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Oleanolic Acid as a Therapeutic Agent in *Drosophila melanogaster* Model of Lead-induced Alpha-synucleinopathies

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ABSTRACT

Alpha-synucleinopathy, characterized by the abnormal clumping of alpha-synuclein, is associated with neurodegenerative conditions like Parkinson's disease. Lead, a widespread environmental contaminant, intensifies oxidative stress and promotes alpha-synuclein aggregation. Oleanolic acid (OA), a biologically active triterpenoid, is recognized for its antioxidant and anti-inflammatory qualities, yet its effectiveness against lead-induced neurotoxicity remains largely unexplored. This research investigated the protective capacity of oleanolic acid against lead-triggered alpha-synucleinopathies within a Drosophila melanogaster model. Drosophila melanogaster (Harwich strain, 1 -3 days old) were initially subjected to oral exposure of lead nitrate (10, 20, and 30 ppm) and oleanolic acid (50, 100, and 200 µM) under controlled conditions for 14-day survival assessments. Subsequently, oleanolic acid (100 µM) was selected to evaluate its therapeutic impact on lead (10 ppm)-induced alpha-synucleinopathies in D. melanogaster following 7 days of oral administration. Neurobehavioral tests, including crawling, climbing, and reproductive rates, were used to assess impairments in locomotion and development. Biochemical analyses quantified oxidative stress markers, enzyme activities, and antioxidant levels. Histological and immunohistochemical examinations were conducted to evaluate brain morphology and alpha-synuclein aggregation. The findings indicated that lead exposure significantly diminished survival, locomotor activity, and reproductive success. Biochemical indicators revealed elevated oxidative stress, decreased antioxidant levels, and increased enzymatic dysfunction. Histological studies demonstrated extensive neurodegeneration, while immunostaining confirmed alpha-synuclein aggregation. Conversely, OA treatment alleviated lead-induced effects, leading to improved survival, enhanced motor functions, and better reproduction. OA also re-established oxidative balance, reduced alpha-synuclein aggregation, and maintained brain histology. Oleanolic acid exhibits neuroprotective attributes by mitigating lead-induced alpha-synucleinopathies.

Keywords: alpha-synucleinopathies, oleanolic acid, neuronal dysfunction, Drosophila melanogaster, Lewy bodies

INTRODUCTION

Alpha-synucleinopathy encompasses a group of neurodegenerative conditions marked by the atypical buildup of insoluble protein aggregates within neuronal or glial cells.[1] For more than two decades, neurodegenerative disorders such as Parkinson's disease (PD), Dementia with Lewy bodies (DLB), Multiple System Atrophy (MSA), Pure Autonomic Failure (PAF), and REM sleep behavior disorder (RBD) have been associated with abnormal protein accumulations, including Lewy bodies (LB), Lewy neurites, and glial cell inclusions.[2-4] These neurodegenerative conditions, characterized by altered alpha-synuclein (aSyn) in neurons and/or glial cells, are collectively known as alpha-synucleinopathies. The presynaptic nerve terminal protein aSyn was first identified in 1988.[5] By 1997, this protein was discovered within Lewy bodies, a defining pathological feature of Parkinson's disease.[6] In the same year, a single point mutation in the aSyn gene (SNCA) was identified as the cause of autosomal-dominant PD.[7] Alpha-synuclein is a small (14 KDa) acidic protein found in neurons of the central and peripheral nervous systems, blood cells, and other organs[8]. Endogenous aSyn typically exists as a folded tetramer of approximately 58 kDa, exhibiting minimal propensity for amyloid-like aggregation, which contrasts with the conventional understanding of it as a 'natively unfolded' monomer.[9] Both forms coexist, though an imbalance favoring the monomer can promote pro-aggregating forms.[10] Lead (Pb) is a carcinogenic heavy metal primarily originating from human activities such as manufacturing, mining, and the combustion of fossil fuels. The accumulation of lead in living organisms can lead to various health issues, including appetite loss, headaches, hypertension, abdominal pain, renal dysfunction, fatigue, sleeplessness, arthritis, hallucinations, and neurodegenerative disorders.[11] The term "heavy metals" has diverse definitions based on specific criteria; generally, they are defined as metals with a density exceeding 5 g/cm³.[12] Lead exposure can harm any human organ (e.g., liver, brain, kidney), but its neurotoxicity is particularly pronounced in the brain, especially during early developmental stages[13]. Human studies have revealed significant gender differences in lead neurotoxicity, documented in research concerning spatial memory, motor behavior, brain gene expression, and dopamine metabolism. Despite long-standing reports of gender disparities in lead neurotoxicity, limited attention has been given to understanding the underlying mechanisms of lead in Alzheimer's disease.[13] A connection between lead exposure and PD has been established, notably in studies indicating that occupational lead exposure increases the risk of PD.[14] Lead promotes the upregulation of aSyn and influences apoptotic pathways linked to tau pathology.[15] The formation of proteinaceous inclusions in response to lead exposure highlights its potential role in the initiation and progression of aSyn pathology. [16,17]

Oleanolic acid (OA) is a naturally occurring compound extracted from various food and medicinal plants, frequently employed as an alternative or complementary treatment for chronic diseases. It is one of the bioactive phytochemicals found in certain plants. This pentacyclic triterpenoid is present in plants of the Oleaceae family, including the olive.[18,19] OA is commonly found in epicuticular waxes, where it acts as a protective barrier against infections and water loss (especially during dry seasons) in plants. Beyond its ecological functions in plants, OA has been associated with various pharmacological activities, such as antioxidant, anti-tumor, anti-inflammatory, anti-diabetic, and antimicrobial properties, demonstrated across several disease models.[20-22] However, to our knowledge, few studies have investigated the impact of lead on aSyn aggregation in cell and animal models, or the associated phenotypic changes. Therefore, this study aimed to comprehensively characterize the effects of lead exposure profiles in *Drosophila melanogaster* models of aSyn

pathology. Ultimately, this research seeks to explore the protective role of oleanolic acid against lead-induced alpha-synucleinopathies in the *Drosophila melanogaster* model.

