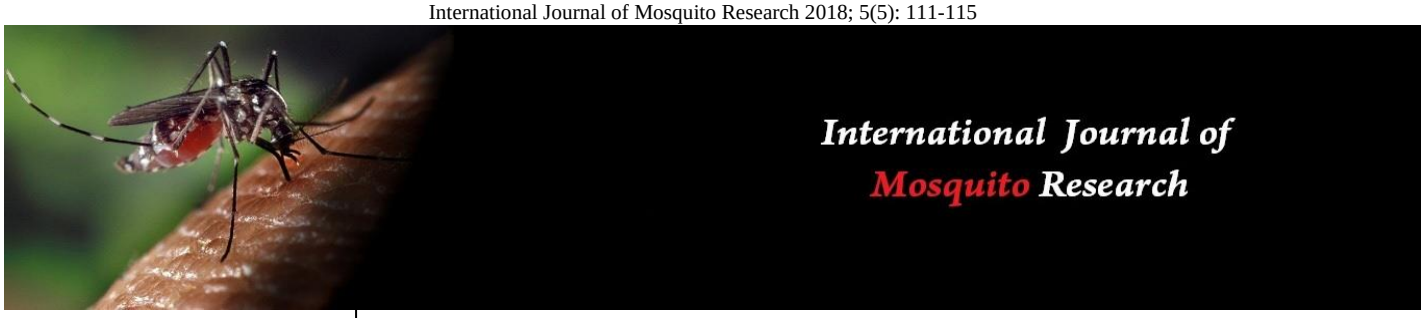

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Toxic and biochemical effects of imidacloprid and tannic acid on the *Culex pipiens* larvae (Diptera: Culicidae)

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Abstract
Resistance of mosquitos to pyrethroid, organophosphate, and carbamate created the need for alternative insecticides. Therefore, 3rd larval instar of *Culex pipiens* was tested with different concentrations of the technical grade insecticides, imidacloprid and tannic acid. Biochemical assays were performed on the same populations of *Culex pipiens* to determine activities of acetylcholinesterase (AChE) and adenosine triphosphatase (ATPase) enzymes. The bioassay test showed that *Cx. pipiens* larvae were susceptible to imidacloprid than tannic acid. Concentrations of 0.01, 0.015, 0.02, 0.04 and 0.05 ppm imidacloprid produced experimental mortalities of 18.5, 31.67, 45, 83.33 and 90%, respectively. Tannic acid in the concentrations of 2500, 5000, 10000, 20000 and 40000 ppm produced experimental mortalities of 13.33, 38.33, 46.67, 85 and 98.33%, respectively. The biochemical assays showed that imidacloprid significantly (P<0.01) decreased the activity levels of both AChE and ATPase, while tannic acid didn't show any significant difference on both enzymes compared to the control group.

Keywords: Imidacloprid, tannic acid, acetylcholine esterase, ATPase, *Culex pipiens*

1. Introduction
Over one million people worldwide die as of mosquito-borne diseases every year. The mosquito, *Culex pipiens*, is considered as one of the dangerous pests not only in Egypt but also worldwide for their capability to transmit many vector-borne diseases such as West Nile virus, Rift valley fever, Saint Louis encephalitis, and Eastern Equine encephalitis [1].

Resistance of mosquitoes to organochlorines, organophosphates, carbamates and pyrethroids directed to regain of concern for the use of some alternative control measures such as neonicotinoid insecticides and tannic acid [2]. Neonicotinoids, synthetic analogues of nicotine, are strong selective agonists of insect nicotinic acetylcholine receptors (nAChRs) which are major excitatory neurotransmitter receptors in invertebrates as well as vertebrates [3].

Imidacloprid, one of the neonicotinoids, is an insecticide that inhibits the transmission of stimuli through the nervous system of insects. It is neurotoxic by mimicking nicotine through its binding to the nicotinic acetylcholine receptor [4]. Imidacloprid was designed to act as an agonist of the post-synaptic nicotinic acetylcholine receptors which cause their overstimulation hence affecting neuronal processes lead to overall impairment and sometimes death [5]. Imidacloprid is more metabolizable than nicotine and accordingly it is 12 times higher in insecticidal activity than nicotine. The advantage of imidacloprid resulted from nonionization, higher hydrophobicity, and consequently penetrability into the target site [6].

