TO INVESTIGATE CYTOPROTECTIVE EFFECT OF PROTEIN X ON DROSOPHILA AGAINST BORIC ACID INSULT

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Abstract : *Drosophila melanogaster* is an excellent model organism to evaluate lethal concentration and the effect of different chemical or bioactive substances due to its short generation time and lifespan. Drosophila is more resistant to oxidative stresses and has a slight but significant increase in their mean lifespan. Boric acid, or sassolite is generally used as insecticide and has proven to be fairly lethal to many pests attacking the food materials and causing various diseases.. Thus, resistance to oxidative stress and lifespan of Drosophila can be manipulated by molecular genetic intervention. Protein X, a hydrolyzed protein based supplement is aimed to make your body stronger, improving stamina and immune system of the body. In present investigation, Drosophila was exposed to different concentrations of Boric acid and Protein X. The present study reveals that prolonged exposure to boric acid at higher concentration causes toxicity which in turn affects the hatching and life cycle of drosophila. The study reveals that the supplementation of Protein X at different concentration increases the resistance ability against oxidative stress generated by boric acid. It acts a cyoprotective molecule thereby significantly normalizing the levels of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase generated by boric acid in drosophila. Also, it helps to maintain the GSH levels in drosophila both larval and adult forms. Thus, Protein X is one of accessible source of antioxidant in pharmaceutical industries and beneficial use in targeted drugs for lowering stress related molecules in organisms. To our knowledge this is the first report on Protein X cyoprotective activity against boric Acid insult.

Keywords: Protein X, Antioxidant enzymes, LC_{50}, Gluathione, oxidative stress

1. INTRODUCTION

Effect of many substances and chemicals are studied on different organisms to check their potency of their toxicity to the cellular structures, physiology, or psychology (Beketov et al., 2008). A model organism is a non-human species that is extensively studied to understand particular biological phenomena (Bonilla et al ., 2012), with the expectation that discoveries made in the organism model will provide insight into the workings of other organisms. These organisms are in vivo models and are widely used in the research human disease where experiments on human would be unfeasible or unethical. This strategy is made possible by the common descent of all living organisms, and the conservation of the metabolic and developmental pathways and genetic material over the course of evolution.Organisms such as E.coli, a bacteria , C. elegans, a worm, zebra fish, a fish, drosophila , a insect, rat, a vertebrate, are most often used (Chelsea et al., 2015).

A weak monobasic lewis acid often known to be used as antiseptic the boric acid can also be used as insecticide and is known to us by many other names such as hydrogen borate, boracic acid, orthoboric acid and *acidum boricum*. It is also known to eliminate fires and act as precursors in many chemical reactions *(*Düzcükoğlu et al., 2009). Boric acid is generally also used as insecticide and has proven to be fairly lethal to many pests attacking the food materials and causing various diseases. Boric acid, or sassolite, is found mainly in its free state in some volcanic districts, for example, in the Italian region of Tuscany, the Lipari Islands and the US state of Nevada. In these volcanic settings it issues, mixed with steam, from fissures in the ground. It is also found as a constituent of many naturally occurring minerals –

borax, boracite, ulexite (boronatrocalcite) and colemanite. Boric acid and its salts are found in seawater. It is also found in plants, including almost all fruits (Schmidt et al., 2010).

Proteins perform a vast array of functions within organisms, including the catalysing metabolic reactions, DNA replication, responding to stimuli, and transporting molecules from one location to another. The protein sources in protein X is peanut protein. Peanuts are rich in protein, an essential nutrient that helps your body build and maintain muscles, hair, skin, organs and other body tissue. It is a complete protein formula containing more proteins than any other health supplement (Watson et al., 2010). Anyone who is expecting a healthy body and stronger muscles can take protein X powder on a daily basis and can see the difference. In the absence of systematic studies in literature, the present study is aimed to evaluate the role of Protein X against Boric acid induced oxidative stress in drosophila.

2. MATERIAL AND METHODS

2.1 Protein X powder: The powder of Protein X was purchased from Nutricia International private Limited, India.

2.2 Preparation of Boric Acid: Boric Acid was purchased from Himedia, India. Different concentrations (0-100 PPM) of Boric acid were used for the experiment.

