Journal of Public Health International

Open Access - Peer-Reviewed - ISSN 2641-4538

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Research Article

Ameliorative Potential of Chlorogenic Acid on Rotenone-Induced Neurotoxicity in Drosophila Melanogaster Model

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Published citation: Oluwatoyin Adeyemo- Salami, Opeyemi Jamiu Afonja, Olamuyiwa Faosiyat Adeleke, Adeola Adedara, Amos O Abolaji (2021) **Ameliorative Potential of Chlorogenic Acid on Rotenone-Induced Neurotoxicity in Drosophila Melanogaster Model**. *Journal of Public Health International* - 4(3):22-33. https://doi.org/10.14302/issn.2641-4538.jphi-21-3993

Article DOI: 10.14302/issn.2641-4538.jphi-21-3993

Abstract

Chlorogenic acid (CA), abundantly found in green coffee beans, is a phenolic compound with antioxidant and anti-inflammatory properties amongst others. Exposure to rotenone, a natural pesticide, induces Parkinsonism (a type of neurodegeneration) through the induction of mitochondrial dysfunction and oxidative stress. Phytochemicals with antioxidant properties may be promising in attenuating this condition. In this research, the ameliorative role of CA on rotenone-induced toxicity in Drosophila melanogaster was evaluated.

Drosophila melanogaster (Harwich strain, 1- 3 days old) was used. 6 groups of five vials each with 50 flies/vial were exposed to CA (0; control (2% ethanol), 7.5, 15, 30, 45 and 60 mg/kg diet) for 28 days in the longevity analysis. A 28-day survival assay was carried out with rotenone (0, 250 and 500 µM). CA (30 mg/kg diet) was selected to evaluate its ameliorative potential on rotenone. For the study, the flies were divided into four groups of five vials each and exposed to CA and rotenone; Group A- control (2% ethanol), Group B- CA only, Group C- rotenone only and Group D- CA (30 mg/kg diet)+ rotenone (500 µM) for 7 days. Thereafter, the homogenate was evaluated for oxidative stress status, rate of emergence, negative geotaxis and acetyl cholinesterase activity.

CA (30 mg/kg diet) extended the lifespan of flies by 21.4%. Also, CA ameliorated rotenone-induced perturbation in catalase, glutathione-S-transferase and acetyl cholinesterase activities, total thiol and glutathione levels, and behavioral deficit ($p < 0.05$).

CA may have an ameliorative effect against rotenone-induced toxicity and Parkinsonism.

Keywords:
Drosophila melanogaster; chlorogenic acid; rotenone; Parkinson's disease; oxidative stress; acetyl cholinesterase

Article Information	
Running Title	ameliorative potential of chlorogenic acid
Academic Editor	Wang Wang, Department of Pediatrics the University of North Carolina at Chapel Hill.
Received Date	11 October 2021
Accepted Date	14 November 2021
Published Date	18 November 2021
Online	18 November 2021
Publication	Volume 4; Issue 3; Pages 22–33
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Introduction

Chlorogenic acid (CA) is a polyphenol from the family of hydroxycinnamic acid. It is one of the main polyphenols in the human diet with many health-promoting properties. CA can be found in foods and herbs such as coffee beans, apples, tea, grapes, tomato etc. [1,2]. Furthermore, it has been found that chlorogenic acid has antioxidant, anti-inflammatory, anticancer, antilipidemic, antidiabetic, antihypertensive and anti-neurodegenerative activities [3,4,5,6,7,8].

Rotenone (ROT) is a commonly used natural pesticide from the roots of *Derris elliptica* and *Lonchocarpus utilis*[9]. It can cross the blood brain barrier, due to its lipophilic property and causes neurotoxicity [10,11]. ROT blocks the mitochondrial electron transport chain through the inhibition of complex I which causes cytotoxicity due to altered redox cycling and oxidative stress [12]. It has been observed that high doses of ROT can induce generalized neurodegeneration; therefore, investigations have been carried out on low-dose systemic administration of this compound in rodents. In this condition, there is slow and specific loss of dopaminergic neurons and impaired mobility in animals treated with ROT, which are attributes of Parkinson's disease [13].

Physical aberrations characterized by Parkinson's disease (PD) (a type of neurodegenerative disease) include progressive impairment in mobility ability such as bradykinesia, tremor and rigidity. Studies have shown that PD resulted from the loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) and the formation of intra neuronal proteinaceous inclusions called Lewy bodies (LBs) in affected brain areas [14,15,16,17]. Moreover, the pathology involves protein aggregates apart from LBs, various neurotransmitters and extensive regions of the nervous system [18].

