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Assessment of Toxicological Effect of Organophosphate, Malathion, on Matrix Metalloproteinase-1 and Matrix Metalloproteinase-2 in 3rd Instar Larvae of *Drosophila melanogaster*

***[1]BANANI BINDHANI; [1]MAITY, S; [1]MAITY, S; [1]CHAKRABARTI, I; [2]SAHA, SK**

[1]Department of Zoology, Dinabandhu Andrews College (affiliated to University of Calcutta), Garia, Kolkata-700084, West Bengal, India
[2]Department of Zoology, West Bengal State University, Barasat, Kolkata- 7000126, West Bengal, India

**Corresponding Author Email: bindhanibanani@gmail.com*
Co-Authors Email: sulagna.maity@gmail.com; snehamaity98@gmail.com; ipsitchakrabarti2011@gmail.com; samir0804@gmail.com

ABSTRACT: Matrix metalloproteinase (MMP) is a type of protease that degrades and removes the extracellular matrix of the cell. Only matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-2 (MMP-2) are found in *Drosophila*, playing different roles in the fruit fly. Malathion is a lethal insecticide which mostly affects the neurological system of *Drosophila*. Hence, the objective of the study was to evaluate the toxicological effect of the organophosphate – Malathion – on MMP-1 and MMP-2 in 3rd instar larvae of *Drosophila melanogaster* using appropriate techniques. A total of 80 third instar larvae of *Drosophila melanogaster* were taken for our experiment (40 larvae for MMP-1 and 40 larvae for MMP-2). For each assessment with 40 larvae, 20 larvae were treated with LC_{50} dose of Malathion and 20 larvae were taken as control sample. Casein and gelatin zymography were run at room temperature to detect MMP-1 and MMP-2 expression in both control and treated larvae sample. Expressions of both matrix metalloproteinases in treated larvae changed substantially. The level of MMP-1 increased from 35.38% to 64.62%. MMP-2 level also rose from 38.37% to 61.63%. Our result implies that an LC_{50} dose of Malathion may activate oxidative stress and upregulate MMP-1 and MMP-2 levels in 3rd instar larvae of *Drosophila*. The deleterious outcome of Malathion could be seen in other non-target organisms, including pollinators and humans. Hence, the use of such compounds should be checked to minimize the risk of insecticide toxicity.

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Farmers use insecticides to protect agriculture products from harmful target insects. But these insecticides contaminate crops and vegetables simultaneously. As a result, non-target organisms that consume these vegetables undergo exposure to such hazardous chemicals (Richmond, 2021). One of the commonly used insecticide categories is the organophosphates. Past studies have indicated the relationship between organophosphate toxicity and insects. Organophosphates inhibit the secretion of acetylcholinesterase enzyme at the synaptic cleft, rendering permanent nerve damage (Rosenthal and Cameron, 1991). In addition to damaging the nervous system (Mehdi and Qamar, 2011), organophosphates reportedly affect the immune system of non-target fruit flies; for example, malathion reduced the number of immune cells (called hemocytes) in adult *Drosophila* (Bindhani et al., 2022 b). In another study, acephate reduced the number of plasmatocytes – which are functionally analogous to vertebrate macrophage – in 3rd instar larvae of *Drosophila* (Rajak et al., 2015). Many studies have demonstrated the effect of organophosphates on the sense of smell and behaviour of non-target insects: chlorpyrifos

**Corresponding Author Email: bindhanibanani@gmail.com*

diminished the learning ability (foraging and pollination) in bees (Urlacher et al., 2016) and disrupted the function of digestive enzymes in silkworms (Kalita et al., 2016); acephate diminished reproductive capacity in *Drosophila melanogaster* (Mandi et al., 2020); dichlorvos prevented male silkmoths from locating pheromone source (Chen et al., 2022). However, few studies have been conducted on the relationship between organophosphates and matrix metalloproteinase enzymes in non-target organisms. Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing protease enzymes that degrade extracellular matrix (ECM) proteins. This protease consists of a propeptide, a catalytic metalloproteinase domain, a hinge region, and a hemopexin domain (Nagase et al., 2006). The genomic sequence of *Drosophila* revealed the similarity between *Drosophila* MMP and vertebrate MMP (Llano et al., 2002). MMP helps in heart development, tissue remodeling, wound healing, glial cell response to nerve degeneration, and ovulation (Bindhani et al., 2022 a). Moreover, these proteases promote cancer and enhance angiogenesis (Beaucher et al., 2007). Data are scarce on whether any insecticide or pesticide can increase the level of matrix metalloproteinase (MMP) and trigger cancer. The effect of organophosphates in vertebrate MMP is yet to be thoroughly examined. We used the fruit fly or *Drosophila melanogaster* as it is one of the non-target organisms of organophosphates and selected third instar larvae for our experiment. Only matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-2 (MMP-2) have been detected in *Drosophila* (Page-McCaw et al., 2003). MMP-2 differs from MMP-1 in that it contains three fibronectin type II repeats in the catalytic domain (Morgunova et al., 1999). The objective of our study was to evaluate the toxicological effect of the organophosphate – malathion – on MMP-1 and MMP-2 in 3rd instar larvae of *Drosophila melanogaster*.

