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Studies on the individual and combined effect of monocrotophos and neem oil on the digestive enzyme activity of female rice Grasshopper *Oxya fuscovittata*

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Abstract
The present investigation has been carried out to study the 10% sublethal concentration of individual and combined monocrotophos and neem oil on digestive enzyme activities of alimentary canal in the female rice grasshopper, *Oxya fuscovittata*. In insects exposed to the 10% sublethal concentration of monocrotophos for 96hrs, the levels of amylase were found to be decreased by 52.67% and 55.19% respectively for foregut and hindgut, but in the hindgut, there was an increase in the levels of amylase was 43.90%. In insects exposed to neem oil for 96hrs, the decrease of 31.98% and 34.85% were observed in foregut and midgut respectively, while in hindgut an increase of 12.19% was observed. In insects exposed to 10% sublethal concentration of monocrotophos for 96 hrs, the activity of protease decreased by 61.87% and 65.40% respectively in the foregut and hindgut while the insects showed increased protease activity (69.38%) in hind tissue. In general, the decrease in protease activity was high in monocrotophos treated insects. In insects exposed to sublethal concentration of monocrotophos for 96hrs, the levels of lipase were found to decrease by 84.61% and 75.80% respectively in the foregut and hindgut while the enzyme activity increased by 59.37% in hindgut. The highest gut enzyme activity is seen in the midget of the control insects. Meager amount of enzyme activity was evident in hindgut than in foregut.

Keywords: monocrotophos, neem oil, combined toxicity, digestive enzyme activity, grasshopper

Introduction
Grasshoppers are foliage feeders; most often but they may at times, feed on any of the other parts of the plant, indeed in some cases, apparently prefer fruits or flowers. The literature on grasshopper food preference includes several research papers and review articles (Ball, 1936; Gangwere, 1961; Misra, 1962; Dadd, 1963; Dadd, 1973; Mulkern, 1967: Gangwere, 1989) [1, 7]. Feeding distinctions have usually been considered to indicate preferences, but it has recently been shown (Bernays and Chapman, 1977) [8] that there is sometimes a matter of avoiding plants which contain deterrents. Gang were (Gangwere, 1967) [9] points out that preference for a particular food plant is the outcome of a complicated evolutionary process of interactions and adaptations between that plant and the grasshopper, which is documented by the fact that the preferred food by a species may vary in different geographical locations. Insects have an intrinsic tendency to feed preferentially on a plant that would ensure their best development, survival and reproduction, but the available experimental evidence on these points is ambiguous (Uvarov, 1997) [10].
In the past two decades, there has been an increased awareness of the need to implement what has come to be known as the Integrated Pest Management Program (IPM), with the aim of making crop production, more efficient by preventing the misuse of insecticides (Subramanian, 1998) [11]. Insecticides are to be used when pest populations reach a level that would lead to economic losses in crops. The post synthetic chemical era has dawned, ready to face the challenges to develop a new service, new technology, new management skills and new concepts of integration to control pests, protect our environment and provide enough food (Narayanasamy, 1998) [12]. Attempts are made to reduce reliance on one method of pest management, and instead make the system more flexible, the new techniques offering a range of options such as approach calls for an integration with crop production, combination of agronomic, genetic, biotechnological and chemical methods in the crop production system (Chanal *et al*., 2006) [13]. Hence, the present investigation has been carried out to study the individual and combined effects of monocrotophos and neem oil on digestive enzyme activity of alimentary canal in the female rice grasshopper, *Oxya fuscovittata* Walker (Orthoptera: Acrididae).

Material and Methods
The adult female rice grasshoppers, *Oxya fuscovittata* were collected in the paddy fields in and around Adirampattinam using sweep net. They were brought to the laboratory and reared in wooden cage (25 x 25 x 30 cm) (Fig. 2) at room temperature (29±°C) and under normal lighting condition (D 12: N 12). The leaves were provided every day and care was taken to remove all excreta and unfed leaves regularly so as to prevent fungal infection and a healthy cage condition. Insects were drawn from this stock culture for all experimental analyses. Mature leaves were provided for all experiments.

