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Larvicidal, biochemical and physiological effects of acetamiprid and thiamethoxam against *Culex pipiens* L. (Diptera: Culicidae).

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ABSTRACT

The resistance of mosquitos to conventional insecticides created an urgent need for using alternative insecticides. In this study, the toxicity of the neonicotinoids (acetamiprid and thiamethoxam) was evaluated against the 3rd larval instar of *Culex pipens*. In the larvicidal assay, acetamiprid and thiamethoxam showed promising larvicidal activity against *Cx. pipiens*, with LC_{50} values of 0.0093 and 0.0305 ppm after 24 hrs, 0.0078 and 0.0206 ppm after 48 hrs and 0.0065 and 0.0137 ppm after 72 hrs of insecticidal exposure. The activity of acetylcholinesterase (AChE), carboxylesterase, (*α*-esterase), (*β*-esterase) and glutathione *S*- transferase (GST) were determined after 72 hrs of insecticidal exposure. Acetamiprid showed a significant increase in the activity level of AChE, GST, carboxylesterase, α, and β-esterases than thiamethoxam. The hemocytes of the treated 3rd larval instar of *Cx. pipens* were sensitive to both insecticides and showed remarkable deformation compared with control. Therefore, both detoxification enzymes and physiological resistance play a great role in neonicotinoids detoxification.

INTRODUCTION

Mosquito control is the most common health problem facing many poor developing countries especially the transmitted diseases by mosquitoes **(Deepak *et al.* 2019)**. Mosquitoes transmit many pathogens which cause dangerous diseases like Zika virus, filariasis, Japanese encephalitis, chikungunya, yellow fever, dengue, and malaria; that pose a real danger not only to human health **(Santhosh *et al.* 2015; Vinoth *et al.* 2019)** but also, extend their impact on the economy worldwide **(Abutaha *et al.* 2018)**.

Culex pipiens L. (Diptera: Culicidae) is one of the most common species in Egypt which causes many human diseases **(Kady *et al.* 2008)**. *Cx. pipiens* is the vector of *Wuchereria bancrofti*, *Bancroftian filariasis* up to 100 million persons every year **(Sayed *et al.* 2018)**, Rift Valley fever **(Abdel-Hamid *et al.* 2009)**. *Bancroftian filariasis* is one of the fastest spreading insect-borne diseases for men in the tropics **(Badawy *et al.* 2015)**.

Mosquito resistance to the common insecticides used in public health programs was markedly increased. Neonicotinoids are new promising insecticide groups with a special mode of action than other insecticides used in public health control programs **(Elamathi *et al.* 2014)**. They have low mammalian toxicity, high selectivity, environmental protection and high effect at low doses **(Tomizawa and Casida, 2003; Kundoo *et al.* 2018)**. The effect of acetamiprid and thiamethoxam on the larval mortality and their impacts on some biochemical and physiological parameters of *Cx. pipiens* larvae were investigated.

MATERIALS AND METHODS

Insect

A laboratory strain of *Culex pipiens* obtained from the Research and Training Center on Vectors of Diseases (RTC), Ain Shams University was used in the bioassay. Larvae were reared in enamel dishes containing 2000 ml of distilled water under the laboratory condition (27±2°C, 75±5 % RH and a 14 hrs/10 hrs light/dark photoperiod) **(Gerberg *et al.* 1994)**. Newly hatched larvae were fed on Tetra- Min, Germany. The adult was reared in wooden cages (30 x 30 x 30 cm). Sucrose solution 10% was provided for adult feeding daily and a pigeon for female blood-feeding.

Insecticides

The commercial formulation of acetamiprid (Acetivot 20% WP) was supplied by King Quenson Co. and thiamethoxam (Actara 25% WG), which was by Novartis Co.

