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ORIGINAL RESEARCH ARTICLE

Biomolecular alterations by chronic sub-lethal exposure of Malathion and Paraquat in *Drosophila melanogaster*: study on pesticide tolerance in insects

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Abstract

Pesticides exert a wide variety of functions for controlling pests. At low doses the pests are not much affected and parallel to that a chance of tolerance has been expected. In this study a population of fruit flies *Drosophila melanogaster* was grown under various doses of insecticide Malathion and herbicide Paraquat. Lower than the sub-lethal doses (0.2 micromolar), various biomolecular alterations in terms of protein oxidation (Advanced Oxidation Protein Products, AOPP), lipid peroxidation (Malondialdehyde, MDA), sialic acids, total thiols, Cupric Ion Reducing Antioxidant Capacity (CUPRAC) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radical scavenging activity have been observed. Positive alteration in AOPP for both Malathion and Paraquat treated fruit flies as well as increased MDA content in Paraquat treated flies are the alterations in the fruit fly population because of oxidative stress. The unaffected MDA, sialic acid content and CUPRAC antioxidant capacity for Malathion treated flies demonstrates the diminished effects of oxidative stress exerted through Malathion. There is increase sialic acid and CUPRAC antioxidant capacity after treatment of Paraquat. There is also an increase in total thiols and ABTS cation radical scavenging activity after treatment of both Malathion and Paraquat. These results evidently show that improved antioxidant mechanisms effectively alleviate the oxidative stress exerted by pesticides. This study is clearly reflecting that oxidative stress generated by the sub lethal doses of pesticides can be diminished and certain tolerance level is also achieved by the insects in terms of increased antioxidant defence and longevity.

Keywords Pesticide tolerance · Protein oxidation · Lipid peroxidation · Total thiols · Antioxidant activity

Highlights
- Tolerance against pesticides is observed when fruit flies are exposed to lower doses of Malathion and Paraquat
- Antioxidant defence system is observed to respond in affirmative manner against the exposure
- Few biomolecules are not affected by the oxidative stress produced
- Comparative longevity is increased in pesticide exposed fruit flies
- Total thiols are positively corroborated against the exposure

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Introduction

Pesticides, that are used to eliminate insects, fungus, unwanted herbs and the other pests, play important role in avoiding diseases and preventing the damage of property (Kumar and Kumar 2019; Yadav and Devi 2017). These compounds, which may be containing an active and inert ingredient, can be broadly divided based on their target organisms like insecticides (for insects), herbicides (for weeds), rodenticides (for rodents) and fungicides (for fungi and molds) (Randall et al. 2008; Sifakis et al. 2011). Pesticides on the basis of action, can be grouped as ruining, repelling and extenuating agents (Speck-Planche et al. 2012). Depending upon the population level, their properties rely upon different factors such as existence, features and time of implementation (Schmolke et al. 2010).

Pesticide tolerance is considered as the natural ability to withstand against action of pesticide and unlike resistance it may not necessarily be the result of genetic changes caused by the insecticide selection pressure (Wu et al. 2017; Zhu 2008).

Resistance becomes an important consideration challenging the pesticide efficacy while maintaining lower concentrations (Barres et al. 2016). An insect population readily developing resistance may continue to develop it for new pesticide applied (Dang et al. 2017; Naqqash et al. 2016). As the pesticide use increases, the ability to rapidly respond to pesticides by increasing tolerance and resistance has important implications for the persistence of non-target organisms (Hua et al. 2014).

Pesticides are sometimes considered only for the economic aspects especially in a country like India, where approximately one third of the crops are damaged by the pests attack (Das 2013). Along with causing toxicity in target species, pesticides, because of their polarity, solubility and heat stability can damage natural flora and fauna including the people associated with industries and public health (Rashid et al. 2010). This may also include the serious negative effects on biodiversity (Agrawal et al. 2010) as well as on avian, terrestrial and aquatic life; eventually leading a long term impact on environment (Mahmood et al. 2016).

