Food and Chemical Toxicology 184 (2024) 114425

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Evaluation of Bis(2-ethylhexyl) phthalate toxicity on the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9}

Kajal Gaur [a], Himanshi Varshney [a], Iqra Subhan [a], Javeria Fatima [a], Smita Jyoti [b], Yasir Hasan Siddique [b,*]

[a] *Laboratory of Alternative Animal Models, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, 202002, Uttar Pradesh, India*

[b] *Department of Zoology, School of Sciences, IFTM University, Moradabad, UP, India*

ARTICLE INFO

Handling editor: Bryan Delaney

Keywords:
Phthalates
Oxidative stress markers
Toxicity
DNA damage
HSP 70

ABSTRACT

Bis(2-ethylhexyl) phthalate, generally known as DEHP is a synthetic compound mainly used as a plasticizer to make polyvinyl chloride products flexible and soft. The present work aimed to study the toxicity of Bis(2-ethylhexyl) phthalate on the third instar larvae of transgenic *Drosophila melanogaster(hsp70-lacZ) Bg*^{9}. The hsp70 gene is associated with the β-galactosidase in our present transgenic strain therefore, the more activity of β-galactosidase will indirectly correspond to hsp70 expression. The third instar larvae were allowed to feed on the diet for 24 h having 0.001, 0.005, 0.01, and 0.02 M of Bis(2-ethylhexyl) phthalate at the final concentration. After the exposure of 24hrs, the larvae were subjected to ONPG assay, X-gal staining, trypan blue exclusion test, oxidative stress markers assays, and comet assay. A dose-dependent increase in hsp70 expression, tissue damage, Glutathione-S-transferase (GST) activity, lipid peroxidation, monoamine oxidase, caspase-9 & 3, protein carbonyl content (PCC), DNA damage and decrease in the glutathione (GSH) content, delta-aminolevulinic acid dehydrogenase (δ-ALD-D) and acetylcholinesterase activity were observed in the larvae exposed to 0.005, 0.01, 0.02 M of Bis-(2-ethylhexyl) phthalate. The dose of 0.001 M of Bis(2-ethylhexyl) phthalate did not showed any toxic effects and hence can be considered as No Observed Adverse Effect Level (NOAEL) for Bis(2-ethylhexyl) phthalate. The study supports the use of *Drosophila* for the evaluation of possible toxic effects associated with synthetic compounds.

1. Introduction

Plasticizers are those compounds that add flexibility, durability, and longevity to products that are made of PVC (Huang et al., 2008). Bis (2-ethylhexyl) phthalate is one of the common plasticizer used in packaging, toys, lubricants, electronics, medical devices, clothing and cosmetics (Huang et al., 2008). Bis(2-ethylhexyl) phthalate is also called dioctyl phthalate or Di-2-ethylhexyl phthalate is an odorless, colorless, viscous, and lipophilic liquid having greater solubility in substances like paint, thinners, petrol, and oils than in water (Rowdhwal and Chen, 2018). More than 8 million tonnes of phthalates were produced annually in 2011, with 1 million tonnes being consumed in Europe and 1.4 million tonnes in China in 2010 (Yuan et al., 2022). Exposure of phthalates to humans and wildlife is inevitable due to their high production, widespread use in consumer products, and because of their non-covalently binding chemical characteristics, they are easily released into the environment, posing a toxicological risk (Ma et al., 2021). A number of studies have been carried out to demonstrate the serious effects of Bis(2-ethylhexyl) phthalate on animal models and humans such as reproductive toxicity, (mainly in males) (Fu et al., 2020), liver as well as kidney toxicity (Zhao et al., 2021; Li et al., 2021) and neurotoxicity (Feng et al., 2020). It is also responsible for promoting a different type of cancer in lungs, breast, and thyroid gland (Kim et al., 2022) by damaging the DNA and Akt/NF-kB signaling pathway activation (Urade et al., 2022; Hsieh et al., 2022). It also leads to high oxidative stress and development defects (Xu et al., 2013; Hamid et al., 2020). Bis (2-ethylhexyl) phthalate is also considered an endocrine disruptor. Humans can be exposed in a variety of ways, through skin contact, ingestion, and inhalation, but food is considered the main source of exposure to humans (Urade et al., 2022). When DEHP is released, it can bind to dust particles that comes in contact with soil and mix with the groundwater which affects the lives of human (Roslev et al., 1998). It has also been

* Corresponding author.
E-mail address: yasir_hasansiddique@rediffmail.com (Y.H. Siddique).

https://doi.org/10.1016/j.fct.2023.114425
Received 12 August 2023; Received in revised form 20 December 2023; Accepted 22 December 2023
Available online 29 December 2023

reported to cause shortening of the anogenital distance (distance between genitals and anus), but such type of effects were not observed in females. It also decreases the testis weight, penile size and changes in vas deferens and epididymis in experimental male rats (Foster et al., 2000; Macleod et al., 2010). In this study *Drosophila* is a commonly used model organism in scientific research due to its short generation time, well-characterized genetics, and similarities to higher organisms in many biological processes. Using this model, we can understand the effect of the potential risks and mechanisms of action associated with the test compound. In the present study we evaluated the toxic potential of Bis(2-ethylhexyl) phthalate on the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lac Z) Bg*^{9}.

2. Material methods

2.1. Fly strain

The transgenic *Drosophila melanogaster* strain used in this study expresses bacterial-galactosidase in response to stress (Lis et al., 1983). The transformation vector of this strain of flies has a P-element, which indicates that the line contains wild-type hsp70 sequence up to the lac Z fusion site. The hsp70 gene is associated with the β-galactosidase in our present transgenic strain therefore, the more activity of β-galactosidase will indirectly correspond to hsp70 expression. According to Nazir et al. (2003a,b) and Siddique et al. (2014), the larvae and flies were cultured on normal *Drosophila* food containing maize powder, yeast, sugar, and agar at 24–25 °C. Bis(2-ethylhexyl) phthalate (purchased from Sigma) was dissolved in food to establish a final concentration of 0.001, 0.005, 0.01 and 0.02 M. The assays were performed after allowing the third instar larvae to feed on it for 24 h. The consumption of the diet having the desired concentrations of Bis(2-ethylhexyl) phthalate was observed separately by mixing the dye in the diet of the third instar larvae and allowing the third instar larvae to feed on it for 24 h.

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

Hsp70 expression can be used to estimate cytotoxicity (Chowdhuri et al., 1999). In this method suggested by Nazir et al. (2003a,b) was used. Larvae were placed in a microcentrifuge tube (30 larvae/tube; five replicates/groups) and washed in phosphate buffer before being permeabilized for 10 min by acetone and being incubated overnight at 37 °C in 600 μl of ONPG staining buffer. The reaction was stopped after incubation by adding 300 μl of Na_2CO_3. By the measurement of absorbance at 420 nm, the extent of reaction was quantified.

2.3. In situ histochemical β-galactosidase activity

The larvae were dissected out in Poles Salt Solution (PSS) using the procedure outlined by Chowdhuri et al. (1999), with 10 larvae per treatment and 5 replicates per group. The tissue explants were stained overnight at 37 °C in the dark with an X-gal staining solution before being fixed in 2.5% glutaraldehyde and cleaned in 50 mM sodium phosphate buffer (pH 8.0).

2.4. Trypan blue exclusion test

The extent of tissue damage in larvae was assayed by a dye exclusion test (Nazir et al., 2003a,b; Krebs and Feder, 1997). Briefly, the internal tissues of larvae (10 larvae/treatment; 5 replicates/group) were explanted in a drop of Pole's salt solution (PSS), washed in phosphate buffer saline (PBS), stained by trypan blue (0.2 mg/ml in PBS) for 30 min, washed thoroughly in PBS, and scored immediately for dark blue staining. The scoring for the trypan blue staining was done on an average composite index per larvae: no color = 0; any blue = 1; darkly stained = 2; large patches of darkly stained cells = 3; or complete staining of most cells in the tissue = 4 (Krebs et al., 1997).

2.5. Preparation of homogenate for biochemical assays

The larvae were homogenized in 1 ml of cold homogenizing buffer (0.1 M phosphate buffer containing 0.15 M KCl; pH 7.4) using 100 larvae per treatment and 5 replicates per group. After centrifugation at 9000*g* supernatant was used for the determination of glutathione content (GSH), glutathione-S-transferase (GST), superoxide dismutase activity (SOD), catalase (CAT) activity, protein carbonyl content (PCC), lipid peroxidation assay (LPO), acetylcholinesterase (AChE), monoamine oxidase (MAO) and Delta aminolevulinic acid dehydrogenase (ALA-D) activities.

