Interaction of Methomyl with Brown Garden Snail, *Eobania vermiculata* (Müller), and White Snail, *Theba pisana* (Müller) Acetylcholinesterase Activity

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ABSTRACT

Comparative toxicity of methomyl against two land snails *Eobania vermiculata* and *Theba pisana* was investigated. The Michaelis constant (K_m) and maximal velocity (V_{max}) values of *E. vermiculata* and *T. pisana* AChE were compared. Finally, the *in vivo* and *in vitro* inhibition of *E. vermiculata* and *T. pisana* acetylcholinesterase by methomyl was studied. Results revealed that, the median lethal concentrations (LC_{50s} values) of methomyl against *T. pisana* were 58.85, 18.71 and 3.87 % after 24, 48 and 72hrs post-treatment,respectively. On the other hand, LC_{50s} values of methomyl against *E. vermiculata* were 2.94, 0.92 and 0.38 % after the same time, respectively. The K_m values were 0.098 and 0.042 mM and V_{max} values were 0.042 and 0.052 mmole/min for *E. vermiculata* and *T. pisana* AChE, respectively. Specific activity of *T. pisana* AChE is 1.43 times the *E. vermiculata* AChE specific activity. When *T. pisana* was exposed to methomyl at concentrations of 375, 750 and 1875 ppm the inhibition percentages of AChE were 88.74, 89.44 and 92.25%, respectively. On the other hand, methomyl revealed inhibition percentages of 42.3, 51.4 and 75.3% of *E. vermiculata* AChE activity at concentrations of 37.6, 75.2 and 188.0 ppm, respectively. The *in vitro* studies showed that, the sensitivity of *T. pisana* AChE (I_{50} = 1.3 µM) is 4.1 times more than the sensitivity of *E. vermiculata* AChE (I_{50} = 5.3 µM) to methomyl.

INTRODUCTION

Snails are the most serious agricultural pests worldwide. The terrestrial snails are economic pests attacking different crops (Glen and Wilson, 1997, and Glen *et al.,* 2000). Among the most serious land snails that attacking the agricultural crops in Egypt are the brown garden snail, *Eobania vermiculata* (Müller), and the white snail, *Theba pisana* (Müller). They are most active during the night and early morning as the environment is damp (El-Okda, 1980).

There are three common methods for controlling these pests: mechanical, biological, and chemical methods. Today chemical control is still one of the most effective methods, particularly over large areas (Moran *et al*., 2004; El-Shahaat *et al*., 2005 and Ghoneim, 2006). Among the chemical pesticides which successfully used in land snails control is methomyl. Methomyl is a carbamate insecticide which inhibiting the acetylcholinesterase (AChE). In this study, the comparative toxicity of methomyl against two land snails *E. vermiculata* and *T. pisana* was investigated. The Michaelis constant (K_m) and maximal velocity (V_{max}) values of *E. vermiculata* and *T. pisana* AChE were compared. Finally, the *in vivo* and *in vitro* inhibition of *E. vermiculata* and *T. pisana* acetylcholinesterase by methomyl was studied.

MATERIALS AND METHODS

Land snails: Adults of both *E. vermeculata* and *T. pisana* were collected for laboratory experiments during April, 2012 from El-Maamoura region, Alexandria. These snails were transferred to plastic cups covered with cloth netting and maintained under laboratory conditions of 27 ° C and 65% R.H. Snails were daily fed on lettuce leaves for two weeks to be acclimatized to these conditions. Dead snails were removed as soon as possible.

Insecticide: The technical grade of methomyl and its formulation; Lannate 90® SP were obtained from E.I.du Pont de Nemours & Co.

Molluscicidal activity of methomyl against *E. vermiculata* and *T. pisana*: Homogenous discs of lettuce leaves were dipped in series of the formulated methomyl (Lannate 90® SP) for 5 minutes and left for dryness. The treated lettuce discs were transferred into plastic cups and 10 adult snails were placed into each cup. Each concentration had three replicates. Untreated lettuce disks were used as check treatment. Mortality percentages were recorded after 24, 48 and 72 h. post-treatments. Mortality values were corrected according to the Abbott equation (Abbott, 1925) and subjected to probit analysis (Finney, 1971). Concentrations which cause 50% mortality of treated snails LC_{50} values were calculated.