2.3 Drosophila Culture and LC_{50}: The wild-type *Drosophila melanogaster* strain was maintained in the laboratory on a standard cornmeal, yeast, dextrose, and agar medium at 25°C (Ford et al., 2007). Eggs were collected from these flies by shaking them without anaesthesia into bottles containing an approximately 2 cm layer of fermenting fresh baker's yeast supplemented with sucrose The egg collection bottles were then kept undisturbed in the dark for 8 h at 25ºC. After removing the parental flies, the egg collection bottles were taken back to 25ºC where they remained at a relative humidity of 65% for the rest of their development. Three days later, the 72 h larvae were collected by washing them out the bottles with tap water at room temperature through a fine-meshed stainless steel strainer. They were thoroughly washed free of yeast with tap water while still in the strainer.

2.4 Estimation of sub lethal toxicity (LC_{50}): The larvae were transferred to vials (20 larvae/vial) containing 0.5 g of *Drosophila* Instant Medium (Carolina Biological Supply Co, NC, USA) prepared with the solutions of the test compounds, Boric Acid at 0 to 200 PPM. Five replications were made for each concentration in five independent experiments for each Boric acid. The treatment vials were kept at 25ºC and at a relative humidity of 65%. The surviving flies were collected from the vials on days 10 to 12 after egg laying and shaken into a flask containing 70% ethanol to quantify mortality. The LC50's for each strain and Boric acid were calculated using logistic regression with all five replications of every concentration. LC50's obtained from the five experiments were analysed with a two-way ANOVA (one factor being the strain, the other the treatment).

2.5 Measurement of antioxidant enzymes: Catalase (CAT, EC 1.11.1.6) activity was determined by the method described by Aebi (1984). Superoxide dismutase (SOD, EC 1.15.1.1) was assayed according to Beauchamp and Fridovich (1971). Glutathione peroxidase (GPx, EC 1.15.1.9) activity was measured by the method of Lawrence and Burk (1976). Glutathione Reductase (GR, EC 1.8.1.7) activity was determined by the protocol of Goldberg and Spooner (1983).

2.6 Statistical analyses

All experiments were repeated at least five times and data presented is average of these replicates. One-way analysis of variance (ANOVA) test associated with the Tukey's test was used to determine the statistical significance of the differences among experimental groups. All the statistical analyses were done using SPSS 17.0 software. A logarithmic trend line was used to calculate the LC_{50} values.

3. Result and Discussion

Man's battle with insects predates the earliest recorded history. Indeed, the history of mankind has been shaped in many respects by its continuing competition with insects for food and warmth (Mawdsley, 2011). During the past century, most efforts at control and eradication of structure dwelling insects has been focused on chemical poisons. Many chemicals are known to be quite effective as insecticides, and some have long lasting residual insecticidal properties (Vopham et al., 2017), DDT, for example, is known to be a very effective insecticide, having residual insecticidal properties for weeks, months or even years after application; However, DDT is also known to cause severe ecological consequences and, consequently, has been banned in the United States and in many other countries. Other insecticides may be highly toxic to insects, but are also highly toxic to humans, pets and warm blooded animals generally, or have very serious ecological impact.

Drosophila melanogaster is considered to be the best known multicellular eukaryote model organism as we can study the interactions between genes and environmental conditions simultaneously. Recently, there have been successful attempts to use this species to investigate the effect of a certain type of diet on viability and lifespan (Choi et al., 2008; Erkosar et al., 2013; Khan et al., 2017).Drosophila use as an excellent model system to evaluate lethal concentration and their effect of different chemical or bioactive substances such as, growth and moulting disruption effects of azadirachtin against Drosophila melanogaster (Diptera: Drosophilidae) by Radia Bezzar-Bendjazia et al, 2015. Other studies on *Drosophila melanogaster* have shown the effect of Asprin and acetaldehyde on longitivity and metamorphosis duration of (Duygu keser and Ayla Karatas, 2012).

In insects, boric acid affects the body differently, making it a very effective insecticide. Boric Acid was first registered as an insecticide in the US in 1948, but had been used prior to this for some time. When an insect consumes boric acid, it poisons the stomach and affects the insects metabolism. The powder is also abrasive, further affecting the exoskeleton of the insect. One of the reasons boric acid is so effective at controlling ants, especially sugar ants and other common household bugs, is that a poisoned insect brings the poison back to their nest, where it spreads to the other insects.

