

Coconut milk ameliorates motor and non-motor deficits in the alpha-synuclein transgenic *Drosophila melanogaster* model for Parkinsonism

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Abstract

Context: Parkinson's disease (PD) is a multisystem neurodegenerative disorder associated with oxidative stress and disrupted mitochondrial function. It is characterized by motor and non-motor symptoms due to multi-factors. Coconut (*Cocos nucifera*) milk contains a complex mixture of highly nutritional constituents such as carbohydrates, vitamins, and minerals which have remarkable health benefits. Lauric acid (LA) which makes up 54.5% of the total nutrient composition of coconut milk (CM) possesses strong antioxidant properties. **Objectives:** This study evaluated the effect of CM on PD-associated motor and non-motor deficits in alpha-synuclein (α -syn) transgenic *Drosophila melanogaster* (*D. melanogaster*). **Methods:** W^{1118} strain (control) and α -syn transgenic (PD strain) of *D. melanogaster* aged between 1 to 3 days were randomized into various groups. The control flies were fed on untreated diets, while the PD flies were orally exposed to varying concentrations (0, 5, 10, and 15% v/v) of the coconut milk diet for 14 days. Subsequently, longevity, behavioral, fecundity, and biochemical analyses were conducted across the groups. **Results:** The results showed that CM significantly increased longevity, climbing ability, egg count, and the rate of emergence ($P < 0.05$). In addition, MDA and NO levels as well as AChE activity were significantly decreased, while GSH levels alongside SOD and CAT activities were increased ($P < 0.05$) in the CM-treated PD flies in a concentration-dependent manner. **Conclusions:** CM ameliorated PD-associated deficits in *D. melanogaster* by prolonging lifespan, and improving locomotor function, fecundity, and redox status.

Keywords: Alpha-synuclein, Coconut milk, *Drosophila melanogaster*, Fecundity, Non-motor dysfunction, Parkinson's disease.

1. Introduction

PD is a devastating neurodegenerative disorder that affects various body systems. It is characterized by various motor and non-motor deficits (Jellinger, 2014). The primary underlying pathophysiology for PD is the degeneration of dopamine-producing neurons in the substantia nigra pars compacta region of the midbrain resulting in alterations of neurotransmitter pathways in the basal ganglia motor circuit (Johnson et al., 2009). It is becoming clearer that PD should no longer be regarded as merely a complex motor disorder characterized by dopamine deficiency, but as a progressive multisystem disease with several neural and non-motor deficiencies

(McIntosh and Wilson, 2018). Among the PD-associated non-motor deficits are reproductive health deficits which may be due to hypothalamic dysfunction associated with decreased libido (Sakakibara et al., 2012). While the mechanisms involved in the etiopathology of PD are not yet clearly understood, neuroinflammation, oxidative damage, mitochondrial dysfunction, and aggregates of α -syn (forming Lewy bodies) traceable to genetic and environmental predisposition have been reported (Gökçal et al., 2017).

Mutations in the α -syn gene such as amino acid substitutions, duplications, and triplications occurring in early-onset of familial PD are causative of dominantly inherited forms of PD (Muñoz-Soriano and Paricio, 2011; Siddiqui et al., 2016). Although there is no known α -syn homolog in *D. melanogaster*, overexpression of transgene of wild-type and mutant α -syn in the flies has reproduced major features of PD, such as Lewy body-like aggregation and locomotor behaviour abnormality (Lu and Vogel, 2009).

D. melanogaster commonly called the fruit fly has been in use for many decades in behavioural and biomedical research due to its strong homology with humans. Owing to its high fecundity, short life cycle, and low cost of maintenance, it has been considered reliable as a model subject for study human neurodegenerative diseases such as PD (Hirth and Targets, 2010; Lee et al., 2014).

