Arch. Bas. App. Med.12 (2024) 131-136
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Research Article

Biflavonoid-rich extract of *Garcinia kola* seed and ascorbic acid are protective against lead-induced oxidative stress in *Drosophila melanogaster*

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Accepted: December 24, 2024.

Abstract

Exposure to toxic metals such as lead causes oxidative damage affecting human health. Sources of exposure could be contaminated water, leaded gasoline, etc. Using *Drosophila melanogaster*, we examined the individual and combined effects of ethyl acetate seed extract (EASE) from *Garcinia kola* and ascorbic acid (AsA) in ameliorating lead acetate-induced oxidative damage. *Garcinia kola* (seeds) was identified and processed to obtain ethyl acetate seed extract (EASE). For the survival study, one to three-day-old flies were exposed to different concentrations of EASE and AsA for 14 days. 150 mg/kg EASE and AsA were selected, while 250 µM of lead acetate (Pb) was used. For the main study, flies were grouped into eight with 50 flies per vial for 5 vials each per group and were treated for 7 days. The flies were thereafter anesthetized and processed to obtain supernatant solutions used to determine oxidative stress, neurological and inflammatory biomarkers. The EASE, AsA, and their combination significantly restored lead-induced oxidative stress, and altered neurological and inflammatory biomarkers. This study suggests the therapeutic potential of the combination of EASE and AsA against lead-acetate-induced toxicity, as there was a reversal of elevated oxidative stress, neurological and inflammatory perturbations.

Key Words: *Garcinia kola seeds; ascorbic acid; lead toxicities; oxidative stress; Drosophila melanogaster; Biflavonoid-rich extract*

INTRODUCTION

Cumulative exposure to non-biodegradable, toxic heavy metals is established to have a deleterious effect on humans' health and wellbeing (Valko *et al*., 2005). Lead, a heavy metal with a long half-life is a cumulative poison of biological systems as it is lethal to most organs via oxidative damage (Mahaffey, 1990). In addition, lead interferes with cellular signalling pathways, impairs enzyme activity, and disrupts intracellular calcium by replacing divalent cations in cellular enzymes. The untoward exposure to lead could be from several sources including contaminated water, leaded gasoline, certain industrial processes, battery recycling, lead-based paints, and so on (Flora *et al*., 2012). The literature is replete with documentation on the utilization of plants and plant-derived products in the treatment of various human diseases.

Garcinia kola seed, otherwise called bitter kola, is a widely cultivated medicinal plant in West Africa. Used in traditional ceremonies as a symbol of long life among the Yorubas in South-West, Nigeria, the seeds are chewed as it provides relief from cold and throat infections (Iwu, 1993). In addition, polyphenolic-rich extract from the seed has been reported to ameliorate various chemically induced oxidative damages. These activities are linked to its ability to neutralize free radicals and reactive electrophilic metabolites and activate the genes and xenobiotic metabolizing enzymes (Farombi *et al*., 2018a; Farombi & Owoeye, 2011).

Ascorbic acid, a cyclic five-membered, conjugated ene-diol, is a water-soluble universal antioxidant. It is a simple sugar molecule and an essential micronutrient in the human diet used to synthesize collagen and intercellular material. A common constituent in most commercially available nutritional supplements and multivitamins, ascorbic acid is well known for its ability to scavenge free radicals (Suh *et al*., 2017). In this study, using *Drosophila melanogaster* (fruit fly), we investigated the individual and combined effects of *G. kola* (ethyl acetate seed extract, EASE) and ascorbic acid in lead acetate-induced oxidative damage. The arthropod *D. melanogaster* is widely used as a model organism in biomedical research. Its high prolificacy, low maintenance cost, and ease of handling and conservation of vital biological

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mechanisms and pathways across evolution between humans and *D. melanogaster* enables its utilization in toxicological studies as a model invertebrate substitute for mammals (Abolaji *et al*., 2013; Rand, 2010).

MATERIALS AND METHODS

Plant material: *Garcinia kola* seed was sourced from markets in Ibadan, Nigeria. It was identified and processed as earlier described to obtain EASE (Idowu *et al*., 2017). Briefly, the outer coats of the seeds were removed, chopped into tiny pieces, dried under shade for four weeks, and thereafter ground into powder. The powder material (450 g) was defatted in n-hexane (1L) for 24 hours. Afterward, the marc was successively macerated in chloroform and ethyl acetate (1L each, twice for 24 hours). Ethyl acetate extract was filtered and concentrated using a rotary evaporator. The concentrated extract was allowed to dry, and the dried extract was stored in the refrigerator until needed.