The insecticide Malathion (Diethyl 2-[(dimethoxyphosphorothioyl) sulfanyl] butanedioate) belongs to a group of organophosphorus compounds which are acetylcholinesterase inhibitor (Costa et al. 2011). It affects the nerve receptors and damage the nervous system of insects (Broughton 1990; Keifer and Firestone 2007; Mehdi and Qamar 2011). A quaternary nitrogen herbicide Paraquat (*N*, *N*′-dimethyl-4, 4′-bipyridinium dichloride) is non-selective in killing green plants tissue by interfering within photosynthetic electron transfer chain and inhibit the reduction of the oxidized nicotinamide adenine dinucleotide phosphate (Cochemé and Murphy 2008). These pesticides are also considered detrimental for higher organisms like potential mutagen of germ cells (Giri et al. 2002) neurotoxicity (Uversky 2004) and can affect the brain regions of animals (Jebali et al. 2006). Malathion and paraquat can also cause the intracellular oxidative stress by the production of reactive oxygen species (Jadavji et al. 2019; Shieh et al. 2019).

Considered as a model organism for more than a century, and been proven as very efficient model even in the neurodegenerative disorders (Auluck et al. 2002; Feany and Bender 2000), *Drosophila melanogaster* is easily maintained and manipulated because of easy handling and short life span (Ong et al. 2015). The present study is conducted to observe the oxidative alterations of biomolecules and antioxidant defence system in *Drosophila melanogaster* after the Malathion and Paraquat low dose sub-lethal chronic exposure, which was achieved by the dose optimization required for their toxicity.

Materials and methods

Drosophila culture

For the present study Oregon-R strain of *Drosophila melanogaster* was used. Drosophila Breeding Laboratory, Department of Biotechnology, Punjab Agricultural University, Ludhiana, Punjab have provided the initial strain of *Drosophila melanogaster*. Maize powder (17%), agar (1.5%), yeast (6%), sucrose (15%), methyl benzoate (1%) and propionic acid (1%) were used for the media preparation. The culture was kept in incubator at a temperature of 25 °C.

Malathion and Paraquat exposure to fruit flies after dose optimization

For Malathion and Paraquat exposure, the adult fruit flies were transferred from the culture medium into different test tubes which were separately containing strips of Whatman filter paper soaked with sucrose solution, Malathion and Paraquat at different concentrations (from 10 μM to 0.2 μM) as mentioned in Table 1 (for Malathion) and Table 2 (for Paraquat). Malathion and Paraquat solutions were prepared in sucrose solution (10% *w*/*v*). The sucrose treated flies were considered for control. The changes in concentration were performed from higher to lower for both Malathion and Paraquat according to the percentage of mortality observed within 24 h of initial exposure. The exposure for same concentration of pesticides was carried to survived fruit flies. In case there were no survived flies, the experiment was restarted with fresh fruit flies at lower concentrations. The flies that survived were employed in further experimentation.

Homogenisation of Drosophila

The flies were primarily kept in refrigerator at 0 °C to make them unconscious for homogenization which was performed in mortar-pestle by diluting with Phosphate buffer saline (PBS, 10 mM). The homogenates were further stored at 4 °C. The homogenate obtained from different groups of fruit flies was further used for various estimations.

Protein estimation

Protein content in all the samples were estimated by the method described by Lowry et al. (1951). Briefly, the homogenate of different group of fruit flies diluted to 1.00 mL by distilled water was added with 5.00 mL of the freshly prepared Lowry's reagent [solution A (2% sodium potassium tartrate),

Table 1 Dose Optimization for Malathion

Concentration (μM)	Average Exposure Time (h)	% Mortality (Within 24 h)	% Mortality (After 24 h)
10.0	24	100	100
5.0	24	100	100
1.0	24	91.6	100
0.20	60	0	0

Table 2 Dose Optimization for Paraquat

Concentration (μM)	Average Exposure Time (h)	% Mortality (Within 24 h)	% Mortality (After 24 h)
10.0	24	100	100
5.0	24	100	100
1.0	24	87.5	100
0.20	60	0	0

solution B (1% copper sulphate) and solution C (2% sodium carbonate dissolved in 0.1 N NaOH) mixed in a ratio 1:1:98]. After proper mixing it was allowed to stand for 10 min at room temperature. After incubation, Folin-Ciocalteu reagent (0.50 mL, diluted 1:1) was added followed by shaking and further allowed to stand for 30 min to develop the colour. The absorbance was measured at 690 nm against a reagent blank. Bovine serum albumin was taken as the standard protein.