The standard drugs for the treatment of PD (such as levodopa) have serious side effects and are only effective for few years, and hence the need for a natural and safer remedy (Sveinbjornsdottir, 2016; Zhuo et al., 2017). CM is known to possess a high content of lauric acid (Suryani et al., 2020). While coconut extract contains other nutrients, LA makes up 41% – 54.5% of its total nutrient composition (Fernando et al., 2015; Suryani et al., 2020). Virgin coconut oil which has the same LA content as CM has also been reported to have a high content of active polyphenol compounds, which are powerful inhibitors of lipid peroxidation (Dosumu et al., 2021). Therefore, this study was designed to investigate the effect of CM on parkinsonisms in α -syn *D. melanogaster* model through the evaluation of longevity, locomotor functions (negative geotaxis), rate of reproduction (fecundity) and biochemical assays for redox status (including malondialdehyde, nitric oxide, glutathione, and acetylcholine esterase, superoxide dismutase and catalase enzyme activities).

2. Materials and Methods

2.1 Preparation of coconut milk

Coconut fruits were purchased from Mushin market, Lagos, Nigeria. The Coconut fruit samples were authenticated at the Botany Department of the University of Lagos, Nigeria. To prepare the CM, the fruits were cracked open to remove the meat, which was washed with clean water and chopped into small pieces. A hundred grams (100g) of the sliced coconut meat was blended in 200ml of distilled water using an electric blender. Thereafter, the ground mixture was sieved with cotton sieve, and the filtrate (CM) was used in the experiment (Benjamin et al., 2017; Dosumu et al., 2021). The extract was freshly prepared for every day of diet preparation throughout the experiment.

2.2 *Drosophila melanogaster* stock and culture

Ethical clearance for this study was obtained from the Animal Care and Use Research Ethics Committee of the College of Medicine, University of Lagos, Nigeria.

Genetically modified strains of *D. melanogaster* (W^{1118} , DDC-GAL4, and UAS-synuclein) were obtained from the Department of Pharmacology and Therapeutics, University of Lagos, Nigeria. All the flies were from Bloomington *Drosophila* Stock Center, Indiana, USA. The flies were cultured on a standard *Drosophila* diet containing corn meal, agar, and yeast at a constant temperature of 25°C under standardized conditions (12 hours/12 hours light/dark cycle) and received adequate care in the *Drosophila* laboratory of the Department of Anatomy, University of Lagos, Nigeria.

2.3 *Drosophila melanogaster* strain crosses

To produce flies with PD features, the methods of D'Souza *et al* (2022) and Mostoufi and Singh (2022) were adopted with modifications. Virgin females of the DDC-GAL4 strains were harvested within 1 - 3 hours as they emerged in the vials, and males of UAS-synuclein strains were introduced to the virgin females of DDC-GAL4 in a ratio of 1 to 3 (1:3; male/female). The offspring (expressing a mutant form of human α -syn in neurons) were referred to as PD flies.

2.4 Experimental Design

Flies of about 1 - 3 days old were firstly divided into two groups (CTR and PD flies). The PD flies were further randomized into four groups (PD, PD/CM5, PD/CM10, and PD/CM15) of 40 flies each with five replicates per group. The control group (CTR) was exposed to an untreated diet, the untreated PD group (PD) was also exposed to an untreated diet, while the CM-treated PD groups (PD/CM5, PD/CM10, and PD/CM15) were exposed to 5, 10 and 15 % CM diets respectively. The flies were transferred to a freshly prepared diet every other day and the administration was continued for 14 days. Subsequently, the total number of deaths was recorded (for longevity assay) and a climbing assay (negative geotaxis) was conducted, after which the flies were anesthetized by freezing, weighed, and homogenized in 0.1M phosphate-buffered saline (pH 7.2) at a ratio of 1 mg:10 μ L, and cryo-centrifuged (4°C) at 2500rpm for 15 min (Abolaji *et al.*, 2018). The supernatant was then transferred into labeled Eppendorf tubes, and used for the evaluation of biochemical parameters including malondialdehyde (MDA), nitric oxide (NO), and glutathione (GSH) levels, as well as acetylcholine-esterase (AChE), superoxide dismutase (SOD) and catalase (CAT) activities. Fecundity assay was also conducted by counting the number of eggs laid and the rate of offspring emergence from the vials.