Biochemical studies on the AChE of *E. vermiculata* and *T. pisana*:

a. **Tissue preparation**: The mollusca shells were removed, then the soft tissues were homogenized in 0.1M phosphate buffer pH 8 (1:10 w/v) using a glass homogenizer. The homogenates were centrifuged at

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5000 rpm for 30 minutes using a cooling centrifuge at 4°C .The supernatant was used as an AChE enzyme source.

b. **Enzyme activity measurements**: The spectrophotometric method of Ellman *et al*. (1961) was used, with acetylthiocholine iodide (ASChI) as a substrate. In a typical assay 150 µl of the enzyme preparation were added to 3 ml of a 1:1 mixture of 2 mM substrate solution and 2 mM dithiodinitrobenzoic (DTNB). The final concentration of substrate and DTNB in the assay mixture was 1 mM. The change in absorption at 412 nm was monitored on Milton Roy spectronic 601. An assay mixture without enzyme was used as blank.

c. Determination of Michaelis constant (K_m) and maximal velocity (V_{max}): Michaelis constant (K_m) and maximal velocity (V_{max}) values of *E. vermiculata* and *T. pisana* AChE were determined by incubating the optimum protein amount with varying concentrations of ATChI. The Line weaver-Burk plot (1/V vs. 1/[S]) was used to determine the K_m and V_{max} values.

d. *In vivo* inhibition of *E. vermiculata* and *T. pisana* AChE by methomyl: *E. vermiculata* and *T. pisana* snails were fed on lettuce leaves dipped in the formulated methomyl (Lannate 90® SP) solutions at 1/10 LC_{50}, 1/5 LC_{50} and 1/2 LC_{50}. The snails in the check treatment; control were fed on untreated lettuce leaves. The samples were prepared to obtain the enzyme source as mentioned above. Enzyme activity in the different treatments was measured as mentioned above and inhibition percentages were calculated.

e**. *In vitro* inhibition of *E. vermiculata* and *T. pisana* AChE by methomyl:** The *in vitro* inhibition of *E. vermiculata* and *T. pisana* AChE by methomyl was determined by incubating the enzyme source with different methomyl concentrations; 0.313, 0.625, 1.25, 2.5, 5.0 and 10.0µM (dissolved in 10 µl acetone) for 15 min at 37ºC. The residual activity was measured spectrophotometrically at 412 nm as described before. The inhibition percentages were calculated and used to calculate the concentrations which cause 50% of the enzyme inhibition (I_{50}).

f. Determination of protein: Protein estimation has been carried out according to the method of Lawry *et al*. (1951). Aliquots (600 µl each) of diluted protein solution were added to 2.5 ml of reagent C (freshly prepared from reagent B [0.5 % $CuSO_4.5H_2O$ in 1% Na-K tartarate] and reagent A [2% Na_2CO_3 in 0.1 M NaOH] in a ratio of 1:50, respectively. The mixture was vortexed and incubated for 20 min at room temperature. At the end of incubation, 250 µl of folin reagent (1 N) was added, vortexed vigorously and incubated for 30 min. and the developed color was measured at 750 nm using spectrophotometer (Milton Roy spectronic 601) against the blank. The standard curve of protein was previously established using different concentrations of bovine serum albumin (BSA) (10-100 µg/ µl).

Statistical analysis: Probit analysis was carried out using a PC probit program adopted by Finney (1971). Whenever appropriate, data were analyzed by using the least significant difference ($LSD_{0.05}$).

RESULTS AND DISCUSSIONS

Molluscicidal activity of methomyl against *E. vermiculata* and *T. pisana*: Terrestrial snails, *E. vermiculata* and *T. pisana* are considered as dangerous species in the Delta region, especially in northern areas of Egypt. They are also known as destructive pests, causing severe damage to vegetables, ornamentals, and citrus trees (EL-Wakil and Attia, 1999). Toxicity of methomyl against *E. vermiculata* and *T. pisana* by the dipping technique, using lettuce discs was evaluated (Tables 1 and 2). The median lethal concentrations (LC_{50s} values) of methomyl against *T. pisana* were 58.85, 18.71 and 3.87 % after 24, 48 and 72hrs post-treatment (Table 1).

