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Evaluation of the toxic potential of lead nitrate on transgenic *Drosophila melanogaster* (hsp-70 lac Z) *Bg*^{9}

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Abstract

Lead is a well-known environmental toxicant that induces cellular damage through oxidative stress and apoptosis. The present study evaluates the toxic effects of lead nitrate at four different concentrations i.e., 0.05, 0.10, 0.20, and 0.40 M on transgenic *Drosophila melanogaster (hsp-70 lacZ) Bg*^{9}. The results revealed a dose-dependent increase in oxidative stress markers indicated by elevated levels of reactive oxygen species along with a decline in antioxidant defense systems. Furthermore, apoptotic markers (caspase-3 and 9) showed a significant increase in the activity suggesting the activation of programmed cell death pathways. This study highlights the utility of *Drosophila* as an alternative model to higher animals for investigating the mechanisms associated with heavy metal toxicity.

Keywords: Lead nitrate; *Drosophila*; Oxidative stress; Toxicity; Tissue damage.

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1. Introduction

Lead (Pb) is among the most pervasive heavy metals, detected in nearly all environmental compartments and biological systems. Its presence in the environment continues to raise significant concerns regarding potential risks to human health [1]. Concern has also been raised about the increasing use of chemicals in the environment, having lead (Pb) as its constituent. In India, the list of possible sources of lead exposure is wide ranging [2]. Conventionally, drinking water systems and metal-lined or tinned food utensils have been considered to be a major source. Moreover, lead could be introduced food by different means, which include painted machine parts, brass fittings, and metal surfaces [3]. A broad spectrum of negative health consequences have been linked to exposure to lead [4]. Various neurological disorders, immunological, renal, hepatic and hematological dysfunctions have been associated with lead exposure [5]. The current studies have suggested that the toxicity of lead is mediated by oxidative stress. This could lead to the production of reactive oxygen species (ROS) which results in the damage of brain, liver, kidney, sperm, and blood cells [6] and also lead to DNA damage by initiating a sequence of apoptotic events [7]. *Drosophila melanogaster* was the experiment model in the current study as it has a long-standing significance in the field of development biology and toxicological research [8-10]. Due to the high genetic homology between humans and Drosophila toxicant-induced effects on the flies are likely to indicate similar biological reactions in humans. The present study was aimed to study the toxic effects of lead nitrate using *Drosophila* as a model.

2. Materials and methods

2.1. *Drosophila* stocks and treatment

Drosophila melanogaster (hsp70-lacZ) Bg^{9} transgenic strain was used in the present study which is genetically modified to produce bacterial β-galactosidase along with Hsp70 in response to stress [11]. Lead nitrate was purchased from SRL (India) and mixed in the diet at final concentrations of 0.05, 0.10, 0.20 and 0.40 M. The third instar larvae were allowed to feed on the diet having desired concentrations of lead nitrate for 24 hrs before performing the assay.

2.2. O-nitrophenyl-β-D-galactopyranoside (ONPG) assay

Hsp70 expression serves as a valuable indicator of cytotoxicity [12]. The methodology proposed by Nazir et al. [13] was employed in this study. Larvae were placed in Eppendorf tubes (30 larvae per tube; five replicates per group) and rinsed with phosphate buffer. They were permeabilized by using acetone and incubated in 600 µl of ONPG buffer. The reaction was stopped by adding 300 µl of Na_2CO_3. The extent of the reaction was quantified by measuring the absorbance at 420 nm.

2.3. *In situ* histochemical β-galactosidase activity

10 larvae per treatment group were dissected in Poles Salt Solution (PSS) according to the method of Chowdhuri et al. [12], with each group having five replicates. The tissue explants tissue samples were then incubated overnight in X-gal staining solution. The samples were then fixed in 2.5% glutaraldehyde solution and subsequently washed with sodium phosphate buffer and observed under microscope.

2.4. Trypan blue exclusion test

The extent of tissue damage in the larvae was evaluated by performing Trypan blue exclusion assay [14]. The explants of 10 larvae per treatment (five replicates per group) were isolated and rinsed in phosphate-buffered saline (PBS). The larvae were then stained with trypan blue for 30 minutes, thoroughly washed in PBS, and assessed for dark blue staining. Scoring was performed using a composite index for each larvae: no color = 0; any blue color = 1; dark blue staining = 2; large patches of dark staining = 3; and complete staining of most cells = 4 (Krebs et al., 1997).

2.5 Analysis of reactive oxygen species (ROS) by 2-7 dicholorofluoroscein-diacetate (DCF-DA)

For the quantification of reactive oxygen species ROS, a solution of 1 mM DCF-DA was prepared in 5 ml of ethanol and 10 ml 50 mM Tris-buffer in each experiment set The larvae from treated and control larvae (five replicate per group). 10 larvae were explanted in a drop of 50 mM Tris-buffer, then 15 µl of the DCF-DA solution was added and the volume was adjusted to 300 µl using the Tris-buffer. The larvae were left at room temperature for 30 minutes. The fluorescence was examined under a fluorescence microscope and the intensity was quantified by using image J software.

2.6. Preparation of homogenate for biochemical assays

The larvae (100 larvae/treatment; five replicates/group) were homogenized in 1 ml of cold homogenizing buffer (0.1 M phosphate buffer containing 0.15 M KCl; pH 7.4). The supernatant after centrifugation at 9000g was used for the determination of glutathione content (GSH), glutathione-S-transferase (GST) activity, protein carbonyl content (PCC), superoxide dismutase (SOD), catalase (CAT) and Thiobarbituric acid reactive acid species (TBARS).

2.6.1. Estimation of glutathione (GSH) content

The glutathione (GSH) content was estimated colorimetrically using Ellman's reagent (DTNB) according to the procedure described by Jollow et al. [15]. The assay mixture consisted of 550 µl of 0.1 M phosphate buffer, 100 µl of supernatant and 100 µl of DTNB. The OD was read at 412 nm and the results were expressed as µ mol of GSH/gram tissue.

2.6.2. Estimation of glutathione-S-transferase (GST) activity

The glutathione-S-transferase activity was determined by the method of Habig et al. [16]. The reaction mixture consisted of 500 µl of 0.1 M phosphate buffer, 150 µl of 10 mM CDNB, 200 µl of 10 mM reduced glutathione and 50 µl of supernatant. The OD was read at 340 nm and the enzyme activity was expressed as µmoles of CDNB conjugates formed/min/mg protein.

2.6.3. Lipid peroxidation assay

Lipid peroxidation was measured according to the method described by Ohkawa et al. [17]. 5 µl of 10 µM butylhydroxy toluene (BHT), 200 µl of 0.67% thiobarbituric acid (TBA), 600 µl of 1% orthophosphoric acid (OPA), 105 µL of distilled water and 90 µl of the sample were taken in an eppendorf, vortexed and kept in the water bath at 90 $^{\circ}$C for 45 min. OD was read at 535 nm and the results were expressed in µmoles of TBARS formed/h/gram tissue.

2.6.4. Estimation of protein carbonyl content (PCC)

The protein carbonyl content was estimated according to the protocol described by Hawkins et al. [18]. The homogenate was diluted to a protein concentration of approx 1 mg/ml. About, 250 µl of diluted homogenate was taken in eppendorf. To it, 250 µl of 10 mM 2,4-dinitrophenyl hydrazine

(dissolved in 2.5 M HCl) was added, vortexed and kept in dark for 20 min. About 125 µl of 50% (w/v) trichloroacetic acid (TCA) was added, mixed thoroughly and incubated at −20 °C for 15 min. The tubes were then centrifuged at 4 °C for 10 min at 8200 g. The supernatant was discarded and the pellet obtained was washed twice by ice cold ethanol: ethyl acetate (1:1). Finally, the pellets were re-dissolved in 1 ml of 6 M guanidine hydrochloride and the absorbance was read at 370 nm.

2.6.5. Estimation of superoxide dismutase activity (SOD) activity

The superoxide dismutase activity **(**SOD) activity was estimated according to the method of Marklund [19]. The reaction mixture consisted of 17 µl of sample and 950 µl of 0.1 M phosphate buffer. The reaction was initiated by adding pyrogallol. An increase in OD was noted at 420 nm for 3 min at 30 s interval and the results were expressed as units/mg protein.