In the present study, an attempt has been made to document the short term toxicity of commonly used commercial boric acid, to the fingerling stages of economically important freshwater fish, Rohu and cytoprotective effect of Protein X powder on these fingerling.

3.1 To measure and evaluate the median lethal concentration (LC_{50})

LC_{50} is defined as the lethal concentration at which 50 % of the population if killed in a given period of time. There can be wide range of tolerance to toxic agents among different population of a species which should be taken into account.

In the following experiment the drosophila was treated with different concentrations of Boric acid ranging from 0 to 200 PPM. At the different concentrations of boric acid viz 0, 5, 10, 50 and 100 PPM percent mortality were 0, 10, 23, 52 and 95 respectively. Figure 1 shows the mortality rates and LD50's for Boric acid was 48.2 PPM (Figure 1). On the other hand, the ANOVA results show significant differences between control ($F = 41.21$, $p = 0.0003$) and Boric acid interaction ($F = 9.62$, $p = 0.0032$). Results revealed an increase in mortality rates directly proportional to increase in different concentrations of Boric Acid (Figure 1). The similar result were observed by insecticide beta cyfluthrin on drosophila studies (Gireesh Naada et al., 2005). The crucial element of drosophila ethanol also showed the concurrent result (You et al., 2004). Results revealed an increase in mortality rates directly proportional to all different concentrations with a sigmoid-type curve

Protein X was also used to test its lethal concentration on drosophila with different concentration (0 – 100 PPM). Drosophila treated with different concentration of Protein X powder does not showed any significant percent mortality. For the further experiments 100 PPM of Protein X powder was used.

In the further experiments three concentrations (10, 50, 100 PPM) of Boric Acid were used in combination with (100 PPM) Protein X powder. Table 1 shows that drosophila flies treated with 10, 50 and 100 PPM boric acid along with 100 PPM Protein X powder showed significant decrease (5.2 ± 1.1; 23.9 ± 1.3; 42.9 ± 1.8 respectively) in the % mortality.

3.2 Morphological Changes in Drosophila Life cycle

Drosophila exposed at different concentrations of boric acid ranging from 0 to 200 PPM showed significant morphological changes in its life cycle. When drosophila exposed at different concentration of Protein X powder showed a considerable increase in the size of the larvae as in contrast to control which suggests the use of Protein powder used as a cytoprotective and growth promoting molecule. Following Observations were made under different treatments:

1. Control showed no significant change in the morphology and percent mortality.

2. Boric Acid (50 PPM) showed significant mortality and the size of emerged larva was almost half compared to control larva.

3. Protein X powder (100 PPM) showed significant mortality and the size of emerged larva was almost 3 times compared to control larva.

4. In combination (50 PPM boric acid + 100 PPM Protein X powder) significantly restored the size of larva as control larval forms.

4. At 100 PPM Boric Acid the larval size was almost 3 times reduced than the control larval forms. Also, the delay in the emergence of larval forms was observed compared to control. The addition of protein X powder (100 PPM) significantly restored the larval size of drosophila.

Interestingly at 200 PPM of Boric acid the life cycle of drosophila was ceased at the stage of eggs which was observed till 15 days. This may be due to inhibitory effect of Boric acid on gonadal development. The inhibition of oviposition is may be a result of imbalanced endocrine system or inhibition of ovarian development or deformities in oviposition organs (Phoebe et al., 2002). The reduced fecundity rate was observed by the effect of beta-cyfluthrin, (Gireesh Nadda et al, 2005). *Spodoptera littoralis* as a sub lethal pyrethroid insecticidal was reported (Radwan 1984) and besides that the life cycle was delayed by 5-6 days as compared to control. i.e prolongation in life cycle. This may be due some cytotoxicity of Boric acid or over production of growth hormone.

At the concentration 200 PPM Boric acid the eggs were not able to hatch from the egg this is may be due to direct impact of Boric acid various tissue such as trophocytes, perifollicular tissue, follicular epithelium and oocyte themselves (Soltani et al., 2016) or hormonal imbalance (Gireesh Nadda, 2005). In the present study it was observed that the egg hatching process and ovicidal action was decreased due to accumulation of Boric Acid in eggs resulting their direct death. The eggs which laid but do not hatched, are may be result of inappropriate incorporation of the yolk so that the embryo failed to complete metamorphosis (Soltani et al., 2016) or may be due antifeedant effect of Boric Acid resulted in weak and non -viable egg production.