On the other hand, LC_{50s} values of methomyl against *E. vermiculata* are illustrated in Table (2). They were 2.94, 0.92 and 0.38 % after 24, 48 and 72 h post-treatment, respectively. It was obvious that the toxicity of methomyl was increased as the time of exposure increased for both *E. vermiculata* and *T. pisana*. The toxicity of methomyl against *T. pisana* was ten times less than against *E. vermiculata* after 72 hrs of exposure.

Table 1. LC_{50} values of methomyl against *T. pisana* after different assay peroides

Exposure time (hr)	LC_{50} (%)	Lower limite	Upper limite	Slope ± S.E	Regression equation
24	58.85	15.64	224.14	0.95 ± 0.07	y = -4.543 + 0.952X
48	18.71	14.34	24.23	1.06 ± 0.01	y = -3.462 + 1.058X
72	3.87	2.74	5.11	1.01 ± 0.01	y = -2.587 + 1.005X

Table 2. LC_{50} values of methomyl against *E. vermiculata* after different assay peroides

Exposure time (hr)	LC_{50} (%)	Lower limite	Upper limite	Slope ± S.E	Regression equation
24	2.94	1.66	5.21	1.81± 0.17	y = -8.072 + 1.806x
48	0.92	0.78	1.08	1.95 ± 0.04	y = -7.745 + 1.954x
72	0.38	0.31	0.46	1.50 ± 0.03	y = -5.371 + 1.503x

The potency of methomyl as an efficient oxime carbamate compound against gastropoda is coincided with Radwan *et al.* (1992) who indicated that the LT_{50} values of aldicarb, methomyl and oxamyl carbamates were 5.7, 2.31 and 3.97 days, respectively.

Biochemical studies on the AChE of *E. vermiculata* and *T. pisana* snails: The cholinesterase enzymes may be used to provide inhibition data which can be of great importance, since the enzymes from different species may differ in their substrates and inhibitor specificities. Significant evidences have been accumulated indicating that AChE possess in addition to an anionic site in the catalytic centre, peripheral anionic sites where ligands bind and exerted a regulatory role on the enzyme activity (Eldefrawi, 1985).

The ability of AChE to undergo ligand-induced conformational changes was first suggested by Changeux (1966), who observed that gallamine inhibition of *Torpedo marmorata* AChE was not strictly competitive in nature and these results were confirmed by Moss and Henderson (1988). This finding led to the proposal that AChE possesses peripheral anionic sites, distinct from the catalytic site, whereby binding of cationic ligand, could influence the catalytic properties of the enzyme.

Michaelis constant (K_m) and maximal velocity (V_{max}): The rate of any enzyme-catalyzed reaction depends directly on the concentration of the enzyme, as well as, it depends on the substrate concentration. With fixed enzyme concentration, an increase of substrate will result at first in a very rapid rise in velocity or reaction rate. As the substrate concentration continued to be increased, however, the increase in the rate of reaction began to slow down until, with a large substrate concentration, no further change in velocity could be achieved. The velocity of the reaction obtained at this high substrate concentration was defined as the maximum velocity of the enzyme-catalyzed reaction under the specified conditions. The substrate concentration that required to yield half the maximum velocity (*V/2*) is an important constant in enzyme chemistry (the concentration of substrate at which V= 1/2 V_{max}). This is value defined as Michaelis constant (K_m). Under suitable conditions of temperature, pH, and ionic strength of the buffer constant, this constant (K_m) approximated the dissociation constant of an enzyme-substrate complex (Conn and Stumpf, 1966).

