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The synergistic effect of octopamine receptor agonists on selected insect growth regulators on *Culex quinquefasciatus* Say (Diptera: Culicidae) mosquitoes

Mohamed Ahmed Ibrahim Ahmed[a,b], Christoph Franz Adam Vogel[b,c,*]

[a] Plant Protection Department, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt
[b] Center for Health and the Environment, University of California, One Shields Avenue, Davis, CA 95616, USA
[c] Department of Environmental Toxicology, University of California, One Shields Avenue, Davis, CA 95616, USA

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ABSTRACT

Synergistic effects of octopamine receptor agonists (OR agonists) have attracted many scientists based on their potent effects on mosquitoes. Herein, we determined the toxicity of selected insect growth regulators (IGRs) on fourth instar larvae of *Culex quinquefasciatus*. We evaluated the synergistic action of OR agonists on the toxicity of IGR insecticides to achieve a better understanding of their mode of action. As a result, pyriproxyfen was the most potent IGR insecticide (EC_{50} = 0.049 ng/ml) followed by lufenuron, novaluron, and diflubenzuron according to the IGR bioassay. Further, based on the acute bioassay, lufenuron was the most toxic IGR insecticide (LC_{50} = 44 ng/ml) after 24-h post treatment followed by pyriproxyfen, novaluron, and diflubenzuron (LC_{50} = 137, 263, and 1127 ng/ml, respectively). Similar tendency was observed after 48 and 72-h post treatment. Furthermore, OR agonists that combined with pyriproxyfen was the most significant effects after 48 and 72-h of exposure. The synergism with amitraz (AMZ) was more significant when co-treated with IGR insecticides compared to chlordimeform (CDM). These findings suggest that OR agonists are promising tools and are important alternative strategies as synergistic compounds in preventing and controlling *Culex quinquefasciatus* mosquitoes.

1. Introduction

Culex quinquefasciatus Say is considered one of the most dangerous mosquito vectors worldwide. Its ability to transmit zoonotic diseases, for instance, St. Louis encephalitis virus, West Nile Virus (WNV), and Zika virus is considered critical [1–3]. Importantly, it is considered a prospective cross between sylvatic arbovirus from birds to man especially in urban areas [4]. However, pesticides are the most effective tools in controlling *Culex quinquefasciatus* [5,6]. Regrettably, the heavy use of conventional pesticides on mosquitoes is linked to numerous issues such as pesticide resistance and health impacts [7–9]. Thus, investigating new strategies are considered paramount and insect growth regulators (IGRs) provide a promising approach. IGRs are mainly disturbing the physiological processes of the insects which causing the death [10–12]. IGRs have many positive properties that make them attractive in pest control programs. Interestingly, IGRs are less toxic to the surrounding environment, and yet more selective and more relevant with integrated pest management (IPM) and integrated resistance management (IRM) [11,13].

Octopamine receptor agonists (OR agonists) such as amitraz (AMZ) and chlordimeform (CDM) are related to formamidine group [14,15]. Formamidine pesticides have a unique mode of action by inhibiting monoamine oxidase (MAO) and interrupting the production of cyclic adenosine monophosphate (cAMP) which triggers a counter effect that lead to behavioral changes in insect [16]. Furthermore, formamidine pesticides are likely to interact with different receptors by binding to the octopamine receptor and acting as OR agonists [14,17]. This is a very important point since many scientists attempt to demonstrate the biochemical and molecular biological pathways of formamidine pesticidal actions on different insect pests.

The synergistic effects of OR agonists on different pesticide groups such as organophosphates, pyrethroids, and neonicotinoids have been reported. Consequently, OR agonists are considered promising components for insect pest control programs [11,18–21].

In the present study, we examined the potency of four IGRs and the acute toxicity on fourth instar larvae of *Culex quinquefasciatus*. Further,

* Corresponding author at: Center for Health and the Environment, Environmental Toxicology Department, University of California Davis, Davis, CA 95616, USA.
E-mail address: cfvogel@ucdavis.edu (C.F.A. Vogel).

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Table 1
Effective toxicity of selected IGR insecticides on fourth instar larvae of *Culex quinquefasciatus*.