2.5 Longevity assay

The determination of longevity was carried out according to a procedure described by Abolaji *et al* (2018) with some modifications. The number of deaths in each vial was recorded daily throughout the experiment. The number of survivors was calculated as a difference between the number of deaths and the initial total fly numbers. The data were collected, analyzed, and plotted as a percentage of live flies.

2.6 Assessment of negative geotaxis assay

To conduct a locomotor performance test, twenty flies from each group were placed into a parafilm-covered vertical tube and allowed to acclimatize for 15 minutes. After which the tube was turned upside down, and the flies were carefully tapped to the bottom of the test tube. The number of flies that climbed above 17.5cm height of the tube within 8 seconds and the flies below the mid mark was recorded. The scores represented the mean of the number of flies at the top (n_{top}) expressed as a percentage of the total number of flies (n_{tot}). This procedure was repeated three times at 1 min intervals (Farombi *et al.*, 2018).

2.7 Fecundity test

To conduct fecundity assays, 1 - 3 days-old flies (15 males and 15 females/vial, $n = 5$) were exposed to CM as previously described in the grouping of the flies. The flies were allowed to lay eggs on the medium for 24 hours before transferring them to a fresh medium. Eggs laid per vial were counted daily for 7 days. Subsequently, the number of flies emerging from the vials were counted for 14 days (Farombi *et al.*, 2018; Hu *et al.*, 2019).

2.8 Determination of malondialdehyde levels

To determine MDA levels, the supernatant from the homogenate was spectrophotometrically analyzed following the method described by Karatas *et al* (2002) with some modifications. The eyes of flies were previously removed to avoid the interference of eye pigments with MDA absorbance. Results were expressed as μ mol/ml (mean \pm SEM).

2.9 Measurement of nitrite and nitrate as indices of nitric oxide production

The level of nitric oxide (NO) was quantified using Griess reagent (1.5% sulphanilamide and 0.15% N-1-naphthyl-ethylene diamine in 1 NHCl), based on the enzymatic conversion of nitrate to nitrite. Samples were

mixed with Griess reagent in a ratio of 1:1, and nitric oxide level (nitrite and nitrate) was quantified from the sodium nitrate standard curve measured at 595 nm (Eleftherianos et al., 2014).

2.10 Determination of glutathione levels

The level of the reduced form of glutathione (GSH) was estimated according to the method described by Sedlak and Lindsay (1968). One millilitre (1.0ml) of supernatant was treated with 0.5ml of Ellman's reagent (19.8mg of 5,5-dithiobisnitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate) and 3.0ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read by spectrophotometry at 412nm. $\Sigma = 1.34 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

2.11 Determination of acetylcholine esterase activity

AChE activity determination was conducted as described by Ellman et al (1961). The reaction was monitored at 15-sec intervals for 5 min at 412 nm spectrophotometry. The data were calculated against blank and sample blank and the results were corrected with the protein content.

2.12 Determination of superoxide dismutase activity

SOD activity assay was carried out according to the method of Sun and Zigma (1978). This is based on the ability of SOD to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480nm. The reference cuvette contained 2.95 ml buffer, 0.03 ml of the substrate (epinephrine), and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 min. $\Sigma = 4020 \text{ M}^{-1} \text{ cm}^{-1}$.

2.13 Determination of catalase activity

Catalase activity was determined as described by Aebi (1984). The decrease in absorbance of H_2O_2 ($\Sigma = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) at a wavelength of 240 nm was recorded at an interval of 10 sec for 2 min, using a SpectraMax plate reader. The activity of catalase was then calculated and expressed in μmol of H_2O_2 consumed/min/mg of protein.