Evaluation of acute toxicity
Acute toxicity bioassay experiments were conducted accordance with standard methods (APHA, 2005) [14]. Stock solution of monocrotophos with a concentration of 1 ml/l (equivalent to 1 ppt) was prepared in distilled water and different dilutions were prepared by adding required amount of distilled water. The stock solution of neem oil with Concentration of 1 ml/l was also prepared in ethanol solvent and desired degree of concentration was prepared. The grasshoppers were starved for 24 hours period to their use in

the experiment. After addition of the pesticide, the mortality was recorded after 24, 48, 72 and 96 hours. Five replicates were maintained simultaneously.

Per cent mortality was calculated by Finney analysis method (Finney, 1971) [15]. Based on acute toxicity (96 hours LC_{50}) test, 10 % sublethal concentration was derived for individual and combined monocrotophos and neem oil. They were used as experimental concentrations in the subsequent experiments.

Enzyme assay

Insects were etherized and fixed on to a wax tray containing Insects Ringer's solution. The dorsal side was cut open under a dissection Wile stereo microscope and the alimentary canal exposed. The adhering trachea was cut and then the alimentary canal was removed and placed on a glass slide. The gut was divided into fore, mid and hind gut regions.

Preparation of tissue homogenate for enzyme analysis

The dissected tissues were placed in labeled Petri dishes containing 2ml of ice cold phosphate buffer. The tissues were then homogenized in a tissue homogenizer by adding another 2 ml of distilled water. The content were centrifuged in a refrigerated centrifuge generally at 5000 rpm unless otherwise specifically mentioned and at 4°C to avoid enzyme denaturation. The resulting supernatant solution was taken as the enzyme solution for all analysis. Ten to fifteen insects were dissected to get sufficient quantity of enzyme solution for each analysis.

Preparation of buffer

The following buffer solution were prepared (1) Phosphate buffer (0.05M) using Di-sodium hydrogen phosphate and Sodium dihydrogen orthophosphate. The buffers prepared had pH ranging from 6.0 and 9.9. (2). Glycine – NaOH buffer at a concentration of 0.03M. The pH ranges prepared were from 7.5 to 12.0. (3) Tris-HCl buffer was prepared at a concentration of 0.05M. The pH ranges prepared were from 2.0 and 9.0. A digital 335 systronics pH meter was used for measuring the pH of the buffer solution. Before measuring the pH, the instrument was calibrated using standard pH buffer tablets to minimize error.

Preparation of dinitrosalicylic acid reagent

The preparation of dinitrosalicylic acid reagent for the carbohydrases was as per Noelting and Bernfield (1986) [16]. 1g of 3, 5-dinitrosalicylic acid, 20ml of 2N NaOH and 50 ml of distilled water were mixed in a glass container, with a magnetic stirrer until all the dinitrosalicylic acid was dissolved. Potassium tartarate (30 g) was added when the solution becomes clear. Then finally distilled water was added to make the final volume to 100 ml.

Characterization of enzymes

Optimum temperature determination

Amylase, protease and lipase enzymes of *Oxya fuscovittata* were characterized for their optimum temperature at which maximum activity could be observed. Enzyme-substrate mixture was prepared under normal assay conditions while the reaction mixture was incubated at different temperature ranges from 25°C to 60°C at 5°C interval. The activity curves were obtained for each enzyme by plotting the data between temperature and the amount of end products formed.

Optimum pH determination

The optimum pH for the various enzymes was estimated from activity curves plotted between various pH ranges and amount of product formed in the unit time. The reaction mixture consisting of suitable substrate, enzyme solution and buffers of varying pH were incubated at 37°C, after which the enzyme reaction was terminated and the end products formed was quantified.

Optimum substrate concentration determination

The digestive enzymes were characterized for their optimum substrate concentration by incubating the enzyme with respective substrates of different concentration while keeping other factors at optimum conditions and finally estimating the amount of end products formed. The graph was plotted between substrate concentration and the product formed in unit time.

