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Genotoxicity and apoptosis in *Drosophila melanogaster* exposed to benzene, toluene and xylene: Attenuation by quercetin and curcumin

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ABSTRACT

Monocyclic aromatic hydrocarbons (MAHs) such as benzene, toluene and xylene are being extensively used for various industrial and household purposes. Exposure to these hydrocarbons, occupationally or non-occupationally, is harmful to organisms including human. Several studies tested for toxicity of benzene, toluene and xylene, and interestingly, only a few studies looked into the attenuation. We used *Drosophila* model to test the genotoxic and apoptotic potential of these compounds and subsequently evaluated the efficiency of two phytochemicals, namely, quercetin and curcumin in attenuating test chemical induced toxicity. We exposed third instar larvae of wild type *Drosophila melanogaster* (Oregon R^+) to 1.0–100.0 mM benzene, toluene or xylene, individually, for 12, 24 and 48 h and examined their apoptotic and genotoxic potential. We observed significantly ($P<0.001$) increased apoptotic markers and genotoxicity in a concentration- and time-dependent manner in organisms exposed to benzene, toluene or xylene. We also observed significantly ($P<0.001$) increased cytochrome P450 activity in larvae exposed to test chemicals and this was significantly reduced in the presence of 3′,4′-dimethoxyflavone, a known Aryl hydrocarbon receptor (AhR) blocker. Interestingly, we observed a significant reduction in cytochrome P450 activity, GST levels, oxidative stress parameters, genotoxic and apoptotic endpoints when organisms were exposed simultaneously to test chemical along with quercetin or curcumin. The study further suggests the suitability of *D. melanogaster* as an alternate animal model for toxicological studies involving benzene, toluene and xylene and its potential in studying the protective role(s) of phytochemicals.

Introduction

Among the organic solvents, benzene, toluene and xylene are extensively used in diverse chemical, industrial and commercial processes (WHO, 1993). Toxicity and/or carcinogenicity of these chemicals due to occupational or non-occupational exposure are of concern. In the environment, these non-oxygenated MAHs are either generated through processing, combustion and evaporation of gasoline or found as constituents of commercial products such as cleaning fluids, paints and glues (Arlien-Suborg, 1992; Indulski et al., 1996). In addition, these compounds are inhaled for recreational purposes by young people around the globe (Greer, 1984; Kozel et al., 1995; Spiller and Krenzelok, 1997).

Benzene-induced toxicity is related to blood disorders, including bone marrow depression, and some types of cancer (Wan and Winn, 2004; Wetmore et al., 2008). Benzene has been shown to induce DNA damage and carcinogenicity both *in vitro* (in HL60 cells; Kolachana et al., 1993; Zhang et al., 1993) and *in vivo* (in mice, and in human as measured through the comet assay; Hiraku and Kawanishi, 1996; Pan et al., 2003; Sul et al., 2005). Recent studies from our laboratory showed induction of heat shock genes (*hsp70*, *hsp83*, *hsp60* and *hsp26*), oxidative stress markers and increased ROS generation in *Drosophila melanogaster*, exposed to benzene, toluene or xylene, individually, or their mixtures, indicating the potential of these chemicals to produce cellular stress (Singh et al., 2009; Singh et al., 2010). A few studies also suggest the DNA damaging potential of toluene and xylene (Al-Ghamdi et al., 2004; Pariselli et al., 2009). Addition of molecules having ROS quenching efficacy suggested that mechanism underlying the DNA damage and carcinogenicity is likely through ROS generation (Bellion et al., 2009; Messner et al., 2009; Becatti et al., 2010).

Programmed cell death or apoptosis is a self-destruction of a given cell due to irreparable damage. Perusal of literature shows that

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benzene, toluene and xylene induced apoptosis and genotoxicity *in vitro* models and also *in vivo* (Smith, 1996; Ross, 2000; Snyder, 2000; Nakai et al., 2003; Al-Ghamdi et al., 2004; Wan and Winn, 2004; 2007; Wetmore et al., 2008). Although several studies have been carried out to test the toxicity of benzene, toluene and xylene, only limited information is available on the attenuation of toxic insults of these test chemicals in the exposed organisms (Emara and El-Bahrawy, 2008). To address this, we used *D. melanogaster*, as an *in vivo* model, as it offers as an excellent alternative animal model [in accordance to European Centre for the Validation of Alternative Methods (ECVAM)] (Festing et al., 1998). Post genomic sequencing, *Caenorhabditis elegans* and *Drosophila* generated much interest to toxicologists as these show functional conservation of majority of genes present in higher mammals. Further, the availability of state-of-the-art molecular tools coupled with well-defined genetics and developmental biology places this organism better for obtaining mechanistic insights. Moreover, from a toxicological/pharmacological perspective, fly and higher mammals were shown to have similar dose–response relationship with four monofunctional alkylating agents (Siddique et al., 2005a). Thus, we believe that laboratory-based experimental evidences using this model is useful in generating information that could be of value for their efficient extrapolation to higher organisms. Therefore, we first studied apoptosis and genotoxicity in *Drosophila*, induced by benzene, toluene and xylene. Subsequently, we used this model to study the cytoprotective effect(s) of two phytochemicals having anti-oxidant properties, quercetin (QC) and curcumin (CUR) (Kottke, 1998; Lodha and Bagga, 2000), against benzene-, toluene- and xylene-induced cellular toxicity in exposed organisms.We show here that QC and CUR do have the potential to protect against the benzene-, toluene- and xylene-induced toxicity *in vivo*.

Material and methods

Flies and maintenance

All experiments were performed using wild type (Oregon R^+) strain of *D. melanogaster*. The flies and larvae were reared at 23 ± 1 °C on standard *Drosophila* food containing agar–agar, maize powder, sugar, yeast, nepagin (methyl-*p*-hydroxy benzoate salt; HiMedia, India) and propionic acid. Additional yeast suspension was provided for their healthy growth.

Chemicals used for treatment

All chemicals, reagents and kits were procured from Sigma, MO, USA except otherwise stated. Analytical grade benzene (99.7% from Ranbaxy Pvt. Ltd, India), toluene (99.5%, SRL Pvt Ltd, India), xylene (mixture of *o*, *m* and *p*, 99.8%, SRL Pvt Ltd, India) and two phytochemicals namely, quercetin (3,5,7,3′,4′-pentahydroxyflavone, 5,7,3′,4′-tetrahydroxyflavonol; QC) and curcumin [active ingredient of the rhizome of the plant turmeric (*Curcuma longa* Linn); CUR] were used in the study. Dimethyl sulfoxide (DMSO) (SRL Pvt Ltd, Mumbai, India) was used as a solvent and final concentration of DMSO in food was restricted to 0.3% (based on a previous study from this laboratory Nazir et al., 2003).

Treatment schedule

Four different concentrations (1.0, 10.0, 50.0 and 100.0 mM), each corresponding to different fractions of the LC_{50} (48 h) of benzene, toluene or xylene were used. Third instar larvae were grown on standard *Drosophila* diet, contaminated with or without different concentrations of the three test chemicals in triplicates for 2–48 h. For genotoxicity studies by alkaline and neutral Comet assay, we used 100.0 mM of benzene, toluene and xylene. Ethyl methanesulfonate (EMS) (1.0 mM) and γ-radiation (40.0 Gy) using Co^{60} source were as used as positive controls for alkaline and neutral Comet assay, respectively. In another set of experiments, 100.0 μM QC or CUR (based on previous studies: Balasubramanyam et al., 2003; Gupta et al., 2007) was added to the food individually, or along with test chemicals for exposing larvae (24 and 48 h).

Quantitative determination of benzene, toluene and xylene

The chemical burden in the larvae exposed to test chemicals was quantified as described previously (Singh et al., 2009). Briefly, third instar larvae were exposed to 100.0 mM benzene, toluene or xylene in food with/without phytochemicals for 48 h. These larvae were homogenized in deionized water in a headspace vial to obtain 2.0 ml homogenate (10% w/v). The vials were heated at 65 °C for 30 min with magnetic agitation. After the equilibrium, septa were pierced with SPME needle and the SPME polydimethyl siloxane (PDMS) fiber was exposed to headspace for 15 min to affect the adsorption of the test chemicals in the sample. SPME fiber was collected and inserted directly into the injection port of Perkin Elmer gas chromatography (USA) at 200 °C. Total time of this chromatographic analysis was 27 min. Blank analysis was carried out to avoid any carry over phenomena and/or external contamination between analyses of samples (Alegretti et al., 2004).

Quantitative estimation of quercetin and curcumin levels

We have used 125 mg of larvae exposed to QC or CUR for extraction. Larvae were homogenized in 1.0 ml of 1× PBS. QC was extracted using 500.0 μl of DMSO–methanol (1:4 v/v) following the method of Schiborr et al. (2010), CUR was extracted using 95% ethyl acetate and 5% methanol (v/v) and the extract was dried at 40 °C and resuspended in 200.0-μl mobile phase (49% acetonitrile, 20% methanol, and 1% acetic acid (Jones et al., 1998). Subsequently, these extracts were analyzed by HPLC system (Water Milford, MA, USA.) containing a reverse phase C-18 ODS analytical column (5 μM particle size). The levels of QC were estimated by injecting 20.0 μl of sample (mobile phase containing 0.5% aqueous solution of orthophosphoric acid and methanol) and separating with a flow rate of 1 ml min^{-1}, for a run time of 15 min, with photoarray detector (PDA) at 375 nm. For CUR estimation, 20.0 μl of the extract was separated with a flow rate of 1.5 ml min^{-1}, for a run time of 5 min, with PDA detector at 420 nm. A blank or control (extract from larvae exposed to control food) injection and the QC or CUR standards were run immediately before each group of samples.

Measurement of oxidative stress parameters

In this study, we measured different oxidative stress parameters such as Reactive Oxygen Species (ROS), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) measured as Malondialdehyde content (MDA) essentially following methods described previously (Gupta et al., 2007; Gupta et al., 2010).

Preparation of microsomes

The method for preparation of microsomes described previously by Johri et al. (2006) was followed with minor modifications. Control and treated larvae were homogenized in ice-cold homogenization buffer (0.25 M potassium phosphate buffer containing 0.15 M KCl, 0.25 mM phenylmethanesulfonylfluoride (PMSF), 0.01 M ethylenediaminetetraacetic acid (EDTA) and 0.1 mM Dithiothreitol (DTT); pH 7.25) to obtain 10% homogenate. The homogenate was centrifuged at 9000 × *g* for 30 min (at 4 °C) to obtain the supernatant, which in turn was centrifuged at 105,000 × *g* for 60 min to sediment microsomes. The pellets were resuspended in microsome dilution buffer (0.1 M potassium phosphate buffer, pH 7.25, 20% (v/v) glycerol, 0.25 mM PMSF, 0.01 M EDTA and 0.1 M DTT) and stored at −80 °C until further use.

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Ethoxyresorufin-O-deethylase (EROD) and methoxyresorfin-O-deethylase (MROD) assay for estimation of cytochrome P450 activity. The activities of EROD and MROD in the isolated *Drosophila* larval microsomes were determined following Johri et al. (2006), with minor modifications. The reaction mixture consisted of 1.0 ml of 0.1 M PBS pH 7.8, 5.0 µl 1.0 mM ethoxy or methoxy resorufin and 25.0 µl microsomal fraction. The reaction was initiated by the addition of 1.0 ml 1.0 mM NADPH and the mixture was incubated at 37 °C for 10 min. Reaction was terminated by adding 2.0 ml of methanol and the mixtures were centrifuged at 2000 × *g* for 7 min. Levels of resorufin in the supernatant were measured using a Perkin Elmer LS 55 Luminescence Spectrometer at excitation wavelength of 550 nm and emission wavelength of 585 nm.

Inhibition of aryl hydrocarbon receptor (AhR). The role of AhR or its homolog was examined using a potent AhR inhibitor 3′,4′-dimethoxyflavone (DMF) (Lin et al., 2006). Larvae were exposed to food containing 100.0 µM DMF together with 100.0 mM of benzene, toluene or xylene as mentioned in Treatment schedule section and subsequently, EROD activity was measured as in Ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorfin-*O*-deethylase (MROD) assay for estimation of cytochrome P450 activity section. We have used larvae exposed to 200.0 ng/ml Tetrachlorodibenzo-*p*-dioxin (TCDD) (Cespedes et al., 2010) together with 100.0 µM DMF as positive control. TCDD was used as a positive control because this is also a halogenated aromatic hydrocarbon and is known to induce cytochrome P450 1AI (CYP1A1) through AhR (Cespedes et al., 2010). Larvae exposed to 100.0 µM DMF alone was used as an additional control.

