In Vitro Biochemical Interactions of some Neonicotinoids on the Activity of AChE Extracted from Different Insects.

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ABSTRACT

The biochemical interactions of five insecticides (nicotine, dinotefuran, acetampirid, thiacloprid and imidacloprid) on AChE isolated from *Spodoptera littoralis*, 4th instar larvae and adults of both *Musca domestica* and *Culex pipienes* were investigated. The effect of the tested insecticides on the enzymatic activity (*in vitro)* proved that nicotine incressed the percentage of AChE inhibition. The results showed reduction in the enzymatic activity of AChE obtained from *S. littoralis*, *M. domestica* and *C. pipienes* was induced by nicotine, dinotefuran, acetampirid, thiacloprid and imidacloprid, respectively. The sensitivity of AChE activity to the five tested insecticides was measured by the I_{50} values. The I_{50} values were 0.17, 0.25 and 0.28µM for nicotine, 0.22, 0.30 and 0.34µM for dinotefuran, 0.27, 0.36 and 0.39µM for acetampirid, 0.33, 0.43 and 0.48µM for thiacloprid and 0.35, 0.46 and 0.52µM for imidacloprid in *S. littoralis*, *M. domestica* and *C. pipienes*, respectively. I_{50} values proved that nicotine, dinotefuran and acetampirid were the stronger inhibitors on AChE activity than thiacloprid and imidacloprid, respectively. Furthermore, the AChE activity parameters such as K_m, V_{max} and the inhibition constant K_i values were determined. The K_i values were 12, 15 and 20µM for nicotine, for *S. littoralis*, *M. domestica* and *C. pipienes*, respectively. Also, K_i values were 18, 20 and 26 µM for dinotefuran for the three tested insects, respectively and K_i values of acetampirid were 23, 28 and 34µM, respectively. These values were 27, 36 and 45µM for three sources, respectively in the case of thiacloprid. On the other hand, K_i values were 34, 40 and 50µM for three sources, respectively in the case of imidacloprid. The data obtained showed that AChE activity was competitively inhibited by all the tested insecticides. The results of the present study may add some forward steps to use these tested insecticides as non conventional insecticides especially against those that would be tested insects. So, the tested compounds can be involved in important steps necessary for successful IPM programmes applied against those insects species.

INTRODUCTION

Most commercially important insecticides are neuro-toxins that act on ion channels, receptors or enzymes within the insect nervous system. The insecticide market has been dominated by the organophosphate, carbamate, (which inhibit AChE) and pyrethroide classes of insecticides which act on valage-gated sodium channels (Argentine *et al*., 2002). However, most of them does not give satisfactory results, probably because of resistance development (Issa *et al*., 1984; Keddis *et a*l., 1988;

El-Sayed and Abdallah, 1988 & Ishaaya and Klein, 1990). There is a continuing need for new safe, effective and economical insecticides for crop protection and public health (Casida and Quistad, 2005). The searsh for new

insecticides is an ongoing process depend on many factors including the ability of insect to develop resistance to conventional materials (Cochran, 1990). High level of resistance to the synthetic insecticides had increased due to the intensive application of such insecticides for controlling *S. littoralis* larvae (Ishaaya and Klein, 1990; Martin *et al*., 2000 & El-Aw *et al*., 2002). More researches were done on insecticides resistance of public health pests such as *M. domestica* (Plapp, 1984 and Keiding, *et al*., 1991 & Soderlund and Knipple, 2003), and *C. pipienes* (Priester and Georghiou, 1979; Mouches *et al*., 1986; Corbel *et al*., 2003 and 2004; Darriet *et al*., 2003; Bonnet *et al*., 2004 & Robyn *et al*., 2004). Neonicotinoids are the most promising insecticides acting on insect nAChRs (Casida and Quistad, 1998; Matsuda *et al*., 2001 & Millar and Denhalm, 2007) such compounds can be effective on those resistant insects.

The present study was undertaken to investigate the effect of four neonicotinoids compared with nicotine against three field populations of the most injurious pests in Egypt, (S. littoralis, M. domestica and C. pipienes) which developed very high levels of resistance to the traditional insecticides used for controlling them. The present study aimed to investigate in vitro the biochemical interaction of five insecticides with AChE activity isolated from S. littoralis, M. domestica and C. pipienes. The kinetic parameters of AChE were also estimated. The attention was given for this new insecticide class as a new aspect that can be used in IPM programs for such deleterious subjected pests.