IGRs	n[a]	EC_{50}[b] (95% CL)[c]	Slope (± SE)	$X^2(df)$[d]	g values[e]	PR[f]
Pyriproxyfen	360	0.049 (0.015–0.14)	5.3 (± 0.11)	3.4 (3)	0.03	1.0
Lufenuron	360	0.22 (0.066–0.93)	4.9 (± 0.11)	2.6 (3)	0.05	4.5
Novaluron	360	2.36 (0.50–5.03)	4.0 (± 0.10)	0.5 (3)	0.09	48.2
Diflubenzuron	360	7.11 (0.97–17.98)	3.4 (± 0.11)	0.2 (3)	0.08	145.1

[a] n = number of larvae tested, including control.
[b] Effective concentration (ng/ml) to cause 50% of treated larvae to fail to emerge as adults. Toxicity was determined as percentage of adult emergence after 10 days.
[c] If the 95% CI of the ratio includes a value of one, then the differences between the two LC_{50} values are insignificantly different.
[d] df = Degree of freedom.
[e] If g value < 0.5, the data are considered to fit the probit model. If not, the data do not fit the probit model and the analysis is invalid.
[f] Potency ratio = LC_{50} value of lufenuron, novaluron, or diflubenzuron divided by the LC_{50} value of pyriproxyfen.

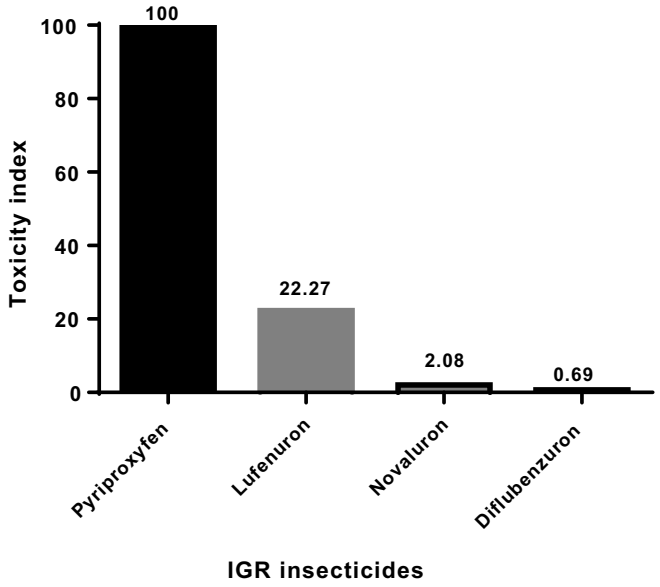

Fig. 1. Toxicity index of four IGR insecticides on 4th instar larvae of *Culex quinquefasciatus* after 10 days of exposure as calculated from EC_{50} values from Tables 1.
Toxicity index = [(EC_{50} of the most toxic tested IGR insecticide/EC_{50} of the tested IGR insecticide) × 100].

we assessed the synergistic action of AMZ and CDM on the selected IGRs to reduce the heavy reliance on conventional pesticides as potential tools for the control of vector-borne mosquitoes.

2. Material and methods

2.1. Mosquitoes

The colony of *Culex quinquefasciatus* was attained from the laboratory of Prof. Walter Leal, University of California Davis (UC Davis) and used in all experiments. This colony, also called the *Davis colony*, was generated from mosquitoes collected in Merced, California in the 1950s. However, the original colony has been maintained in the Kearney Agricultural Center (KAC), University of California for more than six years. The *Davis colony* has been reared at Davis under a photoperiod of 12:12 h (L:D), 27 ± 1 °C and 75% relative humidity [22]. Further, since the UC Davis Institutional Review Board (IRB) ruled that this study did not meet the requirements for human subject research, the IRB approval was not demanded.

2.2. Chemicals

Chlordimeform (CDM; 99.8%), amitraz (AMZ; 96.8%), pyriproxyfen (99%), lufenuron (99.7%), diflubenzuron (98.1%), and novaluron (99.6%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.3. IGR bioassay

IGR bioassays were carried out according to Ahmed and Vogel [11]. The emergence of adults was observed after 10 days of IGR insecticide exposure, since adults in controls had completed the emergence at this time point. The synergistic effects of CDM and AMZ were not included in the IGR bioassays because they inhibited the adults from the emergence at the concentrations that have been applied.

2.4. Acute toxicity bioassay

Acute toxicity bioassays were conducted based on Paul et al. [23]. The larvae were deemed to be dead if they were not responding to the touching of a probe or even if they could not reach the surface of the water. Further, percentage of mortality was determined after 24, 48, and 72-h of exposure because of the slow-acting nature of these IGR insecticides which delayed the acute toxicity that needs to be effective.