Larvicidal Assay

The bioassay was assessed by using the standard method according to **(WHO, 2005)** with some modification. Batches of 20 larvae of *Cx. pipiens* were transferred to six small test cups, by a plastic dropper. Different concentrations of insecticides (0.002, 0.004, 0.008, 0.016, 0.032, and 0.064 ppm for acetamiprid) and (0.004, 0.008, 0.016, 0.032, 0.064 and 0.128 ppm for thiamethoxam) were assayed. Three replicates were usually applied for each concentration including the control. The larval mortality was recorded after 24, 48 and 72 hrs post- treatment.

Preparation of Samples for Biochemical Assay

The laboratory strain of 3rd larval instar of *Cx. pipiens* was treated with LC_{50} concentrations of acetamiprid and thiamethoxam solution and control. Twenty larvae were collected after 72 hrs, from each insecticide the collected larvae were homogenized in distilled water (50 mg /1 ml). The refrigerated centrifuge was used to centrifuge the homogenate at 8000 rpm. for 15 minutes and the supernatant was used as a source of biochemical assays of enzymes.

Acetylcholinesterase Activity (AChE)

Acetylcholinesterase activity was evaluated depending on **(Simpson *et al.* 1964)** method. Acetylcholine bromide (AChBr) was used as a substrate.

Carboxylesterase Activity

The activity of carboxylesterase activity was estimated depending on **(Simpson *et al.* 1964)** method, Methyl n- butyrate (MeB) was used as a substrate.

Nonspecific Esterase Activity

The activity of alpha esterase (α-esterase) and beta esterase (β-esterase) were estimated depending on **(Van Asperen, I962)** method. α-naphthyl acetate or β-naphthyl acetate were used as a substrate, respectively.

Glutathione S-transferase Activity (GST)

The activity of glutathione *S*-transferase (GST) was evaluated depending on **(Kao *et al.* 1989)** method CDNB was used as a substrate.

Hemocytes Study

For hemocyte examination, the LC_{25} and LC_{50} values were used and the haemolymph samples were taken after 72 hrs of exposure. One drop of larval hemolymph of each treatment was smeared on a glass slide. The slides were kept to dry 5 min then stained with 10% Wright's stain for 10 min. Each slide was scanned under a light microscope ×100 magnification. A hundred of hemocytes in the microscopic field were identified according to **(Jones, 1962)**.

Statistical analysis

Mortality and the percentages of enzyme activation were subjected to probit analysis for calculating LC_{50}, LC_{90} **(Finney, 1971)**, other parameters statistic used (LDP-line) for the goodness of fit (Chi -square test) **(Duncan, 1955)**.

RESULTS

1. **Larvicidal activity of tested insecticides**

The LC_{25}, LC_{50} and LC_{90} values were estimated for the susceptible *Cx. pipiens* larval strain treated with acetamiprid and thiamethoxam by dipping method of application. The mortality data was collected after 24, 48 and 72 hrs post- treatment. The obtained data revealed that both tested insecticides showed high efficacy against *Cx. pipiens* larvae, while acetamiprid was highly effective than thiamethoxam, as showed in (Table 1). Acetamiprid showed the highest larval toxicity with LC_{50} values of 0.0093 ppm followed by thiamethoxam with LC_{50} values of 0.0305 ppm 24 hrs after exposure.

Therefore, this toxicity directly proportional with the concentration and time of insecticidal exposure. The slope values of acetamiprid and thiamethoxam were convergent which indicat that the populations used in bioassay tests had somewhat the same homogeneous responses to the insecticides used as seen at (Table 1).

Table 1. Larvicidal activity of acetamiprid and thiamethoxam against 3rd larval instar of *Culex pipiens* after different exposure times.