In this study, using ROT-induced toxicity in *Drosophila melanogaster* as a model for neurodegeneration, the ameliorative effect of CA was evaluated.

Materials and Methods

Chemicals

All chemicals were of analytical grade. ROT and CA were procured from AK Scientific, 30023 Ahern Ave, Union City, CA 94587, United State of America.

Drosophila Melanogaster Culture

Drosophila melanogaster (Harwich strain) obtained from the National Species Stock Centre, Bowling Green, Oklahoma, U.S.A., were maintained and reared in the Drosophila Laboratory, Department of Biochemistry, University of Ibadan, Oyo State, Nigeria on cornmeal medium containing brewer's yeast (1% w/v), agar-agar (1% w/v), and nipagin (preservative, 0.08% v/w) at constant temperature (23 ± 2 °C) under 12 h dark/light cycle.

Rotenone and Chlorogenic Acid Exposure and Survival Rate Analyses

In order to determine the appropriate concentrations and duration of exposure to ROT and CA to be used for the main experiment, *D. melanogaster* (both genders, 1 to 3 days old) were allocated into three (3) groups of five vials with 50 flies each and administered ROT (0- control(2% ethanol), 250, and 500 µM doses respectively) for 28 days for survival assay while another six (6) groups of five vials with 50 flies each were exposed to CA (0- control(2% ethanol), 7.5, 15, 30, 45, and 60 mg/kg diet respectively) for 28-day longevity assay. For both assays, daily mortality was recorded, and data were analyzed and plotted as percentage of live flies. The effect on antioxidant status of exposure of six (6) groups of five vials with 50 flies each to CA (0- control(2% ethanol), 7.5, 15, 30, 45, and 60 mg/kg diet respectively) was determined by assaying in the homogenate for catalase (CAT), glutathione-S-transferase (GST), hydrogen peroxide (H_2O_2), total thiol and reduced glutathione (GSH) after 7 days. Based on these data, 30 mg/kg diet of CA as well as 500 µM dose of ROT were selected for the study and used to determine the effect on biochemical parameters: acetylcholinesterase (AChE) activity (a marker for neurotoxicity) and oxidative stress-antioxidant status ((H_2O_2, total thiol, GSH levels, and CAT and GST activities), as well as the rate of emergence of offspring and negative geotaxis.

Determination of Negative Geotaxis and Rate of Emergence of Offspring

Locomotor performance of CA and ROT-treated flies was investigated using the negative geotaxis assay [19,20]. Emergence rate of *D. melanogaster* offspring after exposure and co-exposure to CA and ROT was evaluated as previously described by Farombi *et al*. [21].

Preparation of Sample for Biochemical Assays

For the determination of biochemical assays, 50 flies (of both gender)/ vial for 5 vials each per group were exposed as described in each of the following groups: Group A (control)-ethanol (2%), Group B- CA only (30 mg/kg diet), Group C- ROT only (500 µM) and Group D- 500 µM ROT + 30 mg/kg diet CA for 7 days. At the end of the treatment period, flies were anaesthetized using CO_2, weighed, and homogenized in 0.1 M phosphate buffer, pH 7.0 (ratio of 1 mg:10 ml), and centrifuged at 4000 g for 10 min at 4^{o}C in a Thermo Scientific Sorval Micro 17R centrifuge**.** Subsequently, the supernatants were separated from the pellets into labeled Eppendorf tubes and used for the determination of the activities of AChE, GST, CAT, and total thiol, H_2O_2 and GSH content. All the assays were carried out in duplicates for each of the five replicates.

Determination of Biochemical Parameters

Estimation of Protein Concentration

The concentration of protein was evaluated by the method of Lowry *et al.*[22]. The sample (25 µl, 1:10 dilution) was mixed with 135 µl of distilled water, and added to 400 µl of alkaline copper sulphate reagent (Lowry reagent) followed by 15 minutes incubation time. Folin ciocalteau solution (1:5 dilution) was added and they were then incubated at room temperature for 20 mins. The absorbance was measured at a wavelength of 660 nm against a blank and protein values were estimated from the standard curve of bovine serum albumin (BSA).