MATERIALS AND METHODS

Drosophila culture medium: Drosophila larvae were reared in a standard culture medium. To prepare the culture medium, we used maize powder (250 g), agar agar (25 g), dried yeast (75 g), brown sugar (250 g), nipagin (2 g), propionic acid (2 ml) and water (2.9 litres) (Poddar et al., 2015). Third instar larvae were chosen for our experiment to assess the effect of Malathion on MMP expression.

Experiment with Malathion: The LC_{50} dose of Malathion was determined (Table 1). 0.80 µg of Malathion insecticide was mixed with 10 ml of *Drosophila* culture medium in a clean vial. The third instar larvae of *Drosophila* were fed food containing the insecticide and observed for 24 hours after the treatment. Control samples were also prepared at the same time.

Hours	Concentration (µl)	$\log_{10}$ (conc.)	% dead	Probit Value	LC_{50}	Final LC_{50} Value
24 hours LC_{50} value determination	2	0	15	3.96	LC_{50}= 1.28	LC_{50}=1.2µl
	1.7	0.113943352	25	4.33		
	1.5	0.176091259	85	6.04		
	1.3	0.230448921	85	6.04		
	1.1	0.301029996	100	7.33		
48 hours LC_{50} value determination	2	0.301029996	99	7.33	LC_{50}= 1.25	
	1.7	0.230448921	85	6.04		
	1.5	0.176091259	75	5.67		
	1.3	0.113943352	50	5		
	1.1	0.041392685	35	4.61		
72 hours LC_{50} value determination	2	0.301029996	99	7.33	LC_{50}= 1.2	
	1.7	0.230448921	90	6.28		
	1.5	0.176091259	85	6.04		
	1.3	0.113943352	60	5.25		
	1.1	0.041392685	40	4.75		
96 hours LC_{50} value determination	2	0.301029996	99	7.33	LC_{50}= 1.1	
	1.7	0.230448921	90	6.28		
	1.5	0.176091259	85	6.04		
	1.3	0.113943352	70	5.52		
	1.1	0.041392685	55	5.13		

Table 1. LC_{50} value determination, 20 Drosophila/vial / 10 ml food

Sample Collection: A total of 40 third instar larvae (20 from the control sample and 20 from the treated sample) were collected from the culture medium for casein zymography and were washed in a few drops of phosphate-buffered saline (PBS) solution separately. Both control and treated samples were homogenized in 1 ml of PBS solution and centrifuged at 10000 rpm for 5 minutes at 4 ^{0}C. For each sample, the protein concentration was determined at 595 nm (Bradford, 1976). The same process was used before gelatin

zymography by taking a total of 40 larvae (20 from the control sample and 20 from the treated sample).

Casein zymography: Casein zymography is an electrophoretic technique to study collagenase or MMP-1 activity in a sample. Gel (SDS-Polyacrylamide gel, 10%) was copolymerized with casein (0.8 mg/ml) (Sigma-Aldrich). Samples were mixed with Novex^{TM} Tris-Glycine SDS Sample Buffer (2X) (Thermo Fisher Scientific).

For each sample, 17 µg of total protein was loaded in each well. Electrophoresis was carried out in a Mini-PROTEAN Electrophoresis Cell (Bio-Rad) at a voltage of 160 V in 1X Novex^{TM} Tris-Glycine SDS Running Buffer (Thermo Fisher Scientific). After electrophoresis, the gel was washed in Zymogram Renaturing buffer (2.5% Triton X-100) for 45 minutes at room temperature and then, incubated at 37 ^{0}C in 1X Zymogram Developing Buffer (50 mM Tris-HCl (pH 7.8), 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% NaN_3) (Liotta and Stetler-Stevenson, 1990) for 24 hours.