Toxic effect of fenoxycarb, dinotefuran, imidacloprid, phenthoate and thiocyclam insecticides on the greenhouses population of the tomato leaf miner, *Tuta absoluta*, was evaluated. Data declared that the five tested insecticides had high toxic effect on 3rd instar larvae. Imidacloprid was the most effective toxicant against larvae and moths, so it had a very low resistance coefficient. And also, it produced a higher induction effect of AChE enzyme activity than the other three insecticides [7].

Tannic acid as a water-soluble polyphenolic is characterized by their ability to bind with proteins and other biological molecules [8]. It is also known to be harmful against some insects [9]. The histopathological effects of tannic acid on the midgut epithelium of some aquatic

dipteran larvae were studied. It was found that all larvae developed severe lesions which affecting primarily the midgut epithelium and secondarily the caeca and the Malpighian tubules [10]. The toxicity of tannic acid against phytophagous lepidopteran larvae has been well documented. However, its effect on aquatic dipteran larvae has been examined for only a few Culicine taxa associated with certain biotopes [11].

The current work was designed to study the toxicological and the biochemical effects of imidacloprid and tannic acid on the activity levels of AChE and ATPase neuro-enzymes in 3rd instar *Cx. pipiens* larvae.

Materials and Methods

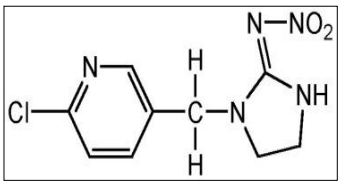
Experimental insect

The culture of the *Cx. pipiens* was reared in an insectary provided by Research and Training Center on Vectors of Diseases (RTC) following the standard methods of Gerberg *et al* [12]. Insects were reared under conditions of controlled temperature (27 ± 1) °C and relative humidity (70 ± 5)% with a constant photoperiod (light: dark = 12 h: 12 h). Pupae were moved from water medium to mosquito rearing cages (30 cm × 30 cm × 30 cm). Afterward, adults were kept in cages and provided with a cotton piece soaked with 10% glucose solution for post-emergence. Blood-fed females were allowed to integrate the blood meals for 24 h. Females were given admission to oviposition sites containing small glass containers lined with filter paper as egg deposition places. Eggs were allowed to hatch in sterilized water. Newly enclosed larvae were reared in plastic trays and fed every two days with a tiny amount of fish food. Late 3rd instar larvae were used for larval bioassays.

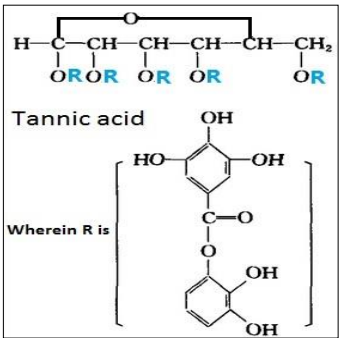
Insecticides

Two commercially available insecticides from different chemical classes and with different modes of action were used based on their generally approving Eco toxicological profiles and availability in the market.

Chemical structure of the insecticides



Common name: Imidacloprid
Trade name: Mallet 35% SC
Chemical formula: $C_9H_{10}ClN_5O_2$



Common name: Tannic acid
Trade name: Gallotannic Acid
Chemical formula: $C_{76}H_{52}O_{46}$

Imidacloprid was obtained from Nufarm limited company Australia and tannic acid was obtained from Oxford Lab Chem.

Toxicological evaluation

Larvicidal activity of imidacloprid and tannic acid on *Cx. pipiens* was assessed by using the standard method according to WHO [13]. In brief, twenty larvae of 3rd instar were taken and treated with different concentrations of insecticides (0.01, 0.015, 0.02, 0.04 and 0.05 ppm for imidacloprid and 2500, 5000, 10000, 20000 and 40000 ppm for tannic acid). Similarly, the untreated larvae were used as control. For each concentration five replicates were maintained at the same time. In order to feed the larvae, all the assay units were supplemented with a diet of finely ground fish food. Mortality data were recorded after 24 hrs in probit regression line and calculate LC_{25} LC_{50}, LC_{90}, and slope function [14].