Glutathione S-transferase (GST, EC 2.5.1.18)

Glutathione *S*-transferase (GST) activity was determined following Habig et al. (1974) with minor modifications. The reaction mixture consisted of 0.2 M sodium phosphate buffer, 75.0 µl larval homogenate, reduced glutathione (1.0 mM) and 1-chloro 2, 4 dinitrobenzene (CDNB) (5.0 mM). An increase in absorbance (340 nm) was measured for 3 min at 30-s intervals and the enzyme activity was calculated as nmol CDNB reduced/min/mg larval protein using molar extinction coefficient of $6.25 \times 10^3\ M^{-1}\ cm^{-1}$.

Trypan blue dye exclusion assay

Tissue damage in the test chemical exposed organisms was examined by trypan blue exclusion assay as described earlier (Krebs and Feder, 1997). In brief, internal tissues of control and treated larvae (50–60/group), explanted in PSS were washed once in 50.0 mM phosphate buffered saline (PBS), pH 7.4 (Dulbecco's, HiMedia Pvt Ltd., Mumbai, India). They were then immersed in trypan blue stain (0.2 mg/ml in 50.0 mM PBS, pH 7.4) and shaken gently for 30 min at 24 ± 1 °C. After staining, tissues were washed thrice in wash buffer (0.1 M PBS pH 7.4) and immediately visualized and scored larvae for trypan blue staining.

Single cell preparation

Midgut tissues of 15 larvae from control and treated groups were incubated in collagenase (0.5 mg/ml of 1.0 M PBS pH 7.4) for 15 min at 24 ± 1 °C. The dissociated cells were then passed through 80 µm nylon mesh to get rid of clumping. Collagenase was removed by washing the cell suspension with 0.1 M PBS (pH 7.4) for at least three times with gentle shaking. Viability of cells was checked by trypan blue staining before the start of the experiment (Phillips, 1973). These cells were processed for different end point measurements as described below.

Assay of apoptosis

All apoptotic end points were measured in single cells prepared from midgut tissues of control and treated organisms except for those of terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay. For TUNEL assay, we used whole midgut tissues and for measuring caspase activity, we used 10% tissue homogenate, to match the published standard protocols.

Flow cytometric determination of cellular Rpr, Hid and Grim (initiator of apoptosis) levels. Previously described flow cytometric detection method was followed with minor modification (Wechsler-Reya et al., 1998). Cells after thorough washing in 0.1 M PBS (pH 7.4) were fixed in PBS containing 0.25% paraformaldehyde for 1 h at 4 °C and then permeabilized by washing twice in PBS with 0.1% Triton X-100 for 10 min at 24 ± 1 °C. Subsequently, cells were rinsed with PBS and incubated with primary antibodies against Rpr, Hid or Grim (anti-goat polyclonal IgG, 1:50 in PBS containing 2% BSA; Santa Cruz, CA, USA) for 1 h at 4 °C. This was followed by incubation of cells with FITC conjugated rabbit anti-goat IgG secondary antibody (1:100 in 0.1 M PBS, pH 7.4 containing 2% BSA) for 1 h at 4 °C. The stained cells were analyzed on Becton Dickinson flow cytometer (BD, NJ, USA) using Cell quest software (Mac OS 8.6). For each sample, 10,000 events were counted and results were expressed in terms of the percentage cells expressing particular protein.

Phosphatidylserine (PS) externalization assay (Annexin V-FITC staining). Apoptotic cells were detected using Annexin V-FITC apoptosis detection kit essentially following the manufacturer's instructions. Briefly, media binding reagent and Annexin V-FITC were mixed with approximately 5×10^5 cells and incubated at 24 ± 1 °C for 15 min. After removing the media, the cells were re-suspended in cold 1× binding buffer and stained with PI. Ten thousand events were acquired per treatment group using Becton Dickinson flow cytometer and data were analyzed with Cell Quest software (Mac OS 8.6). The FITC signal was detected by FL1 (FITC detector) at 518 nm and Pl was detected by FL2 (phycoerythrin fluorescence detector) at 620 nm. The log of Annexin V-FITC and Pl fluorescence was displayed on the *X*- and *Y*-axis of the data report respectively.

Determination of mitochondrial membrane potential ($\Delta\Psi_m$). Depolarization of mitochondrial membrane was analyzed following a previously described method with minor modification (Vayssier-Taussat et al., 2001). A fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) was used for this purpose. The mitochondrial membrane depolarization is associated with a shift in JC-1 fluorescence emission from red to green. Cells of control and treated organisms were suspended in Schneider's *Drosophila* medium containing 10.0 µM JC-1 (prepared in DMSO) for 30 min at 24 °C. Cells were then washed with 0.1 M PBS (pH 7.4) twice and finally re-suspended in 500.0 µl PBS (pH 7.4) for FACScan analysis. Ten thousand events were counted per sample in acquisition and analysis was performed using cell quest software (Mac OS 8.6). The results were expressed as the percentage of cells with disrupted mitochondrial membranes.

Assay of DEVD- and IETD-ase activities. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) obtained after specific action of different cysteine proteases involved in apoptotic pathways on tetrapeptide substrates, respectively. The assay was performed essentially following the manufacturer's protocol (Bio Vision, Inc., CA, USA). Supernatant from the 10% tissue homogenate was mixed with chilled cell lysis buffer, 2× reaction buffer (containing 10.0 mM dithiothreitol) and 200 µM substrates. The reaction mixture was incubated at 37 °C for 1.5 h and absorbance of the colored product was measured at 405 nm on a Cintra 20 ultraviolet spectrophotometer (GBC Scientific Equipment, Melbourne, Australia).

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Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay. TUNEL assay was performed using "*In situ* cell death detection kit" essentially following the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim, Germany). Midgut tissues of control and treated larvae were fixed in freshly prepared 2.5% glutaraldehyde and permeabilized in PBST followed by washing in PBS. They were incubated in primary TUNEL mixture for 1 h at 37 °C and then washed with PBS for 30 min at 24 °C (6 changes of 5 min each) followed by incubation in converter alkaline phosphate (AP) solution at 37 °C. After washing, tissues were incubated in the substrate solution containing nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), specific for AP, for 15 min in dark at 24 °C. The tissues were then washed thoroughly with PBS, mounted on clean glass slides using 50% glycerol and coverglass was placed. The edges of the coverglass were sealed with DPX and the tissues were scored for TUNEL positive cells under Leitz orthoplan light microscope (Wetzlar, Germany). One hundred fifty cells were examined from each group by randomized manual counting (25 cells/gut tissue and 2 gut tissues/experiment and 3 experiments/group/time point).

Poly (ADP-ribose) polymerase (PARP) cleavage. We performed PARP antibody staining in cells of control and treated organisms using "Anti-PARP Cleavage Site Specific Antibody (CSSA) Assay kit" (Invitrogen, USA) essentially following the manufacturer's protocol. The FITC stained cells were analyzed on a Becton Dickinson flow cytometer using Cell quest software (Mac OS 8.6). For each sample, 10,000 events were counted and results were expressed in terms of percent cells expressing PARP.

Evaluation of DNA damage by Comet assay

Single cell preparation and viability test were performed as described in Single cell preparation section. Care was taken to perform all the steps (from single cell preparation to staining) under dim light to avoid any light-induced DNA damage.

Slides preparation. A previously described method for preparation of slides was followed (Tice et al., 2000) wherein frosted slides (with 1.5 cm frosted end) were used. The slides were rinsed in methanol and flame dried. The slides were then coated on non-frosted end with 1.0% normal melting agarose (NMA), prepared in milliQ water and kept at 60 °C) up to two-third of their length by dipping into molten NMA and were allowed to dry for 1 h at room temperature (24 °C).

Evaluation of DNA damage. For the entire group, slides were prepared in duplicate according to the method described earlier (Siddique et al., 2008). All experiments were repeated three times. The cell suspension (80.0 μl) was mixed with 80.0 μl of 1.5% low melting point agarose (LMA; prepared in Ca^{2+} Mg^{2+} free PBS; final concentration 0.75%, 35–40 °C). For each slide, 75.0 μl of the above mixture was immediately layered on a base slide. Cover slip was immediately placed over the second layer. The slide was then placed on a chilled plate for 10 min to allow solidification of agarose. This step was repeated again to place another layer of LMA 0.75%. Finally, the cover slip was removed and the slide was immersed for 2 h in freshly prepared, chilled lysing solution (2.5 M NaCl, 100.0 mM EDTA, 10.0 mM Tris and 1.0% Triton X-100, pH 10). After lysis, the slides were subjected to neutral and alkaline gel electrophoresis as follows.

Electrophoresis for both alkaline and neutral Comet assay. For alkaline comet assay, slides were placed in chilled electrophoresis buffer (1.0 mM Na_2EDTA and 300.0 mM NaOH, pH>13) for 10 min for DNA unwinding. Subsequently, electrophoresis was conducted in chilled electrophoresis buffer (1.0 mM Na_2EDTA and 300.0 mM NaOH, pH>13) for 15 min at 0.7 V/cm (300 mA/25 V) at 4 °C. The slides were then washed three times with 0.4 M Tris buffer (pH 7.5) at 4 °C to neutralize excess alkali and then for staining as described in Staining section.

We followed a previously described method for neutral Comet assay for the detection of double-strand breaks (Fracasso et al., 2009). The slides after lysis were kept in electrophoresis buffer (300.0 mM CH_3COONa, 100.0 mM Tris–HCl, pH 8.5) for 1 h and then transferred to horizontal electrophoresis unit (Life Technologies, Gaithersburg, MD, USA) containing fresh buffer. Electrophoresis was carried out at constant current of 60 mA for 1 h at 4 °C and slides were stained subsequently as described in the following section.

Staining. The slides were stained with ethidium bromide (20.0 μg/ml; 75 μl per slide) for 10 min in dark. After staining, the slides were dipped once in chilled distilled water to remove the excess stain and cover slips were placed over the slides.

Slide scoring. The slides were examined on a Leica DMLB microscope with fluorescence attachment (Leica Germany). The images were transferred to a computer through a charge coupled device (CCD) camera and analyzed using Komet 5.0 software (Kinetic Imaging, Liverpool, UK). One hundred fifty cells from each group (25 cells per slide with two slides/experimental group in triplicates) were examined. The tail length (TL) (μm), tail DNA (%) (TD) and tail moment (TM) (arbitrary units) were used as indicators of DNA damage as described earlier (Olive et al., 1992).

Statistical analysis

Different parameters were analyzed in control and exposed third instar larvae of *D. melanogaster* (Oregon R^+). Analysis of variance (ANOVA) was carried out to find out the significant differences in means considering each end point as dependent variable (with or without QC and CUR) and treatment (benzene, toluene and xylene); concentration (1.0, 10.0, 50.0 and 100.0 mM) and duration of exposure (2, 4, 6, 12, 24 and 48 h) as independent variables. Prior to applying the ANOVA, homogeneity of variance was ascertained using Levene's test of equality error variance. Two-way analysis of variance was carried out for ROS generation, apoptotic and genotoxic markers as dependent variables (Zar, 1984). SPSS 14.0 (SPSS, Mapinfo Max, USA) was used to analysis the data. To identify the degree of relationship between cytochrome P450 activity and oxidative stress parameters analyzed in the present study with test chemical alone or in combination with QC or CUR, simple linear correlation (Pearson *r*) statistics followed significance analysis of correlation coefficient were performed.

Results

We did not observe any significantly altered activities or levels of all the tested parameters at 1.0 mM concentration of the test chemicals throughout the treatment period and till 6 h at 10.0 mM concentration of these test chemicals (data for 1.0 mM test chemicals not shown). Hence, we only included data of 6, 12, 24 and 48 h exposure of 10.0–100.0 mM benzene, toluene and xylene. Unlike apoptotic markers, Comet assay parameters did not show any remarkable damage till 12 h and hence, we presented only 24 and 48 h data in the study. Further, data obtained from parallel control (DMSO) were similar to those of controls and hence, we used the data from controls for comparison.

Detection of chemicals in larvae of D. melanogaster

To ensure the larval uptake of the test chemical and its uptake is not altered in the presence of QC or CUR or vice versa, we estimated the chemical load in exposed organisms. We detected benzene,

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toluene or xylene in larvae exposed to test chemical alone at levels similar ($P>0.05$) to those in combination with QC or CUR. We also detected QC or CUR in organisms exposed to QC or CUR alone at levels similar ($P>0.05$) to those exposed to QC or CUR along with test chemical (please see Supplemental Figs. S1 and S2).