Advanced oxidation protein products detection

The determination of Advanced Oxidation Protein Products was based on spectrophotometric detection followed by Witko-Sarsat et al. (1996). The diluted homogenate [50 μL taken separately from each group of fruit flies and mixed with 950 μL of PBS (10 mM)] was added to 10 μL of potassium iodide (1.16 M) and 20 μL of glacial acetic acid. The absorbance was measured at 340 nm. Chloramine-T solution was used for standard calibration.

Sialic acids determination

The determination of sialic acid level in Drosophila homogenate was performed as followed by Spyridaki and Siskos (1996). The diluted homogenate [50 μL taken separately from each group of fruit flies and mixed with 950 μL of PBS (10 mM)] was added to 100 μL of periodic acid (0.04 M). It was mixed thoroughly and left for 30 min in ice bath. After that 1.25 ml of resorcinol working solution [5 ml of resorcinol (6.0%), 0.125 ml of copper sulphate (0.1 M), 25 ml of hydrochloric acid (10 M) and 19.875 ml of distilled water to make final volume 50 mL) was added. It was again mixed thoroughly and kept in boiling water bath for 5 min. The tubes were cooled in ice bath for 2 min. After addition of 3.25 ml n-butanol, the solutions were mixed thoroughly and tubes were placed in water bath for 3 min at 37 °C. The absorbance of supernatant was measured at 625 nm after centrifugation. Sialic acid was used as standard calibration.

Malondialdehyde (MDA) detection

The MDA contents were estimated by the method as followed by Esterbauer and Cheeseman (1990). The diluted homogenate [50 μL taken separately from each group of fruit flies and mixed with 950 μL of PBS (10 mM)] was added to 1.00 mL of Trichloroacetic acid (10% TCA). It was shaken well and incubated at 37 °C. The supernatant was collected after the centrifuge for 5 min. The boiling was done for 30 min after the addition of 1.00 mL thiobarbituric acid (TBA, 0.67%). The absorbance was measured at 532 nm. MDA content was calculated as thiobarbeturic acid reactive substances (TBARS) using extinction coefficient ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

Total Thiols assay

The total thiol or Glutathione assay was done according to the method described by Pandey et al. (2010). The diluted homogenate [50 μL taken separately from each group of fruit flies and mixed with 950 μL of PBS (10 mM)] was added to test tubes containing 500 μL sodium dodecyl (10%) and 2.50 mL of EDTA phosphate buffer (0.01 M, pH 8.2). It was kept for 10 min and centrifuged for 5 min. After that 2.50 mL supernatant was taken and 100 μL freshly prepared 5,5′-dithiobis-(2-nitrobenzoic acid) solution (10 mM) was added and incubated for 15 min. The absorbance was measured at 412 nm. Glutathione was taken for standard calibration.

Cupric ion reducing antioxidant capacity (CUPRAC) antioxidant activity assay

CUPRAC Antioxidant Activity Assay was done according to Apak et al. (2005). The diluted homogenate [50 μL taken separately from each group of fruit flies and mixed with 950 μL of PBS (10 mM)] was added to test tubes containing 500 μL copper chloride solution (10 mM), 500 μL Neocuproine solution (7.5 mM) and 500 μL ammonium acetate buffer (1.0 M, pH 7.0). The absorbance was measured at 450 nm. CUPRAC Antioxidant Activity was represented as microgram of ascorbic acid equivalent per mg protein.