Biochemical test

Enzymes activities were measured by grinding the whole bodies of treated larvae with the LC_{50} of each compound, then centrifuged at 5000 rpm for 10 minutes at 4ºC. The supernatant from the whole-body extracts were used as the enzymes sources for ATPase and esterase activity [15].

Acetylcholin esterase determination

AChE activity was measured according to the method described by Simpson et al [16] by using acetylcholine bromide (AChBr) as a substrate. The test was carried out for treated and untreated larvae in 3 replicates. The reaction mixture contained 200 µl enzyme solution mix (as previously described), 0.5 ml 0.067 M phosphate buffer (pH7) and 0.5 ml AChBr (3 mM). The test tubes were incubated at 37 °C for precisely 30 min. One ml of alkaline hydroxylamine (equivalent volume of 2 M hydroxylamine chloride and 3.5 M NaOH) was added to each test tube. Then 0.5 ml of HCl (1 part of conc. HCl and 2 parts of ΔH_2O) was added. The mixture shaken vigorously and allowed to stand for 2 min. Half ml of ferric chloride solution (0.9 M $FeCl_3$ in 0.1M HCl) was added and mixed well. The reduction in AChBr resulting from hydrolysis by AChE was read at 515 nm.

ATPase determination

The total enzyme activity of the ATPase was determined according to Amaral *et al* [17] in treated and untreated larvae in 3 replicates. The enzyme mix was incubated at 37 °C, PH 7.5 in a solution (Final volume equal to 0.5 ml) containing ATP.Na2-TRIS 5mM, NaCl 150 mM, KCl 15 mM in histidine HCl-TRIS 30 mM. The reaction was started by the addition of ATP. After 30 min. of incubation at 37°C, the reaction was stopped by 100 ul 5% SDS. ATPase activity was expressed in µ moles of Pi released per minute per milligram protein. The phosphate ion was detected using a commercial kit of Quimica clinica applicada S.A. (Spain). Phosphorus reacts with molybdate to produce phosphor-molybdate, which is finally reduced to a molybdenum blue which is photometrically measured at 650 nm. Zero adjustment was against reagent blank and results obtained after comparison with a reference standard concentration.

Statistical analysis

The data was analyzed with SPSS 19 software followed by one-way analysis of variance (ANOVA) and Tukey's HSD

test. The results were stated as (means ± SE) of untransformed data and considered significantly different at $P<0.01$. Probit analysis was conducted to calculate the estimate LC_{50} values with their limits by Probit analysis software.

Results

Toxicological assay

Different concentrations of imidacloprid and tannic acid were applied to the 3rd instar of *Cx. pipiens* larvae. The mortalities percentages are shown in Tables (1 & 2). Data in these two tables show that as the concentration of any compound increase, the mortalities percentages significantly ($P<0.01$) increased. The least mortality (18.50%) was recorded in 0.01 ppm imidacloprid while the least mortality (13.33%) was recorded in 2500 ppm tannic acid.

Analyses of regression lines of both imidacloprid and tannic acid indicated that the LC_{50} for these two compounds were 0.0205 and 7842.1 ppm, respectively (Figure 1).

From the bioassay test, imidacloprid and tannic acid were effective as larvicide against the 3rd instar larvae but imidacloprid was found to be more toxic than tannic acid as showed in figure (1).

On the other hand, tannic acid caused malformations in the tested larvae by expelling the alimentary canal outside the larvae body (Figure 2).

Table 1: Mortality percentages of 3rd instar *Cx. pipiens* larvae exposed to different concentrations of imidacloprid

Concentrations (ppm)	Average Mortality% ± SE
Control	0.00 ± 0.00[a]
0.01	18.50 ± 6.17[b]
0.015	31.67± 1.67[c]
0.02	45.00 ± 0.00[d]
0.04	83.33 ± 1.67[e]
0.05	90.00 ± 5.00[f]
LC_{25} (ppm)	0.0127
LC_{50} (ppm)	0.0205
LC_{90} (ppm)	0.0516
Slope ± SE	3.21 ± 0.26

Means bearing different letters within column are significantly different ($P<0.01$) ANOVA, Tukey's HSD test.