2.5.1. Estimation of glutathione (GSH) content

According to the method used by (Jollow et al., 1974), the glutathione (GSH) content was determined colorimetrically using Ellman's reagent. In a 1:1 ratio, 4% sulphosalicylic acid was used to precipitate the supernatant. The samples were centrifuged at 4200 rpm for 10 min after being stored at 4 °C for 1hr. The 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) assay combination contained 550 μl of 0.1 M phosphate buffer, 100 μl of supernatant, and 100 μl of DTNB. OD was read at 412 nm and the data was expressed as μ moles of GSH/gram tissue.

2.5.2. Determination of glutathione-S-transferase (GST) activity

The method of (Habig et al., 1974) was used to measure the glutathione-S-transferase (GST) activity. 500 μl of 0.1 M phosphate buffer, 150 μl of 10 mM 1-Chloro-2-4, dinitrobenzene (CDNB), 200 μl of 10 mM reduced glutathione, and 50 μl of supernatant make up the reaction mixture. The enzyme activity was measured by taking an OD at 340 nm and expressed in moles of CDNB conjugates/min/mg/protein.

2.5.3. Superoxide dismutase activity (SOD) activity

For the estimation of SOD activity, Marklund and Marklund's (1974) method was used. The reaction mixture is made up of 950 μL of phosphate buffer and 17 μL of sample. Finally, pyrogallol was added to start the reaction. At 420 nm, an increase in OD was seen for 3 min after every 30 s. The results were expressed in units per milligram of protein.

2.5.4. Catalase (CAT) activity

According to the kinetic approach (Beers and Sizer, 1952), the concentration of catalase in the sample determines how quickly H_2O_2 is dismutated into water and molecular oxygen. 650 μl of phosphate buffer (0.1 M), 333 μl of H_2O_2 (0.05 M), and 17 μl of sample make up the reaction mixture. At intervals of 30 s at a wavelength of 240 nm, a drop in OD was observed for 2 min. The catalase activity was determined and represented as μ moles of H_2O_2 consumed per minute per milligram of protein.

2.5.5. Lipid peroxidation assay

Lipid peroxidation was measured according to the method described by (Ohkawa et al., 1978). The reaction mixture consists of 5 μl of 10 mM butyl-hydroxy toluene (BHT), 200 μl of 0.67%. thiobarbituric acid, 600 μl of 1% O-phosphoric acid, 105 μl of distilled water and 90 μl of supernatant. The resultant mixture was incubated at 90 °C for 45 min and the OD was measured at 535 nm. The results were expressed as μ moles of TBARS formed/h/gram tissue.

2.5.6. Protein carbonyl content

The protein carbonyl content was calculated using the method outlined in Hawkins et al. (2009). To get a protein content of 1 mg/ml, the homogenate was diluted. 250 μl of diluted homogenate with 250 μl of 10 mM 2, 4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was left in the dark for 20 min 125 μl Trichloroacetic acid (TCA), 50% (w/v), was added carefully, mixed, and then incubated at −20 °C for 15 min. After that, the tubes underwent centrifugation (9000 rpm) for 10 min at 4 °C. The pellet was washed twice with chilled ethanol:ethyl acetate (1:1) after discarding the supernatant. 1 ml of 6M guanidine hydrochloride

was taken to re-dissolved the pellet and at 370 nm absorbance was taken.

2.5.7. Determination of acetylcholinesterase (AChE) activity

The activity of acetylcholinesterase was estimated according to the method of Ellman et al. (1961). 100 μl of 10 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 650 μl of 0.1 M phosphate buffer (pH 7.4), and 100 μl of supernatant make up the reaction mixture. The material was properly blended before the absorbance at 412 nm was measured. 10 μl of 0.075 M acetylthiocholine was added when the absorbance value had stabilized, and the change in absorbance per minute was noted in order to determine the enzyme activity.

2.5.8. Estimation of monoamine oxidase (MAO)

The monoamine oxidase activity was estimated using the method outlined by McEwen and Helen (1965). The 400 μl of 0.1 M phosphate buffer (pH 7.4), the 1300 μl of distilled water, the 100 μl of benzylamine hydrochloride, and the 200 μl of homogenate make up the assay mixture. After 30 min of room temperature incubation, 1 mL of 10% perchloric acid was added, and the mixture was centrifuged at 1500 g for 10 min. The OD was read at 280 nm.

2.5.9. Determination of delta aminolevulinic acid dehydrogenase (δ-ALA-D)

The method given by Abolaji et al. (2014) was used to measure the rate of porphobilinogen production in order to assess the δ-ALA-D activity. The reaction mixture, (25 μl of homogenate, 10 μl of 0.5 M Tris-glycine (pH 8.5), 7 μl of distilled water, and 8 μl of 31.25 mM 5-aminolevulinic acid hydrochloride) was incubated at 37 °C for 3 h. The reaction mixture was then mixed with 10% triscarboxylic acid containing 20 mM CuSO4 before being centrifuged at 4500g for 10 min. Following this, 100 μl of the Erlich reagent and 100 μl of each of the produced supernatants were taken and incubated for 30 min at room temperature. The absorbance was read at 555 nm.

2.6. Assay for caspase-9 (Dronc) and caspase-3 (Drice) activities

The test was performed in accordance with the manufacturer's instructions (Bio-Vision, California, USA). The assay was based on the specific caspase-3 and caspase-9 actions on the tetrapeptide substrates, DEVD-pNA and LEHD-pNA, respectively, to yield the chromophore p-nitroanilide (pNA), which was detected spectrophotometrically. 50 μl of mid gut cell suspension and 50 μl of cooled cell lysis buffer was incubated on ice for 10 min. After 1.5 h of incubation, 50 μl of 2X reaction buffer (10 mM DTT) was added, along with 200 μM substrate (DEVD-pNA for Drice and IETD-pNA for Dronc). The OD was read at 405 nm.

2.7. Analysis of DNA damage by comet assay

The comet assay was performed according to the method performed by Mukhopadhyay et al. (2004). In PSS, the midgut was removed from 20 larvae per treatment (3 replicates/group). After replacing the PSS with 300 μl of collagenase (0.5 mg/ml in PBS, pH 7.4), the microcentrifuge tube was placed at 25 °C for 15 min. The cell suspension was prepared in PBS and before starting the experiment, the cell viability was examined by performing the trypan blue assay (Siddique et al., 2012). The precoated slides with 1% normal melting point agarose were covered with approximately 75 μl of cell suspension mixed with 80 μl of 1.5% low melting agarose. The slides were then placed for 2 h at 4 °C in freshly made chilled lysing solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris pH 10.0, and 1% Triton X-100, pH 10). The chilled electrophoresis solution (1 mM Na_2 EDTA and 300 mM NaOH, pH > 13) was then added to in the electrophoresis assembly after placing the slides. To allow DNA to unwind, the slides were placed in this solution for 10 min. The electrophoresis was carried out for 15 min at 0.7 V/cm and 300 mA at 4 °C. The slides were rinsed three times with neutralizing buffer (0.4 M Tris buffer) after electrophoresis. The slides were then stained for 10 min in the dark with ethidium bromide (20 μg/ml; 75 μl/slides). The slides were covered with cover slips after being immersed in cooled distilled water to remove the excess discoloration. The slides were prepared in duplicate, and each experiment was carried out in three copies. Using comet score 1.5 software (Comet ScoreTM v1.5 Software, TriTek Corporation, Sumerduck), 25 cells per slide (3 replicates/group) were randomly selected at a fixed depth of the gel, and mean tail length (a.u.) was computed to evaluate DNA damage.

2.8. Statistical analysis

The data was analysed by performing one-way analysis of variance (ANOVA) followed by post hoc Tukey's test using GraphPad Prism software [version 5.0]. The level of significance was kept at $p < 0.05$. The results were expressed as mean ± SEM.