MATERIALS AND METHODS

Insect :

Spodoptera littoralis: The cotton leafworm egg masses were collected from cotton fields and reared under laboratory conditions of 27±2^{0}C and 65-70%RH. The housefly *Musca domestica,* and mosquito *Culex pipienes,* were collected as adults from fields and reared under the same laboratory conditions. All insects used in this study were obtained from Abeis area at Alexandria Governorate.

Chemicals :

The tested compounds used in this study were nicotine which has been provided as technical grade insecticide from MERCK-Schuchardt, Schuchardt, 8011Hohenbrunn bei Munchen; thiacloprid 48% SC, obtained from MyTrade Co., dinotefuran 20% SG; imidacloprid 35% SC, obtained from NM Agro Egypt and acetampirid 20% SP obtained

from AL-Esraa Co. Egypt. Stock solutions of these formulated insecticides were prepared in distilled water except nicotine which has been prepared in pure acetone.

AChE Preparation and Activity Assay :

The dissected head capsules of *Spodoptera littoralis* (fourth instar larvae) and the adult of either *Musca domestica* or *Culex pipienes* (adults) were homogenized in tris-HCl buffer (pH 7.4) at 30 individuals/30ml buffer, with polytron mixer (at 50% power for 50sec.), then subjected to alow speed centrifuge at 5,000rpm for 15min at 4^0C. The resulting supernatant was centrifuged at 15,000rpm for 20min at 4^0C. The supernatant was them centrifuged at 25,000rpm for 1hr at 4^0C. Pellets were resuspended in 1ml of tris-HCl buffer (pH 7.4) and they were used as enzyme source.

The AChE activity measurements were done according to method reported by Ellman *et al .* (1961). This method is based on the hydrolysis of acetylthiocholine iodide (ATChI) as substrate by enzyme to produce thiocholine and acetic acid. Thiocholine reacts with 5,5-dithio bis-(2-nitrobenzoic acid), "DTNB" to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of color production as a function of enzyme activity is measured spectrophotometrically at λ412nm.

Enzyme specific activity was computed as mg protein/hr. The protein content in prepared homogenates of *S. littoralis* was assayed spectrophotometrically by the method of Lowery *et al*. (1951) at λ750nm using Bovine Serum Albumin (BSA) as a standard protein.

***In Vitro* Inhibition and Kinetics of AChE:**

The inhibition of AChE activity was determined in all tested sources using the LC_{50} values which have been calculated previonsly by a bioassay of each of the five tested insecticides as inhibitors. The inhibition of AChE was evaluated to determine enzyme kinetic parameters. The method of Dixon and Webb (1964) was adopted to draw the Dixon-plots by plotting 1/V versus concentrations of the inhibitor at two concentrations (5 and 10mM) of the substrate acetylcholine iodide (the substrate of AChE).

Estimation of I_{50} value (the concentration of the inhibitor which inhibits 50% of the enzyme activity) was carried out by preincubating the enzyme with the inhibitor for 30min. Also, K_i (the inhibition constant) values for each inhibitor were estimated from Dixon-plot.

Michaelis-Menten Kinetics (K_m & V_{max}) values were calculated by a linear regression of 6 points on each Lineweaver and Burk Plot (1934).

RESULTS AND DISCUSSION

Specific Activity of AChE From Different Insects:

Results recorded in Fig. 1 show the specific activity (λ_{max} 412 mg^{-1} protein hr^{-1}) of the AChE, isolated from different insects. Maximum value for specific activity of the AChE was found in the brain preparations of the *S. littoralis* and *M. domestica* and they

were higher than that value of specific activity of the AChE that isolated from *C. pipienes* which have been calculated in the whole homogenate of insect adults. Activity of AChE was greatest (39.31±0.05 & 31.86±0.05) in *S. littoralis* and *M. domestica*, respectively, and it was moderate in the *C. pipienes* (27.09±0.11). Also, the observed AChE activity level in *S. littoralis* and *M. domestica* was high than that of *C. pipienes.*