MATERIALS AND METHODS

Animal use and maintenance

D. melanogaster (Harwich strain) was obtained from Drosophila Research Laboratory, University of Ibadan and were utilized in this study. Preparation of the diet was performed after the previously described[24] with some adaptations. They were maintained on a corn meal medium (3.08% w/v corn flour, 2% w/v brewer's yeast, 1% w/v agar-agar, 0.08% w/v methyl paraben, and 93.92% Distilled H_2O) at a relative temperature (22-25ºC) and constant humidity (60-70%) under a 12-hour dark/light cycle environment at Drosophila Research Laboratory, University of Ibadan.

Chemicals and materials

Lead nitrate (Pb (NO3) 2) and Oleic acid (OA) [CAS: 508-02-1] were purchased from Sigma, USA.

Experimental design

Flies treatments for survival analysis

To acquire the concentration with respect to the duration of treatment to lead and oleanolic acid, the flies (both genders, i.e., 3-day-old emergent) were divided into different groups of 30 flies each and administered lead (10ppm, 20ppm, and 30ppm), and oleanolic acid (50 μM, 100 μM, and 200 μM) for 14-day survival assays. Daily mortality was recorded, and data were analyzed and plotted as a percentage of live flies. Based on these data, 10ppm of lead and 100 μM of oleanolic acid were selected and exposed to flies for 7 days.

Treatment of *Drosophila melanogaster*

D. melanogaster 1-to 3-day-old emergent, 50 flies per vial (n = 4) were exposed to lead and oleanolic acid of selected concentrations for 7 days

Group A received a diet containing vehicle (2.0% distilled water)

Group B flies were exposed to a diet containing lead (10ppm)

Group C flies were fed with a diet mixed with lead and oleanoic Acid (10ppm and 100μM).

Neurobehavioral Analysis

Determination of negative geotaxis

Locomotion performance of lead and oleanolic acid-treated flies was investigated using the negative geotaxis assay method. Briefly, ten (10) lead+oleanolic acid and control flies were immobilized under mild ice anesthesia and placed separately in labelled vertical glass columns (length, 15 cm; diameter, 1.5 cm). After the recovery period (about 20 min), the flies were gently tapped to the bottom of the column. Following 6 s, the number of flies that climbed up to the 6 cm mark of the column, as well as those that remained below this mark, were recorded. The score of each group was the average of three trials for each group of treated and control flies.[25]

Rate of emergence of offspring

The emergence rate of D. melanogaster offspring after exposure to lead and oleanolic Acid was also evaluated. Thus, a day to three-day-old flies (15 males and females per treatment vial, where n = 4) were treated with the selected concentrations of lead and oleanolic acid for 24 hrs. Thereafter, all the flies were disposed of, and the embryos developed into adults. The pupa and emerged flies' numbers were then recorded over the duration of two weeks.[26]

Crawling activity

The crawling rate of the larvae was evaluated. A day to three days old flies (10 males and 10 females per vial, n = 4) were exposed to

selected concentrations of lead and oleanolic acid for 24 hrs. Thereafter, all the flies were removed from the vials, and the larvae were collected. The larvae were allowed to crawl around in a shaded petri dish for 5 minutes (the analysis was triplicate for each group). The crawled traces were then measured using ImageJ software.

Biochemical analysis

D. melanogaster, 1 to 3 days old, 50 models per vial (n = 4) were induced with lead and oleanolic Acid of selected concentrations for 7 days. The flies were collected into empty vials. Afterwards, models were immobilized with ice, measured, and then integrated in 0.1 M buffer, pH 7.4, and fractionated at 10,000g for 10 minutes at 4°C in a cold Mikro 220R centrifuge (CERID Laboratory, Ogbomoso, Oyo state). Then, surface fluid was aliquoted into named tubes, and utilized for evaluating Protein, Acetylcholinesterase (AChE), Mono amine oxidase-like (MAO) activities including total protein, Glutathione (GSH), Glutathione-S-transferase (GST)Total thiol (T-SH), Nitric oxide (NO, nitrate and nitrate) and Lipid Peroxidation (LPO) levels at Drosophila Research Laboratory, University of Ibadan. Protein content was measured using the method stated by Lowry et al.[27] Total thiol present was evaluated as described by Ellman, which built upon the formation of a relatively consistent (yellow) hue when Ellman's (5,5|-dithiobis-(2-nitro-bezoic acid) is added to sulfhydryl compounds. The coloured compound generated by the reaction of Ellman's reagent with the reduced sulfhydryl groups, which is 2-nitro-5-thiobenzoic acid, contains an absorption coefficient of 412 nm.[28] Glutathione and Glutathione-S-transferase activity was measured using the procedure of Habig and Jakoby, which relies on the fact that all known Glutathione-S-transferases exhibit a comparatively increased action with 1-chloro-2, 4-dinitrobenzene as the next reactant; therefore, the standard analysis for Glutathione-S-transferase action uses 1-chloro-2, 4-dinitrobenzene as the target molecule. When the compound combines with reduced glutathione, its wavelength moves to an extended wavelength of 340nm.[29] Evaluation of Acetylcholinesterase activity was carried out as described by Ellman et al., where thicholine generated by the activity of acetylcholinesterase produces a yellow color with 5, 5' - dithiobis (2-nitrobenzoyl acid). The deepness of the product color, measured at 412nm, is commensurate with the catalysis in the sample.[30] The quantity of nitrite in the overlaying liquid was quantified using the reaction method by Griess. The concentration of nitrite in the overlying liquid or in serum was calculated as described in the Griess reaction by incubating a sample with Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; 1% sulfanilamide in phosphoric acid; 1:1 purched] at room temperature and absorbance at 550 nm.[31] Lipid peroxidation level was measured following Hiroshi Ohkawa et al.'s method, in which the acidic state, malondialdehyde (MDA), obtained from oxidative damage of membrane fatty acid, and the reaction between the chromogenic compound, 2-thiobarbituric acid (TBA), with the food products produce a pink colored complex with maximum absorbance at 532 nm.[32] Monoamine oxidase activity was assayed according to the Tipton and Youdim method, in which the reaction of the sample with Benzylamine hydrochloride and Perchloric acid was centrifuged, and the absorbance was read at 280nm.[33]