Chemicals and reagents: The chemicals, solvents, and reagents used were of analytical grade (Sigma Aldrich, UK). Ascorbic acid was obtained from Merck, Germany.

Culture of *Drosophila melanogaster*: *Drosophila melanogaster* (Harwich strain), primarily sourced from the National Species Stock Center (Bowling Green, OH, USA), was cultured and maintained in the Drosophila Laboratory, Biochemistry Department, University of Ibadan, Nigeria as previously described (Farombi *et al*., 2018).

Survival study: To determine the concentration and duration of exposure to EASE and Ascorbic acid (AsA), the flies (both genders, 1-3 days old), were exposed to different concentrations of EASE (0, 30, 150, and 300 mg/kg diet) and AsA (0, 30, 150 and 300 mg/kg diet), over 14 days with daily mortality rate recorded and percentage survival determined. Based on these studies, a 150 mg/kg diet of EASE and AsA was selected, while 250 µM of lead acetate (Pb) was used based on a previous report from our laboratory. The duration of exposure of the flies to lead acetate to evaluate selected markers for toxicity was seven (7) days.

Preparation of samples for biochemical assays: For the evaluation of the biochemical parameters, the flies were divided into eight (8) groups with 50 flies (both gender) per vial and 5 vials each per group and received the treatments for 7 days as follows: Group A (Control 1, Distilled water); Group B (Control 2, 2% ethanol); Group C (AsA, 150 mg/kg diet); Group D (EASE, 150 mg/kg diet); Group E (Pb, 250 µM); Group F [Pb (250 µM) + EASE (150 mg/kg diet)]; Group G [Pb (250 µM) + AsA (150 mg/kg diet)]; Group H [Pb (250 µM) + AsA (150 mg/kg diet) + EASE (150 mg/kg diet)]. At the end of the experiment, the flies were anesthetized using ice and processed as previously described by Farombi *et al*. 2018. The supernatant solutions obtained were used for the determination of total protein level, total thiol (T-SH), Non-thiol proteins (NPSHs) contents, hydrogen peroxide (H_2O_2), nitric oxide (NO) levels, levels of lipid peroxidation and protein carbonyl contents and glutathione S-transferase (GST), monoamine oxidase-like activity and acetylcholinesterase (AChE) activities.

Evaluation of biochemical parameters

Total protein: The total protein was determined following the procedure of Lowry *et al* (1951). Bovine Serum Albumin (BSA) was used as standard. Briefly, 400 µL of solution C {(mixture of 9.8 mL of solution A (made up of 0.1 M NaOH and 2% sodium carbonate) and 200 µL of solution B (150 µL of 1% $CuSO_4 \cdot 5H_2O$ + 150 µL of 2% Na-K tartrate)} was added to 25 µL of sample (1: 10 dilution) and 40 µl of Folin-Ciocalteu (ratio 1:5) and the absorbance measured at 650 nm. The total protein values were used for the calculation of antioxidant enzymes activities.

Total thiols (T-SH): This was determined according to Ellman (1959). The reaction mixture consists of 170 µL 0.1 M potassium phosphate buffer (PH 7.4), 20 µL sample, and 10 µL 10 mM DTNB, which was incubated for 30 min at room temperature. The absorbance values were taken at 412 nm and the T-SH was expressed as µmol/mg protein.

Non-protein thiols (NP-SHs): The NP-SHs levels were assessed according to Jollow *et al*. (1974). Briefly, the supernatant was precipitated with 4% sulphosalicylic acid (4%) in a ratio of 1:1. Samples were left at 4 ◦C for 1 hour and thereafter centrifuged at 5000 rpm for 10 min at 4 ℃. The assay solution consists of 550 µL of 0.1 M phosphate buffer (pH 7.4), 100 µL of supernatant, and 100 µL of DTNB. Absorbance values were taken at 412 nm and the NP-SHs and expressed as µmole/mg protein.