In the present work, K_m and V_{max} values for *E. vermiculata* and *T. pisana* AChE were conducted under the suitable incubation time and protein concentrations to compare between the AChE from the two sources (Figure 1 & 2). Data obtained in this study indicated that the K_m and V_{max} values of *E. vermiculata* and *T. pisana* AChE were different. The K_m values were 0.098 and 0.042 mM for *E. vermiculata* and *T. pisana* AChE, respectively. This means that the affinity between the *T. pisana* AChE and ATChI was higher than the affinity between the *E. vermiculata* AChE and ATChI. The V_{max} values were 0.042 and 0.052 mmole/min for the AChE of *E. vermiculata* and *T. pisana*, respectively (Figs. 1 and 2). The specific activity of *T. pisana* AChE is 1.43 times the *E. vermiculata* AChE specific activity (Table 3). Therefore, the enzyme from the two sources is different.

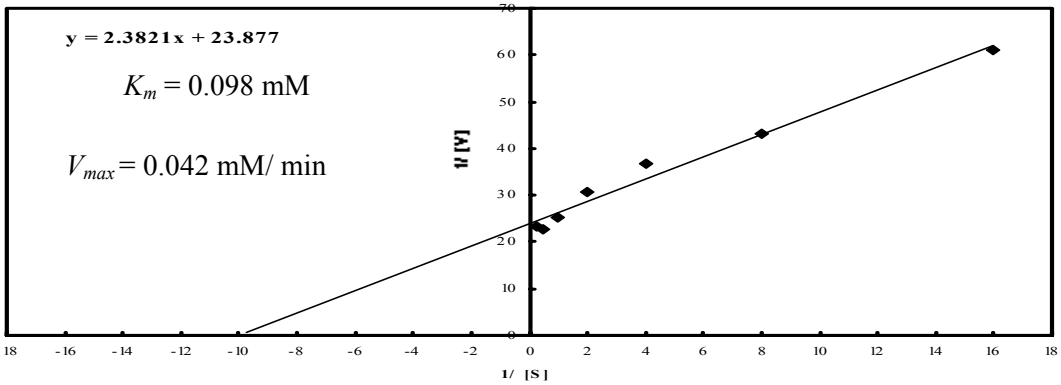

Fig. 1. Lineweavee-Burk plot for AChE from *E. vermiculata*

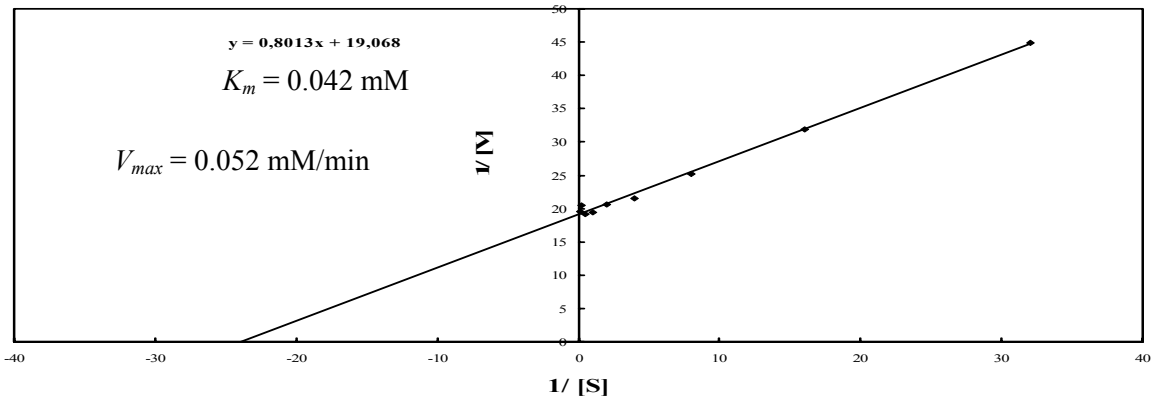

Fig. 2.Lineweaver-Burk plot from AChE from *T. pisana*.