2.5. Synergistic action bioassay

The synergistic action bioassays were determined as described by Ahmed and Matsumura [17] with little modifications. Briefly, our previous study on AMZ and CDM showed that a sublethal concentration of 10 µg/ml was the highest concentration that did not cause mortality during the 72-h post treatment on fourth instar larvae of *Culex quinquefasciatus*. Further, five different concentrations (1000, 100, 10, 1, and 0.1 ng/ml) of each IGR insecticide were used for all bioassays and each bioassay was repeated twice. Every bioassay was held at 25 °C. Percentage mortality was recorded after 24, 48, and 72-h of treatment.

2.6. Statistical analysis

According to Abbott's formula [24] the corrected mortality was adjusted. Further, all bioassay data such as LC_{50}, 95% CL values, slope, X^2, and g values; were pooled by using IBM SPSS Statistics V25 software (SPSS Inc., Chicago, IL). Statistical differences between LC_{50} estimates were determined by using a 95% CI for the ratio of two estimates [25]. However, if the 95% CI for the ratio included 1, then the LC_{50} estimates were not significantly different. Potency ratio (PR) estimated as LC_{50} value of lufenuron, novaluron, or diflubenzuron was divided by the LC_{50} value of pyriproxyfen. Synergistic ratio (SR) was assessed by

Table 2
Acute toxicity of selected IGR insecticides on fourth instar larvae of *Culex quinquefasciatus* after 24, 48, and 72-h of exposure.

IGRs	n[a]	After 24-h				After 48-h				After 72-h			
		LC_{50}[b] (95% CL)[c]	Slope (± SE)	X^2 (*df*) [d]	g value[e]	LC_{50}[b] (95% CL)[c]	Slope (± SE)	X^2 (*df*) [d]	g value[e]	LC_{50}[b] (95% CL)[c]	Slope (± SE)	X^2 (*df*) [d]	g value[e]
Lufenuron	360	44 (10–91)	4.1 (± 0.11)	0.44 (3)	0. 03	15 (4–66)	4.7 (± 0.10)	2.61 (3)	0.09	3 (0.8–12)	4.9 (± 0.11)	4.80 (3)	0.02
Pyriproxyfen	360	137 (36–289)	4.4 (± 0.13)	0.46 (3)	0.09	53 (14–81)	4.5 (± 0.11)	0.40 (3)	0.05	21 (5–30)	4.6 (± 0.10)	0.15 (3)	0.08
Novaluron	360	263 (58–510)	4.1 (± 0.10)	0.27 (3)	0.06	96 (23–105)	4.2 (± 0.10)	0.33 (3)	0.06	38 (10–41)	4.5 (± 0.10)	1.15 (3)	0.07
Diflubenzuron	360	1127 (165–1981)	3.5 (± 0.11)	0.39 (3)	0.02	379 (58–427)	3.5 (± 0.11)	0.59 (3)	0.09	111 (20–329)	3.6 (± 0.09)	1.14 (3)	0.06

[a] n = number of larvae tested, including control.
[b] Concentration is expressed in ng/ml and the response determined after 24, 48, and 72-h of exposure.
[c] If the 95% CI of the ratio includes a value of one, then the differences between the two LC_{50} values are insignificantly different.
[d] *df* = Degree of freedom.
[e] If g value < 0.5, the data are considered to fit the probit model. If not, the data do not fit the probit model and the analysis is invalid.

Table 3
Synergistic action of CDM on the toxicity of selected IGR insecticides on fourth instar larvae of *Culex quinquefasciatus* after 24, 48, and 72-h of exposure.