3. Results

3.1 CM increased longevity and improved locomotor function in *D. melanogaster* model of PD

There was a statistically significant ($p < 0.001$) decrease in the number of survivors flies in the untreated PD flies compared to the control (Figure 1A). PD/CM5 showed no significant difference compared to untreated PD flies. However, PD/CM10 and PD/CM15 showed a statistically significant ($p < 0.05$, $p < 0.01$) increase in the number of survivors compared to untreated PD flies. The results of the locomotor assay showed a statistically significant ($p < 0.05$) decrease in the climbing ability in all the treated and untreated PD flies compared to the control (Figure 1B). When compared with the untreated PD flies, the CM-treated flies showed a significant increase ($p < 0.05$; $p < 0.01$; $p < 0.001$) in climbing activity in a concentration-dependent manner.

3.2 CM improved fecundity in α -synuclein *D. melanogaster* model of PD

In the fecundity assay, there was a statistically significant ($p < 0.01$) decrease in some eggs laid in the untreated PD group compared to the control (Figure 1C). The untreated PD group also showed a statistically significant ($p < 0.001$) decrease in the number of newly emerged flies compared to the control (Figure 1D). Furthermore, there was a statistically significant ($p < 0.05$, $p < 0.01$) increase in the number of eggs laid (in a concentration-dependent manner), and a statistically significant ($p < 0.001$) increase in the number of newly emerged flies in all CM-treated groups compared to the untreated PD group. There was no statistically significant difference ($p < 0.05$) between the control and all CM-treated groups in either egg count or offspring emergence.

3.3 CM improved oxidant/antioxidant status in *D. melanogaster* model of PD

The increase in the levels of MDA in all the PD groups (treated and untreated) was statistically significant ($p < 0.001$, $p < 0.01$, $p < 0.05$) when compared to the control group (Figure 2A). Likewise, there was statistically significant increase in NO levels in the PD ($p < 0.001$), PD/CM5 ($p < 0.05$) and PD/CM10 ($p < 0.05$) groups compared to control (Figure 2B). Moreover, the PDCM15 group showed a numerical but not statistically

significant increase in NO level compared to the control. Contrarily, all CM-treated groups showed a statistically significant decrease in MDA ($p < 0.001$) and NO ($p < 0.001$, $p < 0.01$) levels compared to the untreated PD group. Moreover, there was a statistically significant ($p < 0.001$, $p < 0.01$) decrease in GSH levels across the groups compared to the control (Figure 2C). While all CM-treated groups showed statistically significant increases in GSH ($p < 0.001$) levels compared to the untreated PD group.

3.4 CM regulates enzymatic activities in *D. melanogaster* model of PD

There was a statistically significant ($p < 0.001$) decrease in SOD and CAT activities simultaneously with a significant increase ($p < 0.001$) in AChE activity in all the PD flies compared to the control (Figure 2D - F). However, all CM-treated groups showed statistically significant increase in SOD ($p < 0.001$, $p < 0.01$) and CAT ($p < 0.001$) activities with a significant ($p < 0.001$, $p < 0.01$) decrease in AChE activity compared to the untreated PD group.