3. Results

Fig. S1 shows the consumption of diet having the desired concentrations of phthalate. No significant increase in the activity of β-galactosidase was observed in the larvae exposed to 0.01 M of phthalate compared to control (Fig. 1; $p < 0.05$). A dose-dependent significant increase of 1.43, 3.56 and 4.87 folds, in the activity of β-galactosidase was observed in the larvae exposed to 0.005, 0.010 and 0.02 M of phthalate, respectively, compared to control (Fig. 1; $p < 0.05$). A significant increase of 7.68-fold in the activity of β-galactosidase compared to control was observed in the larvae exposed to 25 μM of EMS (Fig. 1 & Table S1; $p < 0.05$). Fig. 2S showed the results obtained for X-gal staining performed on third-instar larvae. No β-galactosidase activity was observed in the larvae exposed to 0.001 M of phthalate Fig. S2; (P1). A dose-dependent increase in the activity of β-galactosidase activity was observed in the larvae exposed to 0.005, 0.010 and 0.020 M of phthalate compared to control Fig. S2; (P2 to P4). The larvae exposed to 25 μM of EMS showed the highest β-galactosidase activity Fig. S2; (PC). Stress, whether mechanical, thermal, or toxic, is evident in the form of tissue damage, so analyzing tissue damage becomes crucial when studying stress. In this study, the tissue damage was assessed by performing a trypan blue exclusion assay, when we compared control larvae with the treated, a dose-dependent increase in the tissue damage was observed that can be seen in Fig. S3 (P2 to P4). This observation was derived from the noticeable presence of more intensely stained tissues in the phthalate-treated groups indicating the death of cells since trypan blue dye can only penetrate through the lysed cell membranes. The clear differentiation between stained and unstained cells was a direct measure of tissue damage. The same has been quantified in Fig. 2. No significant tissue damage was observed in the larvae exposed to 0.001 M of phthalate compared to the control (Fig. 2; $p < 0.05$). A significant tissue damage of 2.43, 3 and 3.23 folds, was observed in the larvae exposed to 0.005, 0.010 and 0.020 M of phthalate, respectively, compared to the control (Fig. 2; $p < 0.05$). The exposure of 25 μM of EMS exhibited a significant tissue damage of 3.85-fold, compared to control (Fig. 2 & Table S1). The GSH content in the larvae was not reduced significantly at the exposure of 0.001 M of phthalate compared to the control (Table 1 & Fig. S4a, $p < 0.05$). The exposure of 0.005, 0.010 and 0.020 M of phthalate to the third instar larvae showed a significant decrease of 1.36, 1.6 and 1.97 folds, respectively, compared to control (Table 1 & Fig. S4a; $p < 0.05$). The larvae exposed to 25 μM of EMS showed a significant decrease of 3.0 fold in the GSH content compared to control larvae (Table 1 & Fig. S4a; $p < 0.05$). No significant increase in the activity of GST was observed in the larvae exposed to 0.001 M of phthalate compared to control larvae (Table 1 & Fig. S4b; $p < 0.05$), but the larvae exposed to 0.005, 0.01 and 0.02 M of phthalate showed a dose-dependent significant increase of 1.19, 1.38 and 1.62 folds, respectively, in the activity of GST compared to control (Table 1 & Fig. S4b; $p < 0.05$). A significant increase of 1.95 fold in the activity of GST was

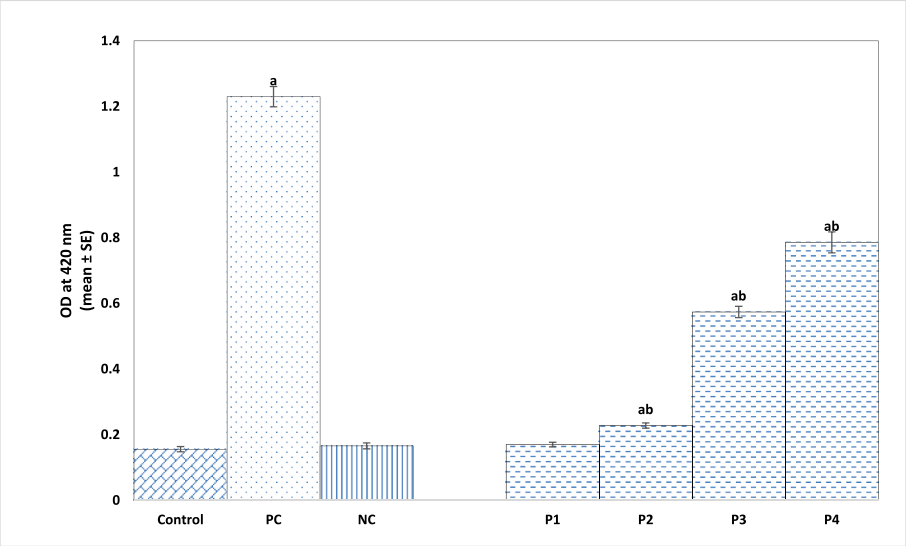

Fig. 1. β-galactosidase activity measured in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} exposed to various doses of Bis(2-ethylhexyl) phthalate for 24 h of duration [(a-significant at $p < 0.05$ compared to control; b-significant at $p < 0.05$ compared to positive control) [C = control; PC = 25 μM of EMS; P1 = 0.001 M; P2 = 0.005 M; P3 = 0.01 M; P4 = 0.02M of Bis (2-ethylhexyl) phthalate; NC = 0.02 M of DMSO].

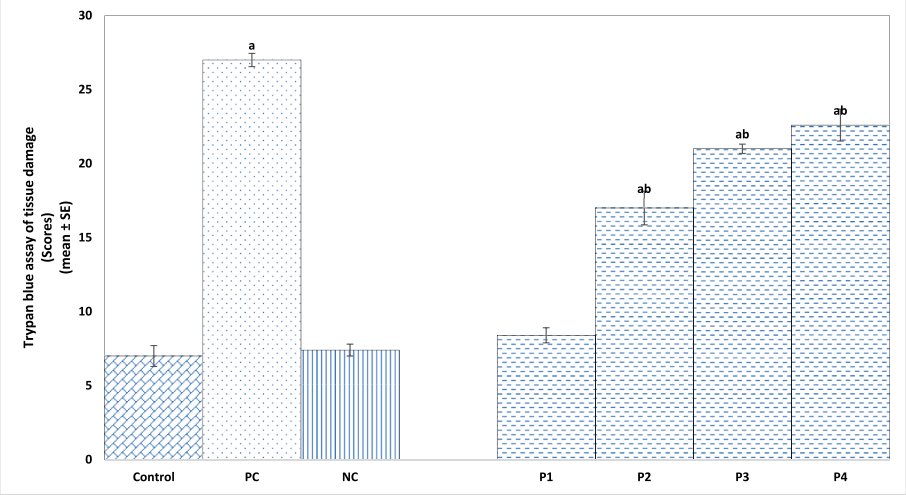

Fig. 2. Quantification of tissue damage in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} exposed to various doses of Bis(2-ethylhexyl) phthalate for 24 h [(a-significant at $p < 0.05$ compared to control; b-significant at $p < 0.05$ compared to positive control) [C = control; PC = 25 μM of EMS; P1 = 0.001 M; P2 = 0.005 M; P3 = 0.01 M; P4 = 0.02M of Bis (2-ethylhexyl) phthalate; NC = 0.02 M of DMSO].

observed in the larvae exposed to 25 μM EMS compared to the control (Table 1 & Fig. S4b; $p < 0.05$). No significant increase in the activity of SOD was observed in the larvae exposed to 0.001 M of phthalate compared to control (Table 1 & Fig. S4c; $p < 0.05$). A dose-dependent significant increase of 1.47, 2.07 and 2.34 fold, in the activity of SOD was observed in the larvae exposed to 0.005, 0.010 and 0.020 M of phthalate, respectively, compared to control larvae (Table 1 & Fig. S4c; $p < 0.05$). The larvae exposed to 25 μM of EMS showed an increase of 3.36 fold in the activity of SOD (Table 1 & Fig. S4c; $p < 0.005$). No significant increase in the activity of catalase was observed in the larvae exposed to 0.001 M of phthalate compared to control (Table 1 & Fig. S4d; $p < 0.05$). A significant increase of 1.44, 2.01 and 2.59 folds in the activity of catalase was observed in the larvae exposed to 0.005, 0.010 and 0.020 M of phthalate, respectively, compared to control (Table 1 & Fig. S4d; $p < 0.05$). The larvae exposed to 25 μM of EMS showed a significant increase of 3.53 fold in the catalase activity compared to unexposed larvae (Table 1 & Fig. S4d; $p < 0.05$). No significant increase in the TBARS was observed in the larvae exposed to 0.001 M of phthalate compared to control larvae (Table 1 & Fig. S4e; $p < 0.05$). The larvae exposed to 0.005, 0.010 and 0.020 M of phthalate showed a significant increase of 1.14, 1.49 and 1.74 folds, respectively, in TBARS compared to control larvae (Table 1 & Fig. S4e; $p < 0.05$). The larvae exposed to 25 μM of EMS showed a significant an increase of 2.16 fold in TBARS compared to control larvae (Table 1 & Fig. S4e, $p < 0.05$). No significant increase in the PCC was observed in the larvae exposed to 0.001M of phthalate compared to control larvae (Table 2 & Fig. S4f; $p < 0.05$). A dose dependent significant increase of 1.44, 1.68 and 2.07 folds was observed in the larvae exposed to 0.005, 0.010 and 0.020 M of phthalate, respectively, in PCC compared to control larvae (Table 2 & Fig. S4f; $P < 0.05$). The exposure of 25 μM of EMS to larvae showed a significant increase of 2.95 fold in the PCC compared to control larvae (Table 2 & Fig. S4f; $p < 0.05$). The larvae exposed to 0.001 of phthalate did not show a significant increase in caspase-3 activity compared to control larvae (Fig. 3 & Table S1; $p < 0.05$). A dose-dependent significant increase of 1.45, 1.97 and 2.52 folds was observed in the larvae exposed to 0.005, 0.010 and 0.020 M of phthalate, respectively, in the