Estimation of Total Thiol Level

The method of Ellman [23] was used to determine total thiol content. The reaction mixture consists of 510 µl of 0.1 M phosphate buffer (pH 7.4), 20 µl of sample, 35 µl of 1 mM 5′,5′ – dithiobis- 2-nitrobenzoate (DTNB) and 35 µl of distilled water. The mixture was incubated for 30 mins at room temperature and the absorbance was measured at 412 nm.

Estimation of Hydrogen Peroxide Level

The level of H_2O_2 in the treatment was determined using the method of Wolff [24]. The reaction mixture consists of FOX 1 (10 ml of 100 mM xylenol orange, 50 ml of 250 mM ammonium ferrous sulfate, 10 ml of 100 mM sorbitol, 5 ml of 25 mM H_2SO_4 and 30 ml of distilled water) mixed with the sample. Incubation was carried out for 30 mins at room temperature and the absorbance was measured at 560 nm. The values, which were deduced from the standard curve, were expressed in micromole per milligram protein.

Estimation of Reduced Glutathione Level

The estimation of the level of GSH was carried out with the method of Jollow *et al.*[25] with little modifications. Sulphosalicyclic acid (4%) in the ratio of 1:1 was used to precipitate the supernatant. The samples were incubated for 1 h at 4°C and then centrifuged at 5000 rpm for 10 mins at 4°C. The reaction mixture consists of 550 µl of 0.1 M phosphate buffer, 100 µl of supernatant and 100 µl of DTNB. The absorbance was read at 412 nm and the results were expressed as moles of GSH/gram tissue.

Estimation of Glutathione-S-Transferase Activity

The method of Habig and Jakoby [26] was used to assess the activity of GST. The reaction mixture comprised of 270 µl of a solution consisting of 20 µl of 0.25 M potassium phosphate buffer, pH 7.0, with 2.5 mM ethylene diamine tetra acetic acid (EDTA), 10.5 ml of distilled water and 500 µl of 0.1 M GSH at 25 °C, 10 µl of 25 mM 1- chloro-2,4- dinitrobenzene (CDNB; as substrate) and 20 µl of sample (1:5 dilution). The mixture was monitored for 5 min with 10 s intervals at 340 nm using a spectrophotometer.

Determination of Catalase Activity

CAT activity was assessed with the method of Aebi [27]. The reaction mixture consists of 50 mM of phosphate buffer (pH 7.0), 300 mM H_2O_2 and sample (1:50 dilution). The loss in absorbance of H_2O_2 was monitored for 2 min at 240 nm. The result was used to calculate CAT activity and expressed as µmol of H_2O_2 consumed per minute per milligram of protein.

Determination of Acetyl Cholinesterase Activity

AChE activity was evaluated using the method of Ellman *et al.*[28]. The reaction mixture contained 135 µl of distilled water, 20 µl of 100 mM potassium phosphate buffer (pH 7.4), 20 µl of 10 mM DTNB, 5 µl of sample, and 20 µl of acetylthiocholine (8 mM). The reaction was monitored at a wavelength of 412 nm (for 5 min, 15 s intervals) in a SpectraMax microplate reader (Molecular Devices, USA). The data were expressed thereafter in µmol/min/mg protein.

Statistical Analysis

All of the experiments were replicated at least two times. The data are presented as the mean ± standard error of mean (SEM). To assess the significant differences among multiple groups under various treatments, One-way analysis of variance (ANOVA) was used. For all of the assays, differences with a p-value < 0.05 were considered statistically significant.

Results

Longevity and Selected Biochemical Indices in Flies Exposed to Chlorogenic Acid

Lifetime exposure to CA (7.5, 30, 45 and 60 mg/kg diet) increased longevity of flies by 3.57%, 21.4%, 14.3% and 7.1% respectively while there was a reduction (-3.57%) at the 15 mg/kg diet (Figure 1). In addition, CA maintained CAT (Figure 2A) and GST (Figure 2B) activities in most of the groups, and reduced H_2O_2 levels in the 15, 30 and 60 mg/kg diet (Figure 2C) after exposure to flies for 7 days when compared with the control flies ($p < 0.05$). CA (30, 45, and 60 mg/kg diet) concentrations maintained total thiol level in flies when compared with the control (Figure 2D). Moreover, 45 mg/kg diet of CA significantly increased GSH (Figure 2E) levels in flies when compared with the control group ($p < 0.05$). Generally, 30 mg/kg diet of CA prolonged longevity and maintained redox balance of the flies significantly ($p < 0.05$) when compared with the other groups and the control flies. Therefore, 30 mg/kg concentration was selected to investigate the ameliorative role on ROT-induced toxicity in the flies.