Tissue processing of the brains of D. melanogaster

10% neutral buffered formalin (10% NBF) was used to fix the models in preparation for staining processes. The brains were dewaxed and prepared for H&E staining. The results were placed on slides and then viewed under a standard optical microscope and interpreted by seasoned experts, oblivious to the treatments administered [34]

Immunohistochemistry staining for a-synuclein

Brain sections (mushroom body and optic lobe) were carried out using an epitope exposure technique. The sections were briefly passed through 88% formic acid for twenty minutes, next was treatment in citric acid (pH = 6.0, at bp) for another twenty minutes. The sections were then kept outside at 25µC and washed through distilled water thrice and also through 3 washes of buffer solution. Sections were heated through 3% normal horse serum and 2% bovine serum albumin for about an hour, then re-incubated in the primary anti-a-synuclein (mouse anti-alpha synuclein, 1:500, Invitrogen) or 3-NT (Millipore Inc; 1:500) antibody for 48 hrs. at 4 °C. The sections were washed in a water bath, then incubated in the secondary antibody (biotinylated horse anti-mouse, 1:200, Vector Laboratories), phosphate buffer saline washes, and the ABC solution (ABC kit, Vectastain Elite, cat # PK-6100). After stain development, the sections were dried overnight, then cover-slipped with the aid of mounting media (23244257, Fisher). All immuno-stained slides were checked and analyzed for the presence and intensity of a-synuclein and 3-NT staining by a blinded rater.[35]

Statistical Analysis

All the experiments were replicated at least three times. The data were analyzed using GraphPad Prism version 9.0.0. (121). The data are shown as the Mean ± SEM. One-way Analysis of variance (ANOVA) was adopted to check the significant differences across all the groups under different treatments, followed by Tukey's post hoc and Fischer's LSD test where appropriate. A p-value of $p < 0.05$ was considered statistically significant. * Represent significance against the control group; # represent significance against the lead group. The crawling test was measured using segmented line measurement in the ImageJ software.

RESULTS

Survival, reproduction, and neurobehavioral results

Survival rate of D. Melanogaster exposed to lead and oleanolic acid for 14 days (Figure 1) showed a reduction in survival rate of D. melanogaster exposed to lead compared to the control group, as shown in Figure 1A. The decreases in survival were more pronounced in the higher concentrations L3 (30 ppm) than in the lower concentrations L1 and L2 (10ppm and 20 ppm). Flies exposed to oleanolic acid (Figure 1B) show an increase in survival rate for the first and second doses OA 1 and 2 (50 and 100 µM), while the third dose OA 3 (200 µM) appeared to reduce the survival rate compared to the control. Oleanolic acid compared to control, in the order C3, C1 and C2 (50, 100 and 200 µM respectively)

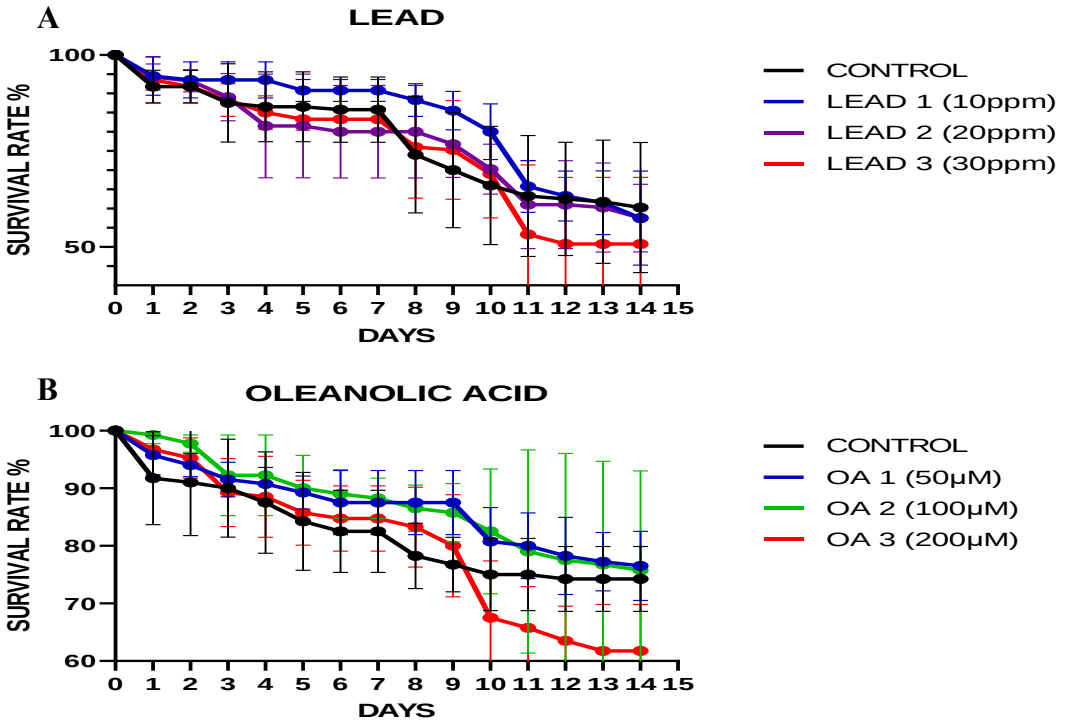


Figure 1. Survival rate of D. Melanogaster exposed to lead (A) and oleanolic acid (B) for 14 days. OA: Oleanolic acid

Reproductive rate results

These graphs showed the neurobehavioral defects on the reproductive rate of the lead-induced flies; the results derived show that the reproductive rates of the lead-induced flies have significantly reduced eclosion rate (Figure 2A-C) of both the numbers of pupae and adults. The administration of oleanolic acid as the ameliorative drug used to counter the effects of lead represents a positive impact in improving the eclosion by balancing the level of reactive oxygen species in the hormonal production that aids development.