Insecticides ppm	Acetamiprid			Thiamethoxam		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
LC_{25}(*F.l. at 95%)	0.0031 (0.0023- 0.004)	0.0027 (0.002- 0.0034)	0.0024 (0.0017- 0.003)	0.0111 (0.0087- 0.0135)	0.0073 (0.0056- 0.0091)	0.0052 (0.0039-0.0065)
LC_{50} (*F.l. at 95%)	0.0093 (0.0077- 0.011)	0.0078 (0.0064- 0.0093)	0.0065 (0.0054- 0.0078)	0.0305 (0.0258- 0.0365)	0.0206 (0.0173- 0.0244)	0.0137 (0.0115- 0.0162)
LC_{90} (*F.l. at 95%)	0.0726 (0.0529- 0.11)	0.059 (0.0438- 0.0874)	0.0454 (0.0347- 0.0646)	0.2094 (0.1513-0.3219)	0.1468 (0.1083- 0.2189)	0.0878 (0.0684- 0.121)
Slope ± SE	1.4324±0.118	1.457±0.120	1.5228±0.123	1.5328± 0.122	1.5018± 0.12	1.59± 0.121
P	0.5862	0.4666	0.1251	0.6959	0.4814	0.0705
(χ^2)	2.8328	3.5749	7.2127	2.2172	3.4767	8.6473

* Fiducially Limits
*Slope of the concentration-inhibition regression line± standard error.
*(χ^2) Chi square value.

2. Biochemical study

The detoxification enzymes involved in insecticide resistance were examined. Acetylcholinesterase (AChE), carboxylesterase, *α*- esterases, *β*- esterases and glutathione *S*-transeferase (GST) activity levels were detected at the sub-lethal concentration (LC_{50}) of tested insecticides in the hemolymph of *Cx. pipiens* larvae, 72 hrs after exposure (Table 2; Fig. 1).

Results revealed that thiamethoxam and acetamiprid significantly increased AChE activity about 32.22% and 39.31%, respectively, than control. The same trend was observed in carboxylesterase activity which, significantly increased by 27.57% and 37.44% for thiamethoxam and acetamiprid, respectively compared with control. Acetamiprid exhibited significant high GST activity compared to thiamethoxam about 58.82% and 35.29%, respectively. The *α*-esterases activity increased significantly after 72hrs of acetamiprid exposure by 82.66%, while, it was only 26.66% for thiamethoxam. Both tested insecticides were greatly induced *β*- esterases activity with a highly significant increase than control, where, the extreme activity achieved by acetamiprid was 174.19%, while thiamethoxam was 82.79%. (Table 2; Fig.1)

The biochemical analysis indicated that the activity of the AChE, GST, carboxylesterase, α and β- esterases were significantly higher after acetamiprid exposure than thiamethoxam. Whereas, β- esterases exhibited the highest enzymes activity after exposure to both insecticides.

Table 2. Effect of acetamiprid and thiamethoxam on the activity of acetylcholinesterase, glutathione S-transeferase, carboxylesterase, α- esterases and β- esterases in 3rd larval instar of *Culex pipiens*.

Enzyme	Activity mean ± SE		Control
	Thiamethoxam	Acetamiprid	
Acetylcholinesterase (µg AchBr/ml/min)	517±15.8[b] (32.22%)	544±31[b] (39.30%)	391±9.5[a]
Glutathione S-transeferase (m mole sub. conjugated/min/g.b.wt)	138±3.5[b] (35.29%)	162±5.8[a] (58.82%)	102±6.4[c]
Carboxylesterase (ng Meb/min/mg protein)	310±16.6[b] (27.57%)	334±13.5[b] (37.44%)	243±14.8[a]
α-esterses (µg α-naphthol released/ml./min.)	9.5±0.5[b] (26.66%)	13.7±1.2[a] (82.66%)	7.5±0.33[b]
β-esterses (µg β-naphthol released/ml./min.)	3.4±0.41[b] (82.79%)	5.1±0.55[a] (174.19%)	1.86±0.15[c]

*Number within the same row with a letter in common is not significantly different to the analysis of variance **(Duncan, 1955).**

* Data are mean ± SE. *Data between ( ) represent the percentage of activation.