After incubation, gel was washed in dH_2O, stained with Coomassie Brilliant Blue (R-250) and destained with destaining solution (50% methanol, 10% acetic acid and 40% water). Areas of collagenase or MMP-1 activity appeared as white bands over blue background.

Gelatin zymography: Gelatin zymography is another electrophoretic technique to study gelatinase or MMP-2 activity in a biological sample. Gel (SDS-Polyacrylamide gel, 10%) was copolymerized with gelatin (0.1%) (HiMedia). Samples were mixed with the same Tris-Glycine SDS Sample Buffer (2X) (Thermo Fisher Scientific) and 14 µg of total protein was loaded in each well. The remaining procedure was as same as casein zymography. Areas of gelatinase or MMP-2 activity appeared as sharp white bands over the blue background.

Analysis of enzyme activity: The gel was scanned and MMP bands were quantified using ImageJ software (downloaded free version from National Institutes of Health). The density or intensity of each band was measured.

Statistical analysis: Mean density and standard error of the mean (SEM) were determined from three measurements. Statistical analysis was performed in MS Excel. To test the significance of difference between control and treated samples, Student's t test followed by one way analysis of variance (ANOVA) was applied.

RESULTS AND DISCUSSION

Analysis of MMP-1 expression: The active MMP-1 bands for control and treated larvae were expressed at 50 KDa with the pro-MMP1 bands being visible at 58 KDa (Plate 1). ImageJ software projects an upregulated expression of MMP-1 in the treated sample when compared with MMP-1 in the control sample (Figure 2). The average band area or density of MMP-1 increased from 35.38±3.23% to 64.62±1.84%. This difference between the two samples is statistically significant ($p<0.05$) (Figure 3) BINDHANI, B; MAITY, S; MAITY, S; CHAKRABARTI, I; SAHA, S. K. (2023). Assessment of Toxicological Effect of Organophosphate, Malathion, on Matrix Metalloproteinase-1 and Matrix Metalloproteinase-2 in 3rd Instar Larvae of *Drosophila melanogaster*.

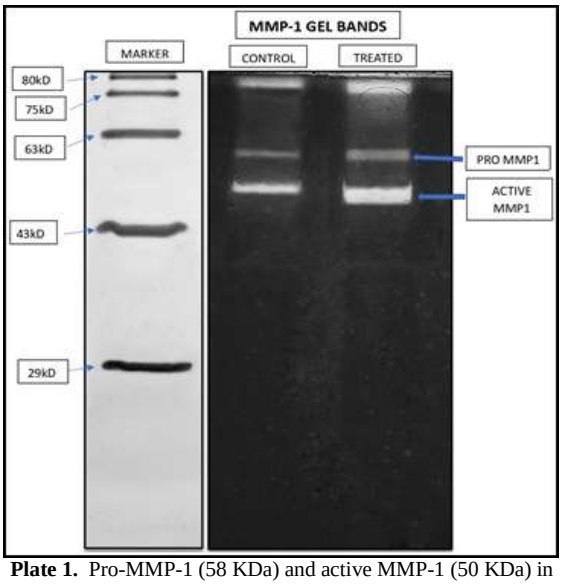

Plate 1. Pro-MMP-1 (58 KDa) and active MMP-1 (50 KDa) in control and treated sample after casein zymography

Analysis of MMP-2 expression: The active MMP-2 bands for control and treated larvae were expressed at 63 KDa with the pro-MMP-2 bands being visible at 72 KDa (Plate 2). Like MMP-1, we observed an increased expression of MMP-2 in the treated sample (Figure 4). The average band area or density of MMP-2 of the control sample was 38.37±2.39% whereas that of the treated sample was 61.63±1.61%. This difference between the two samples is statistically significant ($p<0.05$) (Figure 5). Our present experiment assessed the effect of Malathion in a particular larval stage of *Drosophila* life cycle which is the third instar larvae. We found that both MMP-1 and MMP-2 had higher expression in the treated larval group than the control group. The band density of MMP-1 was increased by 82.64% and that of MMP-2 by 60.62% (Figure 3 and Figure 5).

