption and oxidative stress, thereby highlighting the potent larvicidal properties of the botanical agent, which imposes a metabolic burden that compromises larval viability.

While the current study provides insight into the enzymatic disruptions caused by *Clerodendrum infortunatum* extract at 300 ppm for 24 hours, enzyme profiles do not always show a linear relationship with extract concentration, and their activities can fluctuate over time. As a result, the present study serves as a fundamental baseline for acute exposure at a sublethal concentration close to the LC_{50} level. To fully understand the complex mechanisms underlying these biochemical indicators, future research should include multi-concentration dose-response analyses as well as longer time-course studies.

Conclusions

Continuous use of synthetic insecticides imposes a negative impact on the ecosystem and leads to the emergence of insecticide resistance; therefore, plant-derived compounds are widely used nowadays. This study shows the effect of *Clerodendrum infortunatum* extract against the fourth-instar larvae of *Culex quinquefasciatus*. Following 24-hour treatment, significant enzymatic alterations were observed in treated larvae compared with controls, demonstrating the plant extract's insecticidal potential. Enzymes linked to oxidative stress, SOD, CAT, and GPx decreased, while GST increased, demonstrating oxidative damage and cellular stress, whereas neuroregulatory enzyme AChE and osmoregulatory enzyme Na^+/K^+ -ATPase reduced compared to the controls.

By elucidating enzymatic modulation of antioxidant and non-antioxidant defenses induced by *Clerodendrum infortunatum* extracts in *Culex quinquefasciatus* larvae, the current study addresses a critical gap in understanding the targeted mechanisms of this promising biopesticide, thereby advancing sustainable vector control strategies that avoid the pitfalls of synthetic insecticides. This investigation will contribute to the growing body of knowledge on plant-derived biopesticides, providing a scientifically validated foundation for developing environmentally safe, resistance-resistant control agents against vector mosquitoes. This study aligns with the urgent global need for novel, eco-friendly mosquito-control agents that address the limitations of conventional insecticides and promote sustainable public health interventions through integrated Mosquito Management (IMM) strategies.

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Author contribution

The research plan and article were authored by Sheenu A.S., while Ajitha V.S. coordinated data compilation and discussions. The final article has been reviewed and authorised by all authors.

Conflict of Interest

There are no conflicts of interest in the content of the paper or with the authors.

Abbreviations

AChE, Acetylcholinesterase; CAT, Catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5'-dithiobis (2- nitrobenzoic acid); EDTA, Ethylenediaminetetraacetic acid; GPx, Glutathione peroxidase; GSH, Glutathione-S-Transferase; H₂O₂, Hydrogen peroxide; NADH, Nicotinamide adenine dinucleotide; NaN₃, Sodium azide; ROS, Reactive oxygen species; SOD, Superoxide dismutase; SPSS, Statistical package of social sciences; TCA, Trichloroacetic acid.

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