2.6.6. Determination of catalase (CAT) activity

The catalase activity was estimated according to the method of Beers and Sizer [20] by kinetic method where rate of dismutation of H_2O_2 to water and molecular oxygen is proportional to the concentration of catalase in the sample. The reaction mixture consisted of 650 µl of 0.1 M phosphate buffer, 333 µl of H_2O_2 (0.05 M) and 17 µl of sample. A decrease in OD was measured for 2 min, at 30 s intervals at 240 nm. The activity of catalase was calculated and expressed as µ moles of H_2O_2 consumed/min/mg protein.

2.7. Caspase-3 (Drice) and Caspase-9 (Dronc) activities

The assay was performed according to the manufacturer protocol (Bio-Vision, USA). The assay was based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) obtained after specific action of caspase-3 and caspase-9 on tetrapeptide substrates, DEVD-pNA and LEHD-pNA, respectively. The midguts were collected from 20 larvae per treatment group with 5 replicate for each group. The assay mixture (50 µl of cell suspension and 50 µl of chilled cell lysis buffer) was incubated on ice for 10 min. After incubation, 50 µl of 2X reaction buffer (containing 10 mM DTT) with 200 µM substrate (DEVD-pNA for Drice, and IETD-pNA for Dronc) was added and incubated at 37 °C for 1.5 h. The reaction was quantified at 405 nm.

2.8. Comet assay

The comet assay was performed as per the method of Mukhopadhyay et al. [21]. Midguts were isolated from 20 larvae per treatment group, with three replicates for each group. After replacing the Pole's salt solution (PSS) with 300 µl of collagenase (0.5 mg/ml in PBS, pH 7.4), the samples were incubated and slides were prepared. The cell suspension was prepared in PBS, and cell viability was evaluated using the trypan blue exclusion method as described by Siddique et al. (2012). For electrophoresis, a chilled solution (1 mM Na_2 EDTA and 300 mM NaOH, pH >13) was added to the chamber after placing the slides, which were allowed to unwind for 10 minutes. Electrophoresis was conducted for 15 minutes at 0.7 V/cm and 300 mA at 4°C. After electrophoresis, the slides were rinsed three times with a neutralizing buffer (0.4 M Tris buffer) and stained in the dark for 10 minutes with ethidium bromide (20 µg/ml; 75 µl per slide). The slides were then rinsed with cooled distilled water and covered with cover slips. Using Comet Score 1.5 software, 25 cells per slide were randomly selected, and the mean tail length (in arbitrary units) was calculated to evaluate DNA damage.

2.9. Statistical analysis

The data was analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey test using SPSS 16. The level of significance was kept at $p < 0.05$. The results were expressed as mean ± SEM.

3. Result

The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a dose dependent significant increase of 1.92, 2.84 and 4.23 folds respectively in the activity of β-galactosidase compared to control (Fig1;$p<0.05$). The larvae exposed to 0.05 M of lead nitrate did not showed a significant increase in the activity of β-galactosidase compared to control (Fig 1; $p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 6.69 fold in the activity of β-galactosidase compared to control (Fig 1; $p<0.05$). The results obtained for X-gal staining are shown in Figure 2 (a). The larvae exposed to 0.05 M of lead nitrate did not showed the β-galactosidae reaction compared to control (Fig 2). The larvae exposed to 0.10, 0.20 and 0.40 of lead nitrate showed a dose dependent increase in the X-gal staining compared to control (Fig 2). The larvae exposed to 25 µM of EMS showed an increase X-gal staining in the mid gut region of the larvae compared to control (Fig 2). The results obtained for the trypan blue staining for the tissue damage are shown

in Figure 3 (a) and the same has been quantified in Figure 3(b). The larvae exposed to 0.05M of lead nitrate did not showed a significant tissue damage compared to control (Fig 3a; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a dose dependent increase of 2.09, 4.72 and 7.72 folds, respectively, compared to control (Fig 3b; $p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 9.63 fold compared to control (Fig 3b; $p<0.05$).

The results obtained for DCF-DA staining are shown in Fig. 4 (a) and the same has been quantified in Fig. 4b. The larvae exposed to 0.05M of lead nitrate did not showed a significant increase in the mean grey scale value compared to control (Fig 4b; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40M of lead nitrate showed a dose dependent significant increase of 1.37, 1.75 and 1.99 folds, respectively, compared to control (Fig 4b; $p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 2.58 fold in the mean grey scale value compared to control (Fig 4b; $p<0.05$).

The larvae exposed to 0.05 M of lead nitrate did not showed a significant decrease in the GSH content compared to control (Fig 5a; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a significant decrease of 1.39, 1.68 and 2.04 folds, respectively, in the GSH content compared to control (Fig 5a; $p<0.05$). The larvae exposed to 25 µM of EMS showed a significant decrease of 3.01 fold in the GSH content compared to control (Fig 5a; $p<0.05$). The larvae exposed to 0.05 M of lead nitrate did not showed a significant increase in the GST activity compared to control (Fig 5b; $p<0.05$). the larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a dose dependent significant increase of 1.21, 1.41 and 1.58 folds, respectively, in the GST activity compared to control (Fig 5b;$p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 1.86 fold in the GST activity compared to control (Fig 5b; $p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 1.86 fold in the GST activity compared to control (Fig 5b; $p<0.05$). The larvae exposed to 0.05M of lead nitrate did not showed a significant increase in TBARS compared to control (Fig 5c; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a dose dependent significant increase of 1.06, 1.28 and 1.51 folds in the TBARS compared to control (Fig 5c;$p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 1.79 fold in the TBARS compared to control (Fig 5c; $p<0.05$). The larvae exposed to 0.05 M of lead nitrate did not showed a significant increase in PC content compared to control (Fig 5d;$p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a dose

dependent significant increase of 1.53, 2.13 and 2.66 folds, respectively, in the PC content compared to control (Fig 5d;$p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 3.53 fold in the PC content compared to control (Fig 5d; $p<0.05$). The larvae exposed to 0.05M of lead nitrate did not showed a significant increase in the SOD activity compared to control (Fig 5e;$p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a significant increase of 1.61, 2.82 and 3.91 folds, respectively, in the SOD activity compared to control (Fig 5e;$p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 5.09 fold in the SOD activity compared to control (Fig 5e; $p<0.05$). The larvae exposed to 0.05 M of lead nitrate did not showed a significant increase in the catalase activity compared to control (Fig 5f; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a dose dependent significant increase of 1.74, 3.91 and 5.33 folds, respectively in the catalase activity compared to control (Fig 5f; $p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 6.66 fold in the catalase activity compared to control (Fig 5f; $p<0.05$). The larvae exposed to 0.05M of lead nitrate did not showed a significant increase in the activity of caspase-3 compared to control (Fig 6a; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a significant increase of 1.26, 1.73 and 2.13 folds, respectively, in the activity of caspase-3 compared to control (Fig 6a;$p<0.05$). The larvae exposed to 25 µM of EMS showed an increase of 3.33 fold in the caspase-3 activity compared to control (Fig 6a; $p<0.05$). The larvae exposed to 0.05 M of lead nitrate did not showed a significant increase in the activity of caspase-9 compared to control (Fig 6b; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a dose dependent significant increase of 1.84, 2.53 and 3.23 folds, respectively, in the activity of caspase-9 compared to control (Fig 6b;$p<0.05$). The larvae exposed to 25 µM of EMS showed a significant increase of 4.61 folds in the activity of caspase-9 compared to control (Fig 6b; $p<0.05$).

The results obtained for DNA damage in the midgut cell of the third instar larvae are shown in Figure 7a and the same has been quantified in Figure 7b. A clear dose dependent DNA damage was observed in the midgut cells of the larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate (Fig 7a). The larvae exposed to 0.05 M of lead nitrate did not showed a significant DNA damage compared to control (Fig 7a; $p<0.05$). The larvae exposed to 0.10, 0.20 and 0.40 M of lead nitrate showed a significant increase of DNA damage of 2.18, 3.82 and 6.57 folds, respectively, compared to control (Fig 7b; $p<0.05$). The larvae exposed to 25µM of EMS showed a significant increase of 8.15 fold in the DNA damage compared to control (Fig 7b; $p<0.05$).