3. 3 Evaluation of Antioxidant Enzymes

The Boric Acid is one of the potent ROS generator .which induces oxidative stress use in experiment to generate oxidative stress. The increased level of antioxidant enzymes (CAT, SOD, GPx and GR) indicates the oxidative stress hence act as a biomarker.

3.3.1 Estimation the levels of Catalase (CAT) and Superoxide Dismutase (SOD)

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyses the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). At the concentration of 10, 50 and 100 PPM of Boric acid was increased significantly (Figure 2a) in larva (66.53 ± 6.17 U/mg; 90.32 ± 4.74 U/mg ; 123.58 ± 4.23 U/mg; $p<0.05$) with respect to control (23.71 ± 5.02 U/ mg) and when flies were treated with combinations of 100 PPM of protein powder , the catalase activity reverted back in larvae at the concentrations of 10 , 50, 100 PPM (46.13 ± 5.75 U/mg ; 77.89 ± 4.71 U/mg; 90.30 ± 8.82 U/mg; $p<0.05$).

SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. It is an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. At the concentration of 10, 100, 200 PPMof boric acid the the activity of sod showed gradual increase (55.6 ± 0.6 U/mg ; 80.5 ± 1.7 U/ mg ; 101.1 ± 1.6; $p<0.05$) with respect to control (21.1 ± 0.7 U/ mg) whereas addition of protein 100 PPM in combination with the boric acid shows gradual

increase (Figure 2a) with respect to control and with respect to the concentrations there was decrease in the activity (41.4 ± 1.0 U/ mg ; 61.1 ± 0.8 U/ mg ; 80.8 ± 0.5 U/ mg; $p<0.05$).

3.3. 2 Estimation the levels of Glutathione Peroxidase (GPx), Glutathione reductase (GR)

Glutathione peroxidase (GPx) is an antioxidant enzyme. GPx functions in the scavenging and inactivating of hydrogen and lipid peroxides, thereby protecting the body against oxidative stress. In Figure 2b at the concentration of 10, 50, 100 PPM of boric acid decreased in larvae (1.36 ± 0.03 U/ mg ; 0.75 ± 0.75 U/mg ; 0.47 ± 0.06 U/mg ; $p<0.05$) with respect to control (1.31 ± 0.09 U/ mg), the GPx activity remained almost unaltered with addition of protein X (1.36 ± 0.05 U/ mg) and was brought to normal levels significantly when combined with 100 PPM of protein with 10, 50 and 100 PPM of boric acid (1.07 ± 0.07 U/ mg; 0.07 ± 0.04 U/ mg ; 0.76 ± 0.04 U/ mg ; $p<0.05$).
Glutathione reductase is an enzyme that reduces glutathione disulphide to sulphydryls from GSH, which is an important cytoplasmic antioxidant activity It is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. At concentration of 10, 50 and 100 PPM of boric acid there was steady decline in the activity of GR (0.33 ± 0.032 U/ mg; 0.23 ± 0.02 U/mg ; 0.12 ± 0.015 U/mg ; $p<0.05$) with respect to control (0.56 ± 0.015) and upon addition of protein X powder (100 PPM) to each of the concentration of Boric acid (10, 50 and 100 PPM) the activity of GR showed significant (Figure 2b) elevation (0.45 ± 0.04 U/mg; 0.44 ± 0.038 U/mg ; 0.46 ± 0.04359 U/mg ; $p<0.05$) .
It was observed that the antioxidant defense system of larval stage of *D. melanogaster* is highly responsive increasing the activities of reduced glutathione (GSH), SOD, CAT, GR and GST (Ozata, 2006). Studies of *G. mellonella* showed that the activities of antioxidant systems of SOD, CAT, GST and GPx significantly change in parallel with the increased levels of MDA and PCO in hemolymph and adipose tissue due to increased BA concentrations (Hyršl et al., 2007; Buyukguzel & Kayaoğlu, 2014)
The present study when drosophila was treated with different concentrations of Boric Acid significant increase in the levels of antioxidant enzymes namely CAT, SOD, GPx and GR as observed. Simultaneous treatment with Protein X powder protected and restored the levels of these antioxidant enzymes. Concurrent results were observed by N.Mansa and J.S. Ashadevi (2015). This can be due to increase in activities GPx, SOD and CAT which results in increase in longevity. The relevant result have been reported on Xijnjiang black mulberry fruit on delaying aging (Jiang et al., 2010).