Table 3. Specific activity of *E. vermiculata* and *T. pisana* AChE:

Snail	Specific activity (Δ OD_{412} / mg protein / min)
E. vermiculata	0.0099 ± 0.00039
T. pisana	0.0142 ± 0.00056

***In vivo* inhibition of *E. vermiculata* and *T. pisana* AChE activity by methomyl:**

The *in vivo* inhibition of *E. vermiculata* and *T. pisana* AChE activity by sub-lethal concentrations of methomyl is presented in Tables (4 and 5). When *T. pisana* snails were exposed to methomyl at concentrations of 375, 750 and 1875 ppm the inhibition percentages of AChE were 88.7, 89.4 and 92.3%, respectively (Table 4). On the other hand, methomyl revealed inhibition percentages of 42.3, 51.4 and 75.3% of *E. vermiculata* AChE activity at concentrations of 37.6, 75.2 and 188.0 ppm, respectively (Table 5). From these results, it is clear that the inhibition of *E. vermiculata* and *T. pisana* AChE activity by methomyl is concentration dependent. These results are in agreement with those of Singh and Singh (2003) who stated that the inhibition of AChE activity by methomyl was both concentration and time dependent.

The inhibition of the AChE activity from land snails by methomyl had been studied by Adrian *et al*. (1947), who reported that the primary reason of mortality of poisoned snails by organophosphate and carbamate pesticides was due to the inhibition of AChE activity. In insects, both organophosphate and carbamate insecticides were effective as AChE inhibitors. In contrast, organophosphate and many of the carbamate compounds were ineffective when they were tested as molluscicides (Casida and Quistad, 2004). This information puts a question mark on the mode of action of methomyl as a molluscicide. In the present investigation, although methomyl inhibited *T. pisana* AChE activity (both *in vivo* and *in vitro*) more than with *E. vermiculata* AChE, methomyl proved to be more toxic to *E. vermiculata* than *T. pisana***.** These findings mean that land snails AChE is not the only target of methomyl.

Table 4. *In vivo* inhibition of AChE from *T. pisana* by methomyl

Conc.(ppm)	Specific activity (Δ OD412 / mg protein / min)	Activity	Inhibition
Control (0.0)	0.0142 [a*] ± 0.000558	100	0.0
375	0.0016 [b] ± 0.000197	11.27	88.7
750	0.0015 [b] ± 0.0000283	10.56	89.4
1875	0.0011 [b]	7.75	92.3

	±0. 000076

L.S.D = 0.00083
*Means followed by the same letter(s) are not significantly different.

Table 5. *In vivo* inhibition of AChE from *E. vermiculata* by methomyl

Conc.(ppm)	Speific activity (Δ OD_{412} / mg protein.min)	Activity (%)	Inhibition (%)
Control	0.0099[a*] ± 0.00039	100	0.0
37.6	0.0057 [b] 0.00031±	57.7	42.3
75.2	0.0048 [c] ± 0.00004	48.6	51.4
188.0	0.0024 [d] ± 0.00007	24.7	75.3

L.S.D = 0.00097
*Means followed by the same letter(s) are not significantly different.

Table 6. *In vitro* inhibition of AChE from *E. vermiculata* by methomyl

Conc.(µM)	Specific activity (Δ O.D/mg_{412} protein/min)	Activity (%control)	Inhibition (%)	I_{50} (µM)
Control (0.0)	0.0139 ± 0.000694	100	0.00	5.3
0.313	0.0138 ± 0.000120	99.94	0.06	
0.625	0.0107 ± 0.000300	76.98	23.02	
1.250	0.0105 ± 0.000208	75.54	24.55	
2.500	0.0088 ± 0.000208	63.31	36.69	
5.000	0.0072 ± 0.000267	51.80	48.20	
10.00	0.0056 ± 0.000173	40.29	59.71	

Table 7. *In vitro* inhibition of AChE from *T. pisana* by methomyl.

Conc. .(µM)	Specific activity (Δ O.D/mg_{412} protein.min)	Activity (%Control)	Inhibition (%)	I_{50} (µM)
Control (0.0)	0.0186 ± 0.000609	100	0.00	1.3
0.313	0.0171 ± 0.000067	91.94	8.06	
0.625	0.0143 ± 0.000145	76.88	23.12	
1.250	0.0094 ± 0.000763	50.70	49.30	
2.500	0.0069 ± 0.000385	37.26	62.74	
5.000	0.0045 ± 0.000200	24.19	75.81	
10.00	0.0032 ± 0.000329	17.37	82.63	

This may explain why methomyl has a molluscicidal activity while organophosphorous and other oximecarbamate insecticides do not have. Salama *et al.* (2005) stated that the mode of action of methomyl and carbofuran against land snails could be due to the induction of oxidative stress in addition to their anti-cholinesterase potencies.