Enzyme assay techniques

Amylase and protease activities were determined under optimal experimental conditions as per Ishaaya (1986) [17]. The free aldehydic groups of glucose formed after starch, sucrose and trehalose digestion by respective enzymes were detected using 3, 5 dinitrosalicylic acid reagent. The reduced dinitrosalicylic acid was measured spectrophotometrically at an absorbency of 550 nm. The copper soap method as described by Myrtle and Zell (1975) [18] was followed for rapid assay of lipase activity.

Result and Discussion

Characterization of the digestive enzymes

Optimum pH determination

Each enzyme has a characteristic pH at which the activity is at the peak. Amylase, protease and lipase enzymes were characterized for their optimum pH through a series of pilot experiments. For *Oxya fuscovittata*, amylase showed an optimum pH of 7.0 and protease showed maximum activity at pH 9.0, while lipase had maximum activity at pH 8.0 (Table 1) all the enzymes studied, protease and lipase showed maximum activity in the alkaline range, while amylase was active in the neutral range.

Optimum temperature determination

Insects being poikilothermic, the activity of the enzymes depends on the surrounding environmental temperature, as well as the physiological status of the insects. However, each enzyme has an optimum temperature at which maximum activity is observed studies on the enzyme activity of *Oxya fuscovittata* showed that the optimum temperature for amylase was 45°C, while for protease it was 50°c lipase had an optimum activity at 40° C (Table 1).

Optimum substrate concentration

The optimum substrate concentration for amylase was 1% starch and for protease enzyme it was 2.0% casein whereas lipase had an optimum of 10% olive oil (Table 1).

Time course study

Activity of amylase, protease and lipase enzymes were studied through incubation at 37°C and maintenance of the enzyme mixture at the respective optimal experimental conditions with regard to the pH and substrate concentration. The enzymes were incubated at different time intervals and the quantities of end products formed were

recorded. Amylase incubated at 37°C and in pH 7.0, showed linearity till the end of 60 minutes of incubation time. In case of protease, a catalytic period of up to 60 minutes was found while lipase was able to maintain its linearity only till 30 minutes of incubation.

Amylase activity

The levels of amylase activity were found to decrease in foregut and midgut of the test insects while in hindgut the activity levels were found to increase, compared to controls (Table 2and Fig.1 -3). The decrease of amylase activity in the foregut and midgut and increase in hindgut were time dependent. In insects exposed to the 10% sublethal concentration of monocrotophos for 96hrs, the levels of amylase were found to be decreased by 52.67% and 55.19% respectively for foregut and hindgut, but in the hindgut, there was an increase in the levels of amylase was 43.90%. In insects exposed to neem oil for 96hrs, the decrease of 31.98% and 34.85% were observed in foregut and midgut respectively, while in hindgut an increase of 12.19% was observed. Exposure to mixture of monocrotophos and neem oil elicited same pattern of deviation in the amylase activity.

Protease activity

Normal insects showed high levels of protease activity in midgut (23.2-23.7), lesser level in foregut (13.5-13.9) and least in hindgut (4.5-4.9). In test insects exposed to sublethal concentration of three pesticides, significant decreases were observed in the protease activity in the foregut and midgut tissues except hindgut (Table 3 and Fig.4-6). In insects exposed to 10% sublethal concentration of monocrotophos for 96 hrs, the activity of protease decreased by 61.87% and 65.40% respectively in the foregut and hindgut while the insects showed increased protease activity (69.38%) in hind tissue. In general, the decrease in protease activity was high in monocrotophos treated insects.

In the insect exposed to sublethal concentration of monocrotophos, neem oil and combined monocrotophos and neem oil, maximum decrease of protease activity in foregut and hindgut and maximum increase of protease activity in hindgut was observed at 96hours exposure and minimum decrease or increase in respective tissues at 24 hours exposure.

Lipase activity

Lipase activity was found to be high in midgut, less in foregut and least in hindgut. In insects exposed to pesticides, lipase activity increased in hindgut and decreased in foregut and midgut (Table 4 and Fig. 7-9).

In insects exposed to sublethal concentration of monocrotophos for 96hrs, the levels of lipase were found to decrease by 84.61% and 75.80% respectively in the foregut and hindgut while the enzyme activity increased by 59.37% in hindgut.