Moderate trypan blue staining in tissues of Oregon R^+ larvae exposed to benzene, toluene or xylene

To determine if exposure to benzene, toluene or xylene results in tissue damage, we analyzed trypan blue staining in tissues of *D. melanogaster* larvae exposed to 100.0 mM benzene (Fig. 1B), toluene (Fig. 1C) and xylene (Fig. 1D) for 48 h (Fig. 1). Of the larvae exposed to benzene, 94% of them showed blue staining in their midgut, salivary gland, gastric caeca and brain ganglia while 86% or 84% of the larvae exposed to toluene or xylene exhibited a pale to moderate blue staining in the above mentioned tissues respectively.

Increased levels of Rpr, Hid and Grim (homologues of mammalian SMAC/DIABLO) in benzene-, toluene- or xylene-exposed D. melanogaster larvae

To examine whether apoptosis is indeed induced by the test chemical, we analyzed the levels of pro-apoptotic proteins, Rpr, Hid and Grim (Hay and Guo, 2006). Figs. 2–4 show the level of Rpr, Hid and Grim in control or exposed larvae. Larvae exposed to 10.0–100.0 mM benzene, toluene or xylene for 24 or 48 h showed a significant concentration- and time-dependent increase in Rpr (Fig. 2A–F), Hid (Fig. 3A–F) and Grim (Fig. 4A–F) levels, when compared to control. Interestingly, at most concentrations, organisms exposed to benzene showed a significantly higher level of Rpr, Hid and Grim after 24 h whereas those exposed to similar concentration(s) of toluene or xylene showed significantly increased levels of these proteins only after 48 h. However, no significant difference between Rpr, Hid and Grim levels was observed in toluene- or xylene-exposed organisms at all the tested concentrations. The toxicity trend observed for *Drosophila* larvae exposed to 100.0 mM benzene, toluene and xylene for 48 h was as follows: benzene>toluene≧xylene (Figs. 2–4). We did not observe a significant increase in levels of Rpr, Hid or Grim in larvae exposed to 10.0–50.0 mM benzene, toluene or xylene for 12 h, when compared to their controls (see also Supplemental Fig. S3A–C).

Externalization of phosphatidylserine (PS) in D. melanogaster larvae following treatment of benzene, toluene or xylene

We examined the initiation of apoptosis in benzene-, toluene- or xylene-exposed organism by quantifying the Annexin V positive cells.

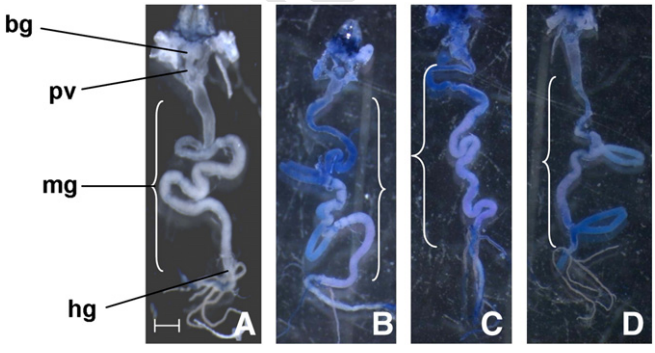

Fig. 1. Trypan blue staining in the internal tissues isolated from third instar larvae of *D. melanogaster* (Oregon R^+) control (A) and in third instar larvae exposed to 100.0 mM benzene (B), toluene (C) and xylene (D) after 48 h. bg = brain ganglia, sg = salivary gland, pv = proventriculus, gc = gastric caeca, mg = midgut, hg = hind gut. Bar represents 100 μm.

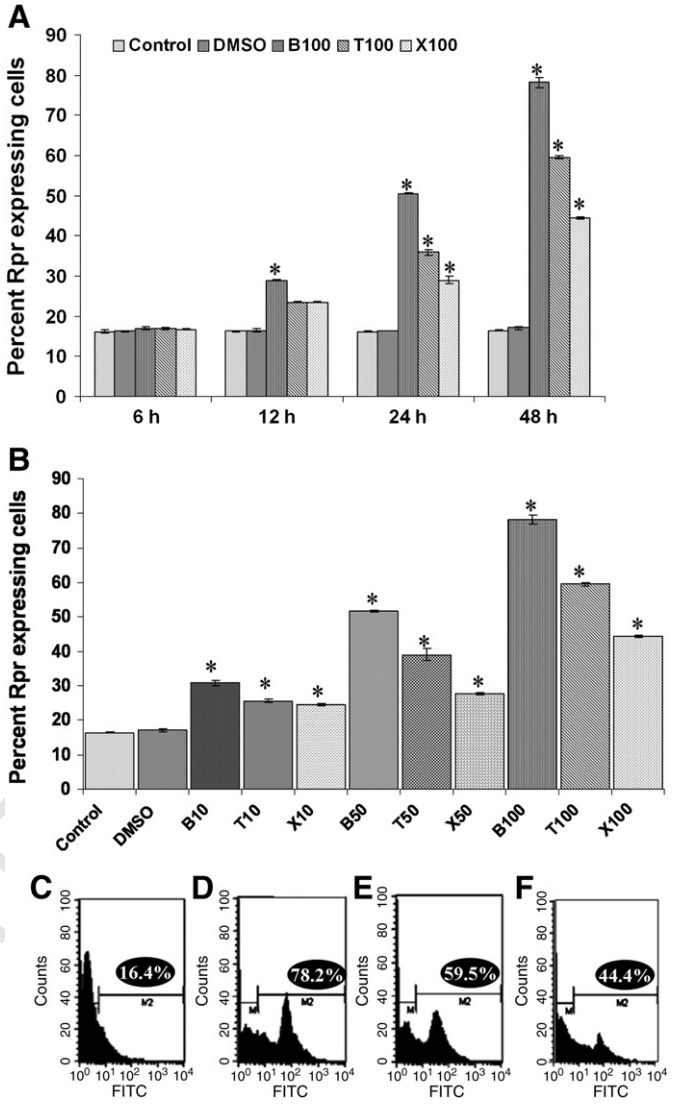

Fig. 2. Levels of Rpr (A–F) in cells from midgut tissues of third instar larvae of *D. melanogaster* (Oregon R^+) exposed to control food, DMSO, benzene, toluene or xylene. Panel A represents levels of Rpr in larvae exposed to 100.0 mM benzene, toluene or xylene for 6, 12, 24 or 48 h. Panel B shows levels of Rpr in larvae exposed to 10.0–100 mM of benzene, toluene or xylene for 48 h. Flow cytometric panels depict level of Rpr in control (C), 100.0 mM benzene- (D), toluene- (E) and xylene- (F) treated *Drosophila* larvae after 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance in comparison to control is ascribed as $^*P<0.01$, $^{**}P<0.001$. B10, B50 and B100 = 10.0, 50.0 and 100.0 mM benzene; similarly T10, T50 and T100 = 10.0, 50.0 and 100.0 mM toluene and X10, X50 and X100 = 10.0, 50.0 and 100.0 mM xylene in this figure and the remaining figures in this paper. Q8

Fig. 5 shows the Annexin V (AV) positive early apoptotic cells in the midgut tissues of control and treated larvae. Larvae exposed to 10.0–100.0 mM benzene, toluene or xylene exhibited a concentration- and time-dependent significant increase ($P<0.01$) in AV positive cells in comparison to control (Fig. 5A–F). When compared to control, larvae exposed to 10.0 mM benzene showed significantly higher AV positive cells after 24 h whereas organisms exposed to the same concentration of toluene or xylene exhibited a significantly higher AV positive cells only after 48 h. Similar trend was observed even in organisms exposed to higher concentrations (50.0 or 100.0 mM) of benzene, toluene or xylene when compared to control: significant increase in the AV positive cells by 12 h in case of 100.0 mM benzene-exposed organisms (40.0% increase) and significant increase of the same in toluene- or xylene-exposed organisms was only after 24 h (43.0% and 30.0% increase respectively) (also see Supplemental Fig. S4).

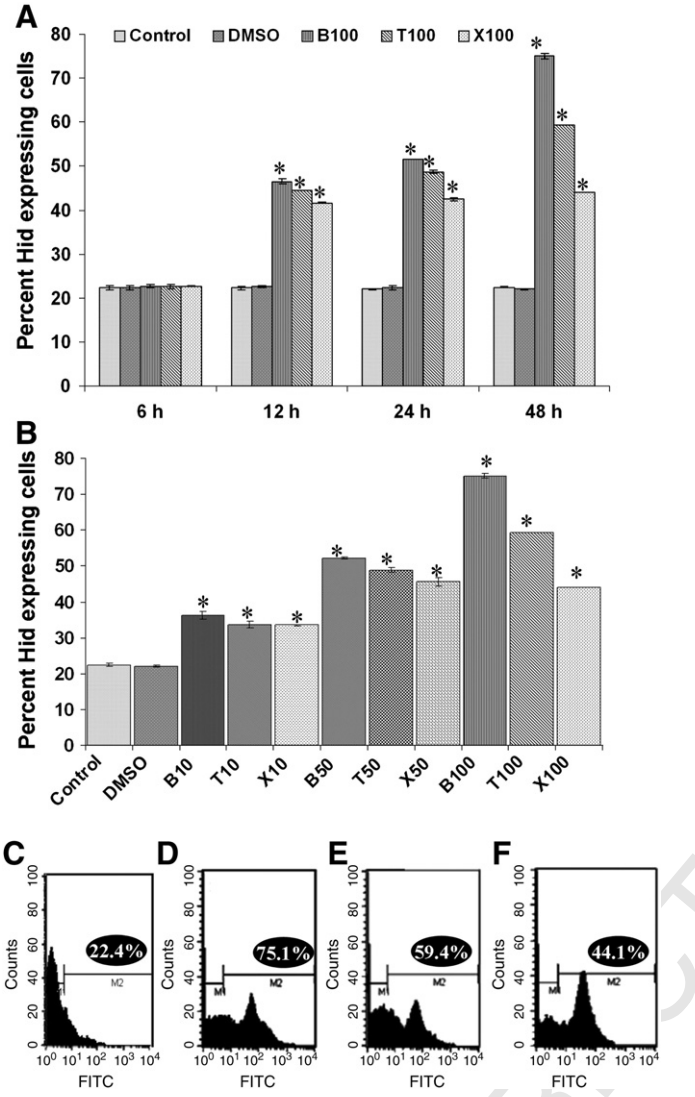

Fig. 3. Levels of Hid (A–F) in cells from midgut tissues of third instar larvae of *D. melanogaster* (Oregon R^+) exposed to control food, DMSO, benzene, toluene or xylene. Panel A represents levels of Hid in larvae exposed to 100.0 mM benzene, toluene or xylene for 6, 12, 24 or 48 h. Panel B shows levels of Hid in larvae exposed to 10.0–100 mM of benzene, toluene or xylene for 48 h. Flow cytometric panels depict level of Hid in control (C), 100.0 mM benzene- (D), toluene- (E) and xylene- (F) treated *Drosophila* larvae after 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance in comparison to control is ascribed as $^*P<0.01$, $^{**}P<0.001$.