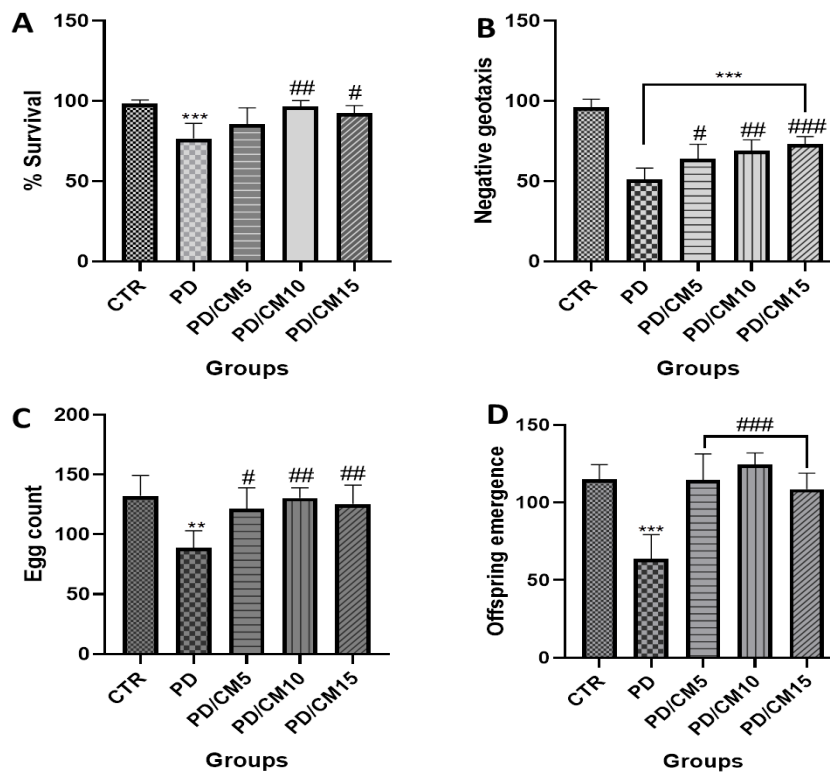


Figure 1: Longevity test (A), Negative geotaxis test (B), Egg count (C), Emergence rate (D). Data is presented as Mean \pm S.E.M. at **: $p < 0.01$, ***: $p < 0.001$ compared to CTR; #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ compared to PD group (CTR: Control, PD: Parkinson’s disease, CM: Coconut milk).