Hydrogen peroxide (H_2O2) generation: Hydrogen peroxide level was estimated following the procedure of Wolff (1994). Briefly, the assay mixture contains 590 µL of FOX1 reagent (100mol/L xylenol orange, 250 mol/L ammonium ferrous sulfate dissolved in 25 mmol/L H_2SO_4 and 100mmol/L sorbitol) and 10 µL of sample. The reagent mixture was incubated at room temperature for 30 minutes, and the absorbance was measured against the blank (distilled water) at 560 nm. The hydrogen peroxide level was calculated and expressed in µmol/L.

Nitric oxide (NO) production: Nitric oxide production was evaluated from the sodium nitrite standard curve (Green *et al*., 1982). The assay mixture consists of 200 µL of sample incubated with 200 µl of Griess reagent (0.1% n-(1-naphthyl)-ethylenediamine, 1% sulphanilamide in 5% phosphoric acid) at room temperature for 20 min. The absorbance values were taken at 550 nm.

Glutathione S-transferase (GST) activity: The GST activity was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Habig and Jacoby, 1981). The reaction mixture consists of 270 µL solution A (0.25 M phosphate buffer, pH 7.0, 2.5 mM EDTA, 0.1 M GSH, and 10.5 mL of distilled water) and 20 µL of the sample and 10 µL of 25 mM CDNB, which was monitored at 340 nm for 2 minutes at 30-seconds intervals. Using a molar extinction coefficient of 9.6 mM-1Cm-1, GST activity was expressed as µmol/min/mg protein.

Lipid peroxidation: Lipid Peroxidation was evaluated following the procedure earlier described by Ohkawa *et al*. (1978). The total reaction volume which consists of 5µL of 10 mM butyl-hydroxytoluene (BHT), 100 µL of 0.67%

thiobarbituric acid, 300 µL of 1% O-phosphoric acid, 55µL of distilled water and 40 µL of the sample, was incubated for 45 min at 90 ℃. Absorbance values were taken at 535 nm and lipid peroxidation levels expressed as µmol of TBARS formed per mg protein.

Protein carbonyl: This was evaluated according to Hawkins *et al*. (2009). Briefly, 250 µL of 10 mM 2, 4-dinitrophenyl hydrazine was added to 2.5 M HCl, and 250 µL of the sample was added, vortexed, and kept in the dark for 20 min. Around 125 µL of 50% (w/v) trichloroacetic acid (TCA) was added, and then mixed thoroughly, and incubated at −20°C for 15 min. The mixture was thereafter centrifuged at 4°C for 10 min at 9000 rpm. The supernatant was discarded, and the resulting pellet was washed twice with ice-cold ethanol: ethyl acetate (1:1) solution and then dissolved in 1 mL of 6 M guanidine HCl. The absorbance values were taken at 370 nm. The protein carbonyl content was calculated and expressed as millimoles of carbonyls formed per milligram of protein.

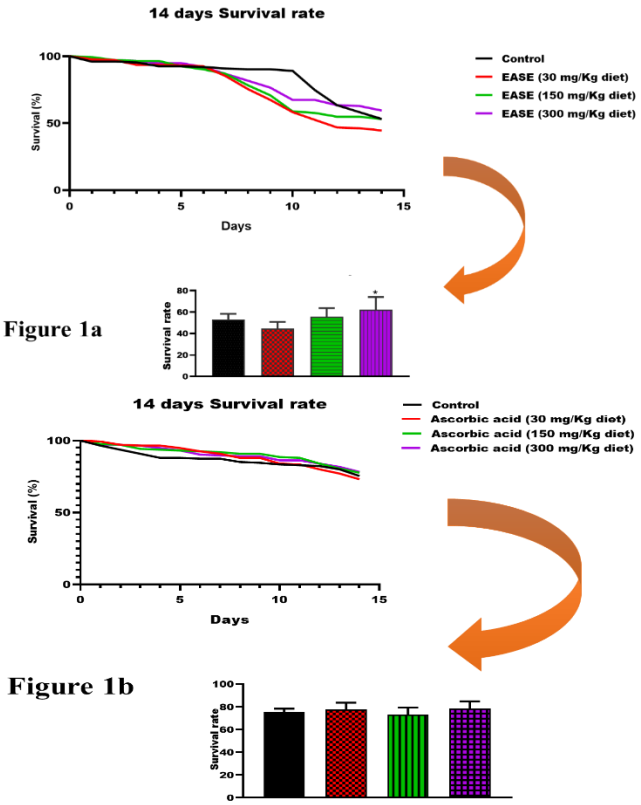
Monoamine oxidase-like activity: The monoamine oxidase-B (MAO-B) activity was determined according to McEwen Jr. (1965). The reagent mixture is composed of 60 µL of 0.1M phosphate buffer (pH 7.4), 195 µL of dH_20, 15 µL of benzylamine HCl, and 30 µL of the sample. The mixture was incubated at room temperature for 30 mins, 30 µL of 10% perchloric acid was added, and then centrifuged at 1500 g for 10 min. The absorbance of the solution was taken at 280 nm. MAO-B activity was calculated and expressed as mmol/mg protein.