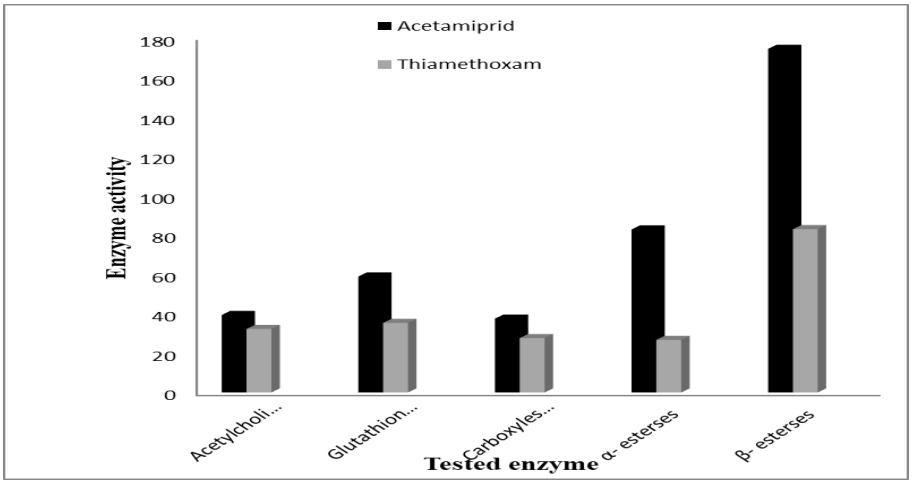

Fig. 1. Effect of acetamiprid and thiamethoxam on acetylcholinesterase, glutathione S-transeferase, carboxylesterase, α- esterses and β- esterses activities in the haemolymph of 3rd larval instar of *Culex pipiens*.

3. Hemocyte index

The hemolymph of 3rd instar larvae of *Cx. pipiens* was reared on control media contained 22 % of prohaemocyte, 30% of plasmatocyte, 38% of granulocyte and 10.5 % of oenocyte (Fig. 2). The effect of acetamiprid and thiamethoxam on the number of different hemocyte types of the 3rd instar larvae of *Cx. pipiens* 72 hrs after exposure are illustrated in Fig.2.

In thiamethoxam treatment, the percentage of the prohaemocytes in relation to the total number of haemocyte types significantly increased (26 and 28.5 %), respectively 72hrs after (LC_{25}) and (LC_{50}) exposure. Also, the percentage of plasmatocytes was significantly increased (45 and 51%), respectively.

Furthermore, the percentage of granulocytes increased significantly (35 and 37.4 %), respectively. The same trend was observed in the percentage of oenocytes, it increased significantly (18 and 24.7%), respectively compared to control. In contrast, acetamiprid caused a reduction in the percentage of prohaemocytes in relation to the total number of haemocyte types (8 and 5 %) 72hrs after (LC_{25}) and (LC_{50}) exposure, respectively. Also, the percentage of granulocytes decreased significantly (20 and 16.3%), respectively. The percentage of plasmatocyte decreased significantly (26 and 22.3 %), respectively. While, oenocyte percentage was increased significantly (8 and 6.5 %), respectively (Fig. 2).