Compounds + CDM[a]	n[b]	After 24-h					After 48-h					After 72-h				
		LC_{50}[c] (95% CL)[d]	Slope (± SE)	X^2 (*df*) [e]	g value[f]	SR[g]	LC_{50}[c] (95% CL)[d]	Slope (± SE)	X^2 (*df*) [e]	g value[f]	SR[g]	LC_{50}[c] (95% CL)[d]	Slope (± SE)	X^2 (*df*) [e]	gv alue[f]	SR[g]
Lufenuron	360	13 (4–50)	4.9 (± 0.11)	0.27 (3)	0.06	3.4*	4 (1−11)	5.6 (± 0.12)	2.30 (3)	0.02	3.8*	0.7 (0.2–2)	5.3 (± 0.17)	2.26 (3)	0.05	4.3*
Pyriproxyfen	360	29 (7–65)	4.5 (± 0.10)	0.46 (3)	0.09	4.7*	8 (2−31)	4.8 (± 0.10)	0.89 (3)	0.02	6.8*	3 (0.9–10)	5.2 (± 0.14)	2.98 (3)	0.03	7.0*
Novaluron	360	71 (16–86)	4.1 (± 0.10)	0.23 (3)	0.07	3.7*	20 (6-93)	4.7 (± 0.11)	1.04 (3)	0.02	4.8*	6 (2-18)	5.6 (± 0.12)	2.90 (3)	0.04	6.3*
Diflubenzuron	360	293 (54–956)	3.8 (± 0.10)	0.19 (3)	0.08	3.9*	78 (19–97)	4.3 (± 0.10)	0.04 (3)	0.09	4.9*	21 (6–87)	4.9 (± 0.11)	0.52 (3)	0.05	5.3*

[a] Concentration of synergist was 10 µg/ml and larvae exposed to insecticide and synergist simultaneously.
[b] n = number of larvae tested, including control.
[c] Concentration is expressed in ng/ml and the response determined after 24, 48, and 72-h of exposure.
[d] If the 95% CI of the ratio includes a value of one, then the differences between the two LC_{50} values are insignificantly different.
[e] *df* = Degree of freedom.
[f] If g value < 0.5, the data fit the probit model. If not, the data do not fit the probit model and the analysis is invalid.
[g] SR, synergistic ratio. Calculated by dividing the LC for IGR insecticide by the LC of insecticide + CDM.
* SR significantly different from control without synergist (=1.0) at ($P \leq .05$).

Table 4
Synergistic action of AMZ on the toxicity of selected IGR insecticides on fourth instar larvae of *Culex quinquefasciatus* after 24, 48, and 72-h of exposure.

Compounds + AMZ[a]	*n*[b]	After 24-h					After 48-h					After 72-h				
		LC_{50}[c] (95% CL)[d]	Slope (± SE)	X^2 (*df*)[e]	g value[f]	SR[g]	LC_{50}[c] (95% CL)[d]	Slope (± SE)	X^2 (*df*)[e]	g value[f]	SR[g]	LC_{50}[c] (95% CL)[d]	Slope (± SE)	X^2 (*df*)[e]	g value[f]	SR[g]
Lufenuron	360	7 (2–26)	5.0 (± 0.11)	0.54 (3)	0.01	6.3*	2 (0.6–7)	5.5 (± 0.12)	1.76 (3)	0.06	7.5*	0.3 (0.03–1)	5.2 (± 0.11)	1.47 (3)	0.08	10.0*
Pyriproxyfen	360	15 (4–66)	4.7 (± 0.11)	1.13 (3)	0.07	9.1*	4 (2 – 12)	5.7 (± 0.12)	2.92 (3)	0.04	13.3*	1 (0.3–4)	5.2 (± 0.13)	2.43 (3)	0.09	21.0*
Novaluron	360	29 (7–166)	4.5 (± 0.10)	0.46 (3)	0.02	9.1*	9 (3-27)	5.4 (± 0.11)	0.32 (3)	0.06	10.7*	3 (0.8-7)	5.5 (± 0.12)	1.62 (3)	0.04	12.7*
Diflubenzuron	360	120 (30–194)	4.4 (± 0.11)	0.41 (3)	0.08	9.5*	30 (9–129)	5.0 (± 0.11)	2.02 (3)	0.05	12.6*	8 (3 – 22)	5.8 (± 0.12)	3.82 (3)	0.02	13.9*

[a] Concentration of synergist was 10 μg/ml and larvae exposed to insecticide and synergist simultaneously.
[b] *n* = number of larvae tested, including control.
[c] Concentration is expressed in ng/ml and the response determined after 24, 48, and 72-h of exposure.
[d] If the 95% CI of the ratio includes a value of one, then the differences between the two LC_{50} values are insignificantly different.
[e] *df* = Degree of freedom.
[f] If g value < 0.5, the data fit the probit model. If not, the data do not fit the probit model and the analysis is invalid.
[g] SR, synergistic ratio. Calculated by dividing the LC for IGR insecticide by the LC of insecticide + AMZ.
* SR significantly different from control without synergist (= 1.0) at ($P \leq .05$).

dividing the LC_{50} value of the test insecticide by that of the LC_{50} obtained for the combined treatment (insecticide + synergist). Plus, toxicity index calculated as [(LC_{50} of the most toxic tested IGR insecticide/LC_{50} of the tested IGR insecticide) × 100].