In insects exposed to the sublethal concentrations of three pesticides, the maximum decrease in lipase activity was observed in foregut and midgut, while the maximum increase in lipase activity was observed in hind gut at 96 hours exposure. Minimum levels of decrease and increase in the same tissues were found at 24 hours of exposure.

Important activities like energy synthesis and metabolism are carried out by enzymes, the active biocatalysts. Toxicological evaluation of some enzymes modified starches and certain other substances was reported by WHO (1972) [19]. In the present study the enzyme activity was significantly altered in the grasshopper, *Oxya fuscovittata*after acute treatment with monocrotophos and neem oil. Lomte and Patil (1989) [20] observed a decrease in amylase activity in the army worm, *Mythimna separata* after pesticide treatment. Nalina Sundari *et al.,* (1986) [21] reported depletion in the amylase, invertase and protease activity of *Spodoptera litura* when exposed to *Vinca rosea* extract. The results of the present investigation are in harmony with the results of some workers like Sontakke (1992) [22] in *Thiara tuberculata*, Bhamre (1993) [23] in *Parreysis favidens*, Jadhav (1993) [24] in *Corbiculata striatella* and Patil (1993) [25] in *Lamellidens marginalis.*

Higher activity of enzymes in foregut and midgut of control insects is due to consumption as well as utilization of large quantities of food. Imbalance in the enzyme – substrate complex and inhibition of peristalic movement of the gut (Hori, 1969) [26], midgut have enhanced the enzyme quantity in hindgut in all the doses studied. Several studies have shown that feeding is necessary for the stimulation of enzyme activities (Sibley, 1981; Broadway and Duffey, 1988) [27-28]. The decrease in the activities of digestive enzymes in monocrotophos and neem oil treatment can be concluded that the role of gut enzymes secretion mainly depends on the peristaltic movement of the gut (Peter and Ananthakrishnan, 1993; Babu and Murugan, 1999) [29-30].

Table 1: Characterization of digestive enzymes of *Oxya fuscovittata*.

S. No.	Enzyme	pH
Optimum pH		
1.	Amylase	7.0
2.	Protease	9.0
3.	Lipase	8.0
Optimum temperature		
1.	Amylase	45°C
2.	Protease	50°C
3.	Lipase	40°C
Optimum substrate concentration		
1.	Amylase	1% starch
2.	Protease	20% BSA
3.	Lipase	10% olive oil
Time course study		
1.	Amylase	60 min
2.	Protease	60 min
3.	Lipase	30 min

Table 2: Effect of sublethal concentration of individual and combined monocrotophos and neem oil on digestive enzyme activity of female rice grasshopper *Oxya fuscovittata* for an exposure period of 24 hours.

Control			
Tissue	**Amylase µg of glucose / mg tissue/ hr**	**Protease µg of tyrosine/ mg tissue/ hr**	**Lipase µg of /mg oleic acid/tissue/ hr**
Foregut	11.7 ± 0.8	13.6 ± 0.9	0.36 ± 0.02
Midgut	23.7 ± 1.6	23.5 ± 0.6	0.58 ± 0.04
Hindgut	3.7 ± 0.3	4.7 ± 0.3	0.32 ± 0.01
monocrotophos			

Foregut % Variation	7.96 ± 0.2-30.78	7.5 ± 0.4-45.25	0.09 ± 0.2-76.31
Midgut. % Variation	15.6 ± 1.4-34.72	11.2 ± 0.6-51.72	0.26 ± 0.03-55.93
Hindgut % Variation	4.6 ± 0.1+27.77	7.6 ± 0.2+58.3	0.49 ± 0.01+53.12
neem oil			
Foregut % Variation	10.5 ± 0.7-10.26	12.2 ± 0.6-10.29	0.28 ± 0.02-22.22
Midgut % Variation	22.5 ± 1.6-5.06	18.4 ± 1.6-21.70	0.51 ± 0.03-12.06
Hindgut % Variation	3.9 ± 0.1+5.40	5.1 ± 0.3+8.51	0.34 ± 0.02+6.25
monocrotophos + Neem oil			
Foregut % Variation	9.85 ± 0.5-15.81	8.4 ± 0.2-38.23	0.19 ± 0.01-47.22
Midgut % Variation	21.5 ± 1.7-9.28	13.4 ± 1.3-42.97	0.43 ± 0.02-25.86
Hindgut % Variation	3.9 ± 0.2+5.40	6.7 ± 0.4+42.53	0.39 ± 0.01+21.87

Values are mean ± SD of six observations. + Or - indicates percent increase or decrease over control.