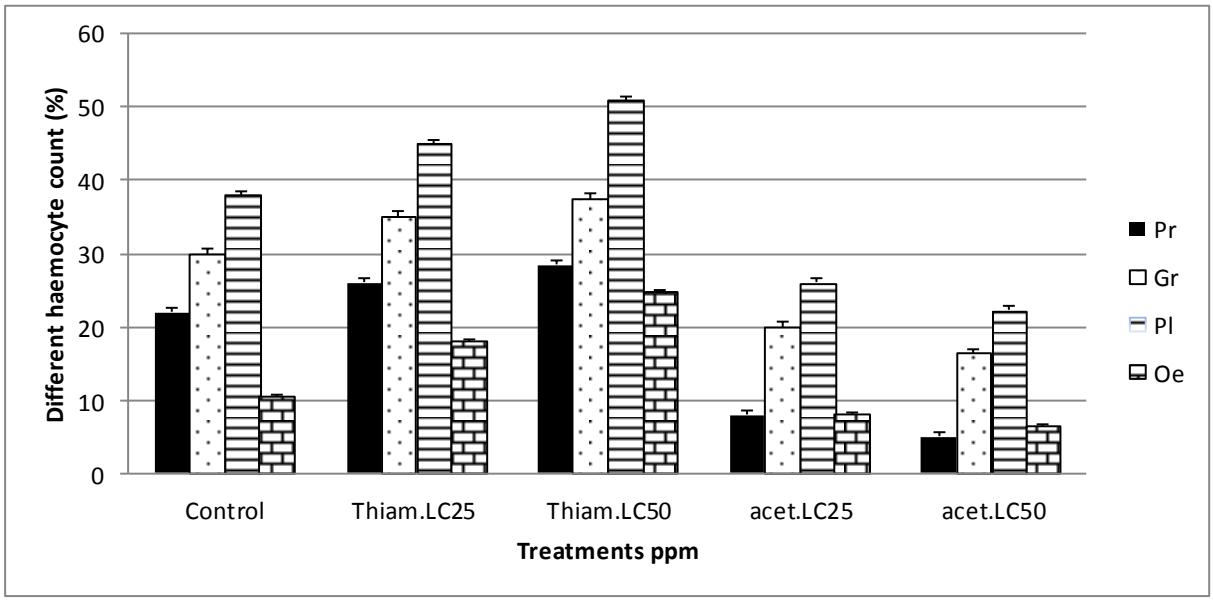

Fig. 2. Effect of acetamiprid and thiamethoxam on the different hemocytes count of 3rd larval instar of *Culex pipiens* 72hrs after exposure.

3.1. Abnormalities in the haemocytes after exposure of two tested insecticides

Certain abnormalities were observed after exposure of two tested insecticides to the haemocytes. These included vacuolization in granulocytes, reducing in cytoplasm, forcing nucleus through the cell membrane, some cells lost their smooth cell membrane to become irregular and some decomposing and apoptotic hemocytes (Fig. 3).