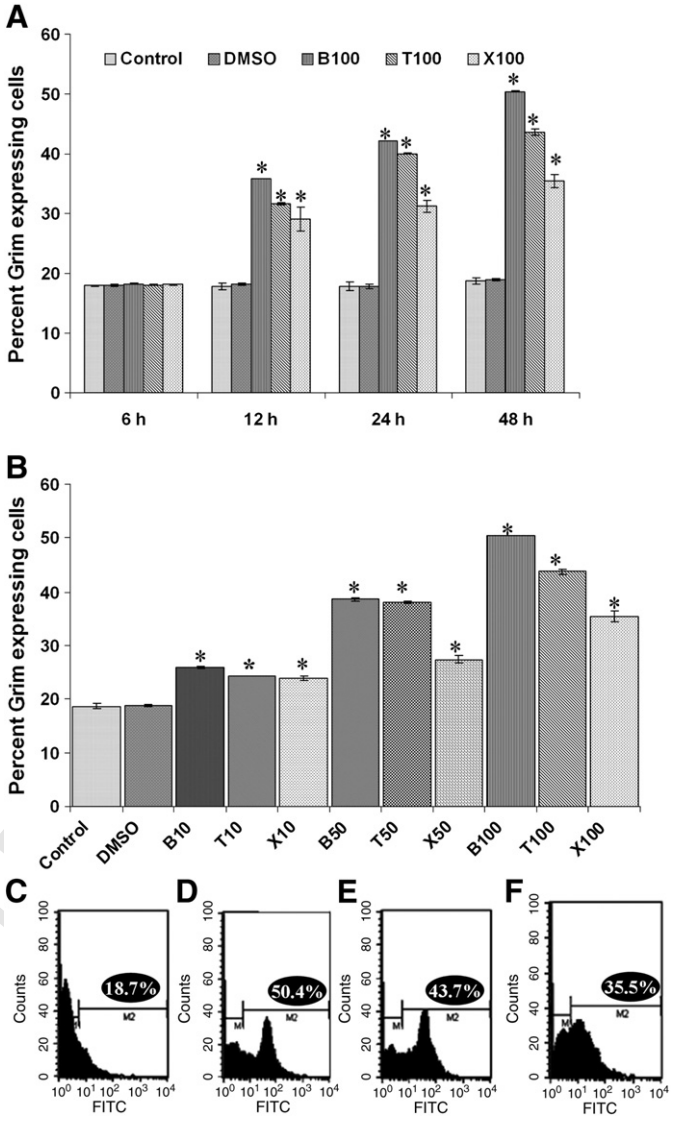

Fig. 4. Levels of Grim (A–F) in cells from midgut tissues of third instar larvae of *D. melanogaster* (Oregon R^+) exposed to control food, DMSO, benzene, toluene or xylene. Panel A represents levels of Grim in larvae exposed to 100.0 mM benzene, toluene or xylene for 6, 12, 24 or 48 h. Panel B shows levels of Grim in larvae exposed to 10.0–100 mM of benzene, toluene or xylene for 48 h. Flow cytometric panels depict level of Grim in control (C), 100.0 mM benzene- (D), toluene- (E) and xylene- (F) treated *Drosophila* larvae after 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance in comparison to control is ascribed as $^*P<0.01$, $^{**}P<0.001$.

Depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) in exposed third instar D. melanogaster larvae

Change in mitochondrial membrane potential is required for caspase activation (Hay and Guo, 2006). Larvae exposed to 10.0–100.0 mM benzene, toluene or xylene showed a concentration- and time-dependent significant ($P<0.001$) depolarization of mitochondrial membrane ($\Delta\Psi_m$) when compared to their respective controls (Fig. 6A–F). However, *Drosophila* larvae exposed to 10.0 mM benzene exhibited significant $\Delta\Psi_m$ after 24 h, whereas toluene- or xylene-exposed organisms exhibited significant $\Delta\Psi_m$ only after 48 h, when compared to controls (also see Supplemental Fig. S5 for details). Organisms exposed to (50.0 or 100.0 mM) benzene, toluene or xylene showed significant $\Delta\Psi_m$ after 24 h when compared to control (Fig. 6A–F). In all these cases, we did not find any significant difference between toluene- and xylene-treated groups.

Increased IETDase, DEVDase and Poly (ADP-ribose) polymerase (PARP) cleavage in benzene-, toluene- or xylene-exposed third instar D. melanogaster larvae

To measure the activation of caspases, we have carried out IETDase and DEVDase assays. The relative intensities of the cleaved chromophore, *p*-nitroanilide, obtained after specific cleavage by IETDase and DEVDase in the larvae exposed to benzene, toluene and xylene are shown in Fig. 7. We observed a concentration- and time-dependent up-regulation ($P<0.001$) of IETDase activity in organisms exposed to 10.0–100.0 mM test chemicals (Fig. 7A). Larvae exposed to 10.0 mM benzene showed a significantly increased IETDase activity after 24 h (2.0-fold increase as compared to control) and while those exposed to toluene and xylene exhibited significantly higher activities only after 48 h (1.8- and 1.6-fold increase as compared to control). Larvae exposed to 50.0 mM of test chemicals showed significantly increased

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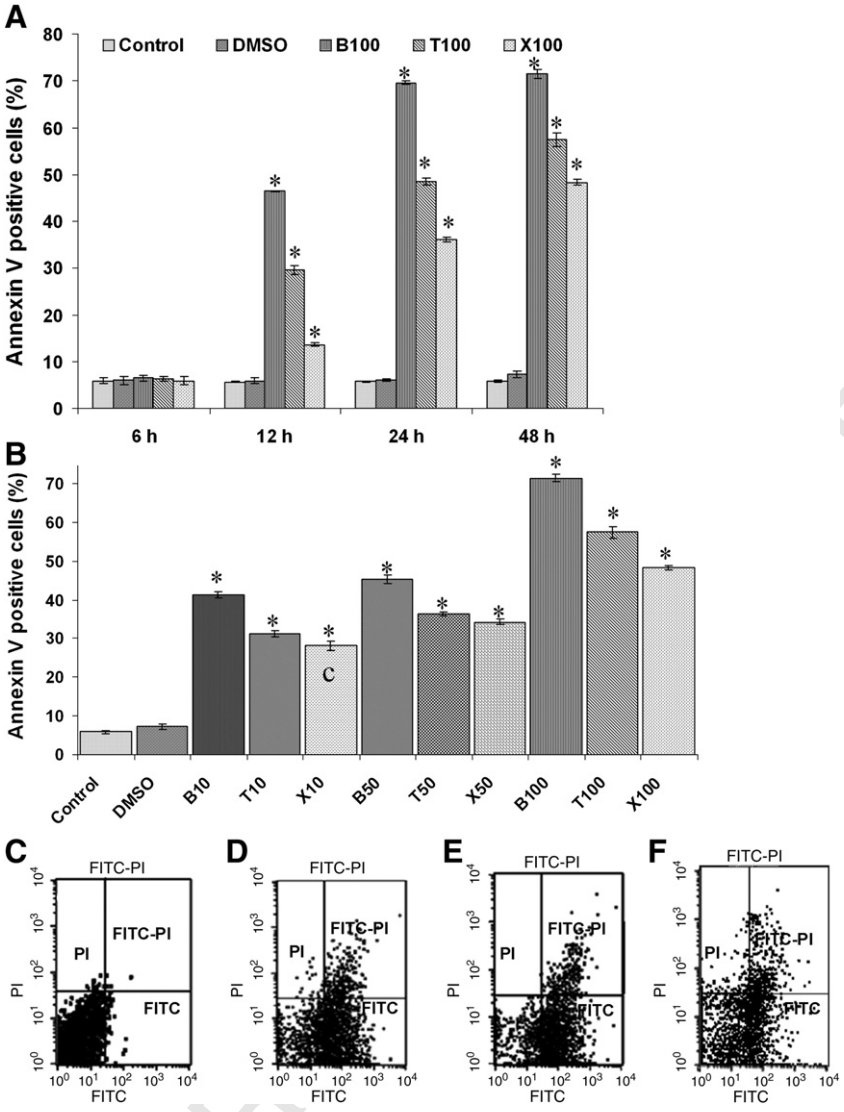

Fig. 5. Annexin V positive cells (A–F) in cells from midgut tissues of control, DMSO-, benzene-, toluene- or xylene-exposed third instar larvae of *D. melanogaster* (Oregon R^+). Panel A represents percentage of Annexin positive cells from midguts of larvae exposed to 100.0 mM benzene, toluene or xylene for 6, 12, 24 or 48 h. Percent Annexin positive cells from midguts of larvae exposed to various concentrations of benzene, toluene or xylene for 48 h is represented in Panel B. Flow cytometric panels depict cells in early apoptotic stage (Annexin V positive cells, FITC quadrant), late apoptotic stage (Annexin V and propidium iodide (PI) positive cells, FITC-PI quadrant) and necrotic state (PI positive, PI quadrant) from midgut tissues of control (C), 100.0 mM benzene- (D), toluene- and (E) xylene- (F) exposed organisms after 48 h. Data represent mean ± SD of three identical experiments made in triplicate and significance in comparison to control is ascribed as $^*P<0.01$, $^{**}P<0.001$.

IETDase activity after 12 and 24 h, respectively, in benzene-, toluene- or xylene-exposed groups as compared to their respective controls. A maximum ($P<0.001$) 4.0-, 3.3- or 3.1-fold increase in IETDase activity was observed in 100.0 mM benzene-, toluene- or xylene-exposed larvae after 48 h, respectively (Fig. 7A). The DEVDase activity in the test chemicals exposed organisms was similar to that of IETDase activity (Fig. 7B). We observed significantly increased number of PARP-FITC positive cells in organisms exposed to 100.0 mM benzene, toluene or xylene for 24 and 48 h (4.3, 3.9- and 3.6-fold increase after 48 h respectively), when compared to their respective controls (Fig. 8A–E). The differences in the number of PARP-FITC positive cells observed among benzene-, toluene- or xylene-exposed organisms for 24 and 48 h were found to be non-significant.

Increased DNA nicking in test chemicals exposed third instar larvae of D. melanogaster

To examine test chemical mediated DNA nicking, a key feature of apoptosis, we performed TUNEL assay in midgut cells of control and 100.0 mM test chemicals treated larvae after 24 and 48 h (Fig. 9). After 24 h, we observed significant increase in TUNEL positive cells only in *Drosophila* larvae exposed to benzene (100.0 mM; 3.3-fold) when compared to control. Organisms exposed to similar concentrations of toluene or xylene showed significantly more TUNEL positive cells than controls only after 48 h (7.7- and 7.9-fold increase). However, even at this time point, the number of TUNEL positive cells in organisms exposed to 100.0 mM of toluene or xylene for 24 and 48 h was significantly lower than that in organisms exposed to benzene (Fig. 9A–C).

Increased DNA damage in the midgut cells of D. melanogaster larvae exposed to benzene, toluene or xylene

To determine the extent of DNA damage, we analyzed Comet parameters in organisms exposed to the test chemical. We observed 95–98% cell viability in controls, and also in 100.0 mM benzene-, toluene- or xylene-exposed groups. Larvae exposed to 1.0 mM EMS (used as a positive control) showed a significant increase ($P<0.001$)

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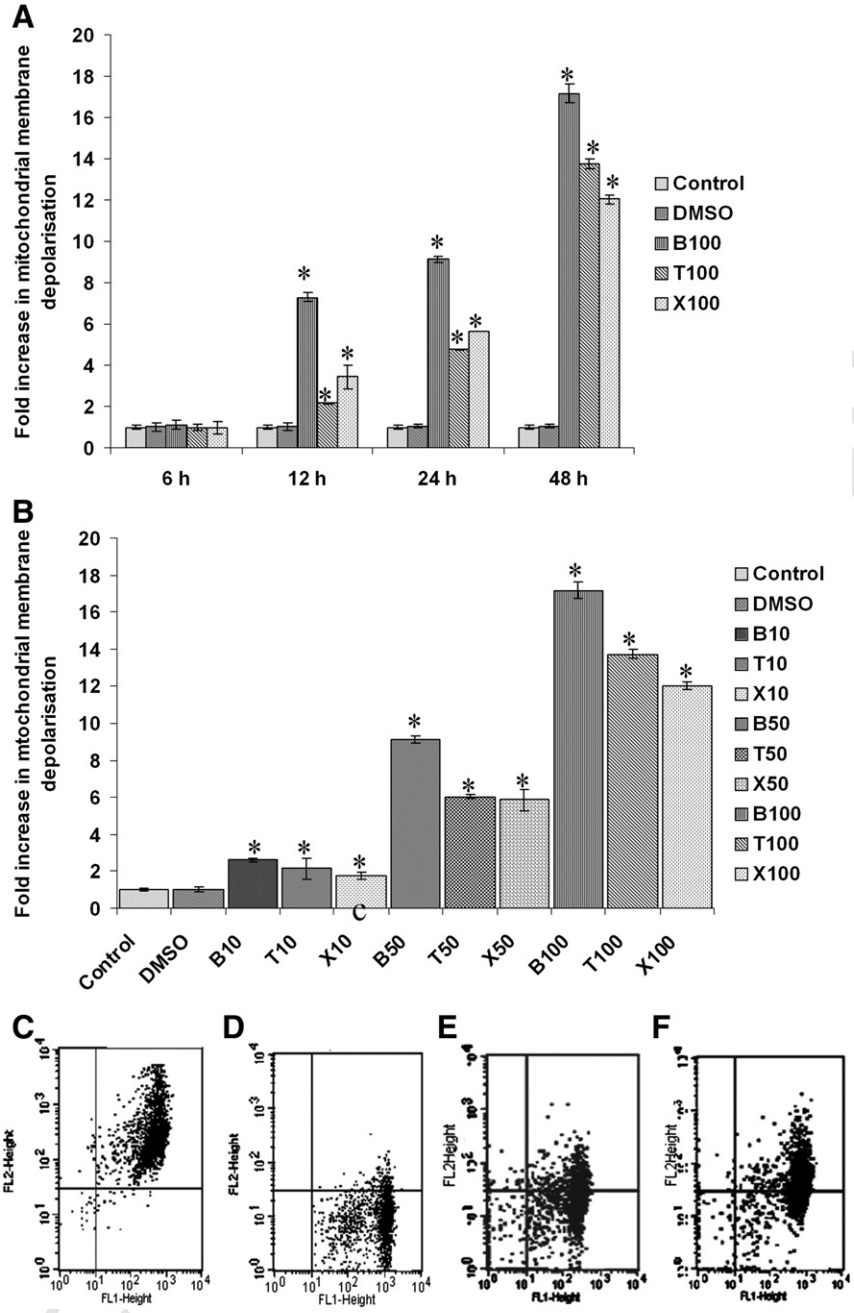