Table 2: Mortality percentages of 3rd instar *Cx. pipiens* larvae exposed to different concentrations of tannic acid

Concentrations (ppm)	Average Mortality% ± SE
Control	1.67 ± 1.67[a]
2500	13.33 ± 3.33[b]
5000	38.33 ± 1.67[c]
10000	46.67 ± 1.67[d]
20000	85.00 ± 2.89[e]
40000	98.33 ± 1.67[f]
LC_{25} (ppm)	4053.54
LC_{50} (ppm)	7842.1
LC_{90} (ppm)	27478.38
Slope ± SE	2.35 ± 0.172

Means bearing different letters within column are significantly different ($P<0.01$) ANOVA, Tukey's HSD test.

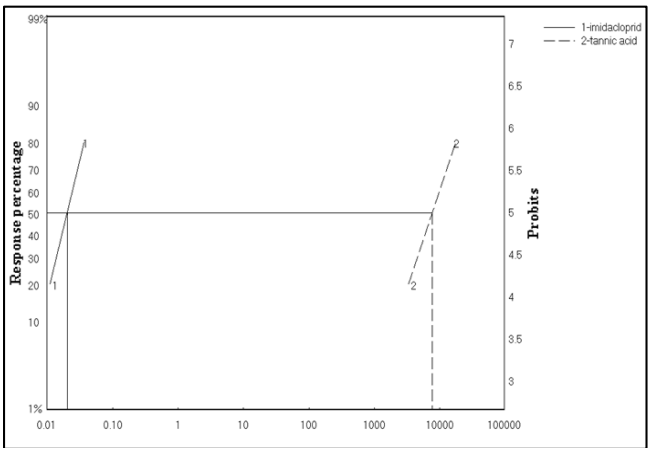


Fig 1: Toxicity regression lines of both imidacloprid and tannic acid against 3rd instar larvae of *Cx. pipiens*.

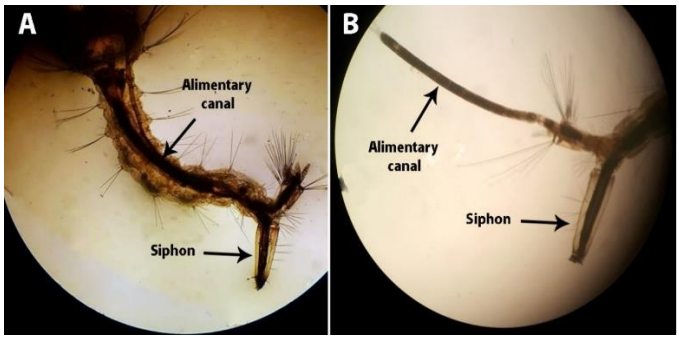


Fig 2: Microscopic photo showing the expelling of the alimentary canal of 3rd instar *Culex pipiens* larvae after treatment with tannic acid (B) as compared with untreated larvae (A).

Biochemical assay

Third instar *Cx. pipiens* larvae were treated with the LC_{50} of imidacloprid and tannic acid. The activity levels of AChE and ATPase enzymes were measured in the treated and control larvae. The activity levels of AChE and ATPase were significantly decreased in treated larvae with imidacloprid. The activity level of AChE was decreased by 42% from 21.5±1.05 to 12.5±1.19 (ug AChBr⁄min⁄mg protein) from the control to the treated samples, respectively. While the activity level of ATPase was decreased in the treated samples by 40% from the control (Table 3). On the other hand, there was no significant differences in the activity levels of AChE and ATPase in treated larvae with tannic acid compared to the control samples. The levels of AChE were 21.5±1.05 and 15.23±1.58 (ug AChBr/min/mg protein) for the control and treaded samples, respectively. The values for ATPase levels were 3.2±0.37 and 3.79±0.39 (n mole Pi/min/mg protein) for the control and treated samples, respectively (Table 4).