Table 1
Glutathione (GSH) content, glutathione-S- transferase (GST), superoxide dismutase (SOD), catalase (CAT) activity and TBARS measured in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} exposed to various doses of Bis(2-ethylhexyl) phthalate for 24 h of duration.

Treatment groups	Total GSH (μ moles of GSH/ gram tissue) (mean ± SE)	GST Activity (μ moles of CDNB conjugates/ min/mg protein) (mean ± SE)	SOD activity Units/ mg protein (mean ± SE)	CAT activity μ moles of H_2O_2 consumed/ min/mg protein (mean ± SE)	LPO n moles of TBARS formed/ hour/ gram tissue (mean ± SE)
Control	10.40 ± 0.37	19.18 ± 0.53	2.82 ± 0.13	3.32 ± 0.12	185.6 ± 3.41
Positive Control	3.46 ± 0.14[a]	37.44 ± 0.14[a]	9.5 ± 0.22[a]	11.72 ± 0.47[a]	401.4 ± 7.13[a]
Negative Control	8.72 ± 0.28	19.86 ± 0.52	3.4 ± 0.15	3.72 ± 0.05	186.6 ± 2.31
P1	9.72 ± 0.29	19.86 ± 0.50	3.14 ± 0.13	3.58 ± 0.12	189 ± 1.09
P2	7.64 ± 0.21[ab]	22.96 ± 0.46[ab]	4.16 ± 0.20[ab]	4.80 ± 0.18[ab]	212.2 ± 2.70[ab]
P3	6.46 ± 0.15[ab]	26.6 ± 0.60[ab]	5.84 ± 0.12[ab]	6.7 ± 0.16[ab]	277.4 ± 9.24[ab]
P4	5.26 ± 0.26[ab]	31.14 ± 0.49[ab]	6.62 ± 0.17[ab]	8.6 ± 0.33[ab]	324 ± 4.03[ab]

a-significant at $p < 0.05$ compared to control; b-significant at $p < 0.05$ compared to positive control.
[C = control; PC = 25 μM of EMS; P1 = 0.001 M; P2 = 0.005 M; P3 = 0.01 M; P4 = 0.02M of Bis (2-ethylhexyl) phthalate; NC = 0.02 M of DMSO].

Table 2
Protein carbonyl content (PCC), Acetylcholinesterase, ALA-D and Monoamine oxidase (MAO) measured in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} exposed to various doses of Bis(2-ethylhexyl) phthalate for 24 h of duration.

Treatment groups	PC Content OD at 370 nm (mean ± SE)	Acetyl cholinesterase activity activity/min/g tissue x 10^{-4} (mean ± SE)	MAO OD at 280 nm (mean ± SE)	ALA-D OD at 555 nm (mean ± SE)
Control	0.158 ± 0.011	3.39 ± 0.20	0.61 ± 0.026	1.348 ± 0.062
Positive Control	0.466 ± 0.010[a]	1.058 ± 0.09[a]	2.01 ± 0.056[a]	0.51 ± 0.037[a]
Negative Control	0.17 ± 0.010	2.91 ± 0.17	0.638 ± 0.032	1.332 ± 0.054
P1	0.18 ± 0.007	3.1 ± 0.11	0.63 ± 0.039	1.296 ± 0.072
P2	0.228 ± 0.005[ab]	2.29 ± 0.04[ab]	0.986 ± 0.055[ab]	1.096 ± 0.012[ab]
P3	0.266 ± 0.005[ab]	2.0 ± 0.07[ab]	1.536 ± 0.054[ab]	0.934 ± 0.012[ab]
P4	0.328 ± 0.011[ab]	1.336 ± 0.12[ab]	1.854 ± 0.030[ab]	0.854 ± 0.029[ab]

a-significant at $p < 0.05$ compared to control; b-significant at $p < 0.05$ compared to positive control.
[C = control; PC = 25 μM of EMS; P1 = 0.001 M; P2 = 0.005 M; P3 = 0.01 M; P4 = 0.02M of Bis (2-ethylhexyl) phthalate; NC = 0.02 M of DMSO].

activity of caspase-3 compared to control larvae (Fig. 3 & Table S1; $p < 0.05$). The exposure of 25 μM of EMS showed an increase of 3.67 fold in the activity of caspase-3 compared to control larvae (Fig. 3 & Table S1, $p < 0.05$). The larvae exposed to 0.001 M of phthalate showed no significant increase in the activity of caspase-9 compared to control larvae (Fig. 3 & Table S1; $p < 0.05$). A significant increase of 1.33, 1.63 and 1.98 folds was observed in the larvae exposed 0.005, 0.01 and 0.020 M of phthalate, respectively, in the activity of caspase-9 compared to control larvae (Fig. 3 & Table S1; $p < 0.05$). The exposure of 25 μM of EMS to larvae showed a significant increase of 3.04-fold in the activity of caspase-9 compared to control larvae (Fig. 3 & Table S1; $p < 0.05$). No significant decrease in the activity of acetylcholinesterase was observed in the larvae exposed to 0.001M of phthalate compared to control larvae (Table 2 & Fig. S5a, $p < 0.05$). A significant decrease of 1.47, 1.70 and 2.54 folds in the activity of acetylcholinesterase was observed in the larvae exposed to 0.005, 0.010 and 0.020 M of phthalate compared to control larvae (Table 2 & Fig. S5a; $p < 0.05$). The larvae exposed to 25 μM EMS showed a significant decrease of 3.20 fold in the activity of acetylcholinesterase compared to control larvae (Table 2 & Fig. S5a; $p < 0.005$). No significant decrease in the activity of ALA-D was observed in the larvae exposed to 0.001M of phthalate compared to control larvae (Table 2 & Fig. S5b; $p < 0.05$). The larvae exposed to 0.005, 0.010 and 0.020M of phthalate showed a significant decrease of 1.22, 1.44 and 1.57 folds, respectively, in the activity of ALA-D, compared to control larvae (Table 2 & Fig. S5b; $p < 0.05$). The exposure of 25 μM of EMS showed a significant decrease of 2.64 fold in the activity of ALA-D compared to control larvae (Table 2 & Fig. S5b; $p < 0.05$). No significant increase in the activity of MAO was observed in the larvae exposed to 0.001 M of phthalate compared to control larvae (Table 2 & Fig. S5c; $p < 0.05$). The larvae exposed to 0.005, 0.010 and 0.020 M of phthalate showed a decrease of 1.61, 2.57 and 3.03 folds, respectively, in the activity of MAO compared to control larvae (Table 2 & Fig. S5c; $p < 0.05$). The exposure of 25 μM of EMS to the larvae showed a significant decrease of 3.29 fold in the activity of MAO compared to control larvae (Table 2 & Fig. S5c; $p < 0.05$). The results obtained from the comet assay performed on the mid-gut cells of the third instar larvae are shown in Fig. 4 and the same has been quantified in Fig. 5. No significant DNA damage was observed in the larvae exposed to 0.001M of phthalate compared to unexposed larvae (Fig. 5; $p < 0.05$). The larvae exposed to 0.005, 0.010 and 0.020 M of phthalate showed a significant DNA damage of 2.20, 4.03 and 6.23 folds, respectively, compared to control larvae (Fig. 5; $p < 0.05$). The exposure of 25 μM of EMS to the third instar larvae showed significant DNA damage of 10.26 fold compared to control larvae (Fig. 5; $p < 0.05$).