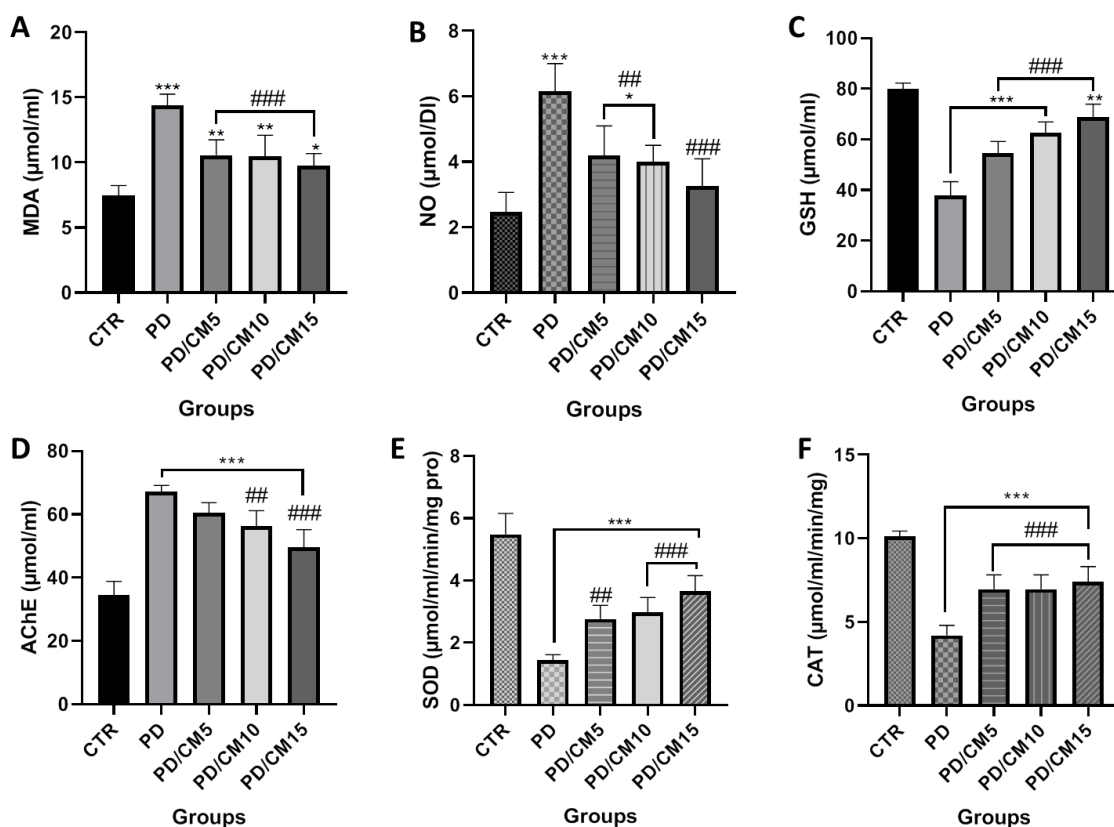


Figure 2: MDA (A), NO (B), GSH (C), AChE (D), SOD (E), CAT (F). Data is presented as Mean \pm S.E.M. at *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared to CTR; #: $p < 0.01$, ###: $p < 0.001$ compared to PD group (CTR: Control, PD: Parkinson’s disease, CM: Coconut milk).

3.5 Statistical analysis

The data were reported as mean \pm SEM. All the statistical analyses and graphs were done using Graph Pad Prism 8 software (Graph pad software Inc., USA). Multiple comparisons were done using a one-way ANOVA with Tukey’s test. Significance was set at $p < 0.05$.

4. Discussion

Mutations in the α -syn gene are one major cause of genetic forms of PD, with an associated increased risk of sporadic forms (Riederer et al., 2019). Transgenic *D. melanogaster* models expressing the mutant form of α -syn are proven to faithfully replicate many features of human PD (Mizuno et al., 2011). Phytochemical analysis has revealed that CM contains several nutritional constituents with remarkable health benefits (Boemeke et al., 2015). This study reveals for the first time as best known to us that CM plays an ameliorative role in α -syn-mediated toxicity in *D. melanogaster*.

CM (10 and 15 % v/v) notably prolonged longevity in α -syn transgenic *D. melanogaster*, this might be accounted for by the presence of polyphenols in CM (Boemeke et al., 2015). It is a fact that polyphenols can modulate several factors associated with aging (Russo et al., 2020). Moreover, in α -syn flies, ubiquitin has been shown to suppress the loss of dopamine-producing neurons and locomotor dysfunction and to extend life span (Lee et al., 2009). With this, it can be suggested that CM possesses ubiquitin-modulating properties.

PD is characterized by progressive death of dopaminergic and cholinergic neurons along with aggregation and accumulation of α -syn (forming Lewy bodies) in the central nervous system (CNS), clinically manifesting through several motor disorders (Ilie et al., 2020; Poewe et al., 2017). From the result of this study, the PD flies showed significantly decreased climbing ability (locomotor deficit) during the negative geotaxis assay. This may be due to over-expression, misfolding (into β -sheet) and aggregation of α -syn which thus inhibits the release of neurotransmitters (such as dopamine and serotonin) in vulnerable neurons (Bendor et al., 2013; Ordóñez, 2018). However, all CM-treated flies showed remarkably improved locomotor performance. Previous studies have shown that LA; a major content of CM plays an important role in behavioural modulation and protects against neuro-inflammation (Casertano et al., 2021). It can also undergo rapid metabolism to produce ketone bodies (an additional source of energy for the neurons) (Cunnane et al., 2016; Nafar et al., 2017). These potentials of CM may be partly responsible for the positive response in the motor skills recorded in the flies.