***In vitro* inhibition of *E. vermiculata* and *T. pisana* AChE activity by methomyl:**

The *in vitro* inhibition of *E. vermiculata* and *T. pisana* AChE activity by methomyl is shown in Tables (6 and 7). Methomyl at concentrations of 0.313, 0.625, 1.25, 2.5, 5.0 and 10.0µM revealed inhibition percentages of 0.06, 23.02, 24.46, 36.69, 48.20 and 59.71%, respectively, on the AChE from *E. vermiculata*. The concentration of methomyl which inhibits 50% of the enzyme activity (I_{50}) is 5.3 µM (Table 6). The same conc. of methomyl revealed inhibition percentages of 8.06, 23.12, 49.3, 62.74, 75.81 and 82.63%, respectively, of AChE from *T. pisana***.** The

I_{50} value is 1.3 µM (Table 7). The sensitivity of *T. pisana* AChE is 4.1 times more than the sensitivity of *E. vermiculata* AChE to methomyl.

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الملخص العربي

تداخل مبيد الميثوميل مع نشاط انزيم الأستيل كولين أستريزفى نوعين من القواقع الأرضيه (*Theba pisana* و *Eobania vermiculata*)

السيد حسن عشرة، محمد سعيد الشحات وشيماء سعيد عبد النبى

تمت مقارنة سمية مبيد الميثوميل على قوقعى *Eobania vermiculata* و *Theba pisana* كما تم تقدير قيم ثابت ميخائيلس(K_m) وأقصى نشاط(V_{max}) لانزيم الأستيل كولين أستريز لذات القوقعين وتم دراسة التثبيط الداخلى والخارجى لنفس الأنزيم ولذات القوقعين ولقد أظهرت الدراسه النتائج الآتيه: التركيز اللازم لموت ٥٠% (LC_{50}) من قوقع *Theba pisana* كانت ٥٨,٨٥، ١٨,٧١ و٣,٨٧% بعد ٢٤، ٤٨و ٧٢ من التعرض ومن ناحية أخرى كانت هذه القيم ٢,٩٤ ،٢,٩٠ و٣٨,% لقوقع *E. vermiculata* لنفس فترات التعرض على الترتيب. كانت قيم (K_m)٠٩٨, و٠٤٢ , مللى مولر وقيم (V_{max}) كانت ٠٤٢,و٠٥٢, ملى مول/دقيقه لانزيم الأستيل كولين أستريز لقوقعى *Eobania vermiculata* و *Theba pisana* على الترتيب. النشاط النوعى لأنزيم الأستيل كولين أستريز لقوقع *Theba pisana* كان ١.٤٣ مرة بالنسبة للنشاط النوعي لقوقع*Eobania vermiculata* . عند تعرض قوقع *T. pisana* لتركيزات ٣٧٥،٧٥٠ و١٨٧٥ جزء فى المليون من مبيد الميثوميل أدى ذلك الى تثبيط انزيم الأستيل كولين أستريز بنسبة ٨٨,٧٤، ٨٩,٤٤، ٩٢,٢٥% مقارنة بالغير معامل على الترتيب ومن ناحية أخرى عند تعرض قوقع *E. vermiculata* لتركيزات٣٧.٦، ٧٥.٢ و ١٨٨ جزء فى المليون لنفس المبيد كانت نسب التثبيط ٤٢,٣، ٥١,٤، ٧٥,٤٢ % على الترتيب. حساسية انزيم الأستيل كولين أستريز لقوقع *Theba pisana*(I_{50} = 1.3 ميكرومولر) وكانت١ ٤ مرة قدر حساسية نفس الإنزيم لقوقع لقوقع *Eobania vermiculat*حيث كانت (I_{50}) هى 5.3 ميكرومولر.