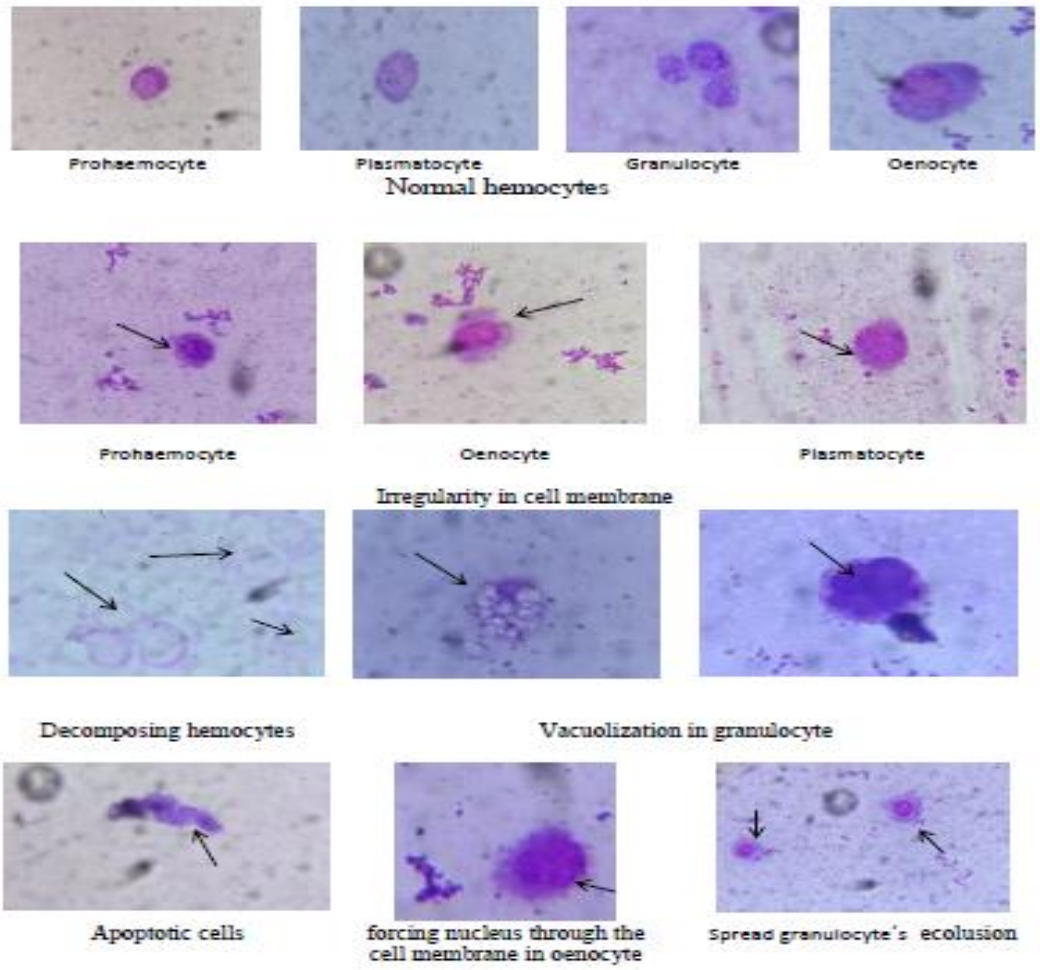

Fig. 3. Effect of acetamiprid and thiamethoxam on the morphology of different hemocytes of 3rd larval instar of *Culex pipiens* 72hrs after exposure. (X100- oil, bar 5 µm).

DISCUSSION

This study explores for the effect of neonicotinoids in the *Cx. pipiens* larvae. The resistance of insects to neonicotinoids may be a result of a modification in the target site of the insecticides, preventing the compound from reaching its action site, or degradation of the insecticides by metabolic enzymes or physiological resistance. These insecticides

affect the nicotinic acetylcholine receptor (nAChR) and inhibited excitatory cholinergic neurotransmission causing death **(Ngufor *et al*. 2017)**.

The obtained results of larvicidal activity of two neonicotinoid insecticides suggested that acetamiprid was highly toxic against the 3rd larval instar of *Cx. pipiens* than thiamethoxam and these results were confirmed by the biochemical and physiological analysis. According to structural analysis, acetamiprid is classified as the 1st generation of neonicotinoid insecticides with a heterocycle of chloropyridine, while thiamethoxam is classified as the 2nd generation with a heterocycle of chlorinated thiazole **(Yang *et al.* 2007)**. Therefore, they probably have a different binding mode with their site of action in the nAChR, and different detoxification enzymes activities and physiological responses. Similar results obtained by **(Darriet and Chandre, 2013)** observed that acetamiprid exhibited higher toxicity against both susceptible and resistant strain of *Aedes aegypti* than thiamethoxam. Likewise, mosquito populations of *Anopheles coluzzii* in Côte d'Ivoire were more sensitive to acetamiprid than imidacloprid **(Mouhamadou *et al.* 2019)**.

Metabolic resistance to neonicotinoids appears to be most common in insects. Elevated levels of glutathione-*S*-transferases and various esterases have been associated with *Aphis gossypii* resistance to this group **(Wang *et al.* 2002)**. Both acetamiprid and thiamethoxam significantly increased AChE activity with different levels. These results agreed with **(Samson-Robert *et al.* 2015)** demonstrated that the neonicotinoids caused an increase in AChE activity. Furthermore, in house flies adult, both thiamethoxam and imidacloprid treatments significantly increased the AChE activity **(Boily *et al.* 2013 and Abdel-Haleem *et al.* 2018)**. The high level of AChE is a result of the inhibitory action of the AChE activity due to the presence of neonicotinoids in the post-synaptic region of the nerves **(Suchail *et al.* 2004)**.