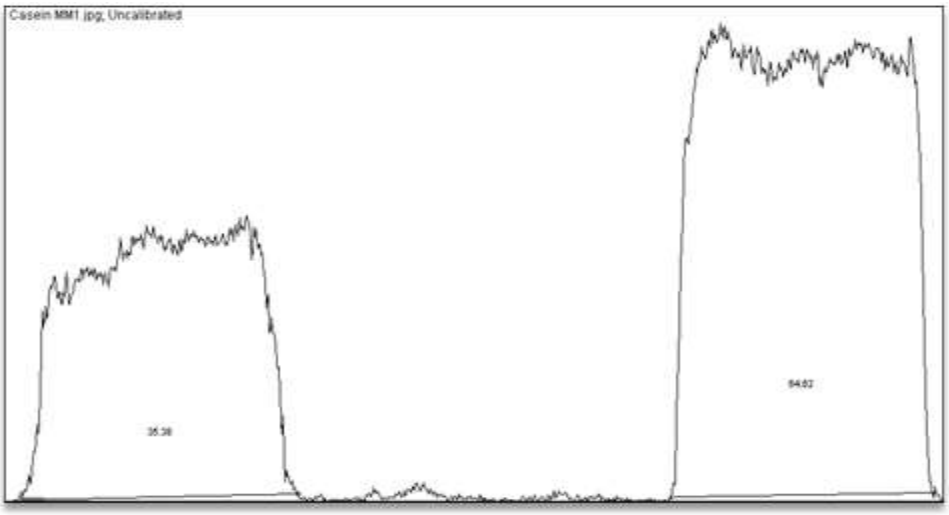

Fig 2. Analysis by ImageJ shows peak in the mean band density of MMP-1 in treated sample.

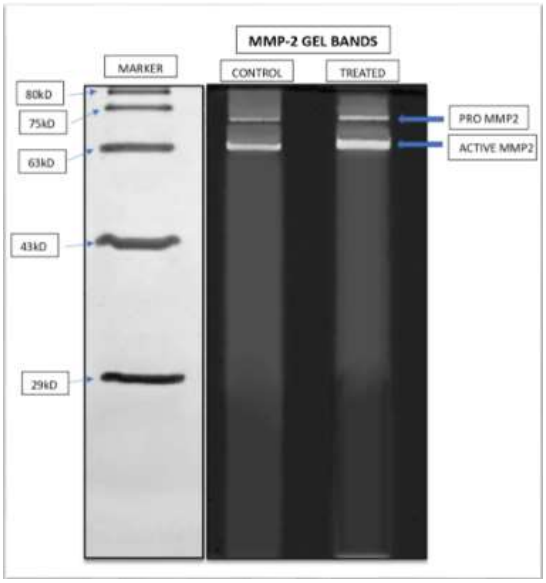

Plate 2. Pro-MMP-2 (72 KDa) and active MMP-2 (63 KDa) in control and treated sample after gelatin zymography

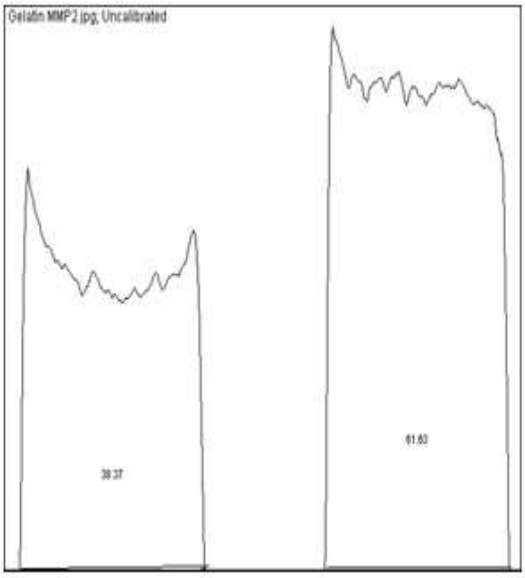

Fig 4. Analysis by ImageJ shows that the mean band density of MMP-2 peaks in treated sample

A recent study indicated the possibility of Malathion to raise oxidative stress by activating Reactive Oxygen Species (ROS) production in *Hermetia illucens* (Abdelfattah and El-Bassiony, 2022). Another study illustrated that the expression of MMP-2 was markedly elevated in the presence of a hypoxic chemical (eg. $CoCl_2$) which, in turn, generated intracellular ROS (Kim et al., 2021). This implies that the presence of ROS may increase MMP-2 level in an organism. Ultraviolet B (UV-B) irradiation is responsible for the formation of ROS which intensifies the level of MMP-1 (Choi et al., 2020). As a result, it leads to skin photoaging due to degradation of collagen by collagenase or MMP-1 (Fisher et al., 2009).