Q6 **Fig. 6.** Alterations in mitochondrial membrane potential ($\Delta\Psi_m$) (A–F) in cells from midgut tissues of control, DMSO-, benzene-, toluene- or xylene-exposed third instar larvae of *D. melanogaster* (Oregon R^+). Panel A represents fold increase in mitochondrial membrane depolarization in cells from midguts of larvae exposed to 100.0 mM benzene, toluene or xylene for 6, 12, 24 or 48 h. Fold change in mitochondrial membrane depolarization in cells from midguts of larvae exposed to various concentrations of benzene, toluene or xylene for 48 h is represented in Panel B. Flow cytometric panels represent alterations in $\Delta\Psi_m$ in (C) control (D) 100.0 mM benzene- (E) toluene- and (F) xylene-exposed organisms after
Q12 48 h. Data represent mean ± SD of three identical experiments made in triplicate and significance in comparison to control is ascribed as $^*P<0.01$, $^{**}P<0.001$.

in DNA migration (TL, % TD and TM) in their midgut cells in alkaline Comet assay while those given γ-radiation (40.0 Gy) showed significantly increased migration of DNA after neutral assay. Similarly, in organisms exposed to test chemicals, we observed a significant increase in DNA migration after 24 and 48 h (in the order of benzene>toluene≥xylene), when compared to control, in both alkaline and neutral Comet assays (Table 1). Interestingly, we observed significantly higher ($P<0.01$) migration of DNA (increased TL, % TD and TM) in cells of the exposed organisms subjected to alkaline Comet assay when compared to those of neutral Comet assay.

Increased EROD and MROD activity in larvae exposed to benzene, toluene or xylene

Cytochrome P450 1A (CYP1A) subfamily is highly conserved and is known to play a vital role in the metabolism of chemical carcinogens and environmental contaminants. To estimate the CYP1A1 and CYP1A2, two common isoforms of CYP1A family, we carried out ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorfin-*O*-deethylase (MROD) assays. Fig. 10 shows EROD and MROD activities in larvae exposed to control or food containing the test chemical. We observed a significant ($P<0.001$) 6.3, 5.3 and 3.7-fold increase in EROD and 3.9, 3.3 and 2.9-fold increase

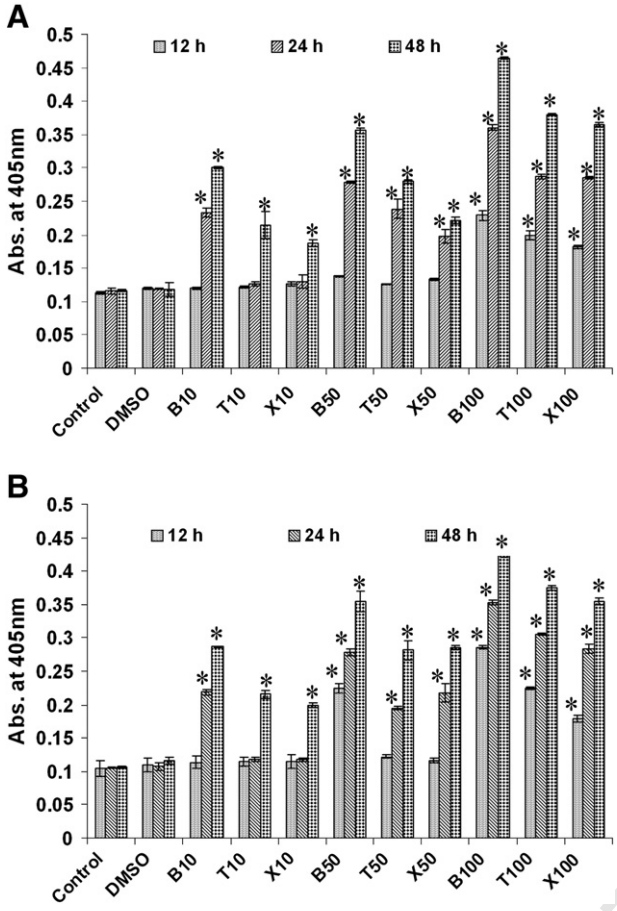

Fig. 7. IETDase (A) and DEVDase (B) activities in midgut tissue homogenate from control, DMSO-, benzene-, toluene- or xylene-exposed third instar larvae of *D. melanogaster* (Oregon R^+) after 12, 24 and 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance in comparison to control is ascribed as $^*P<0.01$, $^{**}P<0.001$.

in MROD activities in organisms exposed to 100.0 mM benzene, toluene or xylene for 48 h, respectively, when compared to their controls (Fig. 10A–B).

Significantly decreased EROD activity in larvae exposed to benzene, toluene or xylene together with DMF

To determine if benzene-, toluene- or xylene-mediated induction of cytochrome P450 enzymes requires Aryl hydrocarbon Receptor

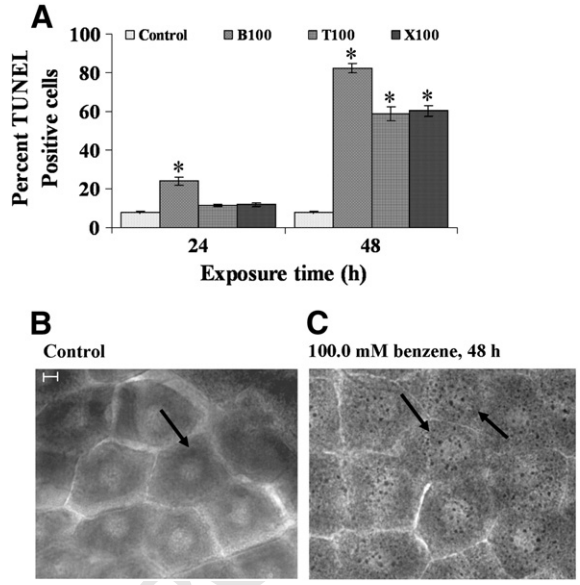

Fig. 9. TUNEL positive cells in midgut tissues of third instar larvae of *D. melanogaster* (Oregon R^+) of control and benzene-, toluene- or xylene-exposed third instar larvae of *D. melanogaster* (Oregon R^+) for 24 and 48 h. Histogram (A) depicts percent TUNEL positive cells in control and benzene-, toluene- or xylene-exposed organisms. Panels B and C represent microscopic images showing TUNEL positive cells in control and 100.0 mM benzene-exposed organisms, respectively after 48 h. Arrows indicate TUNEL positive cells. Bar represents 200 μm. Data represent mean ± SD of three identical experiments made in triplicates and significance in comparison to control is ascribed as $^*P<0.01$.

(AhR) or its homolog, we exposed larvae to DMF, a potent inhibitor of AhR, alone or in combination with test chemical. Exposure to DMF (100 μM) alone did not significantly decrease the EROD activity compared to that in control *Drosophila* larvae. However, we observed significantly decreased EROD activity ($P<0.001$) in organisms exposed to food containing B100, T100, X100, or TCDD (positive control) along with DMF (Fig. 10C), in comparison to that observed in test chemical alone treated group.

Effects of QC and CUR on the adverse effects of benzene, toluene or xylene in exposed Drosophila

As we have observed increase in EROD, MROD activities, oxidative stress parameters and genotoxicity in response to test chemicals, we examined the effect of co-treatment with QC or CUR on the levels of these various parameters.

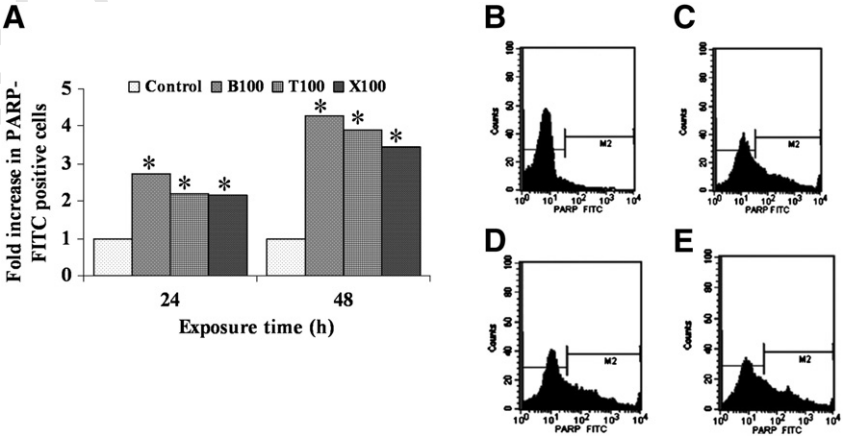

Fig. 8. PARP cleavage in cells from midgut tissues of control and DMSO-, benzene-, toluene- or xylene-treated organisms for 24 and 48 h. Histogram (A) depicts fold increase in PARP-FITC positive cells. Flow cytometric panels represent PARP cleavage in (B) control, (C) 100.0 mM benzene- (D) toluene- and (E) xylene-exposed groups after 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance in comparison to control is ascribed as $^*P<0.01$. $^{**}P<0.001$.

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Table 1
DNA migration in gut cells of *D. melanogaster* exposed to benzene, toluene and xylene and simultaneously with quercetin or curcumin after alkaline and neutral Comet assay.