Glutathion *S*-transeferase is a detoxifying enzyme that protects insect cells against oxidative damage **(Hayes *et al.* 2005)**. **(Yang *et al*. 2009)** revealed that the resistance of some insects to insecticides is a result of elevated GST activity. The present results revealed that GST activity significantly increased after treated with thiamethoxam and acetamiprid. These results are in accordance with **(Badawy *et al.* 2015)** who reported that GST activity significantly increased reaching up to 186 % of control activity at lower levels of insecticides, these results strongly suggest the induction of oxidative stress by acetamiprid against honey bee.

The major enzymes responsible for the metabolism or detoxification of toxins are the carboxylesterase and general (α and β) esterases **(Li *et al.* 2007)**. The increase of detoxification enzymes activities could weaken house flies defence responses to thiamethoxam **(Chen *et al.* 2015)**. In the present results, the treatment of the 3rd larval instar of *Cx. pipiens* with the tested insecticides caused a remarkable increase in

carboxylesterase and (α and β) esterases activity where acetamiprid had the maximum effect on α and β esterases activity level. An obvious correlation of the general esterases activity with IC_{50} suggested that these enzymes could play a significant role in the resistance to neonicotinoids. This approved by **(Kandil *et al.* 2008)** who detected that a high synergistic ratio obtained from mixing of thiamethoxam with esterase inhibitor reflects the role of carboxylesterase in the detoxification mechanism found in thiamethoxam resistance strain. This increase suggests that the mechanism of resistance was due to increased ester hydrolysis caused by higher levels of esterases **(Tian *et al.*2018)**. Carboxylesterase and (α and β) esterase activity could weaken the defense responses of insects against neonicotinoids **(Chen *et al.* 2015)**. Therefore, these enzymes play a fundamental role in detoxification of neonicotinoids.

Insect´s hemocytes have many responses against different insecticides. Their number can increase and decrease under different stress as immune responses against different external exposure **(Gad and El –DaKheel, 2009)**.

It is noteworthy that granulocytes and plasmatocytes have variable responses than other types present under exposure to different insecticides **(Halawa *et al.* 2007; Fatima *et al.* 2014; Gad and Abdel-Megeed, 2006)**. Our results indicated that the tested insecticides significantly affected in the larval hemocytes of *Cx. pipiens.* Acetamiprid significantly decreased the different hemocyte counts while thiamethoxam significantly increased it. These increases in different hemocyte counts may due to a high mitotic, rapid turnover of hemocyte, and the mechanism of releasing its product of their metabolism into the hemolymph **(Arnold and Hinks, 1976)**. Our results agreed with **(Abou-Taleb *et al.* 2015)** observed that oenocytes and spherule cells were significantly increased in the hemolymph *Spodoptera littoralis* while granulocytes and plasmatocytes were decreased after chlorfluazuron and lufenuron treatments. Most of the previous studies confirmed that hemocyte becomes abnormal under insecticide stress **(Bhatti, 1993; Ayub, 1996; Gad and Abdel Mageed, 2006; Abou-Taleb *et al.* 2015)**.

CONCLUSION

Acetamiprid and thiamethoxam have a significant larvicidal effect against the 3rd larval instar of *Cx. pipiens*. Acetamiprid has the highest larvicidal activity than thiamethoxam after 24, 48 and 72hrs of exposure and showed significantly increased in the activity level of AChE, GST, carboxylesterase, α- esterases, and β- esterases than thiamethoxam. Furthermore, the different hemocytes were most sensitive to tested insecticides.

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