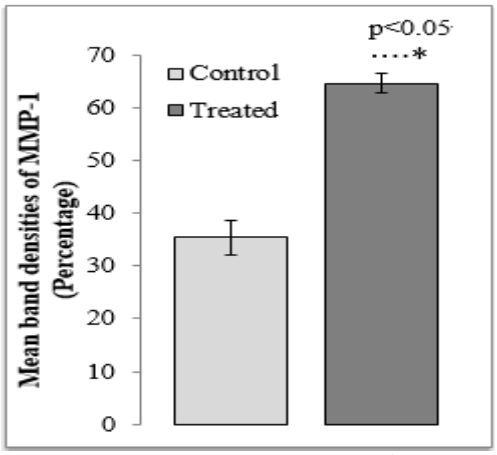

Fig 3. The change between average band areas for control and treated sample is statistically significant ($p<0.05$)

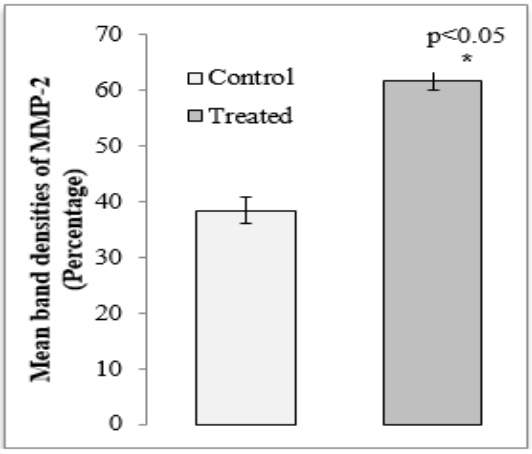

Fig 5. The change between average band areas for control and treated sample is statistically significant ($p<0.05$)

Propoxur, a carbamate insecticide used to control cockroaches, flies and other pests, upregulates MMP-2 protein expression along with ROS overproduction in human breast cancer cells (Shi et al., 2017). Maurya et al. (2014) observed that cypermethrin, a synthetic pyrethroid derived from the chrysanthemum plant, released intracellular Ca^{2+} and ROS. This resulted in enhanced expression of MMP-2 which decreased the viability of astrocytes in rat models. Therefore, we can presume that malathion has induced intracellular ROS production, leading to oxidative stress within the treated 3rd instar larvae of *Drosophila*. This oxidative stress probably increased the protein expression of MMP-1 and MMP-2 in treated larvae of our experiment. Treatment with antioxidants or MEK inhibitors remarkably attenuates MMP-1 and MMP-2 activities (Choi et al., 2020; Shi et al., 2017). Matrix metalloproteinase has majorly been involved with cancer and tumorigenesis (Quintero-Fabian et al., 2019). Chlordane and lindane are organochlorine pesticides that increase MMP-2 activity, leading to angiogenesis (Clere et al., 2012). Chlorpyrifos is an organophosphate insecticide that also increases MMP-2 level and reduces E-cadherin expression, leading to epithelial-mesenchymal transition in breast cancer cells (Lasagna et al., 2020). Although the link between malathion and cancer is not explored yet, malathion has been reported to activate cancer-associated gene expression in human lymphocytes (Anjitha et al., 2020). MMP-1 becomes overexpressed in axon degeneration and other nerve lesions (Purice et al., 2017). Since malathion has projected its neurotoxic effect in rats and *Drosophila* flies (Elmorsy et al., 2022; Mehdi and Qamar, 2011), it is surmised that malathion could have triggered any nerve injury in the treated larvae of our experiment which probably increased the level of MMP-1. Acrolein, a cigarette smoke component, has been found to elevate MMP-1 expression, resulting in atherosclerosis (Lemaitre et al., 2011). Hence, it is observed that the protein expressions of MMP-1 and MMP-2 are intensified under certain stress. Malathion has been found to diminish larval growth rate and upregulate the duration of larval stage in *Megaselia scalaris* (Castillo-Alanis et al., 2022). Whether malathion has any impact on the circulatory system and heart development of *Drosophila* larvae is unknown. However, malathion has shown cardiotoxic effects on early development of zebrafish (Simoneschi et al., 2014).

Conclusion: Our experimental data suggest that Malathion could have comparable effects on human MMP because of the similarity between *Drosophila* MMP and vertebrate MMP. Further research is needed to understand any structural or molecular change in the MMP caused by Malathion. Organophosphates like Malathion may affect non-target organisms like pollinators, impairing their physiological processes and thereby disrupting our ecosystem. Therefore, application of such hazardous chemical in agriculture should be limited in order to diminish the risk of biomagnification – accumulation of chemicals in living organisms.

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