4. Discussion

The results of our study showed that DEHP is toxic at doses 0.005, 0.01, and 0.02 M in third-instar larvae of *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} Phthalates are plasticizers that are not chemically bound to the plastic matrix and gradually leach out of the material, raising concerns about human exposure (Marttinen et al., 2003). Although phthalates possess lower acute toxicity still they can cause toxic effects if exposed to high doses or repeating low doses (Chiellini et al., 2013). Once DEHP was thought to be non-toxic, but later on some researchers found that this compound was responsible for several kinds of toxicity because it can be easily inhaled or ingested which is dangerous to individuals (Gruber et al., 2022). DEHP is required for several medical devices and components in order to make them flexible and it mimics estrogen to disrupt the function of the endocrine system (Casals-Casas and Desvergne, 2011; Kang et al., 2016). The primary indication of the stress response is the upregulation of heat shock protein 70 which is a 70-kDa molecular chaperon that is called to be the guardian of cells because it protects the cell from stressful condition. They are mainly responsible for the proper folding of cellular proteins and maintaining the homeostasis of proteins (Lubkowshka et al., 2021). *Drosophila* was exposed to Bis(2-ethylhexyl) phthalate it showed a stress response by expressing the hsp70 which indicates the toxic effect of Bis(2-ethylhexyl) phthalate. DEHP is responsible for causing hepatotoxicity in quail by decreasing the expression of heat shock transcription factors (HSF1) and (HSF3) after exposure for 45 days, it also downregulated the levels of several HSPs such as HSP 10, HSP 25, HSP 60, HSP 70 and HSP 90 in the liver of quail (Zhao et al., 2019). Li et al. (2018) showed that expression of HSP 10, HSP 40, HSP 70, HSP 90, and HSP 110 was upregulated in the kidney

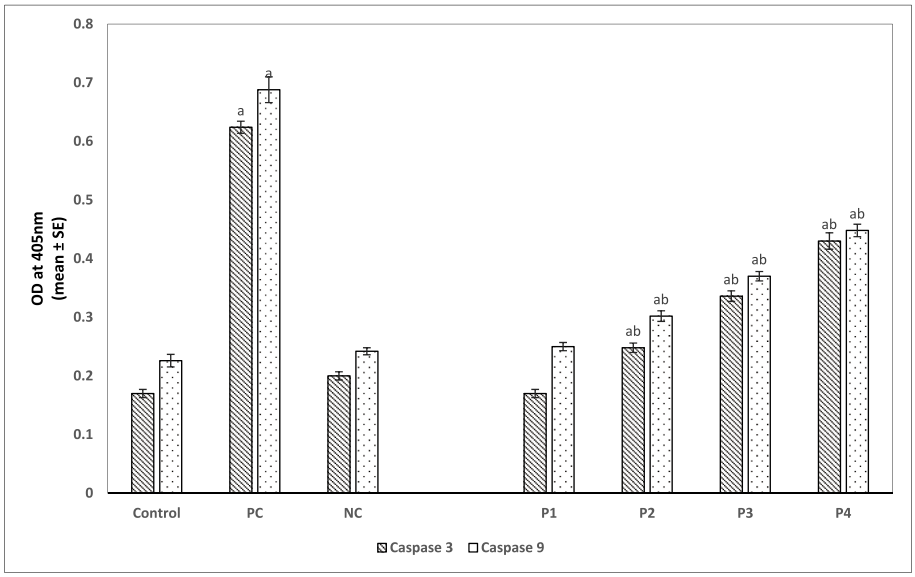

Fig. 3. Caspase-3 (5a) and caspase-9 (5b) activity measured in the third instar larvae of transgenic *Drosophila melanogaster (hsp70 lacZ) Bg*^{9} exposed to various doses of Bis (2-ethylhexyl)phthalate for 24 h of duration (a-significant at $p < 0.05$ compared to control; b-significant at $p < 0.05$ compared to positive control) [C = control; PC = 25 μM of EMS; P1 = 0.001 M; P2 = 0.005 M; P3 = 0.01 M; P4 = 0.02M of Bis (2-ethylhexyl) phthalate NC = 0.02 M of DMSO].

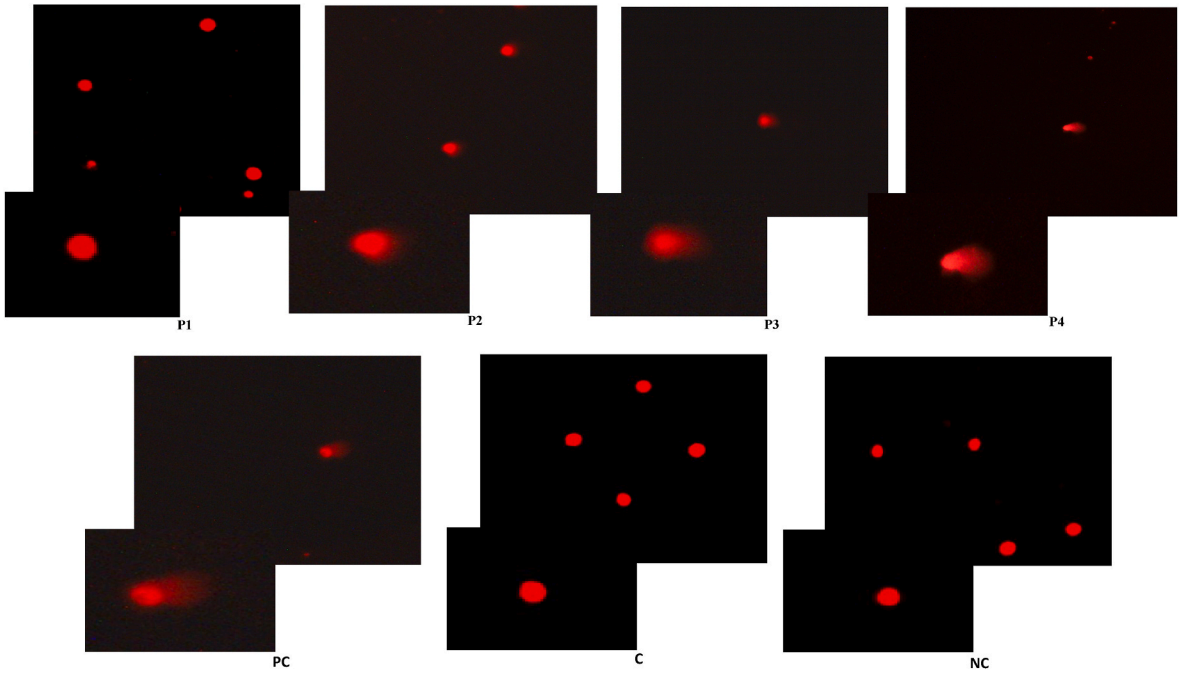

Fig. 4. Comet assay performed on midgut cells of the third instar larvae of *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} shown in Fig. 6 after exposing the larvae to different concentrations of Bis(2-ethylhexyl) phthalate for 24h. [P1 = 0.001 M; P2 = 0.005 M; P3 = 0.01 M; P4 = 0.02M of Bis (2-ethylhexyl) phthalate; PC = 25 μM of EMS; C = control; NC = 0.02 M of DMSO].

of quail when exposed to DEHP for 45 days, it showed that expression of heat shock protein is tissue-specific. Several researchers reported when *Drosophila melanogaster* was exposed to nanoparticles, a phthalimide group of chemicals, antibiotics, and any toxic chemical increased the expression of Hsp 70 to protect the cell from any stressful condition (Nazir et al., 2003a,b; Shakya and Siddique, 2018). The transgenic fly used in the present study contains wild-type hsp70 sequence to the fusion point of LacZ, this showed that hsp70 expression can result in the expression of the Lac Z structural gene which is used as a reporter gene in transgenic strain. Our results showed a dose-dependent increase in the expression of Hsp70 (both quantitative and qualitative). The intense blue color (Gary and Kindell, 2005) in the gut of third instar larvae indicate that the stress response is due to the exposure of DEHP. Hsp 70 expression quantification was carried out through the ONPG assay that was indicated by β-galactosidase activity. This assay utilizes ONPG as a colorimetric substrate to evaluate the β-galactosidase activity. Functioning as a lactose mimic, ONPG is hydrolyzed by β-galactosidase, resulting in the production of galactose and O-nitrophenol. The yellow color generated from O-nitrophenol can be easily quantified through spectrophotometric analysis. Our results showed a significant increase in β-galactosidase activity in response to phthalate. Groups subjected to treatment with four different doses of phthalate from P1 to P4 showed a