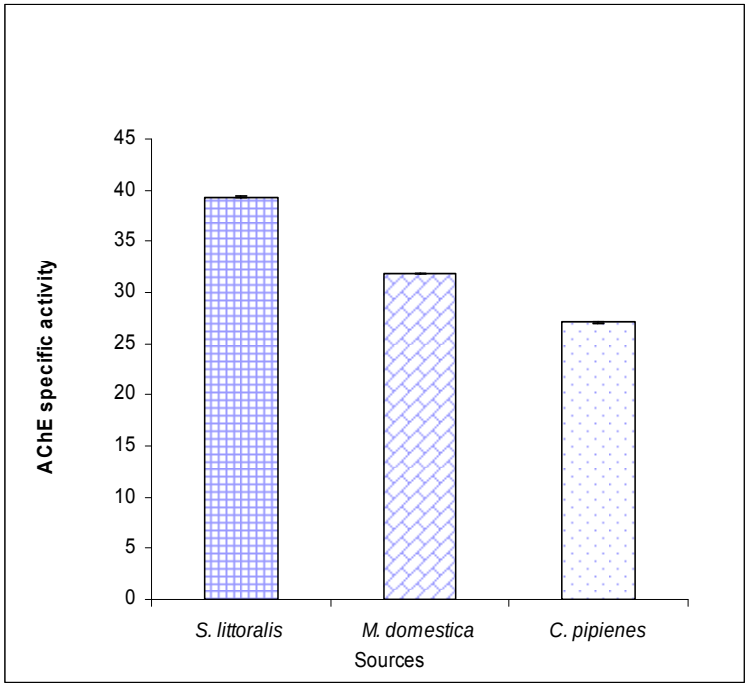

Fig. 1: The specific activity of AChE isolated from three insects.

Kinetic Parameters of AChE Inhibition:

The kinetic studies were conducted to evaluate the effects of nicotine, dinotefuran, acetampirid , thiacloprid and imidacloprid on AChE

activity in those tested field strains. Table 1 shows the obtained Lineweaver-Burk (L-B) plots for AChE in all three tested field strains. Table 1 shows the obtained values of K_m (Michaelis,constant) and V_{max} of the AChE. The changes in K_m values of AChE between the tested field strains indicate changes in the affinities and those results of the present study are strongly emphasized by the kinetic studies of Gonzalez *et al.* (1990) who found that the calculated K_m was 0.22mM for the AChE of the gastropod *Concholepas concholepas.*

Table 1: Michaelis-Menten kinetics of the AChE from different insects.

Insects	K_m(mM)	V_{max}(mM)
S. littoralis	0.1	0.77
M. domestica	0.2	0.59
C. pipienes	0.3	0.53

On the other hand, the present results clearly show that the V_{max} values of AChE are obviously high. These points of high substrate turnover may reflect the physiological importance of AChE in the function of the nervous tissue (El-Aw and Hashem, 2001). The V_{max} values were generally higher in *Spodoptera littoralis* and *Musca domestica* than that of *Culex pipienes*. This fact indicated that there are more active sites on the AChE obtained from the brain.

***In Vitro* Inhibition of AChE Activity from Different Sources:**

Effects of the tested concentrations of each tested insecticide on the *in vitro* inhibition of AChE extracted from different sources were investigated. The obtained results illustrated in Fig. 2 show the *in vitro* interaction of five tested insecticides on AChE activity *S. littoralis, M. domestica* and *C. pipienes*. It is quite clear that nicotine acts as potential inhibitor of AChE obtained from different insects. Also, nicotine, dinotefuran and acetampirid exhibited the highest percentages of reduction on AChE activity. In case of nicotine, the inhibition percentages were 89.5; 85.3 and 74.6% for *S. littoralis*, *M. domestica* and *C. pipienes*, respectively, while in the case of dinotefuran, these values were 85.7; 82.5 and 68.3% for the tested three sources, respectively. Acetampirid, gave inhibition percentages of 79.6; 76.1 and 63.7%. On the other hand, in case of thiacloprid, the reduction of AChE activity were 74.5; 70.6 and 57.5%, respectively for three sources, while those values of imidacloprid were 68.7; 65.7 and 53.8%, respectively.

The results show that the cotton leafworm *S. littoralis* was the most sensitive insect followed by the house fly *M. domestica* and *C pipienes* for the all tested insecticides and that was due to the inhibition of AChE activity of each of the tested insects by those insecticides. The sensitivity of AChE of *S. littoralis* and *M.domestica* (as brain preparations) was high when was compared with *C. pipienes* (whole homogenate).