Acetylcholinesterase (AChE) activity: The acetylcholinesterase activity was evaluated following the procedure earlier described by Ellman *et al*. (1961). Here, the reagent mixture contains 135µL dH20, 20 µL of 0.1M potassium phosphate buffer (pH 7.4), 1mM DTNB (20 µL), 5 µL of sample and 20 µL of 0.8 mM acetylthiocholine as initiator. The reaction was monitored for 3 minutes at 30-second intervals at 412 nm. The AChE activity was calculated as µmol of acetylthiocholine hydrolyzed per minute per mg protein.

Statistical analysis: The biochemical data, expressed as mean ± standard error of the mean (SEM), were analyzed using Graph Pad Prism-6 (version 6 GraphPad Software, USA). Statistical difference was determined by one-way analysis of variance (ANOVA) and $p < 0.05$ was adjudged significant.

RESULTS

Survival of *D. melanogaster* treated with ethyl acetate seed extract and ascorbic acid: Figures 1a and 1b represent the effect of varying concentrations of EASE and AsA on the survival of *D. melanogaster* after 14-day exposure. It was observed that EASE (30, 150, and 300 mg/kg diet) had 44.4%, 53.2%, and 59.2% survival rates, while, AsA (30, 150, and 300 mg/kg diet) increased the survival of the fly by 73.2 %, 76.1% and 78.2 % respectively, relative to the control. The number of days (mean) which corresponds to 50% of mortality was taken at day 14 for the flies treated with EASE and AsA (0, 30,150, and 300 mg/kg), respectively, and 150mg/kg diet of EASE and AsA was selected for the main study.



Figures 1a and 1b:
Effects of 0, 30, 150 and 300 mg/kg diet of EASE and Ascorbic acid respectively, on survival rate. The survival rate in each group represents the percentage of surviving flies.
* Indicate significant differences compared with the control group ($p < 0$ v.05

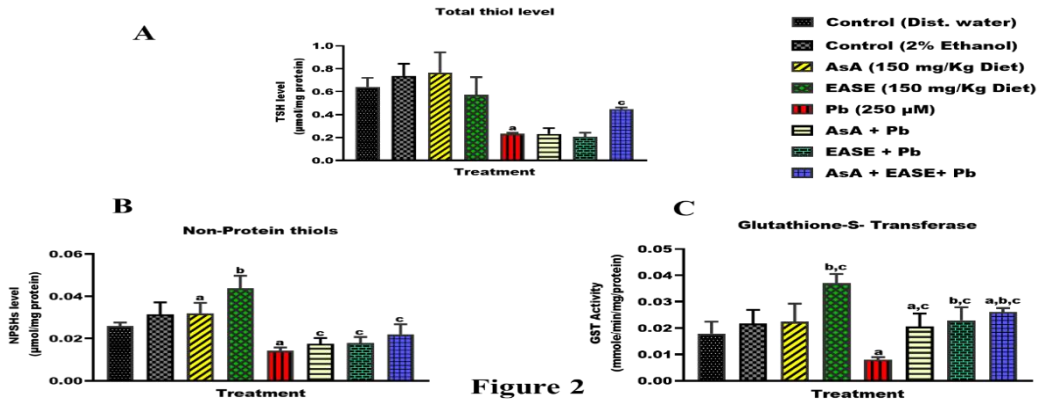


Figure 2

Figure 2:
The effects of antioxidant parameters in *D. melanogaster* treated with AsA (150 mg/Kg diet), EASE (150 mg/kg diet) and Pb (250 µM) for 7 days. Graphs are presented as (A) Total thiol, (B) Non-protein thiols and (C) Glutathione S-Transferase Data are presented as Mean ± SEM of 50 flies/vial with 5 replicates per treatment group. Significant differences from the control water, control ethanol and lead-acetate are indicated by: a, b and c respectively ($p < 0.05$).