4. DISCUSSION

The present study showed that lead nitrate is toxic in nature. The findings of our study indicate that exposure of third instar larvae to the lead nitrate enhances the production of reactive oxygen species (ROS), leading to oxidative stress that disrupts cellular homeostasis and contributes to toxicity. Elevated ROS levels can damage essential biomolecules such as DNA, proteins, and lipids, ultimately impairing cellular function and promoting cell death which further confirms the toxic nature of lead nitrate. Lead (Pb) is a widespread environmental toxin that causes a variety of physiological disorders. Although the precise mechanisms underlying lead nitrate induced toxicity are not fully understood, accumulating evidence indicates that oxidative stress plays a key role. Exposure to lead promotes excessive generation of reactive oxygen species (ROS) while simultaneously weakening the body's antioxidant defense system.

The development of lead toxicity is complex and involves multiple mechanisms. Lead can directly disrupt enzyme activity, interfere with the absorption of essential trace minerals, and bind to sulfhydryl groups in proteins, thereby impairing the synthesis of structural proteins [22]. In the present study transgenic flies were used that contains wild type hsp-70 sequence to the fusion point of lac-Z, which showed the expression of this sequence by the expression of Lac Z structural gene which is used as a reporter gene in the transgenic strain [23]. In our study quantification of hsp-70 expression was done by ONPG assay based on β –galactosidase activity. On the exposure of larvae gut to different doses of lead nitrate a significant increase in the β –galactosidase activity was observed which showed the toxic nature of lead nitrate that induces the oxidative stress and increases the β-galatosidase activity. Similarly, when the gut was exposed to EMS increase in the β-galactosidase activity was observed. As oxidative stress intensifies, cells activate protective stress responses, notably the upregulation of heat shock proteins such as Hsp70 [24]. In *Drosophila*, this response can be visualized using X-gal staining in *hsp70-lacZ* transgenic lines, where increased blue coloration in midgut tissues signifies enhanced transcriptional activation of hsp70. A marked rise in β-galactosidase activity was detected after lead nitrate exposure, suggesting the presence of cytotoxic effects. As a reporter gene, β-galactosidase serves as an indirect indicator of hsp70 expression levels [25]. The upregulation of hsp70 in response to environmental contaminants is generally linked to early stress responses within cells and the onset of toxicity. Consistent with this, multiple studies have utilized β-galactosidase activity as a reliable

proxy to monitor hsp70 expression under stress conditions [26]. The X-gal staining outcomes aligned with the ONPG assay results, showing that larvae exposed to 0.040 M lead nitrate exhibited the highest β-galactosidase activity compared to control. The trypan blue exclusion assay was used to assess tissue damage in third instar larvae of *Drosophila*, revealing a clear dose-dependent increase after lead nitrate exposure. The relationship between hsp70 expression and tissue injury is well documented. In line with this, our results also showed a significant elevation in the tissue damage in larvae treated with lead nitrate compared to the control. When the production of reactive oxygen species exceeds a cell's ability to neutralize them, oxidative stress occurs, leading to potential cellular damage. In response, the antioxidant defense system becomes activated to limit and repair this damage. In the present study, multiple oxidative stress indicators were evaluated to determine the extent of damage induced by lead nitrate [27]. Lead nitrate toxicity in third-instar larvae of *Drosophila melanogaster* can be explained as a sequence of oxidative stress–mediated events occurring in the midgut epithelium, where feeding larvae accumulate Pb^{2+} ions. After uptake, Pb^{2+} disrupts cellular redox balance by inducing excessive production of reactive oxygen species (ROS), as indicated by increased fluorescence in dichlorofluorescein (DCF) staining assays. This rise in ROS serves as the primary trigger for extensive macromolecular damage [28-29]. Intracellular reactive oxygen species (ROS) was measured using 2-7 dicholorofluoroscein-diacetate (DCF-DA), a well recognized probe for detecting and quantifying intracellular hydrogen peroxide (H_2O_2) [30]. DCF-DA enters cells passively, and cellular esterases then remove its acetate groups, converting it, into a non-fluoroscent from that remains inside the cell. Subsequent oxidation by intracellular peroxidase transforms this molecule into the highly fluorescent compound DCF.

The tripeptide glutathione (GSH) plays a crucial role in protecting cells from reactive oxygen species (ROS). It participates in a wide range of cellular functions, such as enzyme activity, transport of molecules, synthesis of proteins and nucleic acids, formation of microtubules, cell signaling, regulation of gene expression, and protection against oxidative damage [31]. Glutathione is present in high concentrations within the cytosol, nucleus, and mitochondria, where it serves as the primary soluble antioxidant in these cellular compartments [32]. GSH is a vital molecule for preserving cellular integrity against reactive oxygen species (ROS); as a non-enzymatic antioxidant, it helps neutralize free radicals and contributes to the detoxification of ROS. In a study showed that the exposure of rat to lead nitrate (100 μ/mol/kg body weight) for 72

hours, leads to the decrease in the GSH content. Similarly, our present study, we found a dose-dependent decrease in glutathione levels upon the exposure to lead nitrate, which confirms the toxic nature of lead nitrate. Additionally, glutathione-S-transferase (GST), which facilitates detoxification by conjugating electrophilic metabolites with GSH, exhibits altered activity, indicating a compromised detoxification system [33]. GST activity relies on a steady availability of GSH, as its primary role is to detoxify xenobiotics by facilitating the nucleophilic interaction with electrophilic compounds. Under conditions of oxidative stress, GST activity is often elevated as part of the cellular detoxification response. The Study by Kansal et al., [34] showed that exposure to mice at 40 mg/kg body weight of lead nitrate for 7 days leads to increase in GST activity. In our study we also found significant dose dependent increase in GST activity. The cellular membrane is a primary site for oxidative damage caused by xenobiotics. Metal-induced production of reactive oxygen species can damage not only nuclear DNA but also other cellular components, particularly the polyunsaturated fatty acid residues in phospholipids, which are highly susceptible to oxidative degradation [35]. Free radicals can target unsaturated lipids within cells, triggering chain reactions that produce breakdown compounds such as malondialdehyde (MDA). Measuring MDA levels is widely used as an indicator of lipid peroxidation (LPO) [36]. Exposure of larvae to lead nitrate showed a dose dependent increase in the level of TBARS. Protein oxidation is an early indicator of oxidative damage and is commonly evaluated by measuring protein carbonyl content (PCC). In a study by Kasperczyk et al. [37] showed an increase in protein carbonyl content. Other studies have also reported the similar findings. In rats exposed to lead nitrate indicated an increase in protein carbonyl content further confirming the toxic narure of lead nitrate [38]. The same results were obtained in our study for protein carbonyl content that showed a dose-dependent increase in the PCC. Superoxide dismutase (SOD) is a key antioxidant enzyme that defends cells against oxidative stress by converting superoxide radicals into oxygen and hydrogen peroxide. SOD contributes to the maintenance of cellular redox homeostasis and protects proteins, lipids, and DNA from oxidative damage. It plays an essential role in cellular defense, as well as in processes such as aging, inflammation, and the development of diseases linked to oxidative stress [7]. Catalase (CAT) is an important intracellular antioxidant enzyme found in the peroxisomes of aerobic cells, where it helps safeguard cells against oxidative stress. In our study, CAT activity was increased in the larvae exposed to lead nitrate, suggesting that lead may disrupt antioxidant defenses by altering CAT activity, in line with earlier reports [39]. H_2O_2 can lead to

oxidative damages on the DNA, proteins and membrane lipids, and this type of damage has been linked to pathogenesis of a number of diseases in living organisms [40]. The larval midgut cells, characterized by high microsomal activity, were subjected to apoptosis assays to assess the effects of lead nitrate on caspase-9 and caspase-3 activation. Exposure of larvae to concentrations 0.05, 0.1, 0.2 and 0.4 M of lead nitrate resulted in a dose-dependent increase in the activities of both caspase-9 and caspase-3. The activation of procaspase-9 initiates a cascade leading to the activation of the downstream effector caspase-3, ultimately triggering cell death. The similar results were obtained in the study performed by Attafi et al., [41].