Table 3: Effect of sublethal concentration of individual and combined monocrotophos and neem oil on digestive enzyme activity of female rice grasshopper *Oxya fuscovittata* for an exposure period of 48 hours.

Control			
Tissue	**Amylase µg of glucose / mg tissue/ hr**	**Protease µg of tyrosine/ mg tissue/ hr**	**Lipase µg of /mg oleic acid/tissue/ hr**
Foregut	11.5 ± 0.5	13.7 ± 0.7	0.38 ± 0.01
Midgut	23.9 ± 1.8	23.2 ± 0.5	0.59 ± 0.03
Hindgut	3.6 ± 0.3	4.8 ± 0.2	0.32 ± 0.02
monocrotophos			
Foregut % Variation	7.96 ± 0.2-30.78	7.5 ± 0.4-45.25	0.09 ± 0.2-76.31
Midgut. % Variation	15.6 ± 1.4-34.72	11.2 ± 0.6-51.72	0.26 ± 0.03-55.93
Hindgut % Variation	4.6 ± 0.1+27.77	7.6 ± 0.2+58.3	0.49 ± 0.01+53.12
neem oil			
Foregut % Variation	10.1 ± 0.2-12.17	10.9 ± 0.7-20.43	0.22 ± 0.04-42.10
Midgut % Variation	21.9 ± 1.2-8.36	16.7 ± 1.8-28.01	0.46 ± 0.02-22.03
Hindgut % Variation	4.1 ± 0.2+13.88	5.3 ± 0.2+10.41	0.36 ± 0.01+12.5
monocrotophos + Neem oil			
Foregut % Variation	9.80 ± 0.4-14.78	7.2 ± 0.3-47.44	0.16 ± 0.04-57.89
Midgut % Variation	20.4 ± 1.6-14.64	12.1 ± 1.2-47.84	0.36 ± 0.01. -38.98
Hindgut % Variation	4.4 ± 0.3+22.22	6.9 ± 0.4+43.75	0.44 ± 0.02+37.5

Values are mean ± SD of six observations.+ or - indicates percent increase or decrease over control.

Table 4: Effect of sublethal concentration of individual and combined monocrotophos and neem oil on digestive enzyme activity of female rice grasshopper *Oxya fuscovittata* for an exposure period of 72 hours.

Control			
Tissue	**Amylase µg of glucose / mg tissue/ hr**	**Protease µg of tyrosine/ mg tissue/ hr**	**Lipase µg of /mg oleic acid/tissue/ hr**
Foregut	11.7 ± 0.7	13.5 ± 0.8	0.37 ± 0.03
Midgut	23.2 ± 0.6	23.4 ± 0.5	0.57 ± 0.02
Hindgut	3.8 ± 0.2	4.5 ± 0.3	0.31 ± 0.04
monocrotophos			
Foregut % Variation	6.58 ± 0.1-43.76	6.4 ± 0.3-52.59	0.05 ± 0.01-86.48
Midgut % Variation	12.7 ± 1.2-45.26	10.1 ± 0.4-56.83	0.18 ± 0.02-68.42
Hindgut % Variation	5.1 ± 0.2+34.21	8.1 ± 0.2+80.00	0.52 ± 0.01+67.74
neem oil			
Foregut % Variation	8.98 ± 0.5-23.24	9.5 ± 0.5-29.63	0.31 ± 0.06-16.21
Midgut % Variation	16.4 ± 1.4.-29.31	15.3 ± 1.2-34.61	0.40 ± 0.05\-29.82
Hindgut % Variation	5.1 ± 0.3+34.21	5.1 ± 0.3+13.33	0.38 ± 0.02+22.58
monocrotophos +Neem oil			
Foregut % Variation	7.75 ± 0.2-33.76	6.7 ± 0.2-50.37	0.12 ± 0.02-67.56
Midgut % Variation	16.2 ± 1.1-30.17	10.8 ± 1.3-53.84	0.29 ± 0.03-49.12
Hindgut% Variation	5.2 ± 0.2+36.84	6.7 ± 0.2+48.88	0.46 ± 0.03+48.38

Values are mean ± SD of six observations.+ or - indicates percent increase or decrease over control.