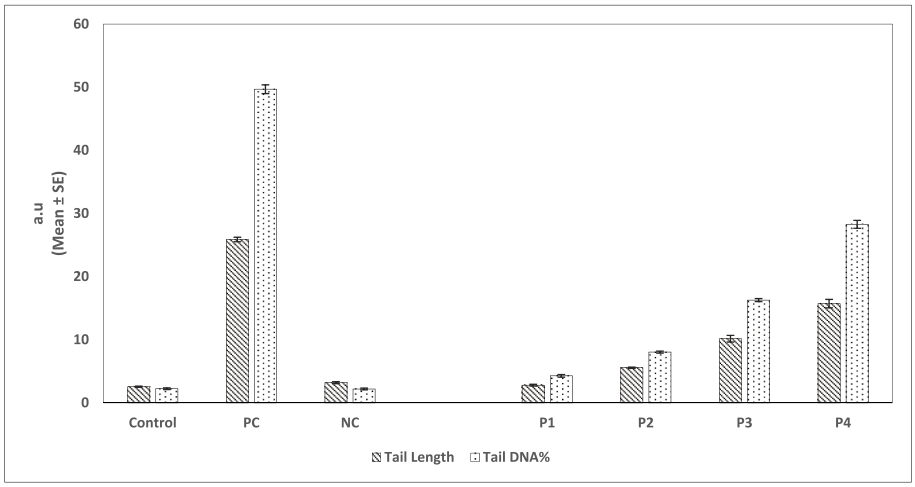

Fig. 5. Quantification of DNA damage in the midgut cells after exposing the larvae to different concentrations of Bis(2-ethylhexyl) phthalate for 24h. [(a-significant at $p < 0.05$ compared to control; b-significant at $p < 0.05$ compared to positive control) [C = control; PC = 25 μM of EMS; P1 = 0.001 M; P2 = 0.005 M; P3 = 0.01 M; P4 = 0.02M of Bis (2-ethylhexyl) phthalate; NC = 0.02 M of DMSO].

dose dependent increase in the expression of β-galactosidase activity and that was correlated with an increase in the optical density (OD) of solution. When a cell's capacity to protect itself from the harm caused by the increased production of reactive oxygen metabolites, it results in a state of oxidative stress; as a result, the antioxidant defense system starts working to reduce oxidative damage. In our research, several oxidative stress markers were measured to assess the oxidative damage caused by DEHP. The most significant antioxidant produced by the cell is GSH which is a tripeptide with a thiol functional group and involved in various biological functions such as cell protection from inflammation, molecular transport, detoxification, signal transduction, formation of microtubules (Forman et al., 2009). Amara et al. (2020) showed in their results that exposure of mice to DEHP affects the Nrf signaling pathway and increases the activity of GSH in mouse kidneys. The exposure of DEHP for 6 and 24h in *Oryza melastigma* showed a decreased level of GSH content in the livers (Yin et al., 2021). The same results were observed in our study on the third instar larvae of *Drosophila melanogaster* when exposed at different doses for 24 h. The decreased activity of GSH compared with the control showed that the detoxification function of GSH was remarkedly inhibited by DEHP. GST is responsible for catalyzing coupling between sulfhydryl of GSH and toxic substance due to which the hydrophobic nature of the substance increase and help in crossing the cell membrane for detoxification, exposure to DEHP showed that there was a significant increase in the concentration of GST in a dose-dependent manner. This conclusion was also supported by Zheng et al. (2022), who showed that *Folsomia candida* exposure to DEHP causes an increase or decrease in a number of oxidative stress markers. SOD is widely distributed in the organism and plays an important role in stress conditions. It is also responsible for catalyzing the conversion of superoxide into hydrogen peroxide and oxygen generated from peroxisomes and mitochondria, so it plays an important role in oxidative stress conditions (Wang et al., 2018). In our findings exposure to phthalate for 24hrs decreased the activity of SOD in higher concentration groups, this showed higher oxidative stress condition. CAT help in catalyzing the conversion of H_2O_2 and is found to be the first-line defense against the toxicity of oxygen. Our study results showed that DEHP decreased the activity of CAT and increased oxidative stress by disturbing cellular homeostasis. Lipid peroxidation is the degradation of lipids with the help of oxygen in which free radicals withdraw the electron from the lipid that is present in the cell membrane and is responsible for damage to the cell. In a study, exposure to DEHP in BaLB/c mice showed that 30 days of continuous administration results in cardiac injury by increased activity of SOD, CAT and lipid peroxidation, and protein carbonyl content (Amara et al., 2019). The same results were obtained in our study for protein carbonyl content and lipid peroxidation that showed a dose-dependent increase in the larvae when exposed to 0.005, 0.01, and 0.02 M compared to the control. The midgut cells of larvae have a high microsomal activity which were subjected to apoptotic test to evaluate the impact of Bis(2-ethylhexyl) phthalate on apoptosis (caspase-9 and caspase-3). When larvae were exposed to Bis (2-ethylhexyl) phthalate at doses 0.005, 0.01, and 0.02 M, showed that there was a dose-dependent increase in the activity of caspase-9 and caspase-3. In order to cause cell death, the cleavage of procaspase-9 causes the cleavage of the downstream effector caspase-3. Earlier studies have shown that prolonged exposure to low doses of DEHP might encourage the liver cells' apoptotic signal, such as by increasing the expression of caspase-3 and Bax (Lee et al., 2020; Li et al., 2022b). The results obtained from our study showed that Bis(2-ethylhexyl) phthalate increased the generation of reactive oxygen species and cause oxidative stress due to which cellular homeostasis gets disturbed and is responsible for inducing toxicity. Within cells, ROS have the ability to damage DNA, proteins, and lipids and this harm can contribute to overall harmful effects by causing cell malfunction and death (Jomova et al., 2023). Acetylcholine is a neurotransmitter hydrolyzed by AchE, so that transmission terminates. Research on Bagrid catfish (*Pseudobagrus fulvidraco)* showed that after exposure to phthalate, there was a significant decrease in the activity of acetylcholinesterase in a dose-dependent manner in the brain and muscle (Jee et al., 2009). Other researchers also showed that AChE activity was decreased by DEHP in Zebrafish and also in *Collembola* (Li et al., 2022a; Zheng et al., 2022). The same results were obtained in our research in which the acetylcholinesterase activity was compared between treated, control, and positive control. Our results showed decrease in the activity of AchE at doses 0.005, 0.01, and 0.02 M of Bis (2-ethylhexyl) phthalate in a dose-dependent manner which confirms its neurotoxic activity. MAO contains iron that helps in catalyzing the monoamines. Our study showed that MAO activity increased with an increase in dose. The study has shown an increase in the activity of MAO in brain tissue on exposure to phthalate in mice (Jiaxin et al., 2022). The DNA damage was also assessed in the third instar larvae exposed to Bis(2-ethylhexyl) phthalate by performing comet assay. The midgut was the cellular target for the comet assay in the larvae because harmful chemicals enter the organism through the gut. A dose-dependent DNA damage was observed in the midgut cells of the larvae exposed to Bis (2-ethylhexyl) phthalate. Alqahtani et al. (2023) showed DNA damage in the liver and kidney of rats exposed to Bis(2-ethylhexyl) phthalate. DNA damage in the lung cancer line of humans due to exposure of Di (2-ethylhexyl) phthalate was confirmed by the comet test by the activation of Akt/NF-kB signaling pathway

(Urade et al., 2022). *In vivo* study also showed that DEHP exposure to Hep3B cells for 24 h led to DNA damage and also increased the rate of replication (Chen et al., 2013). The results indicate that Bis (2-ethylhexyl) phthalate exposure triggers oxidative stress through alterations in the activity of antioxidant enzymes, and this ultimately leads to apoptosis, cytotoxicity and genotoxicity in the third instar larvae of *Drosophila*.