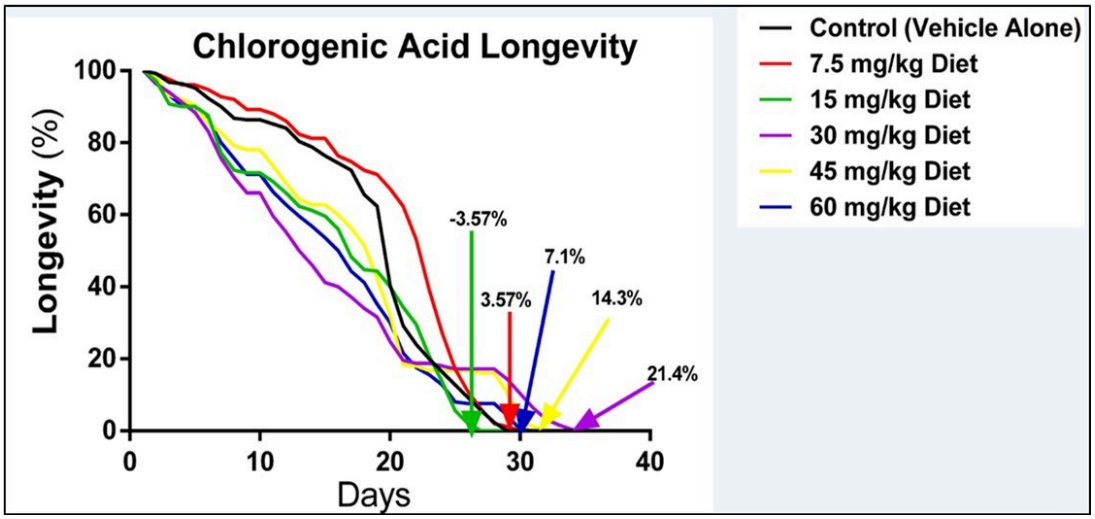


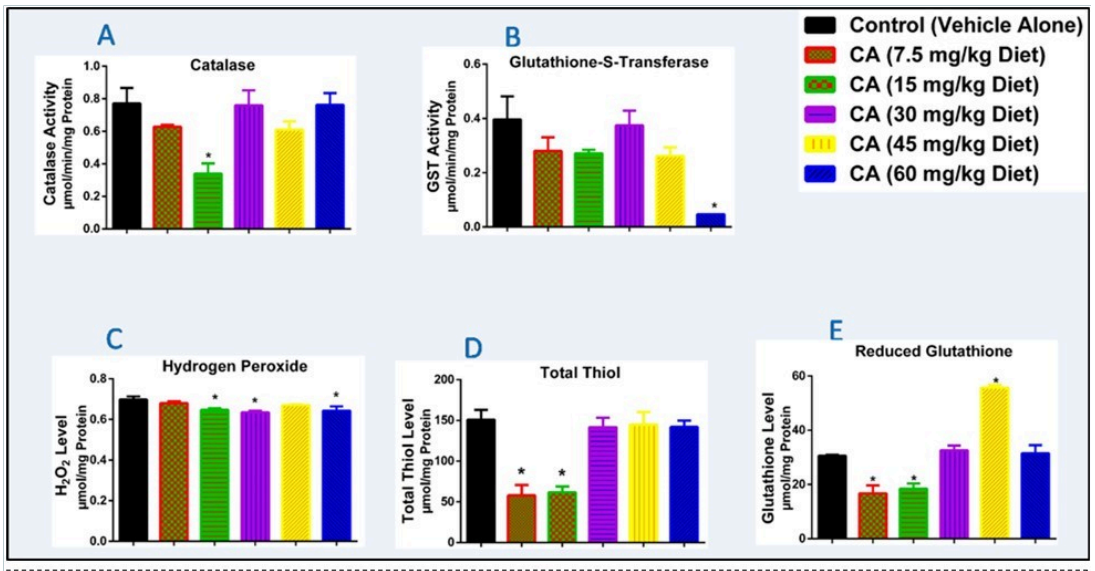
Figure 1. Influence of chlorogenic acid on longevity

Effects of Rotenone on Survival of Drosophila Melanogaster

Figure 3 shows the effects of ROT on survival of *D. melanogaster* after 28 days of treatment with 250 µM and 500 µM of rotenone concentrations. The group treated with 500 µM of ROT concentration shows an increased mortality rate when compared with the control ($p < 0.05$). Hence 500 µM concentration was chosen for the study.