Table 5: Effect of sublethal concentration of individual and combined monocrotophos and neem oil on digestive enzyme activity of female rice grasshopper *Oxya fuscovittata* for an exposure period of 96 hours.

Control			
Tissue	**Amylase µg of glucose / mg tissue/ hr**	**Protease µg of tyrosine/ mg tissue/ hr**	**Lipase µg of /mg oleic acid/tissue/ hr**
Foregut	11.6 ± 0.9	13.9 ± 0.7	0.39 ± 0.04
Midgut	24.1 ± 0.7	23.7 ± 0.6	0.62 ± 0.03
Hindgut	4.1 ± 0.1	4.9 ± 0.2	0.32 ± 0.01
monocrotophos			
Foregut % Variation	5.49 ± 0.2-52.67	5.3 ± 0.2-61.87	0.06 ± 0.03-84.61
Midgut % Variation	10.8 ± 1.4-55.19	8.2 ± 0.6-65.40	0.15 ± 0.01-75.80
Hindgut % Variation	5.9 ± 0.3+43.90	8.3 ± 0.4+69.38	0.51 ± 0.03+59.37

neem oil			
Foregut % Variation	7.89 ± 0.4-31.98	8.7 ± 0.2-37.41	0.16 ± 0.02-58.97
Midgut % Variation	15.7 ± 1.2-34.85	14.6 ± 1.7-38.39	0.33 ± 0.06-46.77
Hindgut % Variation	4.6 ± 0.4+12.19	5.4 ± 0.2+10.20	0.37 ± 0.01+15.62
monocrotophos + Neem oil			
Foregut % Variation	6.51 ± 0.3-43.88	5.6 ± 0.3-59.71	0.09 ± 0.04-76.92
Midgut % Variation	11.1 ± 1.7-53.94	8.7 ± 1.6-63.29	0.18 ± 0.02-70.96
Hindgut % Variation	5.7 ± 0.1+39.02	7.2 ± 0.2+46.93	0.45 ± 0.02+40.62

Values are mean±SD of six observations.+ or - indicates percent increase or decrease over control.

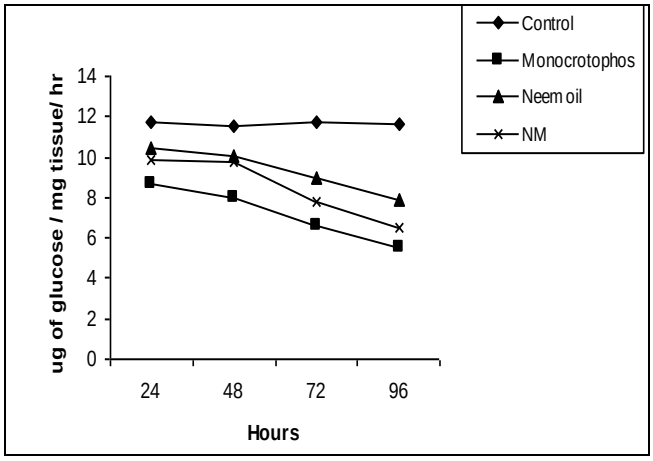

Fig 1: Individual and combined effect of monocrotophos and neem oil on amylase activity in the foregut of *Oxya fuscovittata*.