Ethyl acetate seed extract and ascorbic acid restore the antioxidant status impaired by lead acetate: The effects of Pb, EASE, AsA, and their combination on total thiol, non-protein thiol levels and GST activity in *D. melanogaster* are presented in Fig.2.

The EASE, AsA and their combination significantly prevented lead-induced depletion of T-SH (Fig.2A) and NP-SHs (Fig.2B) levels and ameliorated lead-induced inhibition of GST activity (Fig.2C).

Ethyl acetate seed extract and ascorbic acid attenuate lead acetate-induced oxidative stress: EASE, AsA, and combination restore to normal the elevation of H_2O_2 (Fig.3A) and reduced levels of nitric oxide (Fig.3B, nitrite and nitrate) levels resulting from exposure of the flies to Pb. In addition, they also significantly blocked the elevation of lipid peroxidation (Fig.3C) and protein carbonyl (Fig.3D) relative to flies treated with lead ($p<0.05$).

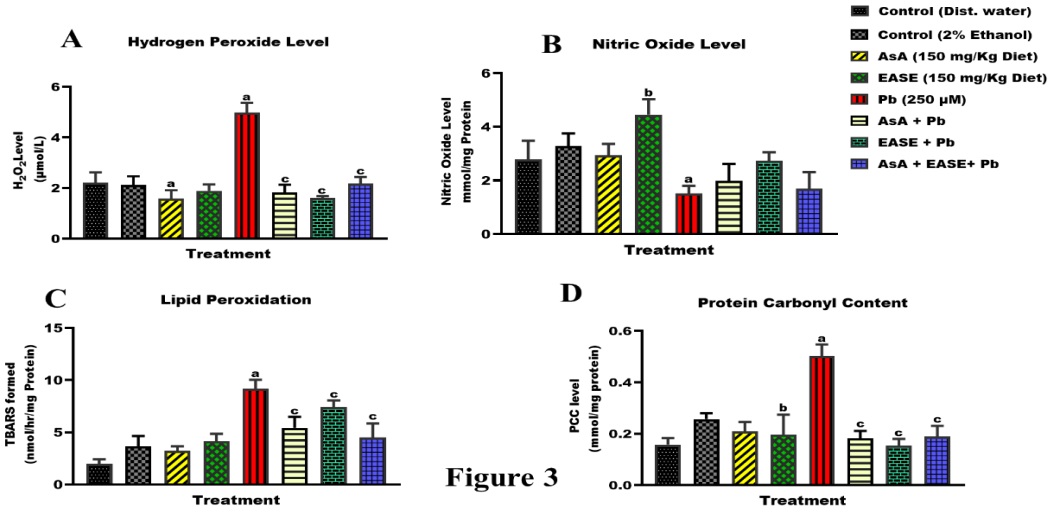


Figure 3

Figure 3:
Effects of AsA (150 mg/Kg diet), EASE (150 mg/kg diet) and Pb (250 µM) on markers of oxidative stress of *D. melanogaster* exposed for 7 days. Graphs are presented as (A) Nitric oxide, (B) Hydrogen peroxide and (C) Lipid peroxidation (D) Protein Carbonyl. Data are presented as Mean ± SEM of 50 flies/vial with 5 replicates per treatment group. Significant differences from the control water, control ethanol and lead-acetate are indicated by: a, b and c respectively ($p < 0.05$).