Effects of Chlorogenic Acid on the Redox State of Rotenone Exposed Flies

Figure 4A shows the effects of CA and ROT on the H_2O_2 level in *Drosophila melanogaster* after 7 days of treatment. There was no significant effect upon administration of CA on the ROT-treated group when compared with control ($p < 0.05$). There was a significant elevation in CAT activity in the ROT-treated group which was ameliorated by CA (Figure 4B; $p < 0.05$). In addition, Figure 4C shows the levels of total thiol and GSH (Figure 4D), and GST activity (Figure 4E) in *D. melanogaster* exposed to ROT and



Values are expressed as mean ± standard error of mean of 50 flies/vial with 5 replicates per treatment group. CA- chlorogenic acid , *-significantly different from control at p < 0.05

Figure 2. Effect of chlorogenic acid on antioxidant status.
Values are expressed as mean ± standard error of mean of 50 flies/vial with 5 replicates per treatment group. CA- chlorogenic acid , *-significantly different from control at p < 0.05

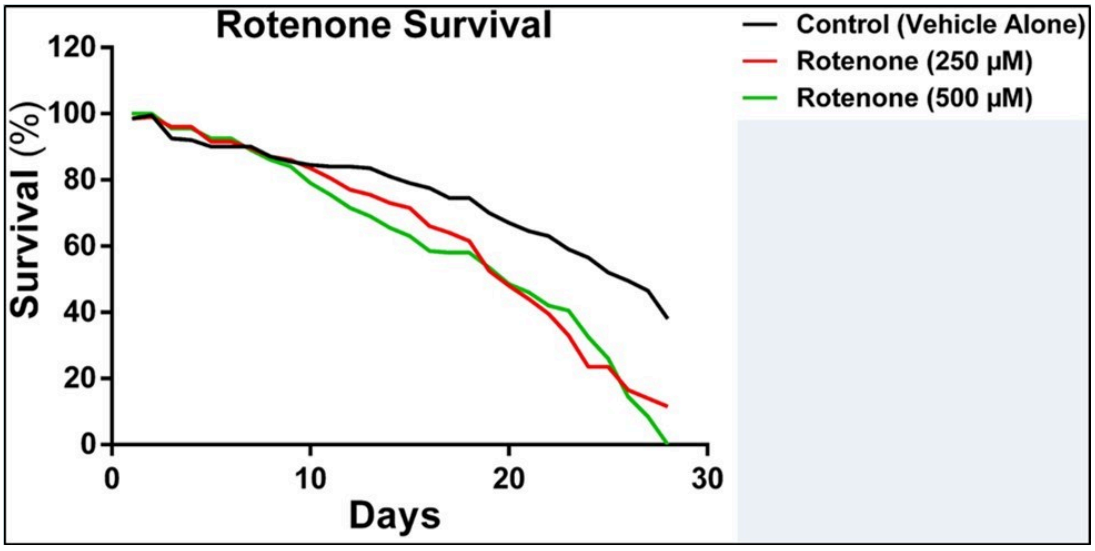
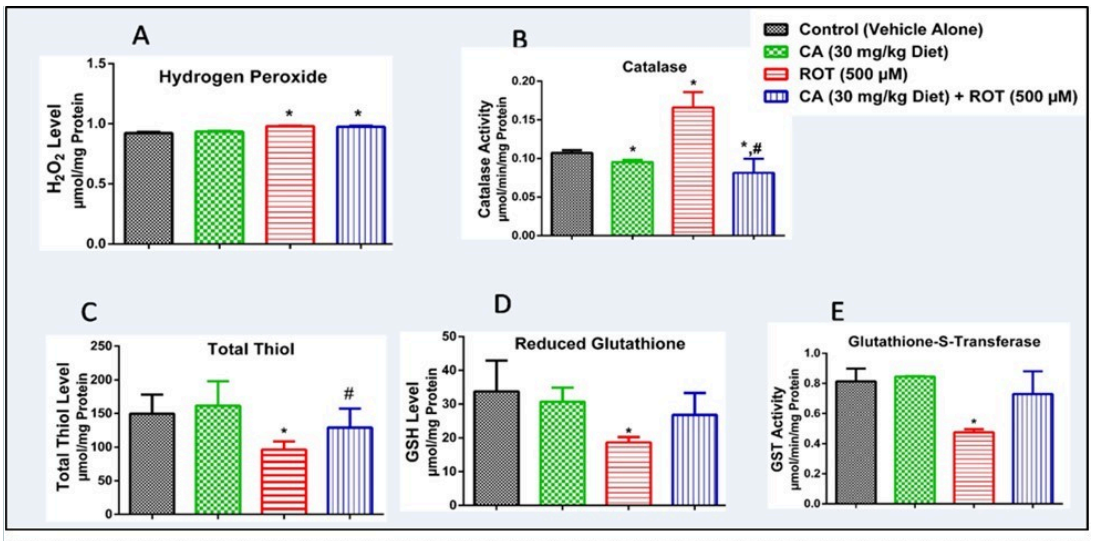