3. Results

The effective toxicity of selected IGR insecticides is shown in Table 1. Pyriproxyfen was the most potent IGR insecticide (EC_{50} = 0.049 ng/ml) followed by lufenuron and novaluron (EC_{50} = 0.22 and 2.36 ng/ml, respectively). However, the IGR with the lowest toxicity was diflubenzuron (EC_{50} = 7.11 ng/ml). Pyriproxyfen was more toxic than lufenuron, novaluron, and diflubenzuron by 4.5, 48.2, and 145.1-fold, respectively (Table 1 and Fig. 1). Lufenuron was more toxic than novaluron and diflubenzuron by 10.7 and 32.3-fold, respectively.

The acute toxicity of selected IGR insecticides on fourth instar larvae of *Culex quinquefasciatus* is presented in Table 2. Lufenuron was the most toxic IGR insecticide (LC_{50} = 44 ng/ml) 24-h post treatment followed by pyriproxyfen and novaluron (LC_{50} = 137 and 263 ng/ml, respectively). Further, diflubenzuron was the least potent IGR insecticide among the tested compounds (LC_{50} = 1127 ng/ml). The same trend of toxicity was held in the same order even after 48 and 72-h of exposure (Table 3).

In the combination with OR agonists, both OR agonists were synergized by all four of the selected IGR insecticides especially after 48 and 72-h of exposure (Tables 3 and 4; Fig. 2). In general, the strongest synergistic effects of CDM and AMZ was found in combination with pyriproxyfen (synergistic ratio was 7 and 21-fold, respectively) after 72-h of exposure. Interestingly, the most distinguished trend was that the synergism was greater when IGRs were co-administrated with AMZ compared to CDM (Fig. 3).

4. Discussion

Insecticide resistance is an essential issue facing insect pest control programs. The combination of insecticides and synergists could be an excellent choice for pest controls. In the current study, the acute toxicity results of the selected IGR insecticides suggest that it may have another mechanism of action other than inhibiting the chitin synthesis or acting at multiple points in the insect life cycle that is responsible for the acute toxicity seen in the fourth instar larvae of *Culex quinquefasciatus*.

The OR agonists demonstrated different degrees of synergism on selected IGR insecticides on fourth instar larvae of *Culex quinquefasciatus* after 24, 48, 72-h of exposure. To date, no reliable data are available focusing on the synergistic action of OR agonists and the toxicity of IGR insecticides on *Culex quinquefasciatus*. In agreement with our results, Ahmed and Vogel [13] revealed that OR agonists synergized the selected IGR insecticides, diflubenzuron, novaluron, and lufenuron and the maximum SR was found for the combination of diflubenzuron and AMZ especially after 24, 48, and 72-h of exposure (SR = 2.6, 3.4, and 4.5-fold, respectively) on fourth instar larvae of *Aedes aegypti*. Further, they found in different study on *Aedes aegypti* adults, OR agonists synergized the potency of selected IGR insecticides especially after 48 and 72-h post treatment [11].

OR agonists are synergized greatly the selected IGR insecticides especially after 48 and 72-h of exposure. Interestingly, SR values were greater with AMZ than CDM on fourth instar larvae of *Culex quinquefasciatus*. These results are consistent with prior studies on *Aedes aegypti* [13,17].

The literature shows that OR agonists synergize with different insecticide groups on different insect pests. For example, in one-day old adults of *Aphis gossypii*, the highest SR in the combination of amitraz with imidacloprid (LC_{10}) was 3.09-fold [26]. Furthermore, Liu and Plapp [18] demonstrated that synergism occurred with a combination