Table 3: Effect of imidacloprid on AChE and ATPase activity levels in treated 3rd instar *Culex pipiens* larvae (Mean±SE)

Samples	AChE (µg AChBr/min/mg protein)	ATPase (n mole Pi/min/mg protein)
Treated	12.5 ± 1.19 [b]	1.9 ± 0.06 [b]
Control	21.5 ⊀ 1.05 [a]	3.2 ± 0.37 [a]

Means bearing different letters are significantly different at $P<0.01$, Tukey's HSD test.

Table 4: Effect of tannic acid on AChE and ATPase activity levels in treated 3rd instar *Culex pipiens* larvae (Mean±SE)

Samples	AChE (µg AChBr/min/mg protein)	ATPase (n mole Pi/min/mg protein)
Treated	15.23 ±1.58 [a]	3.79 ±0.39 [a]
Control	21.5 ±1.05 [a]	3.2 ±0.37 [a]

Means bearing different letters are significantly different at $P<0.01$, Tukey's HSD.

Discussion

Our results indicated that, the 3rd instar larvae of *Cx pipiens* were more susceptible to imidacloprid than tannic acid. Imidacloprid is relatively new class of insecticides and has a mode of action that affects the central nervous system of insects [18]. Imidacloprid was found to be toxic to *Aedes aegypti* larvae with LC_{50} value 0.15 mg/L [19]. On the other hand, it is known that tannic acid can form complexes with proteins; when oxidized to quinones in the midgut of insect, they can produce semiquinones radicals and other forms of reactive oxygen species [20]. Toxicological studies of imidacloprid showed the rapid appearance of neurotoxicity symptoms in honey bee [21]. Imidacloprid considered safe to hu*man* since the partial positive charge in neonicotinoids can distinguish the insect nAChR from the vertebrate nAChR, brain and also peripheral nAChRs in mammals are low in sensitivity to neonicotinoids [22]. The neonicotinoids have an electronegative nitro or cyano pharmacophore and are not protonated. Accordingly, vertebrate have low affinity to neonicotinoids compared with insects which have nicotinic receptors. This is a reason for their favorable toxicological profile. [23]. From the biochemistry test, it was clear that imidacloprid significantly affected the activity of AChE and ATPase enzymes by decreasing their levels. This decrease in the AChE in presence of imidacloprid may be due to the enzyme inability to regenerate its active form due to the formation of strong bond with imidaclorid which cannot easily hydrolysed in post-synaptic region of the nerves. Imidacloprid had the same action in other insects of order Hemiptera. The results showed that the specific activity of AChE in *Myzus persicae* was reduced significantly when treated with different sublethal doses of imidacloprid [24].

Other studies on *Cx. quinqufasciatus* indicated that imidacloprid affected AChE and ATPase enzymes by a different way. High amount of AChE were obtained with many folds in the presence of different metabolites of imidacloprid when compared to control groups. High concentrations of the AChE were obtained in all the different imidacloprid compounds. The amount of AChE was also increased with the increase in the concentration of the imidacloprid metabolites tested. This increase in the AChE in presence of the different metabolites of imidacloprid may be due to the inhibitory action of the AChE activity in post-synaptic region of the nerves **[21]**. Tannic acid causes dramatic lesions, affecting mainly the midgut epithelium, gastric caeca and the Malpighian tubules. Histopathological effects differed qualitatively according to their localization along the midgut and quantitatively according to the concentration assayed, and the duration of the treatment [10]. So, tannic acid showed a little effect on AChE and ATPase enzymes activity levels.

The previous data indicated that the imidacloprid and tannic acid had toxic effects on 3rd instar *Cx. pipiens* larvae.

Conclusion

We concluded that of the two compounds tested in the laboratory, imidacloprid was highly effective larvicides and decreased the activity levels of AChE and ATPase enzymes and efficient in reducing mosquito-transmitted diseases. Both compounds, imidacloprid and tannic acid, can be used as a successful control method.

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