Groups	Tail DNA (%)		Tail length		Tail moment (a.u.)	
	24 h	48 h	24 h	48 h	24 h	48 h
Alkaline Comet parameters						
Control	5.73 ± 0.50	6.01 ± 0.36	6.68 ± 0.57	6.95 ± 1.00	0.70 ± 0.03	0.75 ± 0.15
EMS	25.73 ± 0.45*	26.60 ± 0.52*	28.28 ± 1.27*	28.59 ± 2.60*	7.83 ± 0.33*	10.89 ± 0.49*
DMSO	6.13 ± 0.64	5.97 ± 0.65	6.72 ± 0.38	6.89 ± .84	0.65 ± 0.05	0.78 ± 0.08
B100	21.58 ± 1.22*	24.54 ± 1.19*	28.01 ± 1.30*	32.16 ± 2.01*	8.49 ± 0.10*	10.55 ± 0.32*
T100	19.05 ± 1.18*	22.55 ± 1.99*	26.01 ± 0.91*	29.03 ± 1.19*	7.59 ± 0.09*	7.83 ± 0.11*
X100	17.51 ± 0.86*	21.05 ± 1.45*	22.75 ± 1.32*	24.03 ± 0.05*	7.16 ± 0.11*	7.69 ± 0.09*
QC control	6.17 ± 0.24	6.33 ± 0.15	7.09 ± 0.21	7.15 ± 0.01	0.78 ± 0.02	0.76 ± 0.03
B100 + QC	18.62 ± 0.26*	15.32 ± 0.10*$	24.47 ± 1.28*	15.42 ± 0.31*$	4.85 ± 0.08*$	4.47 ± 0.13*$
T100 + QC	17.23 ± 0.15*	14.93 ± 0.51*$	24.42 ± 0.20*	15.53 ± 0.30*$	4.14 ± 0.08*$	3.90 ± 0.22*$
X100 + QC	15.73 ± 0.25*	14.77 ± 0.73*$	21.63 ± 0.45*	7.12 ± 0.36	3.95 ± 0.15*$	3.22 ± 0.86*$
Cur control	6.09 ± 0.56	6.31 ± 0.20	6.50 ± 0.67	14.93 ± 0.15*$	0.74 ± 0.01	0.76 ± 0.08
B100 + CUR	15.37 ± 0.21*$	14.23 ± 0.15*$	24.77 ± 0.35*	14.17 ± 0.05*$	3.98 ± 0.13*$	3.22 ± 0.03*$
T100 + CUR	15.23 ± 0.15*$	15.13 ± 0.10*$	23.80 ± 0.35*	14.50 ± 0.10*$	3.23 ± 0.19*$	2.31 ± 0.08*$
X100 + CUR	14.11 ± 0.08*$	15.01 ± 0.20*$	21.73 ± 0.50*	14.67 ± 0.21*$	3.27 ± 0.03*$	2.41 ± 0.05*$
Neutral Comet parameters						
Control	5.32 ± 0.10	5.71 ± 0.1	3.45 ± 0.10	3.60 ± 0.05	0.23 ± 0.01	0.23 ± 0.01
γ-Irradiation (40 Gy)	22.61 ± 0.88*	–	13.25 ± 0.25*	–	2.14 ± 0.11*	–
DMSO	5.67 ± 0.33	5.33 ± 0.41	3.58 ± 0.16	3.72 ± 0.11	0.25 ± 0.04	0.21 ± 0.03
B100	16.42 ± 0.21*	19.62 ± 0.21*	11.37 ± 0.38*	15.47 ± 0.12*	1.24 ± 0.03*	1.45 ± 0.12*
T100	13.93 ± 0.15*	16.51 ± 0.30*	9.60 ± 0.10*	14.23 ± 0.15*	0.93 ± 0.02*	1.18 ± 0.02*
X100	13.63 ± 0.21*	16.60 ± 0.36*	9.32 ± 0.10*	14.03 ± 0.72*	0.87 ± 0.01*	1.09 ± 0.02*
QC control	5.5 ± 0.41	6.22 ± 0.64	3.70 ± 0.10	3.90 ± 0.10	0.24 ± 0.01	0.21 ± 0.01
B100 + QC	11.12 ± 0.31*$	10.11 ± 0.12*$	9.01 ± 0.10*	8.73 ± 0.32*$	0.72 ± 0.01*$	0.59 ± 0.01*$
T100 + QC	11.14 ± 0.21*	10.22 ± 0.35*$	8.58 ± 1.46*	8.32 ± 0.10*$	0.62 ± 0.01*	0.54 ± 0.01*$
X100 + QC	10.93 ± 0.18*	10.13 ± 0.58*$	8.02 ± 1.12*	7.93 ± 0.32*$	0.63 ± 0.01*	0.52 ± 0.02*$
Cur control	5.62 ± 0.51	5.81 ± 0.35	3.67 ± 0.21	3.72 ± 0.10	0.26 ± 0.01	0.22 ± 0.01
B100 + CUR	10.21 ± 0.16*$	9.83 ± 0.13*$	8.03 ± 0.32*	7.93 ± 0.38*$	0.58 ± 0.03*$	0.49 ± 0.02*$
T100 + CUR	10.52 ± 0.12*$	9.92 ± 0.51*$	8.30 ± 0.10*	7.97 ± 0.21*$	0.53 ± 0.02*$	0.45 ± 0.01*$
X100 + CUR	10.12 ± 0.11*$	9.70 ± 0.10*$	7.87 ± 0.21*	7.92 ± 0.30*$	0.54 ± 0.02*$	0.45 ± 0.01*$

EMS = 1.0 mM ethyl methanesulfonate, B100, T100 and X100 = 100.0 mM of benzene, toluene and xylene respectively; QC control = 100.0 μM quercetin control, B100 + QC, T100 + QC and X100 + QC = 100.0 μM quercetin along with 100.0 mM of benzene, toluene and xylene respectively; CUR control = 100.0 μM curcumin, B100 + CUR, T100 + CUR and X100 + CUR = 100.0 μM curcumin along with 100.0 mM of benzene, toluene and xylene respectively. Data presented as mean ± SD of three identical experiments made in triplicates and significance is ascribed as *$P<0.01$ vs. control; $^{\$}P<0.01$ vs. individual chemical (B100, T100 or X100).

Reduced EROD, MROD and GST activity

Larvae fed on QC or CUR along with benzene, toluene or xylene in food have shown significant diminution ($P<0.001$) in the EROD, MROD and GST activities (Figs. 10 and 11) when compared to those exposed to test chemical alone for 48 h. We observed 37%, 32%, 11% or 59%, 44%, 21% reduction in EROD activity in B100 + QC, T100 + QC, X100 + QC or B100 + CUR, T100 + CUR, X100 + CUR, respectively, when compared to that of B100, T100 or X100 exposed organisms. Further, we also observed 38%, 34%, 17% or 45%, 34%, 25% reduction in MROD activity in organisms exposed to B100 + QC, T100 + QC, X100 + QC or B100 + CUR, T100 + CUR, X100 + CUR, respectively, in comparison to organisms exposed to B100, T100 or X100 (Fig. 10A–B). Similarly, we observed 50%, 35%, 39% or 64%, 43%, 64% reduction in GST activity in organisms exposed to B100 + QC, T100 + QC, X100 + QC or B100 + CUR, T100 + CUR, X100 + CUR respectively, when compared to those exposed to B100, T100 or X100 alone (Fig. 11).

Reduced oxidative stress levels

Addition of QC or CUR to the food contaminated with benzene, toluene or xylene resulted in the reduction ($P<0.001$) in ROS generation, SOD, CAT activities and MDA content in the exposed larvae when compared to those in test chemical alone treated larvae after 48 h (Figs. 12 and 13). We observed a 65%, 28% and 30% reduction in ROS generation in B100 + QC, T100 + QC or X100 + QC treated larvae as compared to 100.0 mM of benzene-, toluene- or xylene-exposed organisms respectively (Fig. 12) A similar reduction pattern was evident when organisms were exposed to test chemicals along with CUR (B100 + CUR, T100 + CUR and X100 + CUR) (Fig. 12). We observed 41%, 34%, 33% or 39%, 35%, 33% reduction in SOD activity in B100 + QC, T100 + QC, X100 + QC or B100 + CUR, T100 + CUR, X100 + CUR, respectively, when compared to that of B100, T100 or X100 exposed organisms (Fig. 13A). Further, we also observed 52%, 39%, 36% or 46%, 36%, 41% reduction in CAT activity (Fig. 13B) and 52%, 40%, 35% or 56%, 44%, 45% reduction in MDA content (Fig. 13C) in organisms exposed to B100 + QC, T100 + QC, X100 + QC or B100 + CUR, T100 + CUR, X100 + CUR, respectively, in comparison to organisms exposed to B100, T100 or X100.

Reduced apoptosis and genotoxicity

In organisms exposed to test chemical along with QC or CUR, we observed a significantly lower IETDase activity (47%, 46% and 49% lesser activity in B100 + QC, T100 + QC and X100 + QC treated organisms respectively; $P<0.001$; 61%, 59% and 61% reduced activity in B100 + CUR, T100 + CUR and X100 + CUR exposed organisms respectively after 48 h) (Fig. 14A). In these groups, DEVDase activity was also significantly reduced ($P<0.001$; significantly 48%, 54% and 52% lesser activity in B100 + QC, T100 + QC and X100 + QC treated organisms respectively; $P<0.001$; $P<0.001$; 69%, 68% and 65% reduced activity in B100 + CUR, T100 + CUR and X100 + CUR exposed organisms respectively after 48 h) when compared to those exposed to test chemical alone (Fig. 14B). Similar trend was evident when we measured depolarization of mitochondrial membrane potential: 36%, 40% and 52% reduction in B100 + QC, T100 + QC or X100 + QC exposed organisms, respectively, after 48 h and significantly 45%, 43% and 57% reduction ($P<0.001$) in B100 + CUR, T100 + CUR and X100 + CUR treated organisms, respectively, after 48 h (Fig. 15).

We also observed a significant reduction in DNA damage in organisms exposed to B100 + QC, T100 + QC and X100 + QC and

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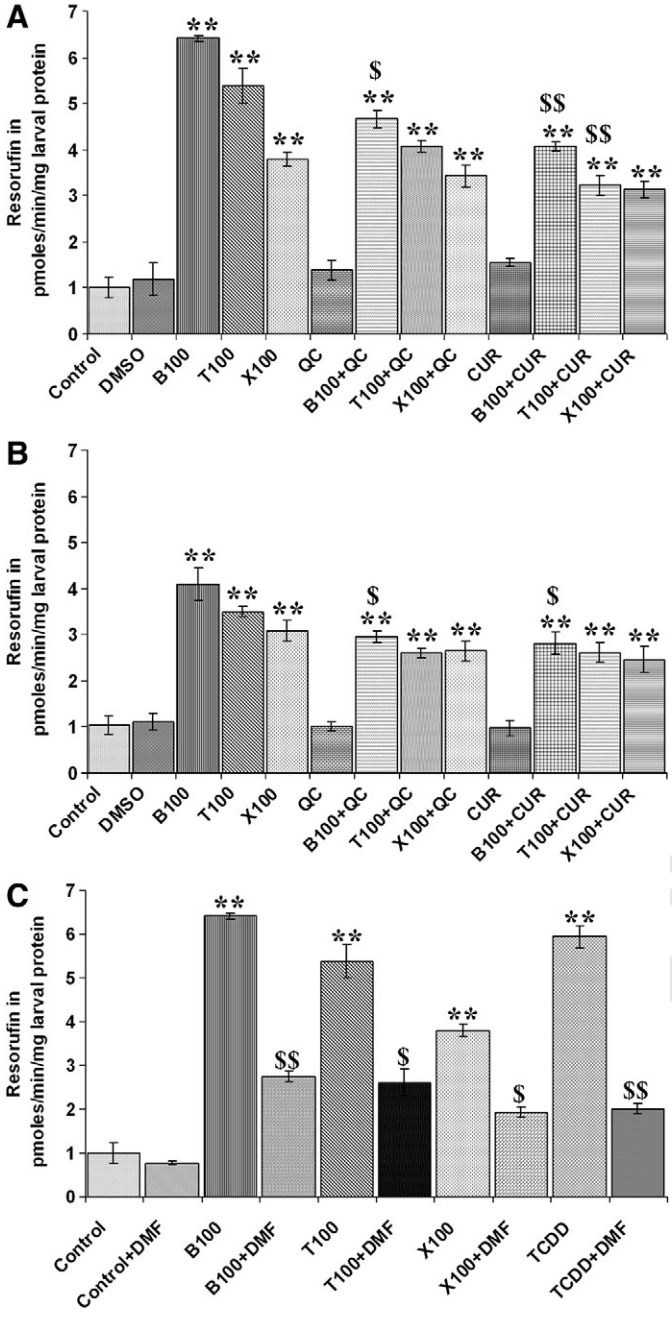

Fig. 10. 7-Ethoxyresorufin-*O*-deethylase (EROD) activity (A) and 7-methoxyresorufin-*O*-de-ethylase (MROD) activity (B) in microsomes isolated from third instar larvae of *D. melanogaster* (Oregon R^+) exposed to benzene, toluene or xylene alone or in combination with quercetin (QC) or curcumin (CUR). B100, T100 and X100 = 100.0 mM benzene, toluene and xylene respectively; B100 + QC, T100 + QC and X100 + QC = 100.0 μM quercetin along with 100.0 mM of benzene, toluene and xylene respectively; B100 + CUR, T100 + CUR and X100 + CUR = 100.0 μM curcumin along with 100.0 mM of benzene, toluene and xylene, respectively, in this figure and the remaining figures in this paper. Panel C depicts the inhibitory effect of 3′,4′-dimethoxyflavone on EROD activity in third instar larvae of *D. melanogaster* exposed to 100.0 mM benzene, toluene and xylene for 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance is ascribed as $^{**}P<0.001$ vs. control; $\$P<0.01$, $\$\$P<0.001$, reduction vs. individual chemical (B100 or T100 or X100).

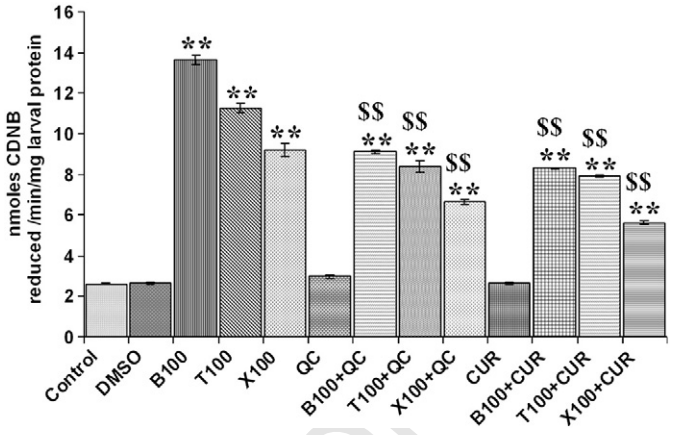

Fig. 11. Glutathione *S*-transferase (GST) activity in larval homogenate from third instar larvae of *D. melanogaster* (Oregon R^+) in control, DMSO and benzene, toluene or xylene alone or in combinations with QC or CUR treatment for 48 h. Data represent mean ± SD of three identical experiments made in three replicates. Data represent mean ± SD of three identical experiments made in triplicates and significance ascribed as $^{**}P<0.001$ vs. control; $\$\$P<0.001$, reduction vs. individual chemical (B100 or T100 or X100).