ABTS Cation radical scavenging activity

ABTS Cation Radical Scavenging Activity (ABTS-RSA) was performed according to Miller and Mitzel (1995). The 5.0 mL of ABTS aqueous solution (7 mM) was prepared and 88 μL potassium persulfate (140 mM) was added to it. This was kept in dark for 12 h to activate ABTS. This solution was diluted to obtain 0.80 unit of absorbance for working ABTS solution. For the measurement of ABTS-RSA, 1.90 mL of working ABTS solution was added to 100 μL diluted homogenate [50 μL taken separately from each group of fruit flies and mixed with 50 μL of PBS (10 mM)]. It was mixed and kept for 5 min. The absorbance was measured at 734 nm after centrifugation. ABTS-RSA was represented as microgram of ascorbic acid equivalent per mg protein.

Statistical analysis

Statistical Analysis was done by the software Graph Pad Prism 5 version 5.01. To assess relationships between parameters, one way analysis of variance (ANOVA) was done with in Bonferroni's Multiple Comparison test as test of significance by considering $p < 0.05$ as significant.

Results and discussion

Pesticides have been used as oxidative stress inducer in model systems for cell and organismal aging or associated neurodegenerative diseases; exerting various physiological and biochemical alterations. The in vivo effects of both the pesticides with low concentrations (around 0.2 μM) was considered after application of a wide range of concentrations (10 μM to 0.2 μM) in *Drosophila melanogaster* as shown in Table 1 (for Malathion) and Table 2 (for Paraquat). The concentrations of pesticides were decreased unless no mortality was observed after 24 h of initial exposure. Malathion and Paraquat have been supplemented after mixing with sucrose solution; for control fruit flies, the doses have been pre-standardized with sucrose as the only dietary supplement. The initial observations regarding the decrease in mortality were prominent and under the low concentration range the fruit flies had shown good survival rate.

The deteriorative or pro-oxidative effects of ROS production have been observed against chronic sub-lethal Malathion and Paraquat concentrations in terms of altered AOPP, sialic acid and MDA (considered as protein, carbohydrate and lipid biomarkers of oxidative stress respectively) as well as total thiol, antioxidant and radical scavenging activity. It is also observed that the chronic non-toxic exposures of Malathion and Paraquat alters few of the oxidative stress parameters in the fruit flies in such a way that may be considered as developing the tolerance against the pesticides. AOPP levels were significantly increased in Malathion treated flies as well as Paraquat treated flies as compared to sucrose treated (control) flies as shown in Fig. 1. The elevated AOPP level can be best explained as effect of oxidative stress generated after the exposure of Malathion and Paraquat. This observation is analogous to previous studies performed for higher organisms (Patil and David 2013; Possamai et al. 2007).

For the carbohydrate content (sialic acid) of Malathion treated flies no significant difference was observed between Malathion treated flies and sucrose treated (control) flies. The sialic acid content of Paraquat treated flies is significantly higher than the sucrose treated (control) flies as shown in Fig. 2. In Drosophila and other insects the N-acetylneuraminic acid phosphate synthase gene is having a capability to produce sialic acids (Kim et al. 2002). It is a diverse family of sucrose units with a nine-carbon backbone

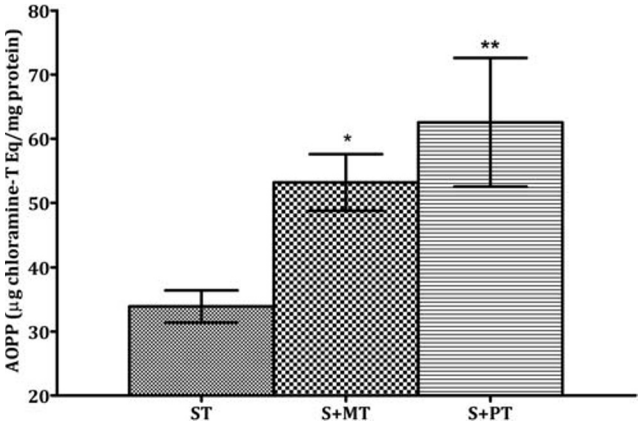

Fig. 1 Advanced Oxidation Protein Products (AOPP) in Drosophila. ST: Sucrose Treated, S + MT: Sucrose + Malathion Treated, S + PT: Sucrose + Paraquat Treated. Values are expressed in terms of mean ± SD. *p < 0.05, **$p < 0.01$ compared to ST.