5. Conclusion

Phthalate exposure can impair Drosophila's antioxidant defense mechanisms and this includes a change in the activity of ROS-neutralizing enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione S- transferase, lipid peroxidation, and protein carbonyl content. In *Drosophila* tissues, phthalate exposure can result in increased ROS levels that cause DNA damage and cause programmed cell death (apoptosis). *Drosophila* offers a potent and adaptable model system for toxicological studies, providing information on how toxic chemicals affect a variety of biological functions. It is an essential tool in the realm of toxicity due to its genetic tractability, short lifespan, and conservation of basic biological processes and can be employed in high throughput screening experiments, which enables scientists to swiftly examine a huge number of substances or situations. Our results showed that Bis(2-ethyhexyl) phthalate is responsible for causing oxidative stress, increased tissue damage in the gut of third-instar larvae, DNA damage in the mid-gut cells of the larvae and increased expression of Hsp 70. The dose of 0.001 M of Bis-2-ethylhexyl phthalate did not show any toxic effects and hence can be considered as No Observed Adverse Effect Level (NOAEL) for Bis-2-ethylhexyl phthalate. In previous research using the *Drosophila* model, researchers showed that Bis(2-ethylhexyl) phthalate disturbs the vision, courtship behavior, and life span and its metabolites, such as MEHP, responsible for the excitation of neurons and disturb the transmission of projection neurons.

CRediT authorship contribution statement

Kajal Gaur: Writing – original draft, Methodology, Data curation, Conceptualization. **Himanshi Varshney:** Investigation. **Iqra Subhan:** Investigation. **Javeria Fatima:** Investigation. **Smita Jyoti:** Software, Data curation. **Yasir Hasan Siddique:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kajal Gaur reports was provided by Aligarh Muslim University.

Data availability

Data will be made available on request.

Acknowledgements

The fellowship received from the University Grants Commission, New Delhi to Kajal Gaur (NTA Ref. No.: 201610028318) is thankfully acknowledged. We are also thankful to the Chairperson, Department of Zoology, AMU for providing laboratory facilities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2023.114425.

References

Abolaji, A.O., Kamdem, J.P., Lugokenski, T.H., Nascimento, T.K., Waczuk, E.P., Farombi, E.O., Loreto, D.S., Rocha, J.B.T., 2014. Involvement of oxidative stress in 4-vinylcyclohexene-induced toxicity in Drosophila melanogaster. Free Radical Biological Medicine 71, 99–108.

Alqahtani, M.A.R., Virk, P., Fouad, D., 2023. Attenuating effect of spirulina (Arthrospira Platensis) against Di(2-ethylhexyl) phthalate induced toxicity in wistar rats. Environmental Pollutants and Bioavailaibility 35 (1). https://doi.org/10.1080/26395940.2023.2197154.

Amara, I., Salah, A., Timoumi, R., Annabi, E., Scuto, M., Trovato, A., Neffati, F., Calabrese, Abid-Essefi, S., 2020. Effect of di(2-ethylhexyl) phthalate on Nrf2-regulated glutathione homeostasis in mouse kidney. Cell Stress and Chaperones 25, 919–928.

Amara, I., Timoumi, R., Annabi, E., Neffati, F., Najjar, M.F., Bouaziz, C., Essefi, S., 2019. Di (2-ethylhexyl) phthalate induces cardiac disorders in BALB/c mice. Environ. Sci. Pollut. Control Ser. 26, 7540–7549.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.

Casals-Casas, C., Desvergne, B., 2011. Endocrine disruptors: from endocrine to metabolic disruption. Annu. Rev. Physiol. 73, 135–162.

Chen, X., Quin, Q., Zhang, W., Zhang, Y., Zheng, H., Liu, C., Yang, Y., Xiomg, W., Yuan, J., 2013. Activation of the PI3-AKT-mTOR signalling pathway promotes DEHP induced Hep3B cell proliferation. Food Chem. Toxicol. 59, 325–333.

Chiellini, F., Ferri, M., Morelli, A., Dipaola, L., Latini, G., 2013. Perspectives on alternatives to phthalate plasticized poly(vinyl chloride) in medical devices applications. Prog. Polym. Sci. 38 (7), 1067–1088.

Chowdhuri, D.K., Saxena, D.K., Viswanathan, P.N., 1999. Effect of Hexachlorocyclohexane (HCH), its Isomers, and metabolites on Hsp70 expression in transgenic Drosophila melanogaster. Pestic. Biochem. Physiol. 63, 15–25.

Ellman, G.L., Courtney, K.D., Andres, J.R.V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Feng, W., Liu, Y., Ding, Y., Mao, G., Zhao, G., Zhao, T., Chen, K., Qiu, X., Tong, X., Zhao, X.F., Wu, X., Yang, L., 2020. Typical neurobehavioral methods and transcriptome analysis reveal the neurotoxicity and mechanisms of di(2-ethylhexyl) phthalate on pubertal male ICR mice with type 2 diabetes mellitus. Arch. Toxicol. 94, 1279–1302.

Forman, H.J., Zhang, H., Rinna, A., 2009. Glutathione: Overview of its protective roles, measurement, and biosynthesis. Mol. Aspect. Med. 30, 1–12.

Foster, P.M.D., Mylchreest, E., Gaido, K., Sar, M., 2000. Effects of phthalate esters on the developing reproductive tract of male rats. Journal of pathology, Microbiology and Immonology 109, 272–277.

Fu, G., Dai, J., Li, Z., Chen, F., Liu, l., Yi, L., Teng, Z., Quan, C., Zhang, l., Zhou, T., Donkersley, P., Song, S., Shi, Y., 2020. The role of STAT3/p53 and PI3K-Akt-mTOR signaling pathway on DEHP-induced reproductive toxicity in pubertal male rat. Toxicol. Appl. Pharmacol. 404 https://doi.org/10.1016/j.taap.2020.115151.

Gary, R.K., Kindell, S.M., 2005. Quantitative assay of senescence-associated β-galactosidase activity in mammalian cell extracts. Anal. Biochem. 343 (2), 329–334.

Gruber, E.S., Stadlbauer, V., Pichler, V., Fauster, K.R., Todorovic, A., Meisel, T.C., Trawoeger, S., Holloczki, O., Turner, S.D., Wadsak, W., Vethaak, A.D., Kenner, L., 2022. To Waste or not to Waste: Questioning potential Health risks of Micro- and Nanoplastics with a Focus on their ingestion and potential Carcinogenicity. Exposure and Health 15, 33–51.

Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M., Jakoby, W.B., 1974. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. Proceedings of the National Academy of Science 71, 3879–3882.

Hamid, N., Junaid, M., Manzoor, R., Jia, P., Pei, D., 2020. Prioritizing phthalate esters (PAEs) using experimental in vitro/vivo toxicity assays and computational in silico approaches. Journal of Hazard Materials 398, 122851.

Hawkins, C.L., Morgan, P.E., Davies, M.J., 2009. Quantification of protein modification by oxidants. Free Radic. Biol. Med. 46, 965–988.

Hsieh, S.H., Hsu, C.Y., Yang, P.J., Chiu, C.C., Liang, S.S., Yang, F.O., Kan, J.Y., Hou, M.F., Wang, T.N., Tsai, E.M., 2022. DEHP mediates drug resistance by directly targeting AhR in human breast cancer. Biomed. Pharmacother. 145 (112400), 753–3322.

Huang, H.C., Tien, C.J., Sun, Y.M., Hsieh, C.H., Lee, C.C., 2008. Occurrence of phthalates in sediment and biota: relationship to aquatic factors and the biota-sediment accumulation factor. Chemosphere 73 (4), 539–544.

Jee, J.H., Koo, J.G., Keum, Y.H., Park, K.H., Choi, S.H., 2009. Effects of dibutyl phthalate and di-ethylhexyl phthalate on acetylcholinesterase activity in bagrid catfish, Pseudobagrus fulvidraco (Richardson). Journal of Applied Ichythyology. https://doi.org/10.1111/j.1439-0426.2009.01331.

Jiaxin, L., Chaoxu, F., Lin, D., Huiling, J., Yanji, X., 2022. Effects of combined exposure of Diethylhexyl phthalate and Bisphenol A on Learning and memory Impairment and Amyloid Precursor protein processing pathway in mice. Asian J. Ecotoxicol. (1), 255–265.

Jollow, D.J., Mitchell, J.R., Zampaglione, N.A., Gillette, J.R., 1974. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology 11, 151–169.

Jomova, K., Raptova, R., Alomar, S.Y., Alwasel, S.H., Nepovimova, E., Kuca, K., Valko, M., 2023. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Arch. Toxicol. 97, 2499–2574.

Kang, Y.T., Huang Q, M., fang, C., Chen, Y., Liu, L., Dong, S., 2016. Accumulation of di(2-ethylhexyl) phthalate causes endocrine-disruptive effects in marine medaka (Oryzias melastigma) embryos. Environ. Toxicol. 31 (1), 116–127.