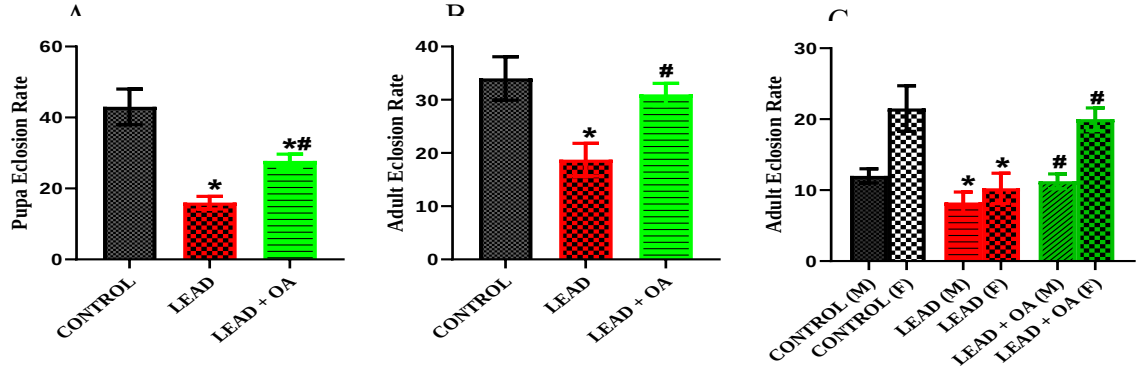


Figure 2. Effects of lead on reproductive rate: pupa rate (A), adult eclosion (B), and adult eclosion (gender specific). Data are observed as Mean ± SEM, * represents a significant difference in relation to the control group; # represents a significant difference in relation to lead ($p < 0.05$). OA: Oleanolic acid; M: Male; F: Female.

Assessment of Locomotive Activities.

The assessment of the movement pattern activities in the third instar larva, as shown in Figure 3A-C, shows that the line indicates the distance covered by each larva on the petri-dish. Control and lead + oleanolic acid show almost the same distance covered quantitatively as depicted in Figure 3D, which shows the improving action of oleanolic acid, but the lead group shows a defect in the locomotive ability (Crawling) as well as the climbing activity (Negative geotaxis) as shown in Figure 4.

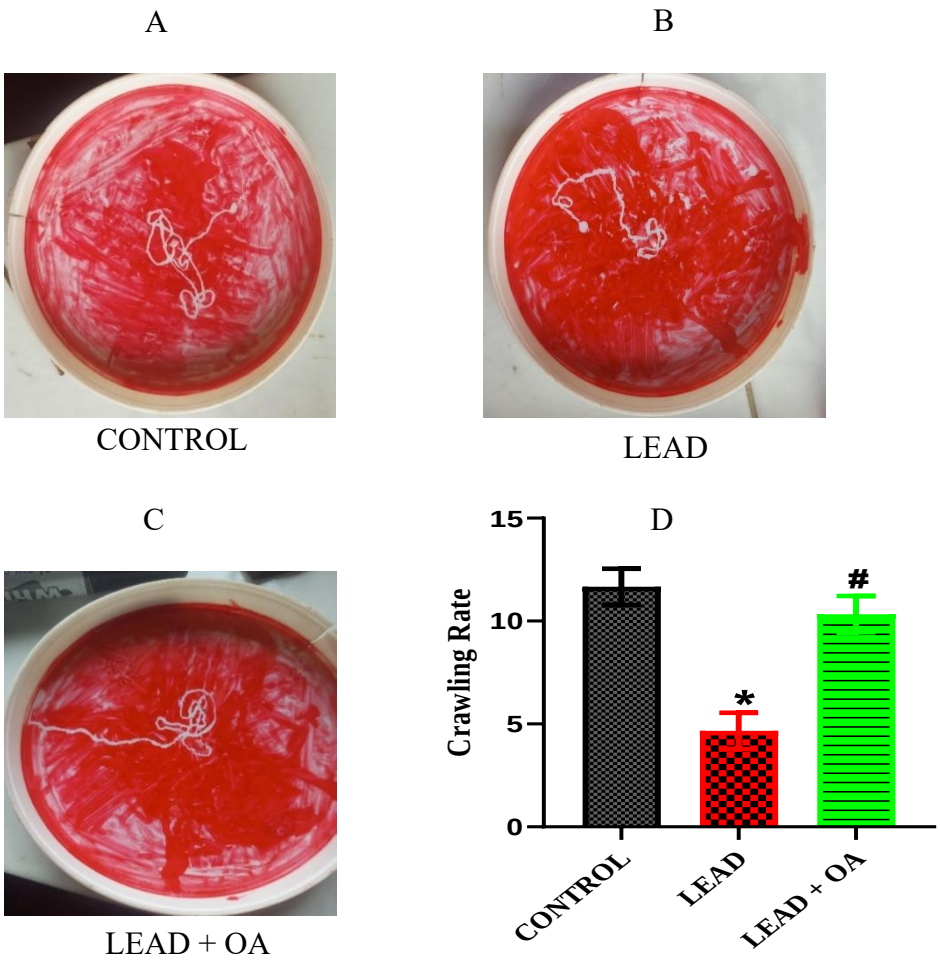


Figure 3. Illustration of the effect of lead and oleanoic acid on the crawling rate of third (3rd) instar larvae (A-C) and quantitative analysis. Data are depicted as Mean ± SEM (D) exposed to lead and lead co-treated with OA for 3 days. 20 flies (10 males and 10 females)/vial with 2 replicates per treatment group. * represents a significant difference in relation to the control group # represents a significant difference in relation to lead ($p < 0.05$). OA: Oleanolic acid.

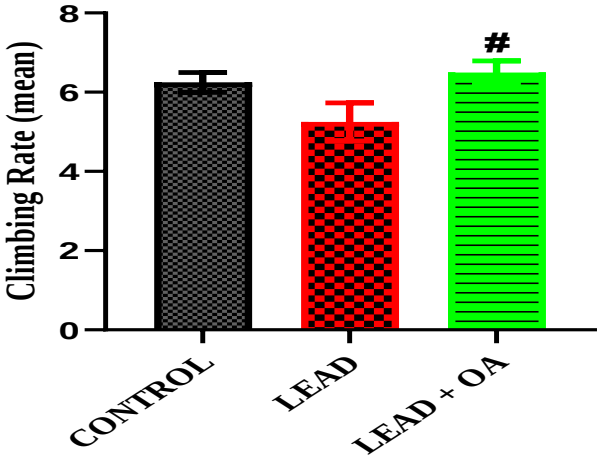


Figure 4. The graph shows the climbing activity of flies exposed to lead and oleanolic acid. Data are shown as Mean ± SEM of 10 flies/vial with 4 replicates per treatment group. * represent significant difference in relation to control group; # represent significant difference in relation to lead ($p < 0.05$). OA: Oleanolic acid.