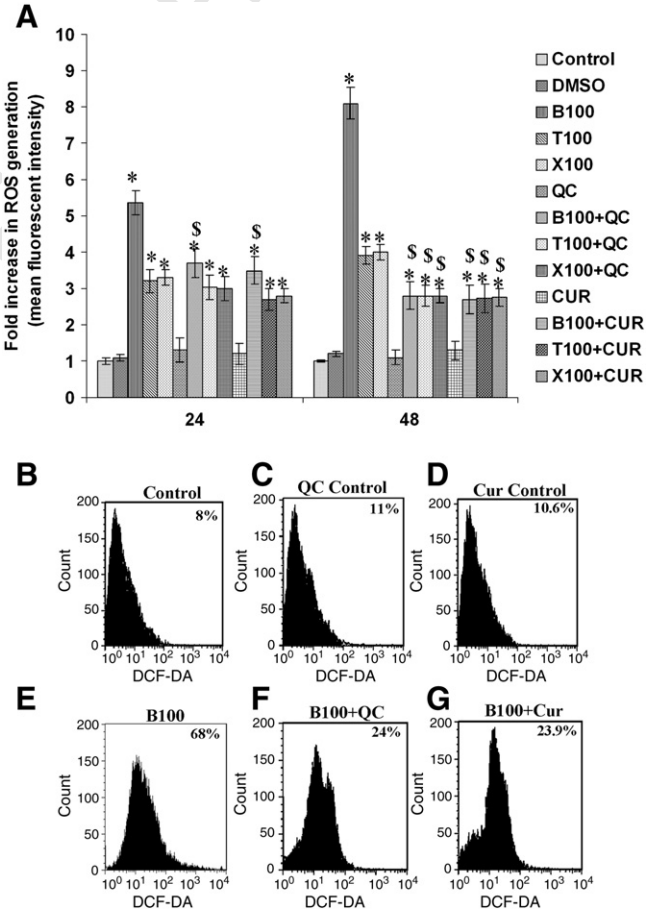

Fig. 12. Levels of reactive oxygen species in cells from midguts of third instar larvae of *D. melanogaster* (Oregon R^+) in control, DMSO and benzene, toluene or xylene alone or in combinations with QC or CUR treatments for 24 and 48 h. Histogram (A) depicts ROS generation in test chemical exposed organisms and flow cytometric panels shows the ROS generation in (B) control, (C) QC control, (D) CUR control, (E) B100, (F) B100 + QC and (G) B100 + CUR exposed organisms after 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance is ascribed as $^{*}P<0.01$ vs. control; $\$P<0.01$, reduction vs. individual chemical (B100 or T100 or X100).

B100 + CUR, T100 + CUR and X100 + CUR. In B100 + QC or B100 + CUR exposed organisms, migration of DNA was significantly less after 24 and 48 h when compared to those in organisms exposed to test chemical alone, in both alkaline and neutral Comet assays (Table 1). We observed a similar trend in T100 + QC, X100 + QC, T100 + CUR or X100 + CUR exposed *Drosophila* larvae when compared to those exposed to toluene or xylene alone (Table 1).

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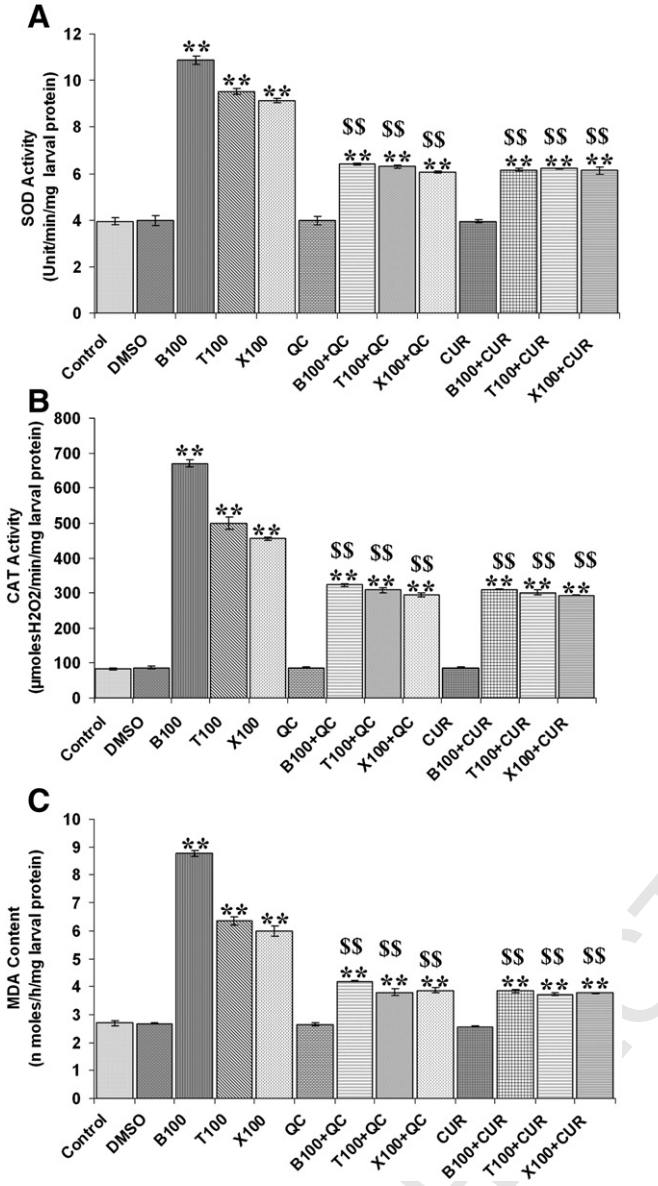

Fig. 13. Cu-Zn SOD (A) and CAT (B) activities and MDA content (C) in the larval homogenate from third instar larvae of *D. melanogaster* (Oregon R^+) in control, DMSO and benzene, toluene or xylene alone or in combinations with QC or CUR treatment for 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance is ascribed as $^{**}P<0.001$ vs. control; $\$\$P<0.001$, reduction vs. individual chemical (B100 or T100 or X100).

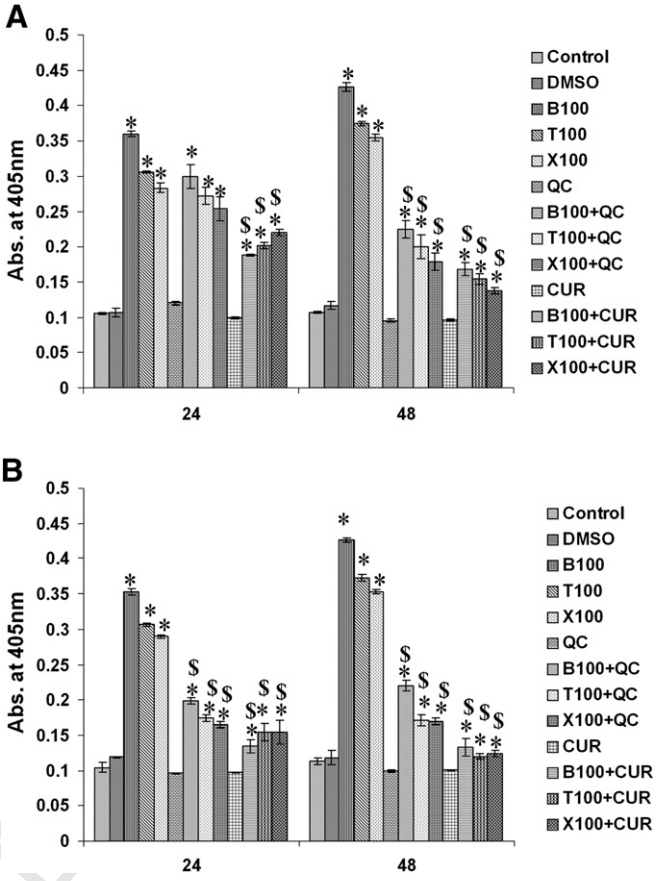

Fig. 14. IETDase (A) and DEVDase (B) activities in microsomes isolated from third instar larvae of *D. melanogaster* (Oregon R^+) in control, DMSO and benzene, toluene or xylene alone or in combinations with QC or CUR treatments for 24 and 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance is ascribed as $^{*}P<0.01$ vs. control; $\$P<0.01$, reduction vs. individual chemical (B100 or T100 or X100).

Discussion

Continuously high levels of exposure of benzene, toluene and xylene to the organisms from occupational/non-occupational sources cause major health hazards. These contaminants are well known to disrupt various cellular processes (Gerin et al., 1998; Chambers et al., 2006). Previous studies showed that benzene, toluene and xylene have potential to induce oxidative stress, an imbalance of the anti-oxidant system, such as SOD, glutathione peroxidase (GP), glutathione (GSH) and MDA content in mammalian cell line or petrochemical workers (Croute et al., 2002; Georgieva et al., 2002). Weaver et al. (2007) and Weaver and Liu (2008) demonstrated benzene-induced apoptosis in epithelial cells of respiratory tracts as evidenced by nicking of DNA, endonucleolytic degradation of genomic DNA as well as increased caspase activity. Similar observations were made *in vivo* using murine models (Smith, 1996; Ross, 2000; Snyder, 2000; Weaver et al., 2007). Cell death is the ultimate fate of the cell with irreparable damage. DNA damage can also occur by chemicals, which if not repaired, can have serious consequences leading to carcinogenesis. Benzene, being classified as class I carcinogen, has been shown previously to cause DNA damage both *in vitro* and *in vivo* (Sul et al., 2005; Weaver et al., 2007; Weaver and Liu, 2008). Singh and Winn (2008) and Faiola et al. (2004) showed significant increase in chromosomal breaks in K-562 cells and hematopoietic stem cells (HSC) exposed to benzene and its metabolites. Similarly, *in vivo* studies using mouse bone marrow cells showed significant increase in sister chromatid exchanges and clastogenicity (Erexson et al., 1986; Zhang et al., 2002). Using alkaline Comet assay, Chen et al. (2008) and Galvan et al. (2008) showed increased Comet parameters in the human lymphocytes and HeLa cells respectively, exposed to benzene and its metabolites. Limited available information suggests toluene and xylene can also induce apoptosis (Nakai et al., 2003; Al-Ghamdi et al., 2004). Despite this vast knowledge on the adverse effects of benzene, toluene and xylene, only a few compounds have been tested for their protective roles against benzene, toluene and xylene induced toxicity. In this study, we first analyzed the apoptotic and genotoxic potential of these three monocyclic hydrocarbons using *D. melanogaster*, an alternate to animal model system. Further, we examined the role of potential candidates/receptors involved in mediating the benzene-, toluene- or xylene-mediated toxicity. Subsequently, we utilized this model to evaluate the protective effects of two well-known phyto-chemicals viz. QC and CUR [quercetin (a well-known antioxidant (Chen and Kang, 2005; Kanupriya et al., 2006) and curcumin (a known

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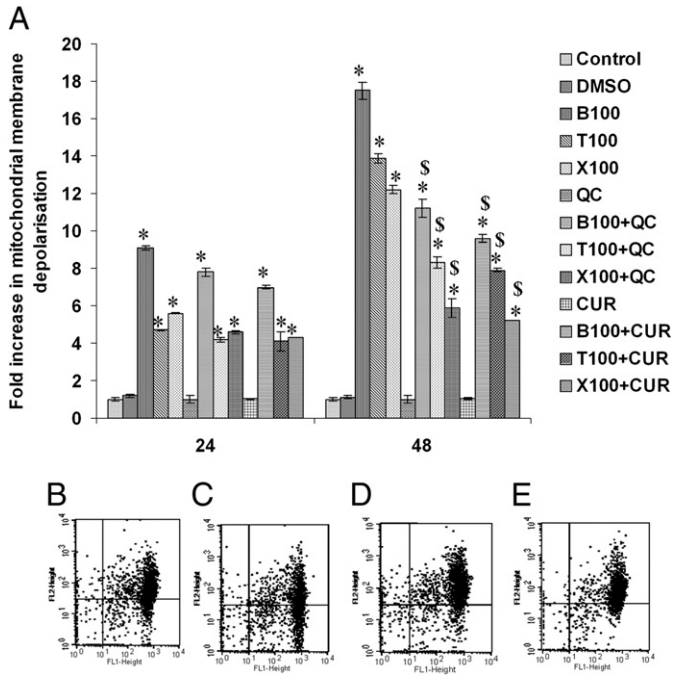

Fig. 15. Alterations in mitochondrial membrane potential ($\Delta\Psi_m$) in cells from midgut tissues of third instar larvae of *D. melanogaster* (Oregon R^+) in control, DMSO and benzene, toluene or xylene alone or in combinations with QC or CUR treatments for 24 and 48 h. Histogram (A) depicts fold increase in mitochondrial membrane depolarization. Flow cytometry panels represent $\Delta\Psi_m$ in (B) control, (C) B100, (D) B100 + QC and (E) B100 + Cur exposed organisms after 48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance is ascribed as $^*P<0.01$. $\$P<0.01$, reduction vs. individual chemical (B100 or T100 or X100).

antioxidant and anti-cancer agent (Becatti et al., 2010; Biswas et al., 2010)] against benzene-, toluene- and xylene-induced toxicity.