and it can mediate a wide variety of physiological and pathological processes; extenuating the elevated levels as a protective mechanism. In earlier studies, Drosophila supplemented with diet containing lipofuscin, advanced glycation end products (Heinrichsen et al. 2014) or carbohydrates like D-galactose have shown reduced life span and increased oxidative stress (Cui et al. 2004).

Malondialdehyde (MDA) is a biomarker of lipid peroxidation. For the MDA (TBARS) content, the difference was not significant between Malathion treated flies and sucrose treated (control) flies but the level of MDA was increased in Paraquat treated flies as compared to sucrose treated (control) flies as shown in Fig. 3. The effects of oxidative stress can be explained bringing increase in MDA levels as lipid peroxidation product in Paraquat exposed fruit flies. The unaffected MDA in Malathion exposed fruit flies can be further explained as an elevated antioxidant response in fruit flies against the lower doses of Malathion. It is contrary to the response observed for the higher organisms like freshwater fish in which an increase is detected for lipid peroxidation against sub-lethal exposure of Malathion (Chitra and Abdu 2013).

The unaffected MDA levels observed in our studies can be further compared with the study conducted in greater wax moth (*Galleria mellonella*) and its parasite *Pimpla turionella*. The unaffected MDA levels along with increase in the antioxidant enzyme superoxide dismutase (SOD) activity in the parasite has been observed with the lower concentration of Malathion treatment. At higher concentrations the MDA level is increasing in both host and parasite (Büyükgüzel 2006). Another study on Malathion in the greater wax moth (*Galleria mellonella*) displayed unaffected MDA content at very low concentrations (0.10 ppm), although higher concentrations (1.0 ppm) have shown increased MDA and superoxide dismutase (SOD) activity (Büyükgüzel 2009). The larvae exposed to another organophosphate insecticide methyl parathion have also displayed increased MDA content even at

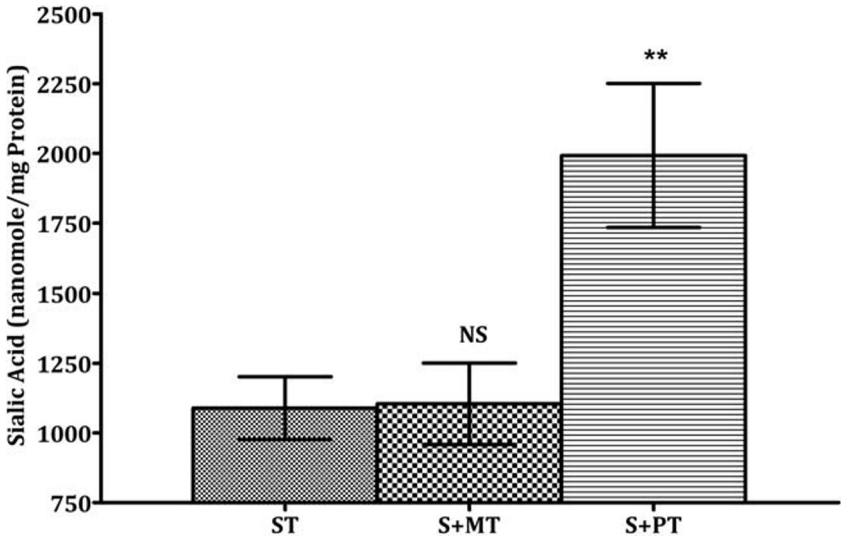

Fig. 2 Sialic acid in Drosophila extract. ST: Sucrose Treated, S + MT: Sucrose + Malathion Treated, S + PT: Sucrose + Paraquat Treated. Values are expressed in terms of mean ± SD. $^{**}p < 0.01$ compared to ST.

lower concentrations (İçen et al. 2005). Studies on compound like minocycline, which have shown decreased MDA formation along with increased life span and motor activity in fruit flies, may suggest the reduced lipid peroxidation as a protective mechanism against reactive oxygen species (ROS) (Bonilla et al. 2012).