Biochemical analysis

Oxidative stress makers

Significant increase in the level of Malondialdehyde (MDA) and Mono-amine oxidase (MAO) was observed in the group exposed to lead compared to the control group (Figure 5A and B). Also, Nitric oxide (NO), which is an indirect oxidative stress marker, shows the same trend as depicted in Figure 5C. Co-treatment with Oleanolic acid significantly decreased the levels of this marker. This suggests that lead causes the production of free radical oxygen, leading to higher oxidative stress, while oleanolic acid possesses a great antioxidant property.

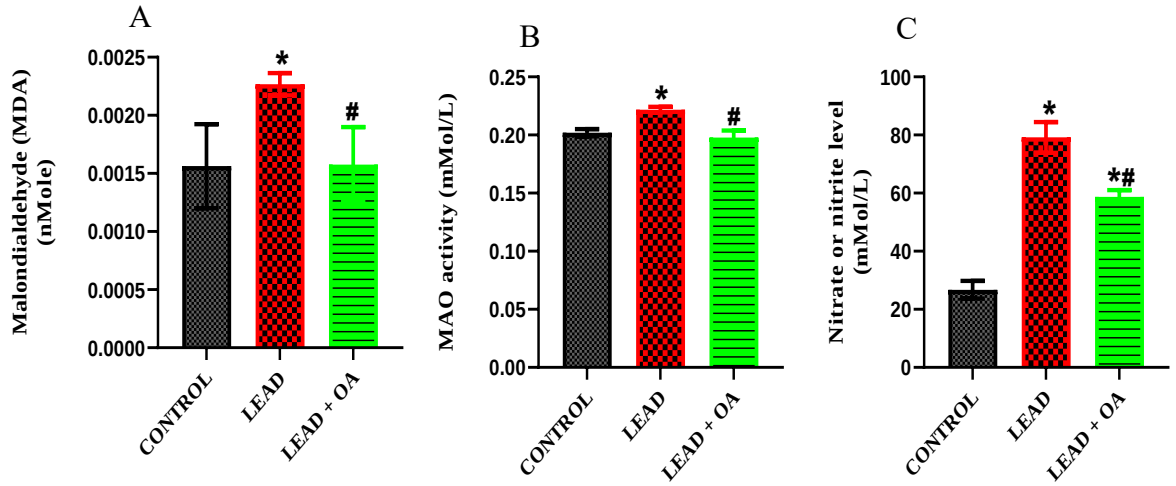


Figure 5. The graphs show the level of oxidative stress present: Malondialdehyde (MDA) (A), Monoamine oxidase (B), and nitrate or nitrite level (C). Data are depicted as Mean ± SEM of 50 flies/vial with 4 replicates per treatment group. * represent significant difference in relation to control group; # represent significant difference in relation to lead ($p < 0.05$). OA: Oleanolic acid.

Enzymatic activities

The Glutathione-s-transferase activity was significantly elevated in the group treated with lead compared to the control group (Figure 6A). However, co-treatment with oleanolic acid significantly decreases the GST activity, as the enzyme is responsible for the breakdown of glutathione levels in the body. In contrast, acetylcholinesterase activity depicted in Figure 6B was significantly reduced in the lead-exposed group when compared to the control group; acetylcholinesterase breaks down acetylcholine, of which its accumulation leads to neurotoxicity and impaired locomotion of the flies. Co-treatment with oleanolic acid significantly elevates the activity of AChE. This suggests oleanolic acid's ability to rescue locomotor deficits incurred by lead.

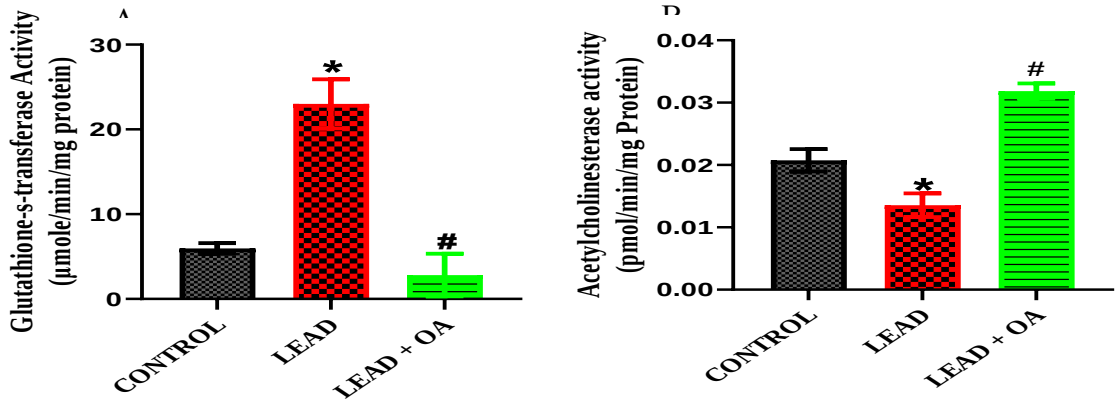


Figure 6. The graphs showed the level of enzymatic activities of Glutathione S-transferase (A) and Acetylcholinesterase (B). Data are presented as Mean ± SEM of 50 flies/vial with 4 replicates per treatment group. * represent significant difference in relation to control group; # represent significant difference in relation to lead ($p < 0.05$). OA: Oleanolic acid

Anti-oxidative markers

The antioxidant markers (Glutathione and total thiol) level, as depicted in Figure 7A and B, revealed the same trend, as the lead-exposed group significantly reduced the antioxidant level by increasing the oxidative stress level in the flies. However, co-treatment with oleanolic acid significantly rescued the antioxidant marker levels in the flies. This result suggests that oleanolic acid possesses antioxidant properties to reduce the oxidative stress caused by lead toxicity.