At the genomic level, excessive ROS and impaired antioxidant defenses culminate in DNA damage, which can be quantitatively assessed using the comet assay. A dose dependent DNA damage was also observed in the midgut cells of the third instar larvae of transgenic *Drosophila melanogaster (hsp-70 lac Z)Bg*^{9} compared to control. Midgut cells from Pb-exposed larvae showed subsequent increased in DNA damage [42]. The larvae exposed to 0.05 M of lead nitrate did not showed the toxic effect therefore could be considered as NOAEL (No observe adverse effect level) for the third instar larvae. This genotoxic stress further amplifies cellular dysfunction by interfering with transcription and replication processes.

5. Conclusion

The present study demonstrates that exposure to lead nitrate induces significant toxicity in *Drosophila melanogaster*, as evidenced by increase tissue damage, increased β-galactosidase activity alterations in oxidative stress and apoptosis markers. Elevated levels of oxidative stress indicators suggest that lead nitrate disrupts cellular redox balance, leading to excessive generation of reactive oxygen species (ROS). This oxidative imbalance is further associated with the activation of apoptotic pathways, as indicated by increased expression or activity of apoptosis-related markers. Together, these findings indicate that oxidative stress plays a central role in mediating lead nitrate-induced cellular damage, ultimately triggering programmed cell death. The results highlight the usefulness of *Drosophila melanogaster* as a model for studying heavy metal toxicity and provide insights into the molecular mechanisms underlying lead-induced cytotoxicity. Overall, lead nitrate can be concluded to exert its toxic effects through a synergistic interaction between oxidative stress and apoptosis pathways.

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Authors contribution

IS: wrote the main manuscript text, performed experiments; HFK: wrote the main manuscript text; JF: performed experiments; AS: Performed experiments; SK: performed experiments; SJ: Draw figures, Statistical analysis of the data ; YHS: concept, wrote and reviewed manuscript

Availability of data and materials

The data will be made available on request.

Declarations

Ethics approval and consent to participate Not applicable.

Competing interests

The authors declare no competing interests.

Consent to Publish declaration

Not applicable

Consent to Participate declaration

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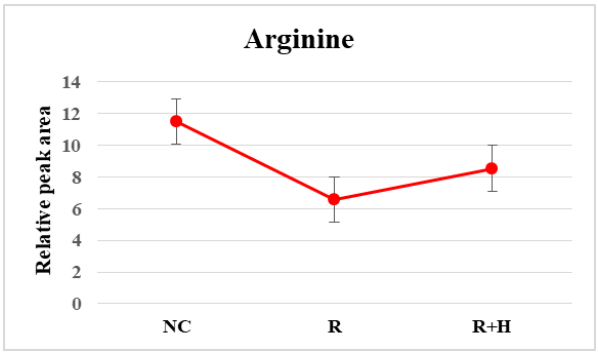
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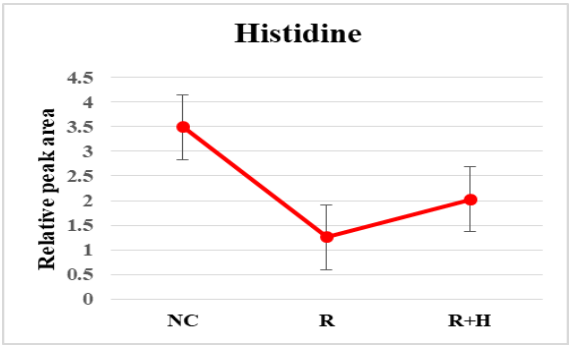
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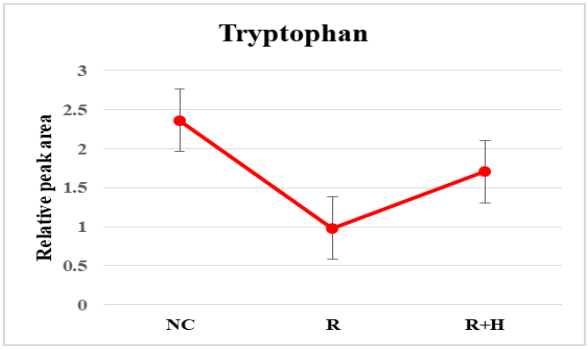
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Arginine
Relative peak area
14
12
10
8
6
4
2
0
NC
R
R+H

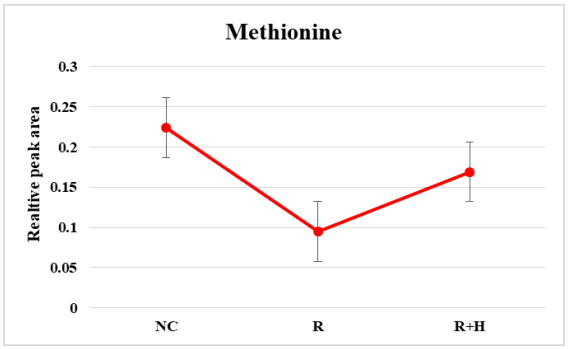
(a)


Histidine
Relative peak area
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
NC
R
R+H

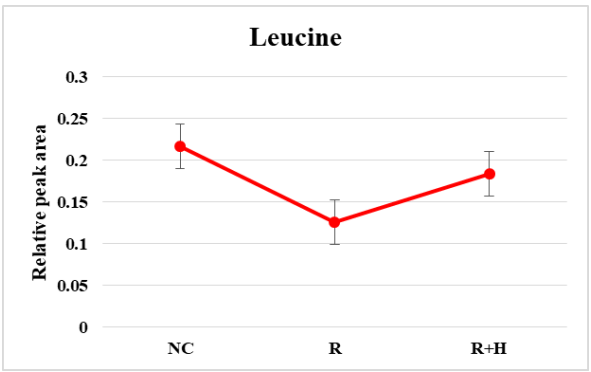
(b)


Tryptophan
Relative peak area
3
2.5
2
1.5
1
0.5
0
NC
R
R+H

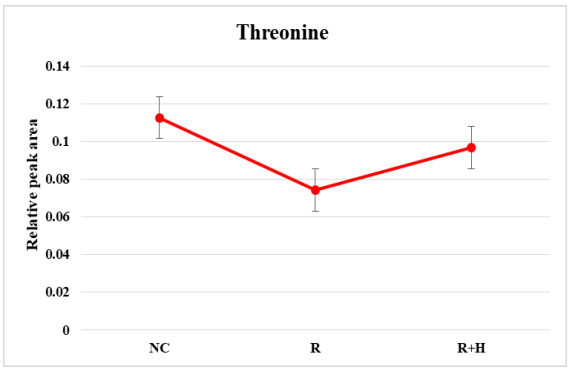
(c)


Methionine
Realitive peak area
0.3
0.25
0.2
0.15
0.1
0.05
0
NC
R
R+H

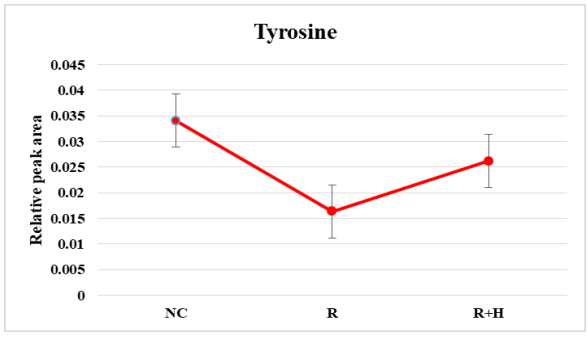
(d)


Leucine
Relative peak area
0.3
0.25
0.2
0.15
0.1
0.05
0
NC
R
R+H

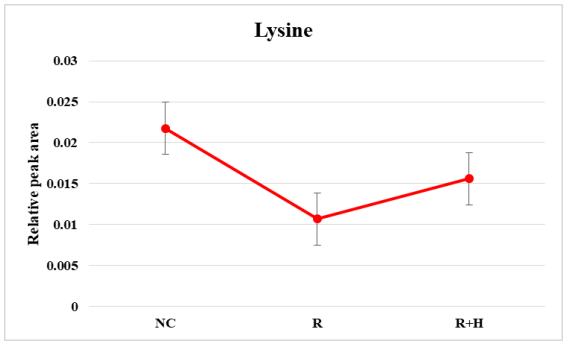
(e)


Threonine
Relative peak area
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
NC
R
R+H