There is significant increase in the level of total thiols in exposed Drosophila shown in our results. In Malathion treated flies there is increase in total thiols as compared to sucrose treated (control) flies. In Paraquat treated flies there is more significant and prominent increase in total thiols as compared to sucrose treated (control) flies as shown in Fig. 4. Contrary to previous studies where glutathione (GSH) was decreasing (Büyükgüzel 2009), the elevated total thiol levels can be best explained as a protective mechanism in our study. This alteration can be further supported by the study performed for glutathione supplementation which showed increased survival rate of Malathion treated fruit flies (Bonilla et al. 2006). Studies also show that manganese superoxide dismutase (MnSOD) demonstrate an important role in defence against oxidative stress caused by Malathion (Wu et al. 2017). In earlier studies, at lower doses Paraquat was causing an enhanced levels of antioxidant enzyme peroxidase and superoxide dismutase (SOD) activity (Krůček et al. 2015).

For CUPRAC antioxidant capacity, there is no significant difference observed in the Malathion treated and sucrose treated (control) flies. There is significant increase in Paraquat treated flies as compared to the sucrose treated flies as shown in Fig. 5. The scavenging effect on ABTS cation radical was increased in Malathion treated flies as well as in Paraquat treated flies as compared to the sucrose treated (control) flies as shown in Fig. 6. Our observations can be further supported by the study on the effect of another herbicide roundup on fruit fly where rapid activation of antioxidant system along with unaltered lipid

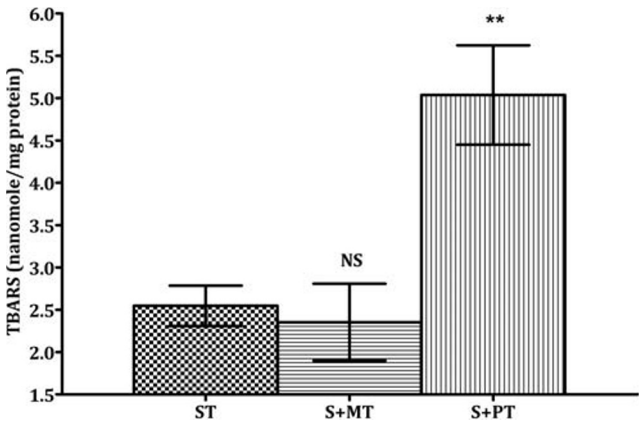

Fig. 3 Malondialdehyde (MDA) in Drosophila extract. ST: Sucrose Treated, S + MT: Sucrose + Malathion Treated, S + PT: Sucrose + Paraquat Treated. Values are expressed in terms of mean ± SD. **p < 0.01 compared to ST.

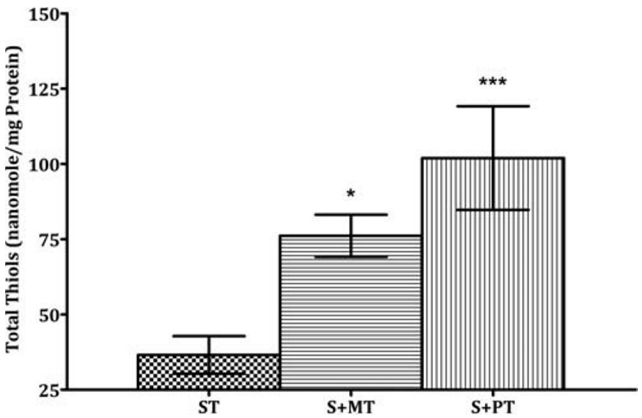

Fig. 4 Total thiols in Drosophila extract. ST: Sucrose Treated, S + MT: Sucrose + Malathion Treated, S + PT: Sucrose + Paraquat Treated. Values are expressed in terms of mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to ST.