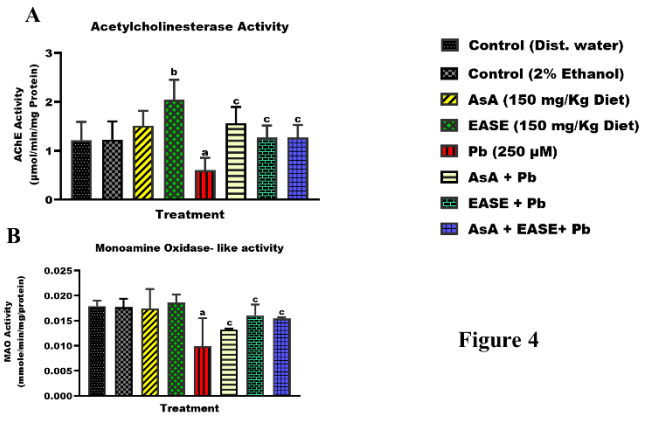


Figure 4

Figure 4:
Effects of AsA (150 mg/Kg diet), EASE (150 mg/kg diet), and Pb (250 µM) on acetylcholinesterase activity and monoamine oxidase–like activity in *D. melanogaster* for 7 days. Graphs are represented as (A) Acetylcholinesterase activity, (B) Monoamine Oxidase–like activity. Data are presented as Mean ± SEM of 50 flies/ vial with 5 replicates per treatment group. Significant differences from the control water, control ethanol and lead-acetate are indicated by: a, b and c respectively ($p < 0.05$).

Ethyl acetate seed extract and ascorbic acid improved acetylcholinesterase activity and monoamine oxidase-like activity disrupted by lead acetate: Given the exposure to Pb, we investigated the effects of Pb and its administration with EASE, AsA, and their combination on neurotoxicity via assessment of the activities of AChE and MAO. The results showed a significant decrease in activities of AChE (Fig.4A) and MAO (Fig.4B) in the flies. However, flies co-treated with EASE, AsA and combination improved the activities compared to groups treated with Pb alone.

DISCUSSION

Lead, obviously one of the most hazardous environmental and industrial contaminants, is of global concern on account of its toxicity to human life and the ecosystem at large. Accumulation of lead results in several organ toxicities such as blood, kidney, liver, born-marrow, lung, and brain among others. Several literatures have shown an association between environmental exposure to lead and many pathological conditions including cancer and neurodegenerative diseases. A major mechanism through which these pathological conditions spring up is through the generation of free radicals such as reactive oxygen and nitrogen species which result in oxidative stress; and eventually damage biologically important biomolecules like DNA, lipids, and proteins (Flora *et al*., 2012; Gillis *et al*., 2012). The use of medicinal plants represents a significant means of intervention to mitigate or prevent these damages. In an earlier study, Idowu *et al* (2017) showed that there were variations in polyphenolic composition present in the biflavonoid fraction of *G. kola* seed obtained by varying solvent extraction processes. Of the three extraction processes evaluated, the EASE gave the highest antioxidant

capacity value of 1500/g. Hence, the choice of EASE for this study.

Thiols, which are molecules containing carbon-bonded sulfhydryl (–C–SH) group, were found to decrease significantly in lead-acetate exposed flies. This is important as the maintenance of cellular thiol level is critical for normal cell function and viability (Yuksel *et al*., 2016). In consonance with the total thiols reduction, there was also a significant decrease in the glutathione (GSH) levels and inhibition of glutathione-s-transferase enzyme. Glutathione is a thiol containing cellular antioxidant. Since, lead has high affinity for sulfhydryl groups through covalent binding, this inactivates GSH and other thiol containing enzymes (Flora *et al*., 2012). Thus, the low level of these thiols. The EASE, AsA and combination were able to prevent this action of lead perhaps due to their ability to competitively scavenge it and thus safeguarding the GSH and other thiol-protein enzymes. Overall, hexavalent lead limits the antioxidant barrier, increases the rate of apoptosis and destroys the glutathione antioxidant system. Glutathione transferase is an important sensitive biomarker for indicating an early warning physiological response of organisms to metal (Dobritzsch *et al*., 2020).