Figure 3. Influence of rotenone on survival of D. melanogaster after 28 days of treatment

CA. CA significantly mitigated ROT-induced decrease ($p < 0.05$) in the levels of total thiol and GSH when compared with the control ($p < 0.05$). Also, CA significantly increased ROT-induced inhibition of GST activity to levels comparable with the control ($p < 0.05$). Comparing the CA treated ROT group with the ROT only group, administration of CA significantly ($p < 0.05$) reduced CAT activity (Figure 4B) and significantly increased total thiol level ($p < 0.05$) (Figure 4C).

Acetyl Cholinesterase and Behavioural Assays

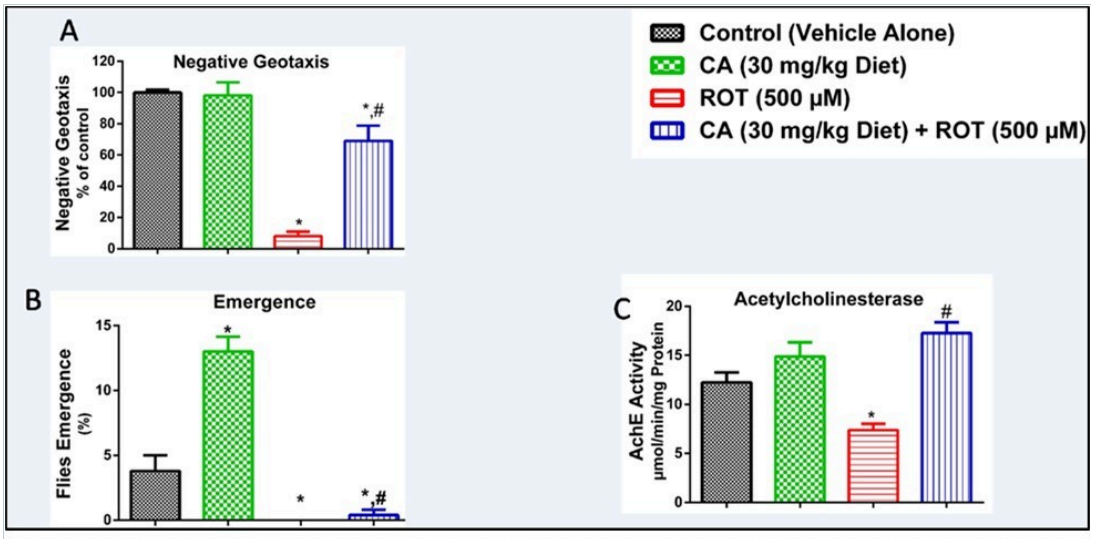
A significant reduction in the climbing rate of flies was observed (Figure 5A, $p < 0.05$) in ROT-treated flies after 7 days when compared with the control. However, CA reversed ROT-induced behavioural deficit depicted by the climbing rate ($p < 0.05$). In addition, ROT had a significant adverse effect on the fertility of flies; however, CA alleviated ROT-induced alteration in fertility depicted by eclosion rate after 7 days of treatment (Figure 5B, $p < 0.05$). CA also significantly ($p < 0.05$) alleviated ROT-induced inhibition of AChE activity to levels comparable to control (Figure 5C). Comparing the CA



Values are expressed as mean ± standard error of mean of 50 flies/vial with 5 replicates per treatment group. CA- chlorogenic acid, ROT- rotenone, *-significantly different from control flies at $p < 0.05$,

#-significantly different from rotenone-treated flies at $p < 0.05$.