Kim, J.H., Zhang, J., Hwang, S., Cho, S., Yu, W.J., Jeong, J.S., Park, H., Lee, B.C., Jee, S. H., Lim, Youngja H.P., 2022. Transcriptome-metabolome-wide association study (TMWAS) in rats revealed a potential carcinogenic effect of DEHP in thyroid associated with eicosanoids. Environ. Res. 214 (2), 113805. ISSN 0013-9351.

Krebs, R.A., Feder, M.E., 1997. Tissue-specific variation in Hsp70 expression and thermal damage in Drosophila melanogaster larvae. J. Exp. Biol. 200, 2007–2015.

Lee, C.Y., Suk, F.M., Twu, Y.C., Liao, Y.J., 2020. Long-term exposure to low-dose Di-(2-ethylhexyl) phthalate impairs cholesterol metabolism in hepatic stellate cells and exacerbates liver librosis. International Journal of Environvironment and Research Public Health. https://doi.org/10.3390/ijerph17113802.

Li, X., Wang, Q., Wang, C., Yang, Z., Wang, J., Zhu, L., Zhang, D., Wang, J., 2022a. Ecotoxicological response of zebrafish liver (Danio rerio) induced by di-(2-ethylhexyl) phthalate. Ecol. Indicat. 143, 109388.

Li, H., Zhang, J., Xia, Y., Pan, W., Zhou, D., 2022b. Antagonistic effect of nano-selenium on hepatocyte apoptosis induced by DEHP via PI3K/AKT pathway in chicken liver. Ecotoxicology and environmental Safety 218, 112282.

Li, M.Z., Zhao, Y., Wang, H.R., Talukder, M., Li, J.L., 2021. Lycopene preventing DEHP-induced Renal cell damage is targeted by Aryl Hydrocarbon Receptor. Journal of Agriculture and Food Chemistry 69 (43), 12853–12861.

Li, P.C., Li, X.N., Du, Z.H., Wang, H., Yu, Z.R., Li, J.L., 2018. Di (2-ethylhexyl)phthalate (DEHP)-induced kidney injury in quail (Coturnix japonica) via inhibiting HSF1/HSF3-dependent heat shock response. Chemosphere 209, 981–988.

Lis, J.T., Simon, J.A., Sutton, C.A., 1983. New heat shock puffs and β=galactosidase activity resulting from transformation of Drosophila with an hsp70-lacZ hybrid gene. Cell 35, 403–410.

Lubkowshka, A., Pluta, W., Stronska, A., Lalko, A., 2021. Role of heat shock protein (Hsp70 and hsp 90) in viral infection. International Journal Of Molecular science 22 (17), 9366.

Ma, Y.B., Manzoor, R., Jia, P.P., Bian, W.P., Hamid, N., Xie, Z.Y., Pei, D.S., 2021. Transcriptome and in silico approaches provide new insights into the mechanism of male reproductive toxicity induced by chronic exposure to DEHP. Environmental Pollution 289, 269–7491.

Macleod, D.J., Sharpe, R.M., Welsh, M., Fisken, M., Scott, H.M., Hutchinson, G.R., Drake, A.J., Driesche S, V.D., 2010. Androgen action in the masculinization programming window and development of male reproductive organs. Int. J. Androl. 33, 279–287.

Marklund, S., Marklund, G., 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. FEBS J. 47, 469–474.

Marttinen, S.K., Kettunen, R.H., Sormunen, K.M., Rintala, J.A., 2003. Removal of bis(2-ethylhexyl) phthalate at a sewage treatment plant. Water Res. 37 (6), 1385–1393.

McEwen, W.K., Helen, R.S., 1965. Rheology of the human sclera Ophthalmologica 150, 321–346.

Mukhopadhyay, I., Chowdhuri, D.K., Bajpayee, M., Dhawan, A., 2004. Evaluation of in vivo genotoxicity of cypermethrin in *Drosophila melanogaster* using the alkaline Comet assay. Mutagenesis 19, 85–90.

Nazir, A., Krishna, D., Saxena, Chowdhuri, D.K., 2003a. Induction of hsp70 in transgenic *Drosophila*: biomarker of exposure against phthalimide group of chemicals. *Biochimica et Biophysica Acta (BBA)*. General Subjects 1621 (2), 218–225.

Nazir, A., Mukhopadhyay, I., Saxena, D.K., Siddiqui, M.S., Chowdhuri, D.K., 2003b. Evaluation of toxic potential of captan: Induction of hsp70 and tissue damage in transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9}. J. Biochem. Mol. Toxicol. 17, 98–107.

Ohkawa, H., Ohishi, N., Yagi, K., 1978. Reaction of linoleic acid hydroperoxide with thiobarbituric acid. JLR (J. Lipid Res.) 19, 1053–1057.

Roslev, P., Madsen, P.L., Thyme, J.B., Henriksen, K., 1998. Degradation of phthalate and Di-(2-Ethylhexyl) phthalate by indigenous and inoculated microorganisms insludge-amended soil. Appl. Environ. Microbiol. 64 (12), 4711–4719.

Rowdhwal, S.S., Chen, J., 2018. Toxic Effect of Di-2-ethylhexyl Phthalate: an Overview. BioMed Research International. https://doi.org/10.1155/2018/1750368. Article ID 1750368 |.

Shakya, B., Siddique, Y.H., 2018. Evaluation of the toxic potential of arecoline toward the third instar larvae of transgenic Drosophila melanogaster (hsp70-lacZ) Bg^{9}. Toxicology Research 7 (3), 432–443.

Siddique, Y.H., Ara, G., Afzal, M., 2012. Effect of the steroid K-canrenoate on hsp70 expression and tissue damage in transgenic Drosophila melanogaster (hsp70-lacZ) Bg^{9}. J. Insect Sci. 12 https://doi.org/10.1673/031.012.9201.

Siddique, Y.H., Jyoti, S., Naz, F., 2014. Effect of epicatechin gallate dietary supplementation on transgenic *Drosophila* model of Parkinson's disease. J. Diet. Suppl. 11, 121–130.

Urade, R., Chou, C.K., Chou, H.L., Chen, B.H., Wang, T.N., Tsai, E.M., Hung, C.T., Wu, S. J., Chiu, C.C., 2022. Phthalate derivative DEHP disturbs the antiproliferative effect of camptothecin in human lung cancer cells by attenuating DNA damage and activating Akt/Nf-kB signalling pathway. Environ. Toxicol. 38 (2), 332–342.

Wang, Y., Branicky, R., Noe, A., Hekimi, S., 2018. Superoxide dismutase: Dual roles in controlling ROS damage and regulating ROs singnalling. JCB (J. Cell Biol.) 217, 1915–1928.

Xu, H., Shao, X., Zhang, Z., Zou, Y., Wu, X., Yang, L., 2013. Oxidative stress and immune related gene expression following exposure to di-n-butyl phthalate and diethyl phthalate in zebrafish embryos. Ecotoxicol. Environ. Saf. 93, 39–44.

Yin, X., Zeb, R., Wei, H., Cai, L., 2021. Acute exposure of di(2-ethylhexyl) phthalate (DEHP) induces immune signal regulation and ferroptosis in oryzias melastigma. Chemosphere 265, 45–6535.

Yuan, L., Liu, J., Huang, Y., Shen, G., Pang, S., Wang, C., Li, Y., Mu, X., 2022. Integrated toxicity assessment of DEHP and DBP toward aquatic ecosystem based on multiple trophic model assays. Environmental Science and pollution Research 29, 87402–87412.

Zhao, Y., Fan, J.H., Luo, Y., Talukder, M., Li, X.N., Zuo, Y.Z., Li, J.L., 2019. Di-(2-ethylhexyl) phthalate (DEHP)-induced hepatotoxicity in quail (Coturnix japonica) via suppression of the heat shock response. Chemosphere 228, 685–693.

Zhao, Y., Bao, R.K., Zhu, S.Y., Talukder, M., Cui, J.G., Zhang, H., Li, Xn, Li, J.L., 2021. Lycopene prevents DEHP-induced hepatic oxidative stress damage by crosstalk between AHR–Nrf2 pathway. Environmental Pollution 285, 269–7491.

Zheng, Y., Zhou, K., Tang, J., Liu, C., Bai, J., 2022. Impacts of di-(2-ethylhexyl) phthalate on Folsomia candida (Collembola) assessed with a multi-biomarker approach. Ecotoxicol. Environ. Saf. 232, 147–6513.