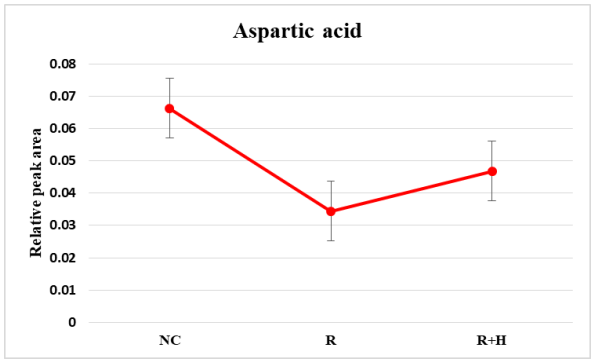
(f)


Tyrosine
Relative peak area
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
NC
R
R+H

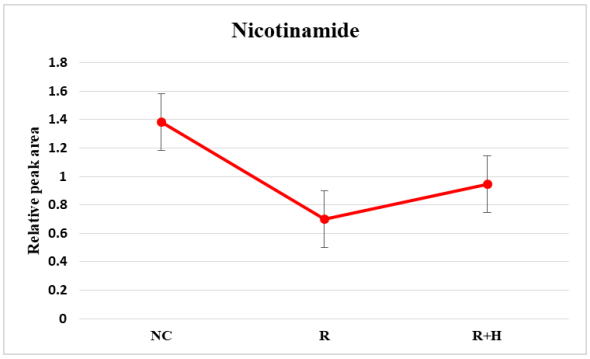
(g)


Lysine
Relative peak area
0.03
0.025
0.02
0.015
0.01
0.005
0
NC
R
R+H

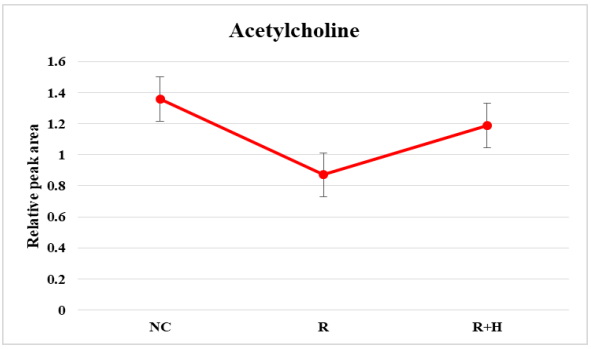
(h)


Aspartic acid
Relative peak area
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
NC
R
R+H

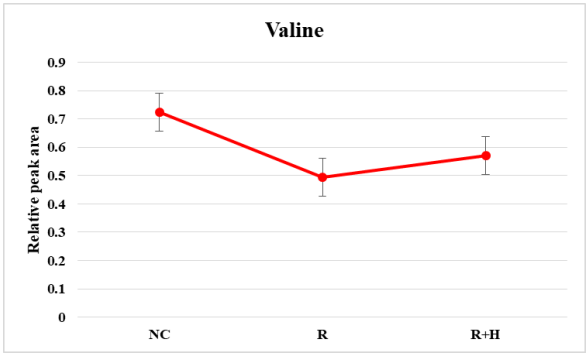
(i)


Nicotinamide
Relative peak area
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NC
R
R+H

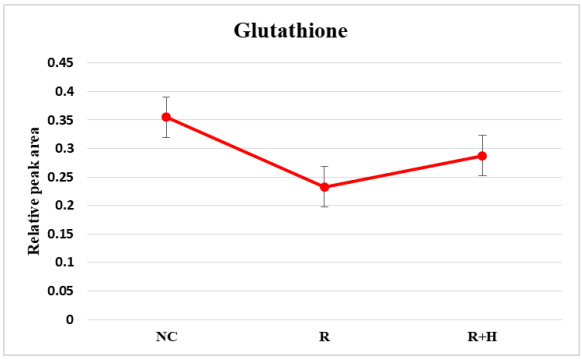
(j)


Acetylcholine
Relative peak area
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NC
R
R+H

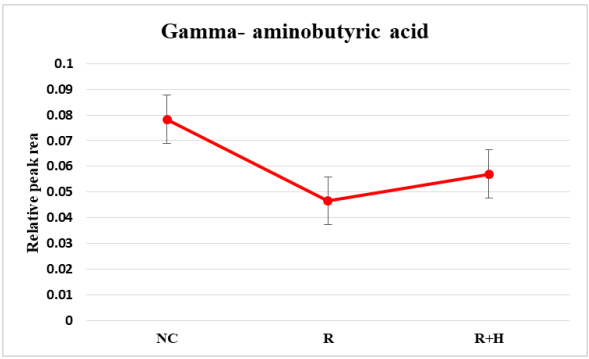
(k)


Valine
Relative peak area
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NC
R
R+H

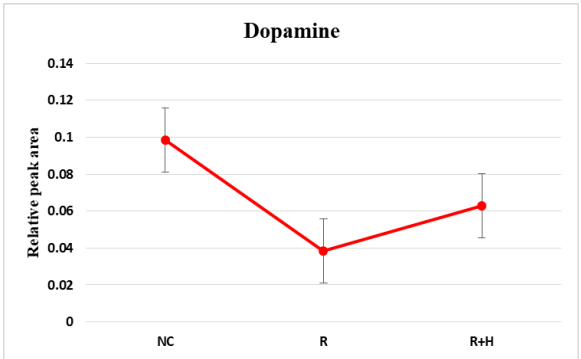
(l)

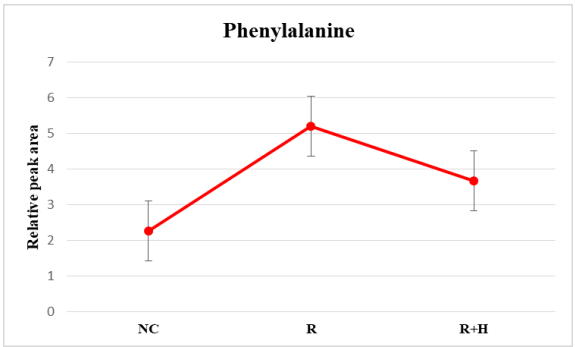

Glutathione
Relative peak area
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
NC
R
R+H

(m)

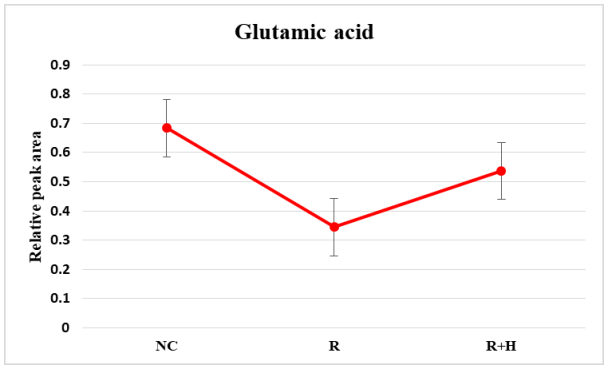

Gamma- aminobutyric acid
Relative peak rea
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
NC
R
R+H

(n)


Dopamine
Relative peak area
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
NC
R
R+H