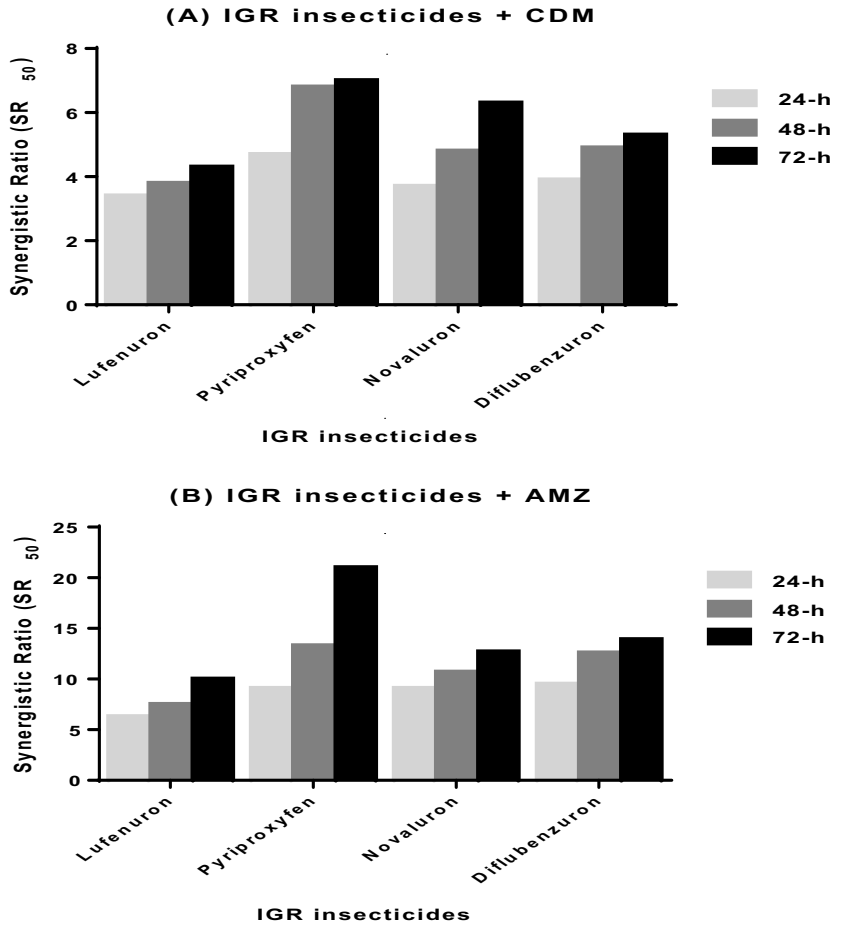

Fig. 2. Time-dependent changes in the synergistic ratio (SR_{50}) as calculated from LC_{50} values from Tables 2-4 for combined treatments with (A) CDM and (B) AMZ on fourth instar larvae of *Culex quinquefasciatus* as assessed 24, 48, and 72-h after their initial exposure.

of cypermethrin plus formamidines on houseflies. They found that the SR value was up to 11.8-fold and was even greater in susceptible strains compared to the more resistant strains. Moreover, they revealed that BTS 27271, a monomeric derivative form of amitraz, was the most active synergist among the tested synergists.

OR agonists have also shown strong effects with various insecticides on ticks. On adult ticks of *Rhipicephalus sanguineus*, the SR values of fipronil mixing with amitraz were > 7.3-, 137-, and 97-fold at 6-h, 24-h, and 48-h of exposure, respectively [19]. Further, Rodriguez-Vivas et al. [20] emphasized that the mixtures of cypermethrin + amitraz (87.0–89.7%) were more effective than cypermethrin alone (76.3–80.5%) on *Rhipicephalus microplus*.

The effects of OR agonists that contribute synergistic action to selected IGR insecticides are likely due to deactivation of detoxification enzymes, promotion of penetration or uptake, and/or reduction in the activities of the nervous system [11]. However, further physiological processes could be responsible for the strong effects observed by the interaction of the IGR insecticides with respective endogenous hormones or OR agonists. For instance, neurotransmitters, neuromodulators, and/or neurohormones are known to regulate diverse physiological and behavioral processes in insect pests [27]. Therefore, it is likely that the synergistic action of OR agonists is due to its effects on the elevation of blood-sugar levels, which could result in robust excitation that causes anorexia in insect pests and accelerated energy exhaustion as shown by hyper-excitation to the given IGR insecticides [16,28]. Another possible explanation is the constant burst of mandibular movements which may result in antifeeding in the affected insect pests [29]. However, further investigation should be carried out to demonstrate the biochemical and molecular biological effects on the synergistic action of OR agonists with IGR insecticides on *Culex quinquefasciatus*.