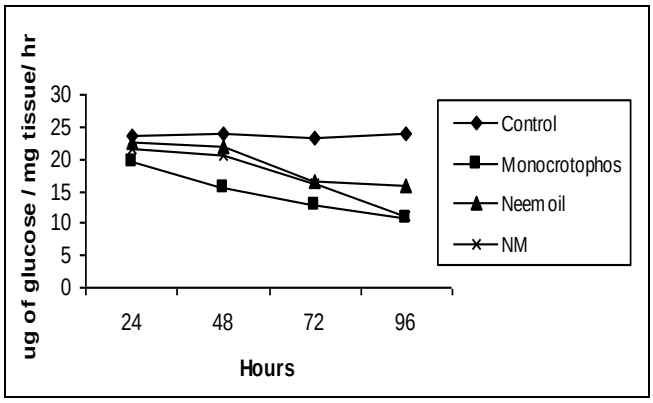

Fig 2: Individual and combined effect of monocrotophos and neem oil on amylase activity in the midgut of *Oxya fuscovittata*.

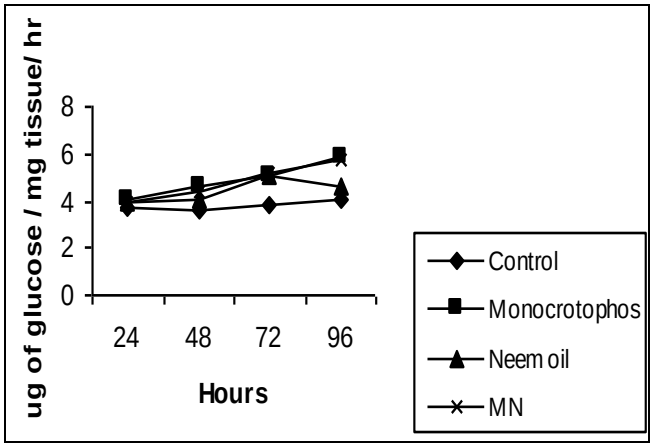

Fig 3: Individual and combined effect of monocrotophos and neem oil on amylase activity in the hindgut of *Oxya fuscovittata*.

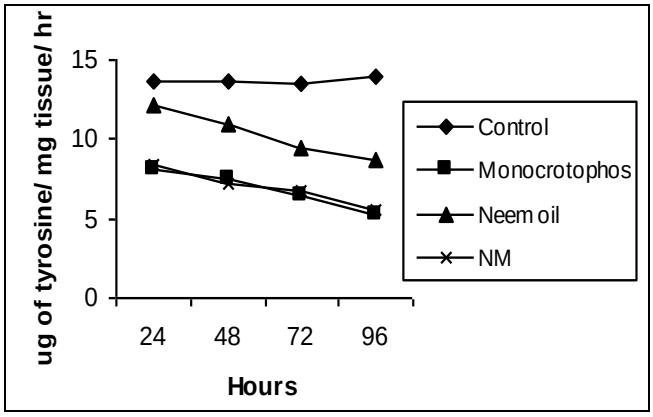

Fig 4: Individual and combined effect of monocrotophos and neem oil on protease activity in the foregut of *Oxya fuscovittata*.

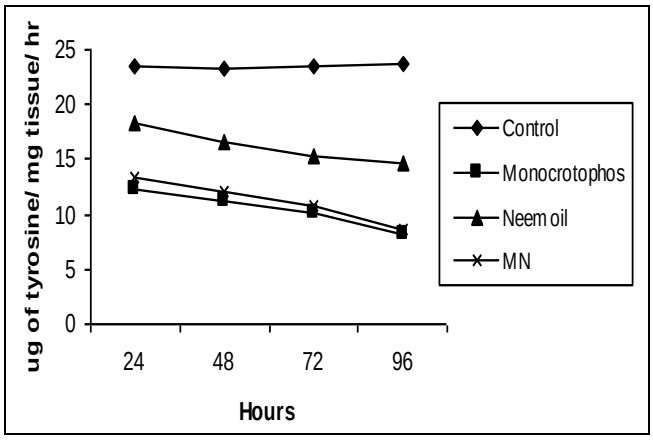

Fig 5: Individual and combined effect of monocrotophos and neem oil on protease activity in the midgut of *Oxya fuscovittata*.