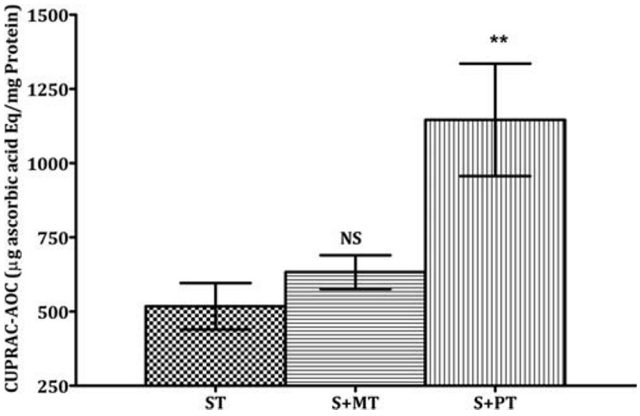

Fig. 5 CUPRAC levels in three different sets of fruit flies. ST: Sucrose Treated, S + MT: Sucrose + Malathion Treated, S + PT: Sucrose + Paraquat Treated. Values are expressed in terms of mean ± SD. **p < 0.01 compared to ST.

peroxidation suggest the resistance for the damage caused by ROS (de Aguiar et al. 2016). Elevated antioxidant response can be considered as a state of tolerance developed by physiological system of the insect. The antioxidant supplementation of γ-orizanol (Araujo et al. 2015) and *Valeriana officinalis* (Sudati et al. 2013) have shown to diminish the toxicity induced by insecticide rotenone in fruit flies.

The response in terms of antioxidant defence and enzymatic alterations against different types of pesticides can be somehow correlated with the individual concentrations which may be toxic or adaptable by the insects at its different physiological and developmental stages. The study on larval amphibians demonstrate that induced tolerance (induced by an early exposure to low concentrations of a pesticide) also provides induced cross-tolerance which is not restricted to pesticides with the same mode of action (Hua et al. 2014).

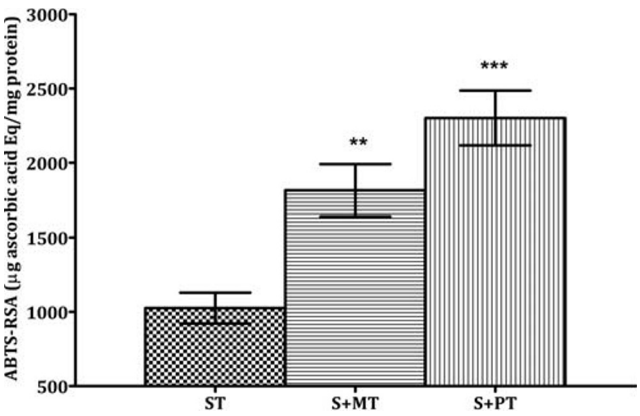

Fig. 6 ABTS cation radical scavenging activity of Drosophila extract. ST: Sucrose Treated, S + MT: Sucrose + Malathion Treated, S + PT: Sucrose + Paraquat Treated. Values are expressed in terms of mean ± SD. **p < 0.01, ***p < 0.001 compared to ST.

Conclusion

The present study is an attempt to correlate the effects of chronic exposure of Malathion and Paraquat in terms of oxidative stress exerted on *Drosophila melanogaster* considering various alterations in biomolecules and antioxidant defence system. The selected doses of pesticides which were given to adult flies, reared under controlled conditions in the present study, have evidently shown tolerance developed by the fruit flies countering the stress exerted by the pesticides at sublethal doses. Although, these pesticides have higher toxicity and some of the oxidative alterations like protein oxidation and lipid peroxidation are affirmative, majority of the results are promising in terms of an increase in antioxidant defence system for diminishing the effect of oxidative stress. This study can be considered for tolerance shown by the fruit flies in terms of countering oxidative stress through increased antioxidant defence and with the decreased mortality.

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Compliance with ethical standards

Conflict of interest None.

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