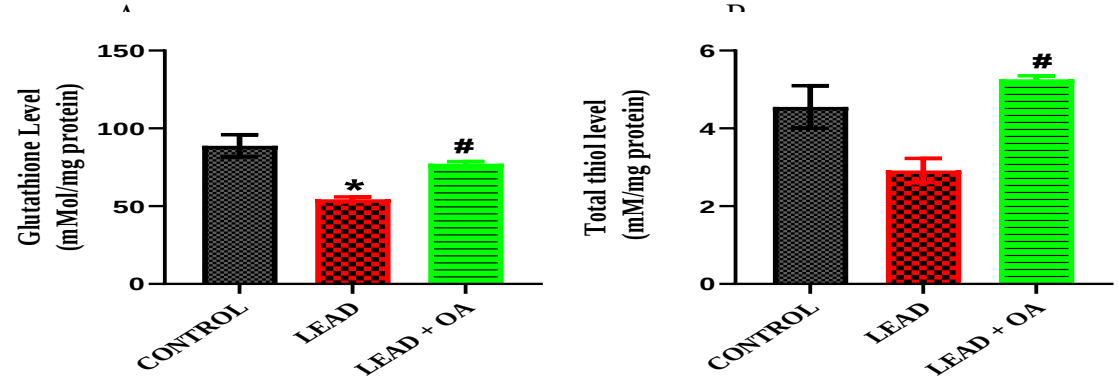
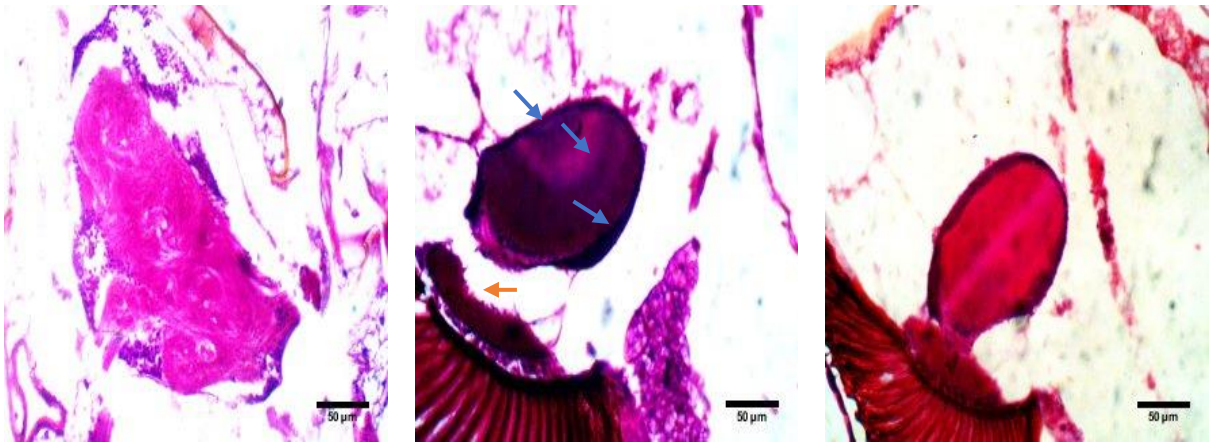


Figure 7. The graphs showed the level of antioxidants present, Glutathione (A) and Total thiol level (B). Data are shown as Mean ± SEM of 50 flies/vial with 4 replicates per treatment group. * represent

significant difference in relation to control group; # represent significant difference in relation to lead ($p < 0.05$). OA: Oleanolic acid.

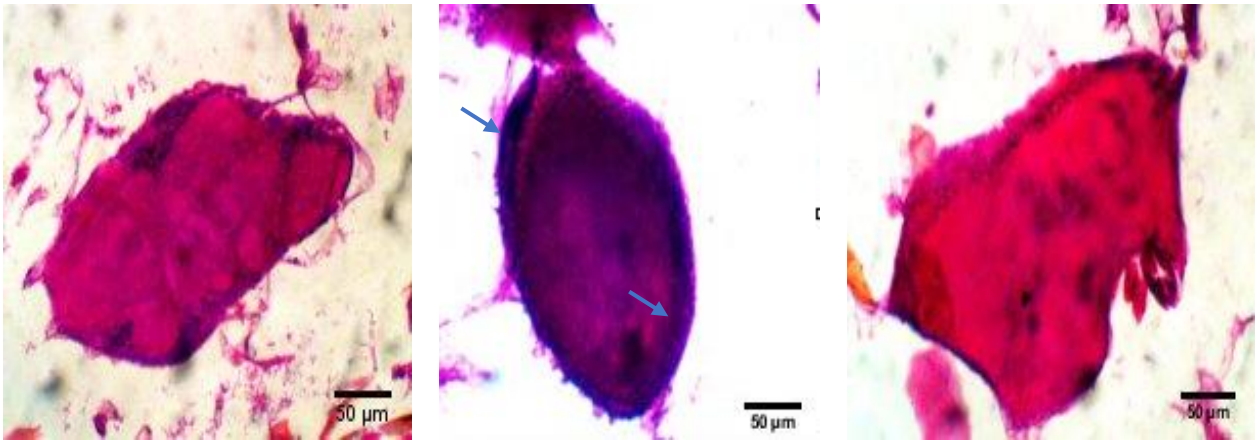
Histological analysis

Photomicrograph of the fly's optic lobe and mushroom body treated with calabash chalk and turmeric (H&E) at x400 magnification is depicted in Figures 8 and 9. The control group fairly showed a well-preserved histoarchitecture, while the flies exposed to lead showed connectome degeneration coupled with pyknotic cells (blue arrows) and eosinophilic rarefaction. However, when the flies are co-treated with oleanolic acid, recovery of cells and eosinophil staining was observed.



CONTROL LEAD LEAD + OA

Figure 8. Optic lobe region. D. melanogaster. Control: Has a normal histoarchitecture. Lead: indicates atrophy of the connectome with bad refraction of the eosin on the white matter of the brain (pink arrow) and sporadic pyknotic cells (Blue arrows). Lead + oleanolic acid group: indicates the optic lobe region regaining its eosinophilic reaction with fewer pyknotic cells and proper connectome. Magnification at x400, Scale bars=50µm. OA: Oleanolic acid.



CONTROL LEAD LEAD + OA

Figure 9. Mushroom body region of D. melanogaster brain. Control: Has a normal histoarchitecture. Lead: indicates a high neurodegeneration leading to sporadic pyknotic cells (Blue arrows). Lead +

oleanolic acid group: indicates restoration of the morphological state of the kenyons cells with reduced pyknotic cells. Magnification at x400, Scale bars =50µm. OA: Oleanolic acid.