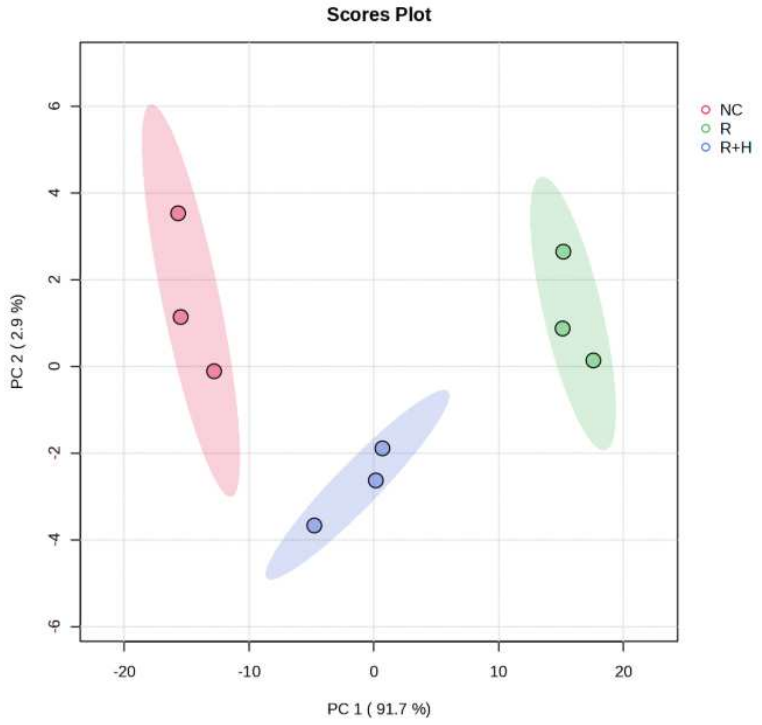

Phenylalanine
Relative peak area
7
6
5
4
3
2
1
0
NC
R
R+H

(o) **(p)**

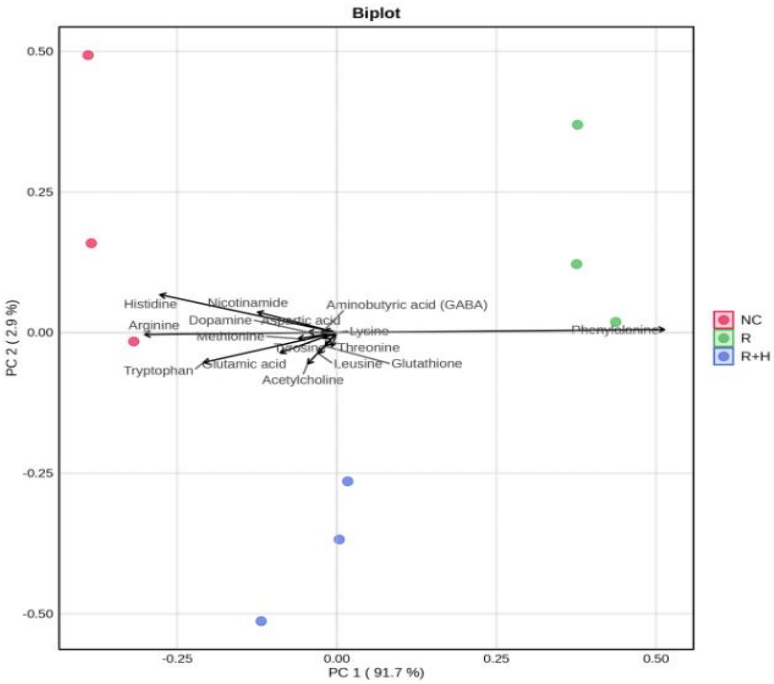


(q)

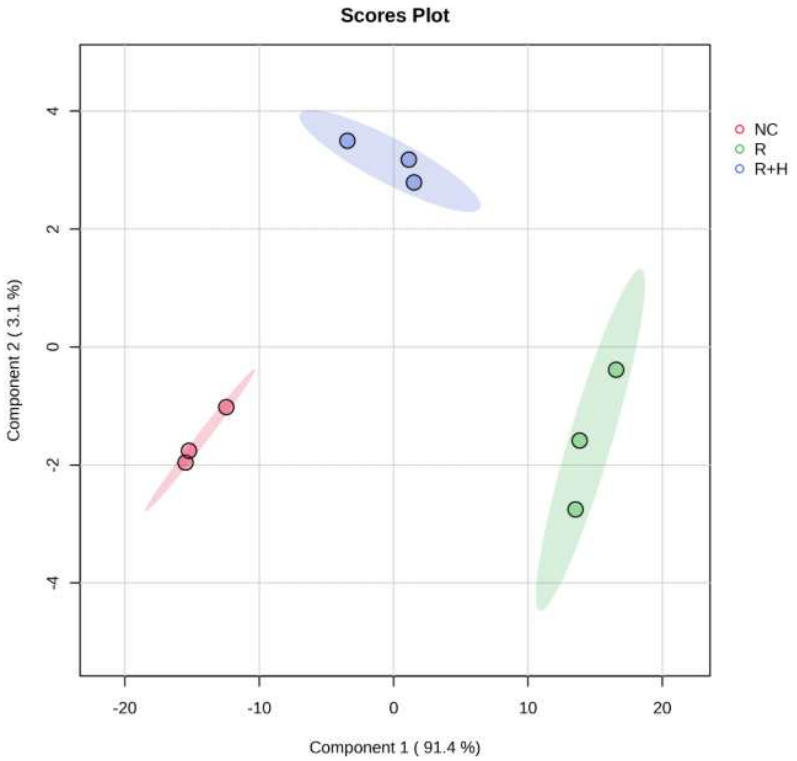
Fig.1. (a-r) Metabolic profile of selected 17 metabolites in the brain homogenate of rotenone (500µM), Negative control and rotenone flies exposed to hesperidin (100 µM).


Scores Plot
NC
R
R+H
PC 2 (2.9 %)
PC 1 (91.7 %)
6
4
2
0
-2
-4
-6
-20
-10
0
10
20

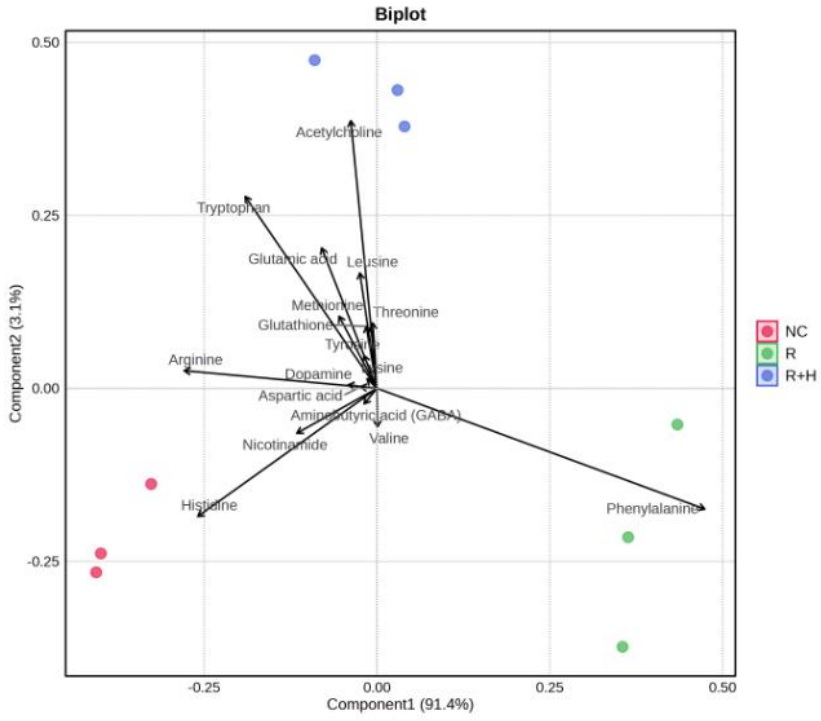
(a)


Biplot
NC
R
R+H
PC 2 (2.9 %)
PC 1 (91.7 %)
0.50
0.25
0.00
-0.25
-0.50
-0.25
0.00
0.25
0.50
Histidine
Nicotinamide
Aminobutyric acid (GABA)
Arginine
Dopamine
Aspartic acid
Lysine
Methionine
Threonine
Tryptophan
Glutamic acid
Leusine
Glutathione
Acetylcholine

(b)



(c)



(d)

Fig 2: PCA analysis of metabolites in this study: (a) PCA Score plot, (b) PCA biplot of metabolites in rotenone treated flies, rotenone flies exposed to hesperidin and negative control flies (c) PLS-DA analysis of metabolites: (a) score

plot and (d) PLSDA biplot of metabolites in rotenone treated flies, rotenone flies exposed to hesperidin and negative control flies.