The levels of hydrogen peroxide (H_2O_2) in this study was observed to be increased in flies exposed to Pb and this is an indication of a system overwhelmed by oxidative stress due to overproduction of hydroxyl radicals and this is in accordance with the finding that some elements such as arsenic present in the environment trigger the generation of free radicals leading to oxidative stress if left unchecked (Oyibo *et al*., 2021), however, we also observed that this increase was significantly mitigated when co-administered with EASE and AsA. Hydrogen peroxide has been linked to the oxidation of protein, nucleic acid and lipids components causing oxidative damage (Ighodaro & Akinloye, 2018). Hence, we further evaluated other markers of oxidative stress, assaying for levels of protein carbonyl content (PC) and lipid peroxidation (LPO). protein carbonylation products are formed from oxidative assault to side chains of amino acids (Dalle-Donne *et al*., 2003), while, lipid peroxidation is a well-known indicator of oxidative stress and is caused by the accumulation of ROS in cells, leading to damage to cell membranes and other structures. The observed increase in lipid peroxidation and protein carbonyl levels in flies exposed to lead acetate (Pb) is consistent with previous studies that have reported the role of lead in inducing oxidative stress and damaging cellular membranes (Adedara *et al*., 2022). Both markers are strongly linked to ageing and neurodegenerative diseases, but interestingly, EASE and AsA were able to reverse Pb-induced elevated levels of PC and LPO in the fruit flies.

The interaction of excessive amounts of oxygen radicals with nitric oxides has been reported to lead to the formation of peroxynitrite anion (NO3-), which oxidizes low-density lipoprotein cholesterol (LDL-C) and damages tissues. These implicated effects are attributed to its cytotoxic radical nature, oxidation of some proteins, and DNA and its decomposition to nitrogen dioxide and hydrogen peroxide radicals resulting in inflammation (Pacher *et al*., 2006). This study demonstrated that exposure to lead-acetate reduces the level of nitric oxide (NO) in flies and that the administration of EASE and AsA counteracted this effect although there was not a significant difference with co-administration of EASE and AsA. The increase in NO level observed in flies exposed to EASE and AsA is consistent with other reports in the literature. NO is an important molecule that regulates various physiological functions, including vasodilation, neurotransmission, and immune response. NO also has antioxidant properties and can scavenge ROS (Radi, 2018). Therefore, the decrease in NO level observed in flies exposed to lead-acetate is consistent with the oxidative stress caused by Pb exposure. The significant effect of EASE may be due to their ability to scavenge ROS and/or enhance the endogenous antioxidant defence system. This suggests that the compounds in EASE have a protective effect against Pb-induced oxidative stress.

Heavy metals and organophosphorus have been confirmed to possess neurotoxic effects even on fruit flies and they cause alteration in the activity of AChE, a key enzyme involved in the hydrolysis of acetylcholine to choline and acetate (Guilhermino *et al*.,1998), thus, plays an essential role in the neuronal functions, affecting memory and behaviour (Sarter and Lustig, 2020). Our study revealed that AsA and EASE significantly alleviated Pb-induced inhibition of the activity of AChE in relation to the control, suggesting that effects of EASE and even standard drug AsA on AChE may improve impairments caused by exposure to heavy metals. This further affirms the involvement of heavy metals in neurotoxicity, as Pb caused inhibition of AChE involved in neuronal functions (Adedara *et al*., 2022).

Monoamine oxidase is an enzyme that catalyzes the oxidation of neurotransmitters such as dopamine and serotonin, and its activity is often used as a marker for neurodegeneration (Kalia, 2013). The observed decrease in MAO levels in flies exposed to lead-acetate suggests that lead exposure may lead to neuronal impairment or neurodegeneration. This is consistent with previous studies that have reported the role of lead in inducing neurotoxicity and neurodegeneration (Sanders *et al*., 2009). The observed decrease in lipid peroxidation and increase in MAO levels in flies treated with EASE and AsA suggests that it may have a protective effect against leadinduced oxidative stress and neurodegeneration. AsA has been reported to protect cells from oxidative stress (Sies and Stahl, 1995). Treating lead-acetate flies with EASE and AsA resulted in a significant decrease in lipid peroxidation and an increase in MAO levels, suggesting that both protect against lead-induced oxidative stress and neurodegeneration. However, their combination does not translate to synergistic effects. This is in line with previous studies that have reported the synergistic effects of ascorbic acid and other antioxidants in protecting against oxidative stress (Sies and Stahl, 1995).

CONCLUSIONS

This study affirms the observation made by several literature that the presence of lead in biological systems interferes with the reactions to maintain redox balance in cells by disrupting innate antioxidant defence systems, thereby generating excess free radicals and hence inducing oxidative stress. It also suggests the therapeutic potential of the combination of ethyl acetate seed extract of *G. kola* and ascorbic acid against lead-acetate-induced toxicity, as there was a reversion of elevated oxidative, neurological, and inflammatory biomarkers.

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