Immunostaining result (alpha-synuclein)

Photomicrograph of alpha synuclein (aSyn) staining at X1000 magnification of the optic lobe and mushroom body of flies for seven days, as depicted in the Figure. 10 and 11. Lewy body (black arrows) aggregation was observed in the group treated with lead, indicating α-synucleinopathies in the flies. But when co-treated with oleanolic acid, no aggregation was seen in the flies, similar to the control groups.

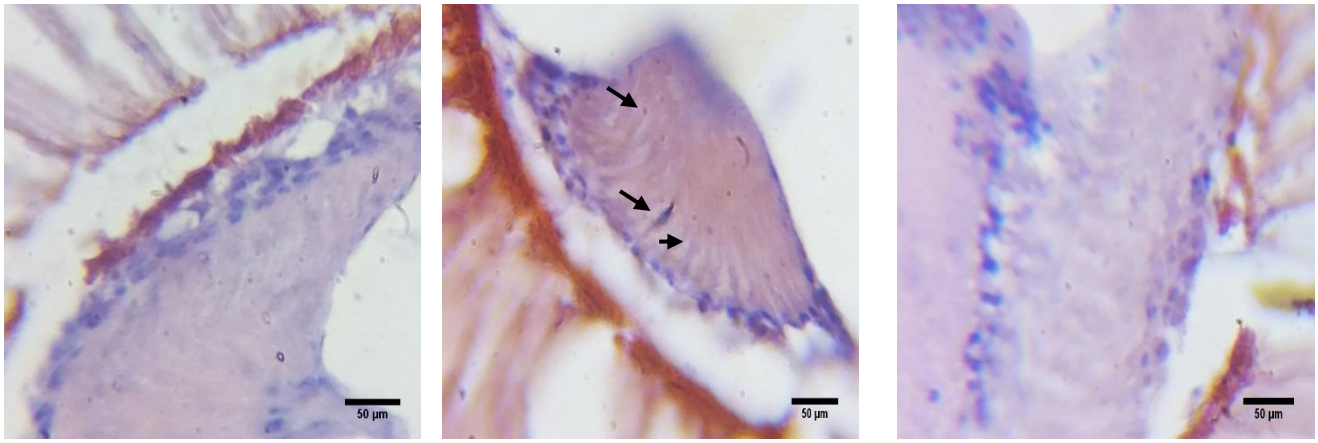


Figure 10. Optic lobe region of D. melanogaster brain. Control: Has a normal histoarchitecture. Lead group: shows degeneration of the connectome, as well as aSyn plaque (black arrow). Lead + oleanolic acid group: indicates the optic lobe region of the brain, where it connects with other neuropils and the calyx regenerating. Magnification at x1000, Scale bars =50µm. OA: Oleanolic acid.

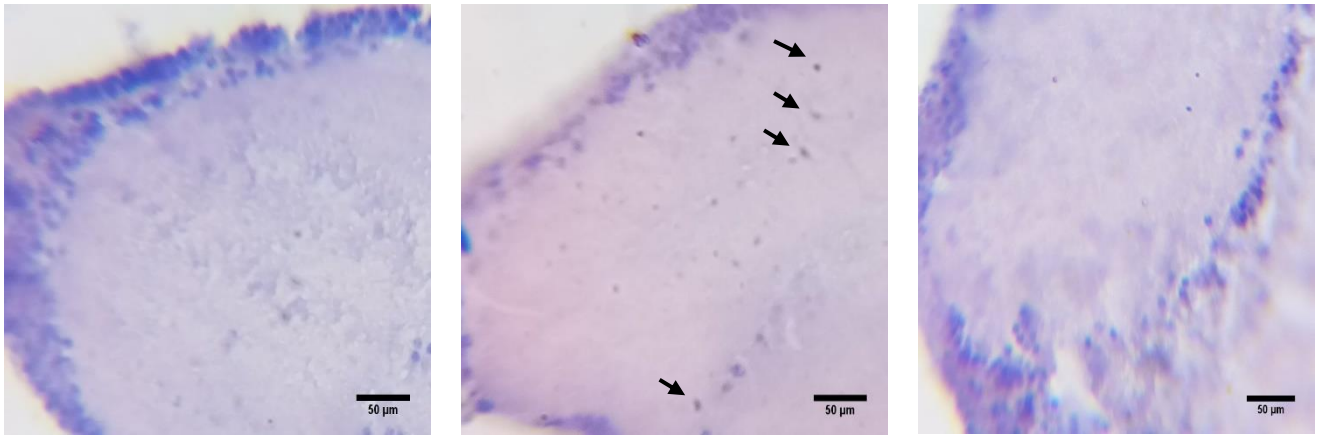


Figure 11. Mushroom body region of D. melanogaster brain. Control: Has a normal histoarchitecture. Lead: Show the excessive expression of α-Synuclein aggregations in the mushroom body revealed synucleinopathies (Black arrows). Lead + oleanolic acid group: indicates restoration of the morphological state of the kenyon cells. Magnification at x1000, Scale bars = 50µm. OA: Oleanolic acid

DISCUSSION

Lead exposure is recognized for its detrimental effects on various human organs, including the liver, brain, and kidneys. Its neurotoxicity is particularly pronounced in the brain, especially during the early developmental stages of exposed individuals. Human studies have demonstrated significant gender differences in lead neurotoxicity, as evidenced by research on spatial memory, motor behavior, brain gene expression, and dopamine metabolism, all of which are symptoms associated with Alzheimer's disease (AD).[36] Early-life lead exposure can lead to behavioral changes such as anxiety, locomotor dysfunction, and memory impairment, which may persist long after the exposure.[37-40] In the context of neurodegenerative diseases, most previous studies have focused on AD, specifically examining lead's influence on Aβand tau proteins, epigenetics/DNA methylation, protein expression and regulation, and RNA biology.[41-43] Human studies have also indicated that even short-term exposure to lead can increase the risk of Parkinson's disease[44]. In this study, we conducted extensive neurobehavioral, biochemical, and microanatomical investigations to evaluate the therapeutic efficacy of oleanolic acid in a lead-exposed *Drosophila melanogaster* model relevant to alpha-synucleinopathies. Our observations revealed that lead-exposure paradigms significantly increased aSyn inclusion formation.