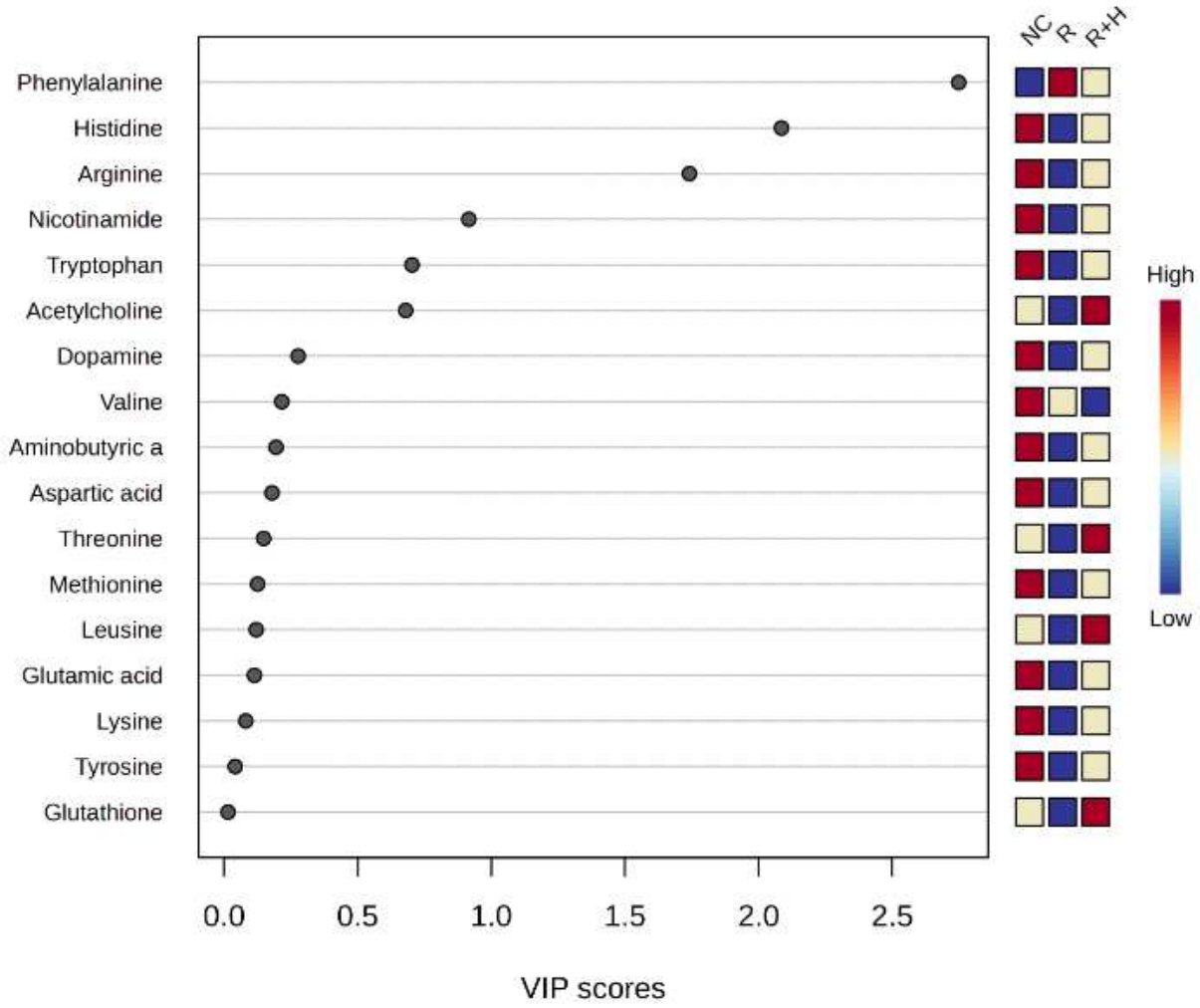


Fig. 3 VIP score plot of metabolites in rotenone treated flies, rotenone flies exposed to hesperidin and negative control flies.

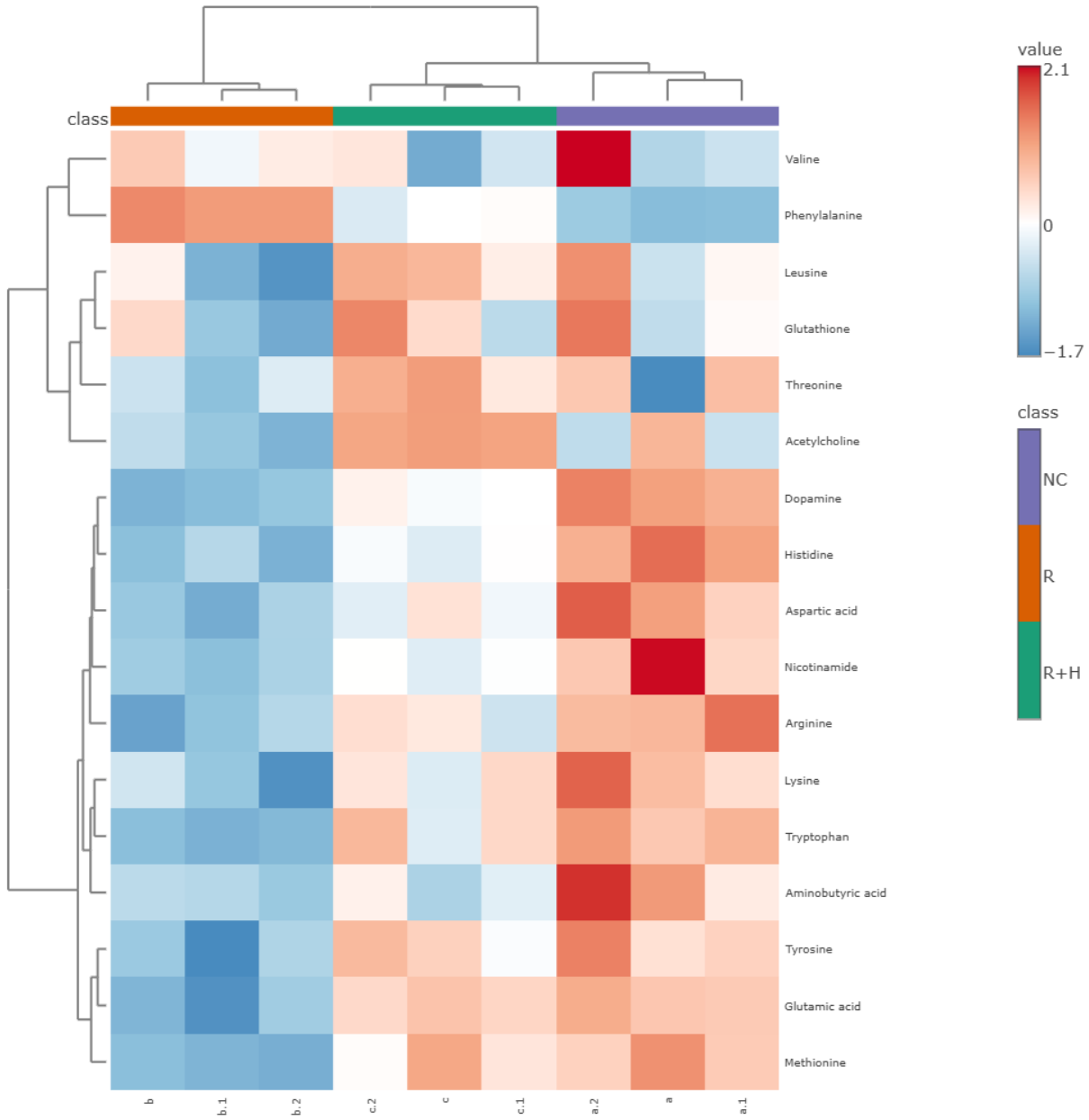
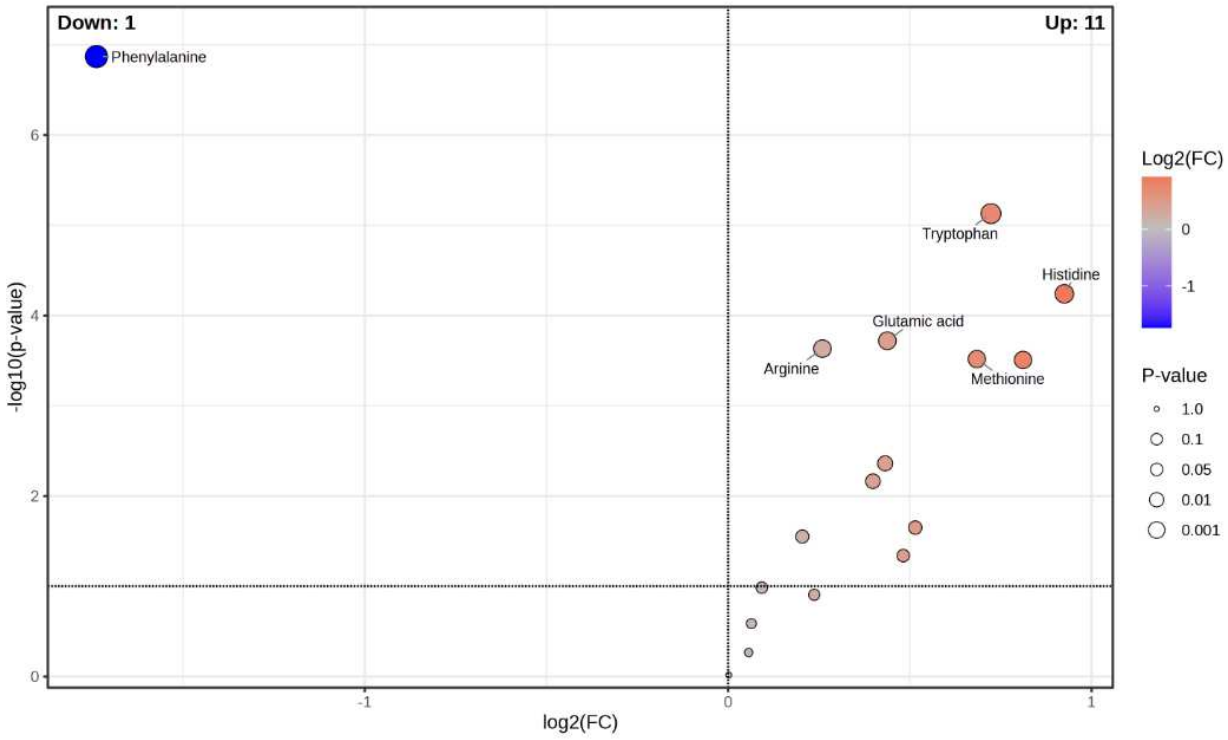
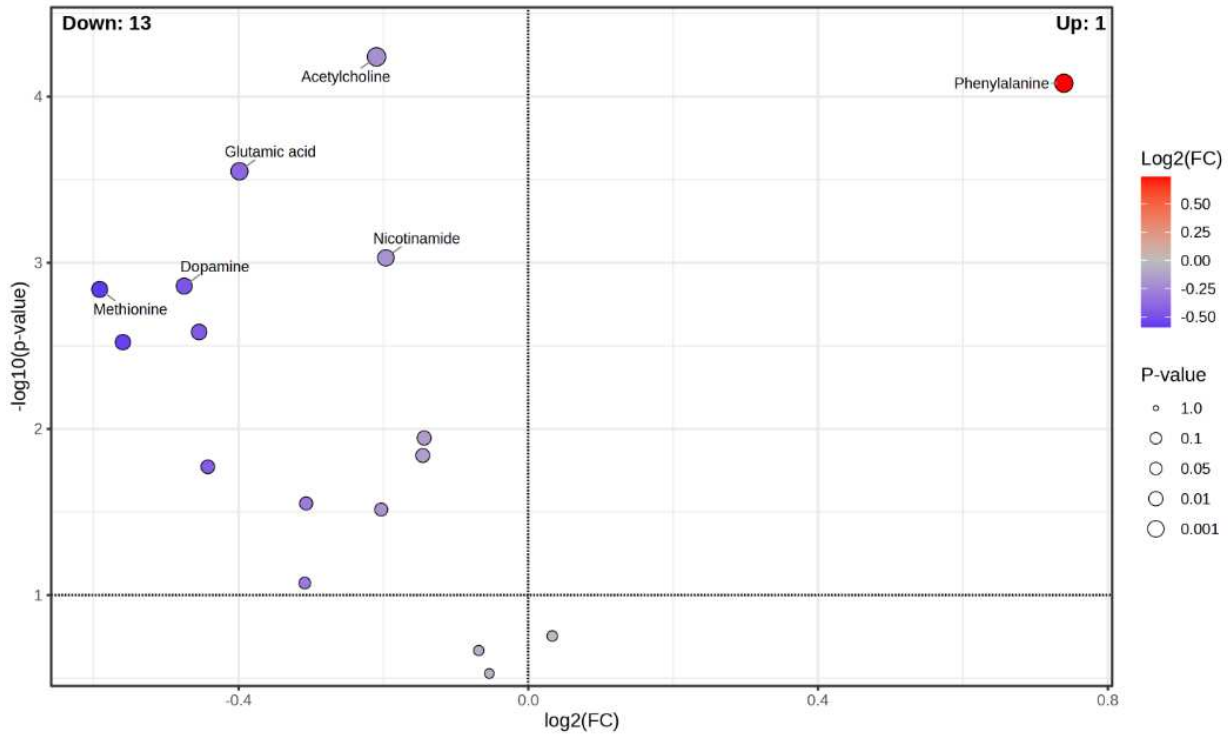