Figure 4. Effect of chlorogenic acid on rotenone induced alterations on oxidative stress markers.



Values are expressed as mean ± standard error of mean of 50 flies/vial with 5 replicates per treatment group. CA- chlorogenic a cid, ROT- rotenone, *- significantly different from control flies at $p < 0.05$, #- significantly different from rotenone-treated flies at $p < 0.05$.

Figure 5. Influence of chlorogenic acid on negative geotaxis, flies' emergence and acetyl cholinesterase activity in rotenone-treated flies.
Values are expressed as mean ± standard error of mean of 50 flies/vial with 5 replicates per treatment group. CA- chlorogenic acid, ROT- rotenone, *- significantly different from control flies at $p < 0.05$, $^{\#}$- significantly different from rotenone-treated flies at $p < 0.05$.

treated ROT group with the ROT only group, administration of CA significantly ($p < 0.05$) increased the climbing and eclosion rates, and the AChE activity.

Discussion

In this study, it was demonstrated in *Drosophila melanogaster* that CA ameliorated the deleterious effects of ROT on AChE and GST activities, total thiol and GSH levels, and climbing and eclosion rates.

PD is an age-related neurodegenerative disease affecting more than 1% of the human population. It is the second most common neurodegenerative disorder with prevalence increasing with age, mostly among the population above 60 years [29]. The symptoms of PD include progressive impairment in locomotive ability, tremor, rigidity, and bradykinesia [17]. Currently, a combination of genetic changes and environmental factors has been observed to be responsible for this condition, though controversially [30,31]. Some of the environmental factors are the exposure to chemicals in pesticides and herbicides such as ROT, permethrin, MPTP (1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine), organochlorines, paraquat and 2, 4-dichlorophenoxyacetic acid [32,33,34]. In this study, ROT was used to induce Parkinsonism in Harwich strain of *Drosophila melanogaster*.

Currently, the modulatory role of several nutraceuticals to attenuate endogenous oxidative stress status has been considered as an effective approach to achieve neuroprotection [35,36,37]. As stated earlier, CA is a phenolic compound found in food of plant origin which has anti-diabetic, antioxidant, anti-inflammatory, anticancer, antilipidemic, and anti-neurodegenerative properties [3,4,5,6,7,8]. Moreover, studies have shown that consumption of diets rich in antioxidant property contribute to extension of lifespan in an organism [38]. The fly-survival and longevity experiment based on CA showed that among the concentration range tested (7.5- 60 mg/kg diet), 30 mg/kg of CA was the most effective (Figure 1). Therefore, in regard to the ingestion of CA, moderate levels would be more beneficial to increase longevity. Moreover, CA significantly reduced the level of H_2O_2 with the 30 mg/kg diet and did not show any adverse effect on the activities of CAT and GST as well as the levels of total thiol and GSH (Figure 2).

In this study, it was observed that 28-day exposure of *D. melanogaster* to ROT elicits alterations in the levels of antioxidant enzyme activities (such as CAT and GST), perturbations in reduced total thiol and GSH levels, increased H_2O_2 level, induced behavioural deficit as well as decreased AChE activity. All the aforementioned detrimental effects observed (with the exception of H_2O_2 level) were mitigated by CA (Figures 4 and 5).

CAT catalyzes the conversion of H_2O_2 to water (H_2O) and oxygen. The most common one is a monofunctional heme-containing enzyme [39]. It is an intracellular antioxidant enzyme that protects biological macromolecules from oxidation by H_2O_2[39]. This study shows an elevated CAT activity in flies exposed to ROT which was mitigated to levels comparable to control by CA (Figure 4B). This reveals the modulatory role of CA in obtaining an optimum level of H_2O_2 molecule in the cell, which is also essential in cellular signaling and this is supported by the observation with the CA diets (Figure 2C).

H_2O_2 is a reactive oxygen species (ROS). Elevated levels of H_2O_2 indicate that a system is plagued by oxidative stress [39]. In this study it was observed that there was a reduction in the level of H_2O_2 in flies treated with CA alone. However, ROT caused a significant accumulation of H_2O_2 which was mitigated, but not significantly, by CA (Figure 4A). Antioxidants play protective roles by scavenging reactive oxygen species and reducing the insults of oxidative damage [40]. Although CA did not significantly reduce H_2O_2 level in the co-administered flies, the reduction in CAT activity upon co-administration shows that CA has an ameliorative effect on ROT-induced toxicity but was not distinct enough on H_2O_2 level upon conclusion of the experiment.