This study demonstrated that oleanolic acid extended the lifespan and increased the survival rate of the flies. Although the anti-aging properties of this compound were not the primary focus of this study, the observed increase in survival rate suggests that these compounds possess anti-aging characteristics. Aging is a progressive step leading to functional decline and elevated susceptibility to diseases like Alzheimer's and Parkinson's, ultimately resulting in death.[45,46] While aging is a complicated process, it has been revealed that age-related reductions in longevity are linked to factors such as the generation of reactive oxygen species (ROS).[47] Lead significantly reduces the survival rate and causes mortality in a majority of flies. This finding aligns with Mathew & Krishnamurthy (2018), [48], who reported that lead drastically shortens the lifespan of *Drosophila melanogaster* even at low concentrations.

Neurodegenerative illnesses induced by heavy metals commonly affect motor activity, with a gradual decline in learning, memory, and motor skills being clinical hallmarks of PD and AD.[49] In this study, neurobehavioral tests were performed to determine how OA could ameliorate lead-impaired locomotor activities, including crawling, climbing, and emergence rates. One method to quantify locomotor activity in *Drosophila* larvae involves measuring the distance they cover within a specified timeframe. This study revealed that larvae raised on a lead-containing diet exhibited significantly reduced motor function. This observation is consistent with Shvachiy et al. (2024), .[50] whose research indicated that lead exposure profoundly impacts motor behavior readouts, even in the absence of aSyn pathology. When comparing third-instar larvae raised on a lead-induced diet co-treated with OA to those on a lead-only diet, the former group displayed enhanced crawling activity.

Biochemical analyses in the current study investigated OA's therapeutic effect on levels of ROS, GST, AChE, GSH, and TSH in AD flies. It has been reported that a reduction in AChE levels can impair cholinergic neurotransmission, a cognitive symptom of AD and a contributor to motor dysfunction in PD.[51] Flies exposed to lead showed a significant decrease in climbing behavior, accompanied by a reduction of Acetylcholinesterase activity. AChE hydrolyzes

acetylcholine, a crucial neurotransmitter for regulating locomotive function.[52] Immediately, lead gained access to the brain, which can hinder Acetylcholinesterase activity and elevate acetylcholine levels in the basal ganglia, causing abnormal cellular function.[53] Thus, the observed inhibition of AChE activity in this study could have hindered normal neurotransmission in the flies, resulting in impaired climbing activity[54]. This clearly indicates that dietary administration of OA helps scavenge free radicals and enhances AChE activity. Glutathione-S-transferases (GSTs) are phase II enzymes that catalyze the conjugation of glutathione (GSH) with electrophiles. GSH plays a vital role in protecting organisms from oxidative damage.[25,55] However, in this study, OA restored lead-induced inhibition of GSH in *D. melanogaster*, thereby confirming its antioxidative property. Thiols are compounds containing a carbon-bound sulfhydryl group.[56] OA also restored lead-induced inhibition of Total thiol (TSH) level in *D. melanogaster*.

Although nitric oxide (NO) serves a physiological role in the brain, its accumulation can also promote the production of mitochondrial ROS and RNS, exerting cytotoxic effects by disrupting cellular respiration.[57] These radicals are known to induce neuronal death through various pathways. For instance, 4-Hydroxynonenal (4-HNE), a product of lipid peroxidation secondary to these oxidant species, can trigger apoptosis via the intrinsic pathway, involving the release of cytochrome C into the cytosol and subsequent activation of associated caspases.[58] Similarly, proinflammatory cytokines released by astrocytes in response to pathological aSyn aggregates can activate the caspase/cytochrome C signaling cascade[59]. Furthermore, in this study, OA prevented the lead-induced increment of NO levels in the flies. While NO participates in various functional steps, it also acts as a pro-inflammatory mediator when it aggregates and reacts with superoxide anion to incur additional toxic nitrite anion, causing various disease challenges.[60] This suggests OA's ability to mediate inflammation in the flies.

This study found that lead reduced the flies' cell viability. Obviously, oxidative stress is partially mediated by MAO-B through the mitochondrial release of cytochrome C and actual activation of caspases 3 and 9, ultimately causing the death of neurons.[61,62] Notably, in this study, OA administration protected against cell death by reducing lipid peroxidation and MAO-like activity in lead-induced flies. Additionally, lead treatment reduced offspring emergence, which might be attributed to elevated cell death. However, the flies co-exposed with OA showed an increased emergence rate comparable to the control group, thus suggesting its valuable role in lead-induced flies. Generally, the results of this study suggested that elevated oxidative stress levels could bring about the alpha-synucleopathies. This was similar to the findings of Scudamore & Ciossek[63], who demonstrated that high levels of intrinsic oxidative stress may influence a-synuclein aggregation in vivo and cause synucleinopathy to worsen. However, there is still room for discussion as to how precisely it elevates oxidative stress, causing α-synuclein aggregation.

The larval neuromuscular junction has demonstrated an astonishing conservation of many important synaptic molecules observed in higher animals.[64] Drosophila might prove to be an ideal model system for the study of lead's effects on similar synapses in mammals. Lead has been established to incur alteration at the synapse between neurons and their respective targets in mammals[65] and flies.[66] Lead-induced histological alterations, characterized by the rarefaction of eosin and white matter in the fly's brain, together with neuronal atrophy and connectomes with numerous pyknotic cells. Nevertheless, OA remarkably restored these lesions, corroborating its protective role against lead-induced toxicity. Alpha-synuclein immunostaining revealed that lead-induced toxicity activated and aggregated alpha-synuclein. OA administration was able to

dissolve these plaque aggregations and restore the normal cognitive function of the flies, thereby confirming the therapeutic effect of this bioactive compound.

CONCLUSION

This study demonstrates that oleanolic acid (OA) confers significant neuroprotection against lead-induced alpha-synucleinopathies in flies. By targeting oxidative stress pathways and restoring antioxidant homeostasis, OA effectively mitigated lead-induced neurobehavioral deficits, biochemical disruptions, and histopathological alterations.

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