Conclusion

The studies reveals that the supplementation of Protein X powder increases the resistance ability against oxidative stress generated by Boric Acid and thus being a cytoprotective drug. It reduces the oxidative stress generated by Boric Acid in drosophila. Its acts a cytoprotective molecule by reducing the levels of antioxidant enzymes such as catalase and superoxide dismutase. Also, it helps to maintain the GSH levels in drosophila (larval forms) by restoring the Glutathione peroxidase and Glutathione reductase levels. Thus, Protein X powder is one of accessible source of antioxidant in pharmaceutical industries. However, Disruption of growth and development in flies and other insect species under Boric acid treatment have yet to be fully investigated.

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Conflict of Interest

Authors declare no conflict of interest.

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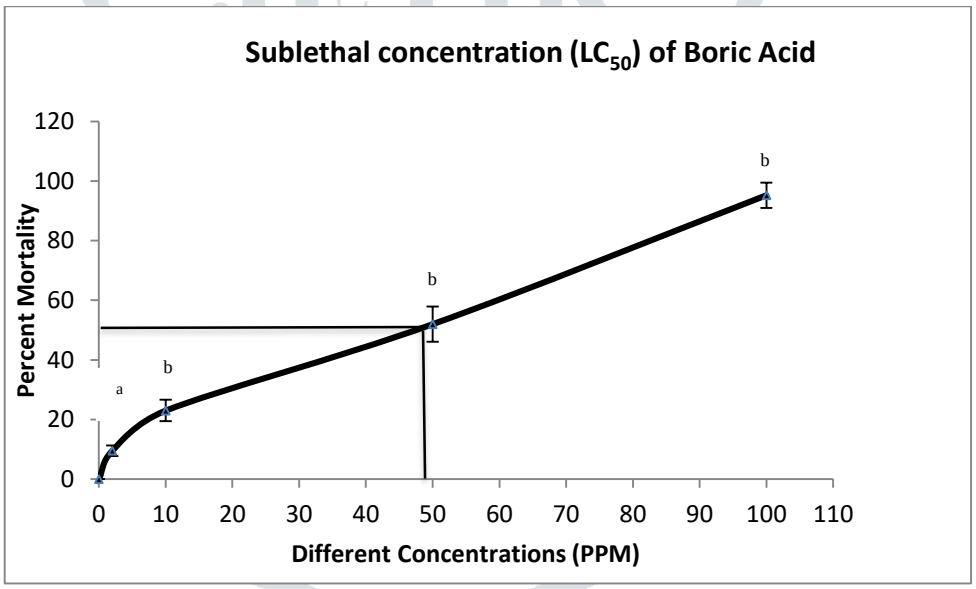
Figures and Tables

Figure 1: Median Lethal Concentration (LC_{50}) at different concentrations of Boric Acid



Dissimilar alphabets a and b in superscript in the figure indicate statistically significant difference at 0.05 level.

Figure 2 (a): Specific activities of different antioxidant enzymes at different conditions