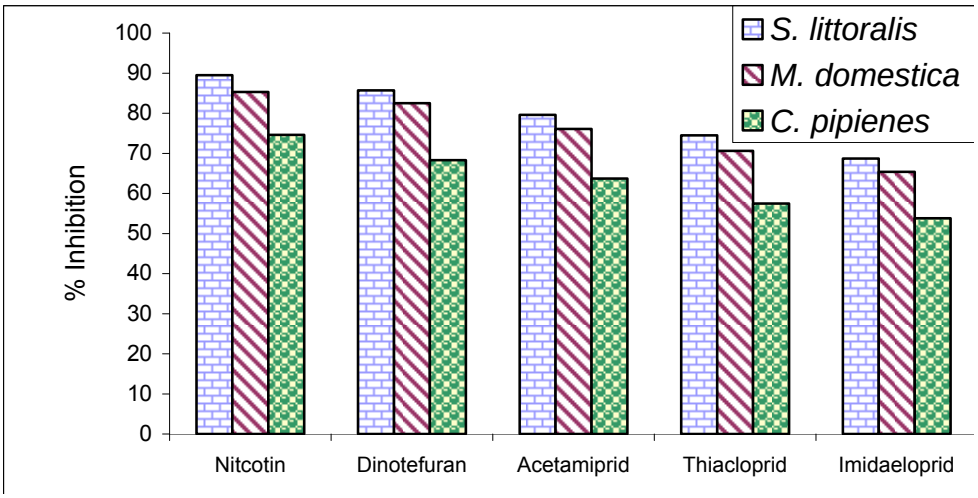

Fig. 2: *In vitro* inhibition of AChE of different insect sources by some insecticides

To characterize more details about the *in vitro* inhibition of AChE by the tested inhibitors, the K_i values of each inhibitor were estimated from the graphical method of Dixon and Weep (1964) (Table 2). The obtained data proved that each of the five tested insecticides showed competitive inhibition on AChE activity and the K_i values were 12, 15 and 20μM for nicotine, for *S. littoralis*, *M. domestica* and *C. pipienes*, respectively. Also, K_i values were 18, 20 and 26 μM for dinotefuran, respectively and those K_i values of acetampirid 23, 28 and 34μM, respectively. These values were 27, 36 and 45μM for three sources, respectively in the case of using thiacloprid. On the other hand, K_i values were 34, 40 and 50μM for three sources, respectively in case of imidacloprid.

Table (2): *In vitro* inhibition of different insects AChE by certain insecticides.

Insecticides	I_{50} (µM)			K_i (µM)		
	S. littoralis	*M. domestica*	*C. pipienes*	*S. littoralis*	*M. domestica*	*C. pipienes*
Nicotine	0.17	0.25	0.28	12	15	20
Dinotefuran	0.22	0.30	0.34	18	20	26
Acetamiprid	0.27	0.36	0.39	23	28	34
Thiacloprid	0.33	0.43	0.48	27	36	45
Imidacloprid	0.35	0.46	0.52	34	40	50

The I_{50} values for the potential tested inhibitors were determined (Table 2). The recorded I_{50} values were 0.17, 0.25 and 0.28µM for nicotine for *S. littoralis*, *M. domestica* and *C. pipienes*, respectively. Meanwhile, the determined I_{50} values for dinotefuran were 0.22, 0.30 and 0.34µM, respectively. I_{50} values for acetampirid, against AChE activity were 0.27, 0.36 and 0.39µM. for three sources, respectively. In the case of thiacloprid the I_{50} values were 0.33, 0.43 and 0.48µM, respectively. Moreover, I_{50} values for imidacloprid against AChE activity were 0.35, 0.46 and 0.52µM for the three tested sources, respectively.

The present findings suggest that the increase of AChE sensitivity to the inhibition by the tested neonictinoids might be due to the interaction of these insecticides with cholinergic proteins however, nicotinic acetylcholine receptor is recognized as the primary target for the insecticidal action of nicotinoide (Aziz and Eldefrawi, 1973; Sattelle *et al*., 1976; Mansour *et al*., 1977; Gepner *et al*., 1978; Harrow *et al*., 1979; Cattell *et al*., 1980 & Sherby *et al*., 1988). Neonictinoids are reported to be nicotinic acetylcholine receptors (nAChRs) agonists (Millor and Denholm, 2007).

CONCLUSIONS

Serious problems of development of resistance to insecticides, arised and growers rarely use any tactics to prevent insecticide resistance and some of their practices actually enhanced insecticide failure. Nevertheless, more rational practices, adopted recently, have helped in delaying the onset of resistance to conventional insecticides. Neonictinoids can serve as a new insecticide class and can be as alternatives insecticides to be involved in Integrated Pest Management (IPM), especially against pests that have developed resistance to conventional

insecticides such as the cotton leafworm (that damage agricultural crops), The house flies and mosquito (that threaten public health). Neonictinoids were proved to be good AChE inhibitors for the tested insects. Therefore, it could be conclutded that use of such compounds instead of conventional hazardous insecticides may play an important role in future of insect pest management programs.