5. Conclusion

In summary, we have shown significant synergistic effects of OR agonists with the selected IGR insecticides on fourth instar larvae of *Culex quinquefasciatus*. These findings indicate the importance of synergism to reduce the amount and use of key insecticides which will reduce the risk of adverse health effects and improve the environment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

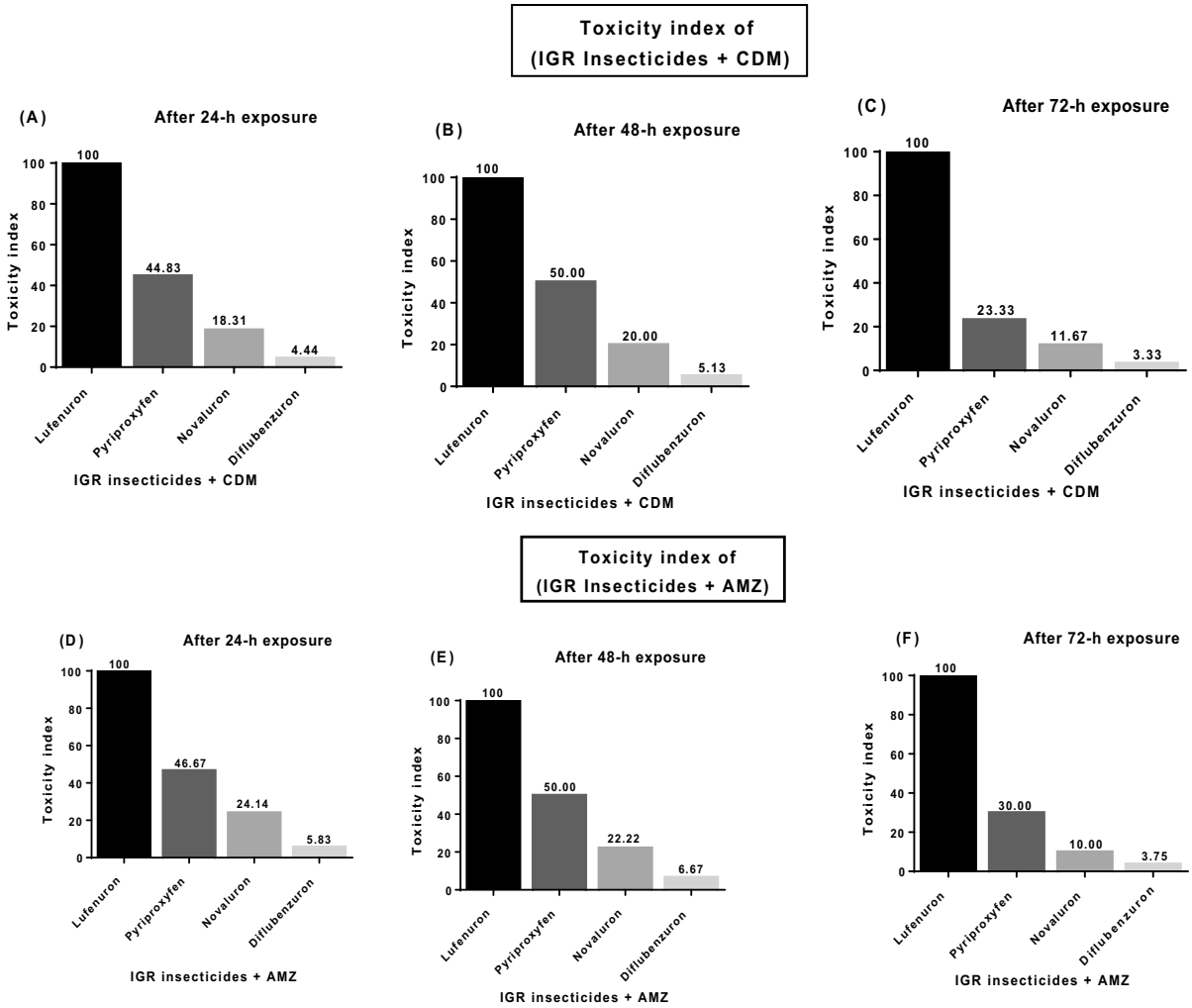

Fig. 3. Toxicity index of selected IGR insecticides in combined with CDM (A, B, and C) and in combined with AMZ (D, E, and F) on fourth instar larvae of *Culex quinquefasciatus* after 24, 48, and 72-h of exposure.
Toxicity index = [(LC_{50} of the most toxic tested IGR insecticides + synergist/LC_{50} of the tested IGR insecticides + synergist) × 100].

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