Although the pathogenic mechanisms involved remain yet unclear, α -synucleinopathies including PD have been recently linked to several reproductive health dysfunctions (Magerkurth et al., 2005; Sakakibara et al., 2012). Ragonese et al. (inconclusive), reported a relationship between PD and the factors that reduce estrogen stimulation during life, these support the speculation that endogenous estrogens may play a role in PD development (Ragonese et al., 2004). Overproduction of free radicals has also been reported to inhibit the synthesis of steroids, the development of follicles and cause a reduction in testicular mass (Degger et al., 2015; Izaguirry et al., 2017; Sun et al., 2016). The reduced fecundity (egg count and offspring emergence) in this study confirms a relationship between α -syn toxicity and reproductive dysfunctions in PD. CM however significantly improved fecundity in all the adjunct treated groups. This outcome may be traceable to the high polyphenol content of CM which has a modulatory effect on reproductive hormones (Pang et al., 2021). In addition, a previous study reported improved testosterone levels in Sprague Dawley rats treated with coconut oil (which has the similar chemical composition to coconut milk) (Dosumu et al., 2010). Sule et al., also reported that coconut milk improved semen motility in West African dwarf bucks (Sule et al., 2007). These findings suggest that CM may be of great importance in the maintenance of reproductive health.

As a well-known secondary product of lipid peroxidation, MDA has been largely used as a marker for cell membrane injury (Grotto et al., 2009). Overproduction and accumulation of NO have been linked to pro-inflammatory mediation (Eleftherianos et al., 2014). Increased AChE activity and degradation of acetylcholine have also been implicated in the progression of PD (Alabi et al., 2019). The present study showed that α -syn mutation increased MDA and NO levels concurrently with increased AChE activity in the PD flies. The elevated MDA and NO levels observed in this study might be associated with disruption of DNA integrity and tissue lipid components, and initiation of some inflammatory processes (Fu et al., 2017). While the upregulated AChE activity might result from dopaminergic neuronal death (Rao et al., 2016; Zhang et al., 2013) manifesting as loss of locomotor function in the PD flies. Interestingly, the elevated MDA, NO, and AChE were attenuated by the CM diet at all concentrations, and this might be accounted for by the neuro-modulating property of CM (Casertano et al., 2021).

GSH is the most plenteous and important antioxidant in living organisms that prevents damage to cellular components caused by free radicals, ROS, and peroxides (Dringen, 2000). Primarily SOD forms the frontline defense mechanism against ROS-mediated cell injury in living organisms (Kangralkar et al., 2010). CAT is a crucial antioxidant enzyme that breaks down hydrogen peroxide to produce water and oxygen, its deficiency is strongly linked to the pathogenesis of PD (Nandi et al., 2019). The depleted GSH levels and inhibited SOD and CAT activities observed in this study might be the consequence of the α -syn-mediated elevation of MDA and NO levels which remarkably disrupted the oxidant/antioxidant balance as observed in the PD flies. However, the depleted GSH level and inhibited activities of SOD and CAT which were consequences of α -syn toxicity were ameliorated in all the CM-treated groups. This confirms the antioxidant property of CM, attributable to its high LA content. Moreover, it may be suggested that the natural antioxidants in CM play an important role in mitochondrial bioenergetics. The ability of CM to improve GSH production can also be traced to its high content

of polyphenols which enhance the uptake of L-cysteine (the limiting substrate for GSH synthesis) (Dröge and Breitkreutz, 2000; Maher et al., 2008).

Although the exact mechanism of action of CM remains largely unclear, it is possible that the protective mechanism elicited is due to its high LA content which has free radical scavenging, antioxidant, anti-inflammatory, neuro-modulatory, and hormonal modulatory properties, and may as well boost the body's immune system (Kumar et al., 2022). Hence this study contributes to the existing knowledge of pharmacological importance of coconut milk and the mechanism underlying PD. In addition it reveals an insight into the importance of lauric acid as a potential therapeutic candidate which can be adopted for a translational research.

5. Conclusion

In conclusion, this study revealed that the CM diet ameliorated the detrimental effects of α -syn-mediated motor and non-motor deficits associated with PD in transgenic *D. melanogaster* by prolonging lifespan, improving locomotor function, fecundity, and redox status.

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