Apoptosis is a physiological mode of cellular suicide that plays a vital role during embryogenesis, development, and normal tissue homeostasis (Brill et al., 1999; Vaux and Korsmeyer, 1999; Fulda and Debatin, 2006). The hallmarks of apoptosis include depolarization of the plasma membrane, cell shrinkage, alterations in intracellular ion concentrations, mitochondrial membrane depolarization, chromatin condensation, and DNA fragmentation. We, therefore, examined several candidates involved in the apoptotic pathway in *Drosophila* exposed to benzene, xylene or toluene.

During early apoptosis, a cell loses its membrane asymmetry (Chen et al., 2008). Phosphatidylserine (PS), normally present on the inner cytoplasmic leaflet of the plasma membrane of healthy cells, is translocated and exposed on the outer leaflet (Seigneuret and Devaux, 1984; Connor and Schroit, 1987). We used AV, a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS (Comfurius et al., 1996). The significantly increased ($P<0.001$) AV positive cells in the present study suggested the initiation of apoptosis in benzene-, toluene- or xylene-exposed organisms.

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either at extra-cellular or intra-cellular level (Chang et al., 1998; Budihardjo et al., 1999; Arya et al., 2007). To identify the apoptotic pathway being followed in the cells of the exposed organism, we analyzed mitochondrial membrane potential, DEVDase activity (caspase-3), IETDase activity, and poly ADP ribose polymerase (PARP) cleavage (Chiarugi and Moskowitz, 2002). We observed significant increase in DEVDase and IETDase activities, PARP cleavage and depolarization of mitochondrial membrane in organisms exposed to test chemicals, indicating the induction of mitochondria-mediated caspase-dependent cell death pathway in *Drosophila* larvae exposed to benzene/toluene/xylene. Similarly, a number of earlier studies also documented increased IETDase and DEVDase activities in organisms/cells exposed to organic solvents containing benzene, toluene or xylene [in Jurkat cells by chlorinated biphenol (Inayat-Hussain et al., 2001; Inayat-Hussain and Ross, 2005), HL60 and $CD34^+$ cells by benzene metabolites (Hiraku and Kawanishi, 1996; Moran et al., 1996)]. Caspases are normally rendered inactive by Inhibitor of Apoptosis Proteins (IAPs) and this inhibition is overcome by p53-mediated transcription leading to increased levels of Smac/DIABLO orthologs (Hid, Rpr and Grim in *Drosophila*) (Brenner and Kroemer, 2000; Brodsky et al., 2000; Schuler and Green, 2001; Brodsky et al., 2004; Kornbluth and White, 2005; Wichmann et al., 2006). The increased levels of Hid, Rpr, Grim and TUNEL positive cells observed in benzene-, toluene- and xylene-exposed groups suggest the activation of pro-apoptotic genes in organisms leading to removal of IAP-mediated inhibition of caspases and also activation of apoptotic pathway.

Increased PARP cleavage is usually associated with oxidative DNA damage (Satoh and Lindahl, 1994; Miller et al., 2004; Babich et al., 2009; Deng et al., 2009). Since we also observed increased PARP cleavage in benzene-, toluene- and xylene-exposed organisms, we analyzed DNA damage through Comet assay, which has been adapted for genotoxicity assessment (Miloshev et al., 2002; Rajaguru et al., 2003; Mukhopadhyay et al., 2004; Siddique et al., 2005b; Deguchi et al., 2008). Of the two variants of the Comet assay, the neutral version detects double-strand DNA breaks (Yasuhara et al., 2003) whereas the alkaline version allows to reveal single- and double-strand breaks as well as alkali labile sites (Moller, 2006). In our study, we found significantly increased migration of DNA (TL, % TD and TM) in the order of benzene > toluene ≥ xylene in the exposed organisms in alkaline as well as neutral Comet assays. Interestingly, alkaline Comet parameters showed statistically significant higher levels ($P<0.01$) as compared to neutral Comet parameters in the test chemical exposed organisms. These results suggest benzene, toluene and xylene are potentially genotoxic causing double-strand breaks to the exposed larvae. These observations parallel the earlier studies on human population (epidemiological data) and experimental models (both *in vivo* and *in vitro*) exposed to benzene, toluene or xylene (Carere et al., 1995a; Carere et al., 1995b; Chen et al., 2008; Pandey et al., 2009). Interestingly, the observed genotoxicity (benzene > toluene > xylene) is inversely related to lipophilicity (benzene < toluene < xylene; Snyder et al., 1993; ATSDR, 1997) and this is consistent with our previous observations in mixture toxicity studies that the higher the lipophilicity, the lower is the toxicity (Singh et al., 2010).

Reactive oxygen species (ROS) are generated in biological systems either by normal metabolic pathways or as a consequence of exposure to chemical (Iqball et al., 2002, Sun et al., 1990). Recent studies from our laboratory showed induction of heat shock genes (*hsp70*, *hsp83*, *hsp60* and *hsp26*), oxidative stress markers and increased ROS generation in *D. melanogaster* exposed to benzene, toluene or xylene, individually, or their mixtures indicating the potential of these chemicals to produce cellular stress (Singh et al., 2009; Singh et al., 2010). In addition, we observed significant induction of cytochrome P450 enzymes in benzene-, toluene-, or xylene-exposed organisms when compared to controls. Induction of cytochrome P450 enzymes in response to benzene, toluene or xylene exposure is well documented (Day et al., 1992; Nakajima and Wang, 1994; Seaton et al., 1994). Further, AhR has been shown to be the key component in the metabolic response to aromatic hydrocarbons, including benzene (Schmidt and Bradfield, 1996; Yoon et al., 2002; Nishiumi et al., 2007). AhR, a cytoplasmic bHLH-PAS transcription factor, upon binding to an aromatic hydrocarbon (TCDD for example), translocates to the nucleus. Within the nucleus, forms a complex with the Aryl hydrocarbon receptor nuclear translocator (ARNT), another bHLH-PAS protein (Hoffman et al., 1991) and together bind to xenobiotic response element to control the expression of specific target genes including certain cytochrome P450s (Swanson et al., 1995). Based on this, we hypothesized a similar role for *spineless* (*Drosophila* homolog of Aryl hydrocarbon receptor Cespedes et al., 2010) in the benzene-,

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xylene- and toluene-mediated toxicity in *Drosophila*. To test if the observed induction of cytochrome P450s is mediated by AhR pathway, we exposed *Drosophila* larvae to test chemical together with the blocker for AhR (DMF, Li, 2007) and measured the activity of cytochrome P450s. The observed reduction of cytochrome P450s (in particular EROD) in exposed organisms in the presence of blocker, clearly indicated that like in mammals, toxicity of benzene, in part, is mediated by AhR in Drosophila. In addition, our study provides an evidence for the involvement of AhR also in toluene- and xylene-mediated toxicity.

The other objective of our study was to look for the agents, which can attenuate the toxicity conferred by benzene, toluene or xylene. As cytochrome P450 enzymes, AhR and oxidative stress underlie the observed benzene-, toluene- or xylene-mediated toxicity in the present study, we looked for nutraceuticals that are known to act on the above. We examined the efficiency of two phytochemicals, namely, QC and CUR for their protective roles against benzene-, toluene- or xylene-induced toxicity. QC is a polyphenolic flavonoid and has protective effects on different adverse cellular events like carcinogenesis through unknown mechanism of action yet reducing LPO and ROS generation or having anti-proliferative activity in cells/organisms (Dihal et al., 2006; Dihal et al., 2008; Larson et al., 2010) indicating its anti-oxidant and anti-cancer properties. Similarly, CUR (diferuloyl methane) has been shown to possess anti-oxidative and free radical scavenging properties and anti-neoplastic properties (Reddy and Lokesh, 1994; Motterlini et al., 2000; Messner et al., 2009). In addition, both CUR and QC were found to degrade both AhR and ARNT to inhibit cytochrome P450 enzymes. Based on these reports (on anti-oxidative, free radical scavenging, inhibiting properties, anti-cancer properties of QC and CUR), we hypothesized that addition of QC or CUR in the exposure regimen will reduce the toxic effects of the test chemicals to the exposed organisms. To test this, we analyzed their efficacy in modulating the cytochrome P450 and GST activities, ROS generation, apoptosis and genotoxicity in the test chemical exposed organism. We observed a significantly reduced cytochrome P450 activities, oxidative stress, GST activity, ROS generation, lower intensity of apoptosis and DNA damage in organisms exposed to QC or CUR along with test chemicals in comparison to those observed in test chemical alone group. Presence of chemicals in organisms exposed to benzene, toluene or xylene alone at levels similar to those in combination with QC or CUR suggested that this reduction in assayed parameters is not due to reduced intake of test chemical by the organisms in the presence of QC or CUR but indeed due to the protective roles of QC or CUR. These results demonstrated the potential of CUR and QC to attenuate the benzene-, toluene- or xylene-mediated toxicity. Intriguingly, a previous study from our group had shown that the co-treatment of QC with dichlorvos (DV) enhanced the oxidative stress and apoptosis compared to that of DV alone (Gupta et al., 2007). This discrepancy may be attributed to the test chemical used and QC may confer protection in a compound specific manner. To confirm, if this is the case, we analyzed the ROS levels in organisms exposed to DV, DV + QC, benzene, benzene + QC or QC alone. Interestingly, we observed that ROS levels are increased in organisms exposed to DV + QC [as in agreement with Gupta et al., 2007] and decreased in benzene + QC (as in the present study), when compared to their respective chemical controls (please see Supplemental Fig. S6). These results indicate that QC may play protective roles in a compound specific manner.

Several molecular mechanisms by which CUR and QC may play protective roles are envisaged. Given their documented roles in modulating cytochrome P450 activities (Firozi et al., 1996; Thapliyal and Maru, 2001) and their anti-oxidant properties (Chan et al., 2005; Messner et al., 2009) they may act either at the level of metabolism or alternatively they may act on the anti-oxidant defence system. During Phase I xenobiotic metabolism, CUR and QC may either 1) inhibit cytochrome P450 enzymes directly by acting as a competitive substrate (Appiah-Opong et al., 2007), 2) interfere with AhR mediated induction of cytochrome P450 enzymes (Rinaldi et al., 2002) or 3) act at the level of GSTs, which play an important role in the phase II xenobiotic metabolism (Oetari et al., 1996). The reduction in cytochrome P450 activities in organisms exposed to test chemical along with QC or CUR and the observed involvement of in the present study point to the second possibility. In addition, we observed significant ($P<0.05$) positive correlation (please see Supplementary Table S1 for details) between activity of cytochrome P450s and GSTs, ROS and other oxidative stress parameters analyzed. Based on these, we believe that the reduced levels of GSTs, ROS and other oxidative stress parameters might be a consequence of reduced activity of cytochrome P450 in response to CUR or QC in exposed organisms. These ultimately might have resulted in reduced apoptosis and genotoxicity in organisms exposed to test chemical along with CUR or QC.

Taken together, the present study suggests that MAHs particularly benzene, toluene and xylene can cause genotoxicity and apoptosis in the exposed Drosophila larvae *in vivo*, through mitochondria mediated caspase dependent pathway of cell death. The study also showed that AhR homologue plays a key role in the induction of cytochrome P450 in response to benzene, toluene or xylene in *Drosophila*. Among the three tested chemicals, benzene is highly genotoxic (possibly due to higher uptake) while toluene and xylene are less genotoxic than benzene. The study further suggests that quercetin and curcumin inhibit cytochrome P450, probably influencing AhR mediated pathway, which eventually may result in the reduction of the genotoxicity and apoptosis in test chemicals exposed *D. melanogaster*.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2011.03.006.

Conflicts of interest

None.

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