Fig 4. Heatmap of metabolites in flies exposed to rotenone, flies exposed to rotenone along with hesperidin and negative control flies.



(a)



(b)

Fig. 5 Volcano plot analysis of metabolites in rotenone versus negative control flies **(a);** volcano plot analysis of metabolites in rotenone flies versus flies exposed to rotenone along with hesperidin **(b).**

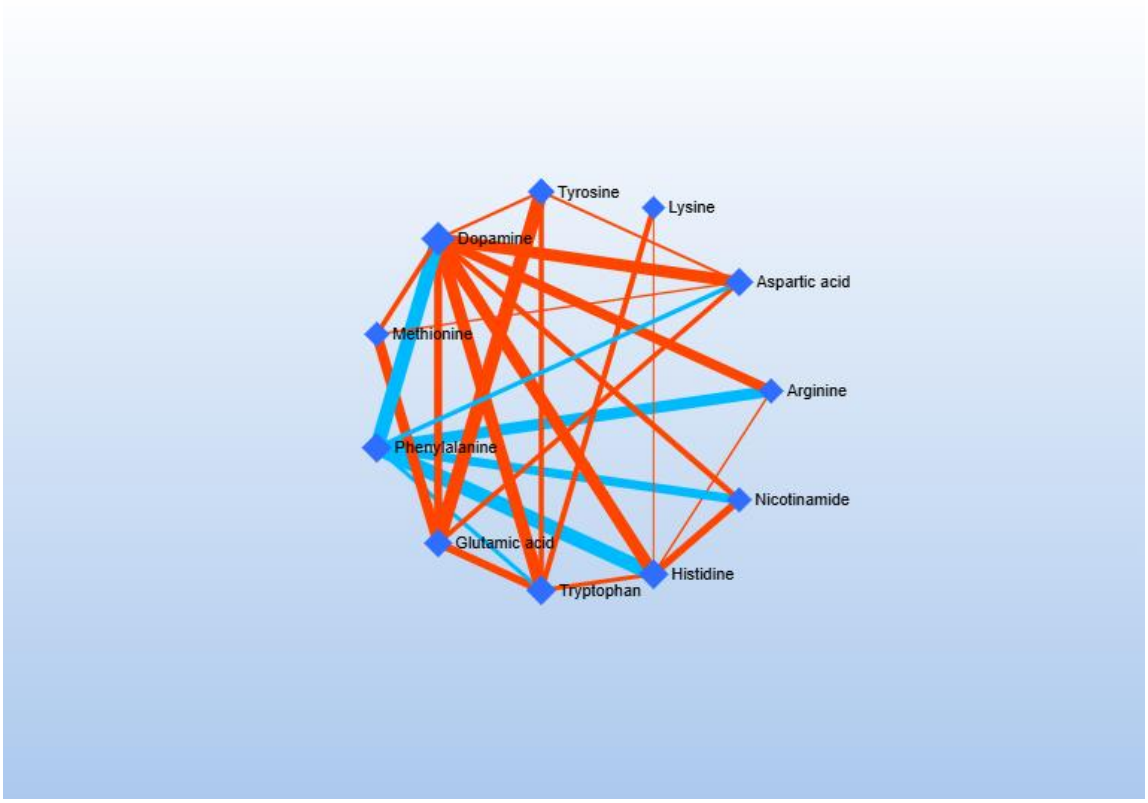


Fig. 6: Metabolic network analysis of metabolites used for the study.