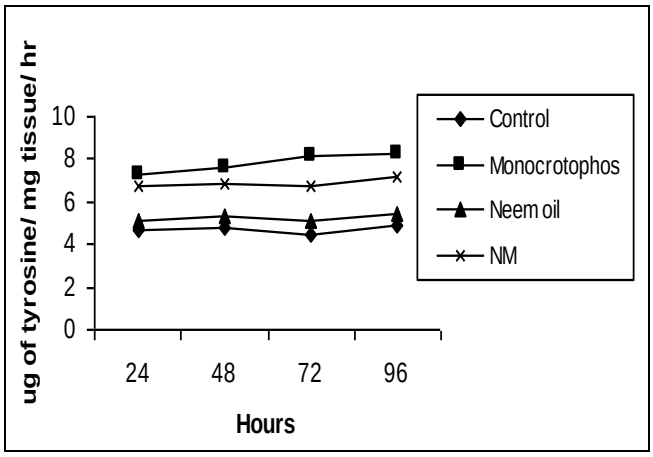

Fig 6: Individual and combined effect of monocrotophos and neem oil on protease activity in the hindgut of *Oxya fuscovittata*.

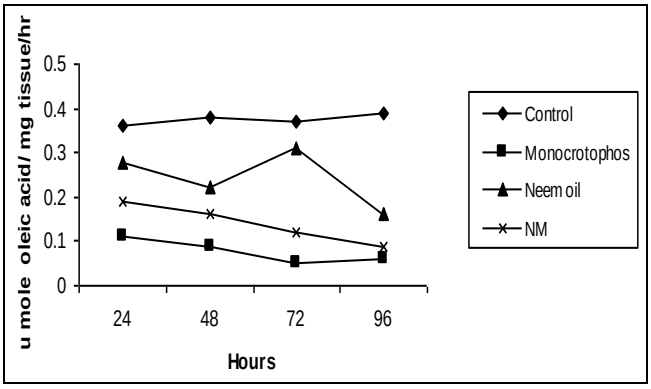

Fig 7: Individual and combined effect of monocrotophos and neem oil on lipase activity in the foregut of *Oxya fuscovittata*.

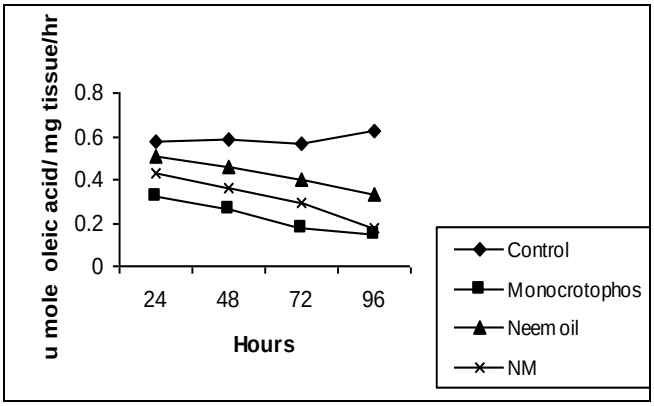

Fig 8: Individual and combined effect of monocrotophos and neem oil on lipase activity in the midgut of *Oxya fuscovittata*.

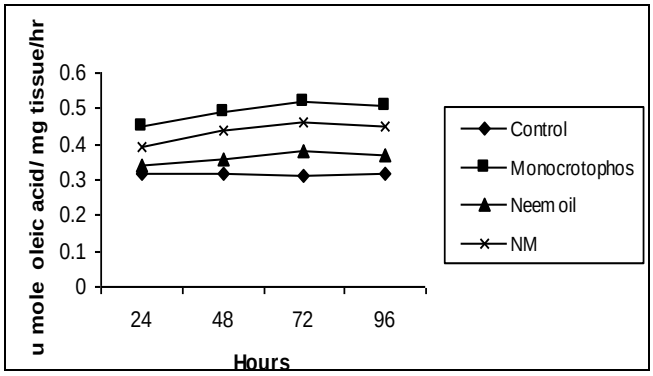

Fig 9: Individual and combined effect of monocrotophos and neem oil on lipase activity in the hindgut of *Oxya fuscovittata*.

Conclusion

In conclusion, the present study revealed that the analysis of digestive enzymes such as, amylase, protease and lipase may be useful to find out the toxicity index of monocrotophos and neem oil in grasshoppers.

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