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الملخص العربى

التأثيرات الكيموحيوية لبعض المبيدات الحشرية الحديثة على نشاط أنزيم AChE المستخلص من حشرات مختلفة. خارجيا"

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المعمل المركزى للمبيدات- الصبحية- الأسكندرية- مركز البحوث الزراعية. *قسم كيمياء المبيدات- كلية الزراعة- جامعة الأسكندرية

تم دراسة التداخل البيوكيميائى لخمسة من المبيدات الحشرية (نيكوتين و دينوتفيوران و أسيتامبريد و ثياكلوبراييد و ايبداكلوبراييد) على أحد أهم الأهداف البيولوجية فى الحشرة وهو الأنزيم المحلل لأستر الأسيتايل كولين AChE حيث تم أستخلاص الأنزيم من يرقات العمر الرابع لدودة ورق القطن ومن الحشرة الكاملة لكلا" من الذباب المنزلى و الباعوض. وكذلك تم دراسة المقدرة التثبيطية للمبيدات المختبرة على النشاط الأنزيمى لأنزيم AChE فقد تم أختبار تأثير التركيز القاتل لـ 50% من الحشرات وذلك للخمس مبيدات المختبرة على النشاط الأنزيمى للـ AChE *in vitro* كما تم تقدير الـ I_{50} (تركيز المبيد اللازم لتثبيط 50% من النشاط الأنزيمى). أوضحت النتائج أن قيم I_{50} فى حالة النيكوتين كانت0.17 و 0.25 و 0.28ميكرومولر لدودة ورق القطن والذباب المنزلى و الباعوض على التوالى بينما بالنسبة

لمركب الدينوتفيوران كانت 0.22 و 0.30 و 0.34ميكرومولر وذلك لثلاثة حشرات المختبرة على الترتيب بينما كانت 0.27 و 0.36 و 0.39ميكرومولر فى حالة مركب الأسيتامبريد أما بالنسبة لمركب ثياكلوبراييد فكانت القيم 0.33 و 0.43 و 0.48ميكرومولر للثلاثة حشرات على التوالى وكذلك فى حالة مركب ايبداكلوبراييد كانت 0.35 و 0.46 و 0.52ميكرومولر للحشرات المختبرة على التوالى. وقد أظهرت المركبات المستخدمة والمختبرة تأثير تثبيطى على نشاط الأنزيم حيث أن النيكوتبن والدينوتفيوران والأسيتامبريد كانت أقوى المثبطات بالمقارنة بالمركبات ثياكلوبراييد و ايبداكلوبراييد و أيضا تم دراسة بعض الثوابت الأنزيمية مثل ثابت ميخائيل (K_m) و أقصى نشاط نوعى (V_{max}) وثابت التثبيط (K_i) فأظهرت هذه المبيدات المختبرة تثبيط تنافسى على نشاط أنزيم AChE. حيث كانت قيم الـK_i فى حالة النيكوتبن 12 و 15 و20ميكرومولر لدودة ورق القطن والذباب المنزلى و الباعوض على التوالى بينما بالنسبة للدينوتفيوران كانت 18 و 20 و 26ميكرومولر وذلك للثلاثة حشرات على الترتيب بينما كانت 23 و 28 و 34ميكرومولر فى حالة الأسيتامبريد أما بالنسبة لمركب ثياكلوبراييد فكانت القيم 27 و 36 و 45ميكرومولر وكذلك فى حالة مركب ايبداكلوبراييد فكانت القيم 34 و 40 و 50ميكرومولر للحشرات المختبرة على التوالى.

.ومن خلال نتائج هذه الدراسة فأنه يمكن إلقاء الضوء على إمكانية تلاشى التأثير الضار على البيئة نتيجة أستخدام المبيدات التقليدية الشائعة لمكافحة هذه الحشرات وبذلك يمكن إدراج المركبات المختبرة ضمن قائمة المركبات التى يمكن أستعمالها فى برامج المكافحة المتكاملة لهذه الأفات المختبرة الضارة بالمزروعات (دودة ورق القطن) والضارة بالصحة العامة (الذباب المنزلى والباعوض).