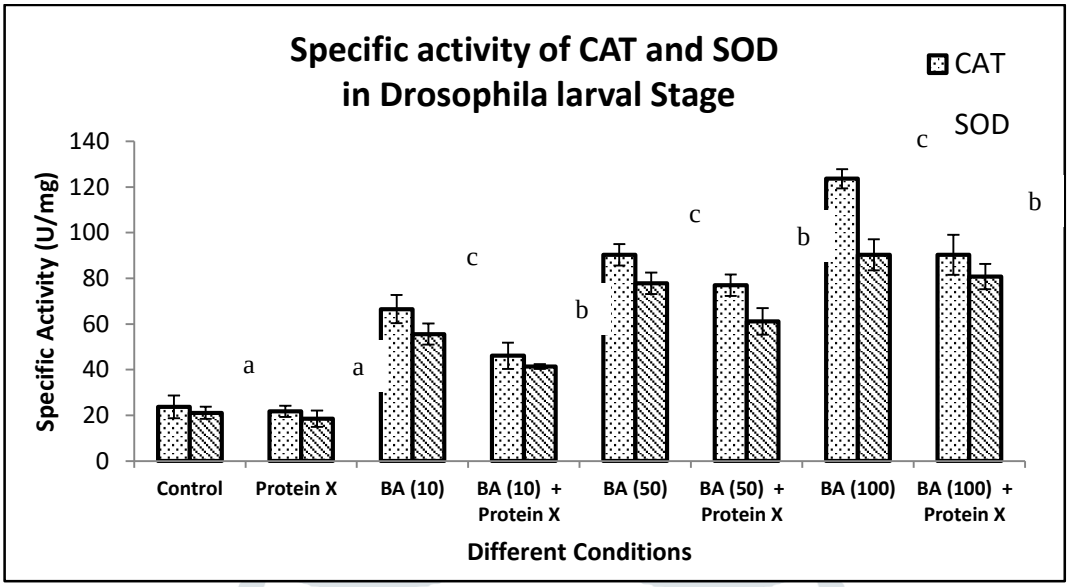
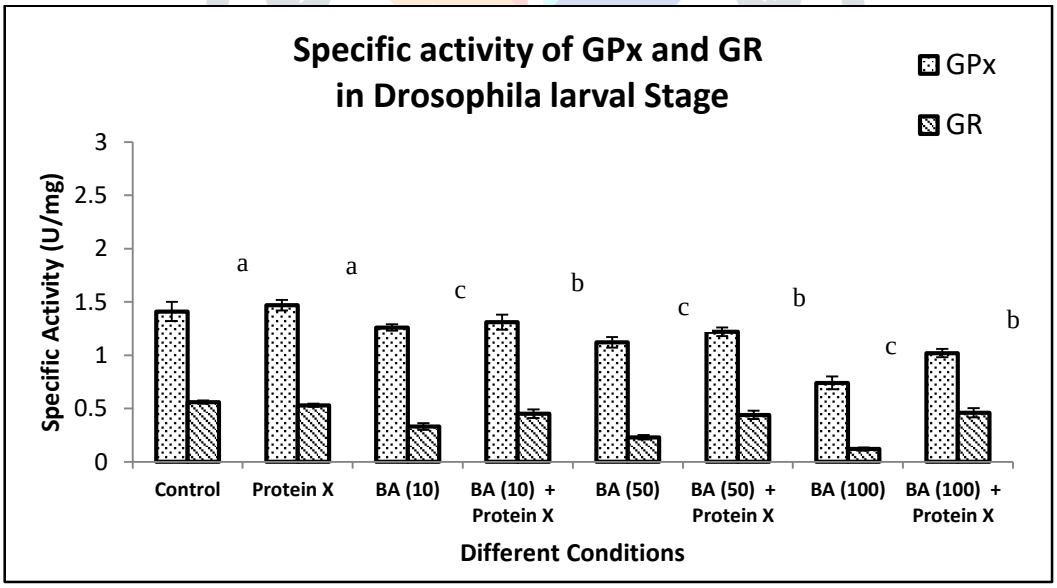


Figure 2 (b): Specific activities of different antioxidant enzymes at different conditions



BA (10):10 PPM Boric Acid, BA (50):50 PPM Boric Acid, BA (100):100 PPM Boric Acid. Dissimilar alphabets a and b in superscript in the figure indicate statistically significant difference at 0.05 level.

Table 1: Percent Mortality of flies at different concentrations of Boric Acid and (100 PPM) of Protein X powder

Different Conditions	% Mortality
Control	0 ± 0^{a}
Protein X (100 PPM)	0 ± 0^{a}
BA (10 PPM)	10 ± 1.8^{c}
BA (10 PPM) + Protein X (100 PPM)	5.2 ± 1.1^{c}
BA (50 PPM)	52.6 ± 5.9^{b}
BA (50 PPM) + Protein X (100 PPM)	23.9 ± 1.3^{c}
BA (100 PPM)	95.1 ± 4.2^{b}
BA (100 PPM) + Protein X (100 PPM)	42.9 ± 1.8^{c}

Control, BA: Boric Acid**.** Dissimilar alphabets a, b and c in superscript in the figure indicate statistically significant difference at 0.05 level.