Total thiols (protein and non-protein thiols) defend the system against oxidative damage [41]. GSH functions as part of the first line of antioxidant defense system in living organisms. In conjunction with GSH-dependent enzymes, it helps to block the propagation of free radical chain reactions and detoxify deleterious ROS [42]. GSH is an important antioxidant in living organisms that prevents oxidative damage to cellular components [42,43,44]. Depletion of cytosolic GSH in neurons is one of the hallmarks in PD [45]. The decrease in levels of total thiols and GSH in the ROT challenged group evidenced from our study suggests a compromised defense system in *D. melanogaster*. However, this study shows that CA modulates ROT-induced reduction of thiol containing compounds, thus confirming its anti-oxidative capacity. Hence, CA may be an ameliorative agent against diseases associated with altered redox state of a system.

GST is a major phase II detoxification enzyme, which comprises a large and multi-functional enzyme family primarily involved in the detoxification of endogenous substrate by catalyzing the conjugation of the nucleophilic sulphur atom of GSH with a variety of reactive electrophilic exogenous compounds [34,46,47]. The result from our study shows that CA protected against ROT-induced inhibition of GST activity. Since GST catalyses the conjugation of GSH with electrophilic molecules, GSH was rapidly used up in order to protect the flies. Therefore, the ameliorative mechanism of CA in ROT-induced toxicity may be by further enhancing activity of thiol-containing proteins like GST.

AChE is a vital enzyme of the cholinergic system that modulates learning, sleep and wakefulness, memory, attention, stress response and locomotor activities. It hydrolyzes acetylcholine to choline and acetate thereby terminating cholinergic neurotransmission between synapses [48,49]. Our study revealed that CA significantly alleviated ROT-induced inhibition of AChE activity when compared with control. This finding suggests that the modulatory action of CA on AChE activity may improve cognitive impairment evident in neuro-related diseases.

In the negative geotaxis assay, it was observed that ROT decreased the climbing rate of the flies but the effect was mitigated in groups co-treated with CA. This shows that CA has an ameliorative potency against bradykinesia, which is one of the effects of ROT toxicity and a hallmark in PD. This is similar to the study conducted by Farombi *et al*. [21], in which kolaviron was able to modulate the effect of ROT in flies.

Flies' emergence was highly increased in the group treated with CA only but a barren effect was recorded in the group treated with ROT only as compared with the control group. Also, substantial flies' emergence was observed in the co-administered group treated. This shows that CA may have a highly ameliorative effect against reproduction deficit common in PD patients. This is in line with the

study conducted by Namula *et al*. [50] in which CA, in addition to caffeic acid, was able to improve the quality of frozen-thawed boar sperm [50].

Conclusion

The present study demonstrates that chlorogenic acid showed substantial ameliorative potential on rotenone-induced Parkinsonism in *Drosophila melanogaster* as shown by the reversal of the detrimental effects of rotenone on the levels and activities of total thiol, reduced glutathione, glutathione-S-transferase and acetylcholinesterase. The negative geotaxis and developmental assays further buttressed the ameliorative effect of chlorogenic acid. These findings indicate that chlorogenic acid might have therapeutic effects for Parkinson's disease. Hence, majorly consumed home diet should be fortified with chlorogenic acid or there should be an adequate intake of foods rich in chlorogenic acid to prevent or modulate the effect of rotenone exposure in our environment as well as delay or possibly halt the onset of neurodegenerative diseases such as Parkinsonism.

Data Availability Statement (DAS)

The data is available upon request from the Corresponding author.

Abbreviations

CA- chlorogenic acid, ROT- rotenone, PD- Parkinson's disease, SNpc- substantia nigra pars compacta, LBs- Lewy bodies, AChE- acetylcholinesterase, DTNB- 5′,5′ – dithiobis- 2- nitrobenzoate, GST- glutathione - S - transferase, CAT-catalase, CDNB-1- chloro-2,4-dinitrobenzene, EDTA- ethylene diamine tetra acetic acid, GSH- reduced glutathione, ROS- reactive oxygen species, MPTP- 1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine.

Competing Interests

The authors declare that there is no conflict of interest.

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