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Research

Toxicological Evaluation of Novel Butenolide Pesticide Flupyradifurone Against *Culex quinquefasciatus* (Diptera: Culicidae) Mosquitoes

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Abstract

The impact of increasing resistance of mosquitoes to conventional pesticides has led to investigate various unique tools and pest control strategies. Herein, we assessed the potency of flupyradifurone, a novel pesticide, on fourth instar larvae of *Culex quinquefasciatus* Say. Further, we evaluated the synergistic action of piperonyl butoxide (PBO) and the octopamine receptor agonists (OR agonists) chlordimeform (CDM) and amitraz (AMZ) on the toxicity of flupyradifurone in comparison with sulfoxaflor and nitenpyram to increase their toxicity on *Cx. quinquefasciatus*. Results demonstrated that flupyradifurone was the most potent pesticide followed by sulfoxaflor and nitenpyram. Further, the synergetic effect of PBO, CDM, and AMZ was significant for all selected pesticides especially flupyradifurone. However, AMZ had the most significant effect in combination with the selected pesticides followed by CDM and PBO. The toxicity of the pesticides was time-dependent and increased over time from 24, 48, to 72 h of exposure in all experiments. The results indicate that flupyradifurone is a promising component in future mosquito control programs.

Key words: flupyradifurone, *Culex quinquefasciatus*, mosquito control, piperonyl butoxide (PBO), octopamine receptor agonists (OR agonists)

Culex quinquefasciatus Say is a peridomestic mosquito which easily feeds on plenty of hosts including avian and human (Lopes et al. 2019). It is considered the principal vector of numerous viral pathogens such as West Nile Virus (WNV), lymphatic filariasis, avian malaria, St. Louis encephalitis, Western equine encephalitis, and Zika (Kothera et al. 2019, McInnis et al. 2019). For many decades, pesticides are remaining the most efficient practice in mosquito control (Sadanandane et al. 2018, Rai et al. 2019). Unfortunately, the great increase in the use of conventional pesticides causes great concern about their effect on human health and safety (Ahmed and Othman 2020). However, pesticide resistance is considered a serious and growing problem worldwide (Kothera et al. 2019, Yuan et al. 2019, Ahmed and Vogel 2020). In this interim, developing new chemical classes of pesticides for efficient mosquito control is of utmost importance to overcome resistance issues (Ahmed and Vogel 2016a, b).

Flupyradifurone is a new pesticide stimulated by the butenolide scaffold naturally occurring in stemofoline (Nauen et al. 2015). Further, flupyradifurone's mode of action is similar to neonicotinoides acting as an agonist on the insect nicotinic acetylcholine receptor (nAChR) in the nervous system (Ihara et al. 2017, Bell et al. 2020). However, it displayed an excellent and fast action against a wide spectrum of sucking pest insects, plus, it exhibits a powerful action toward certain pests that developed a pesticide resistance population such as selected neonicotinoid-resistant pests (whiteflies and aphids that expressing metabolic resistance mechanisms) (Roditakis et al. 2017, Liang et al. 2019). Interestingly, in spite of flupyradifurone's common mode of action with neonicotinoids and sulfoximines, it is chemically different in which it is the first nAChR insecticide containing the stemofoline-derived (natural compound) butenolide pharmacophore that affects the insects' nAChR (Nauen et al. 2015).

Importantly, flupyradifurone is an effective pesticide against numerous pest insects feeding on the underside of leaves for instance, *Aphis gossypii* and *Myzus persicae* (Raupach et al. 2012). Furthermore, flupyradifurone demonstrated an excellent potency

against whitefly species in laboratory bioassays compared to neonicotinoids and other homopteran feeding blockers (Smith et al. 2016, Liang et al. 2019).

In this study, we evaluated the toxicity effects of flupyradifurone in comparison with nitenpyram and sufloxaflor against the fourth instar larvae of *Cx. quinquefasciatus* mosquitoes under laboratory conditions. Further, we assessed the synergistic actions of piperonyl butoxide (PBO) and octopamine receptor agonists (OR agonists), amitraz (AMZ) and chlordimeform (CDM), on these pesticides to demonstrate the possibility of maximizing their toxicity on *Cx. quinquefasciatus* mosquitoes.

Materials and Methods

Mosquitoes

The laboratory colony (susceptible colony) of *Cx. quinquefasciatus* was obtained from the laboratory of Prof. Walter Leal, University of California Davis (UC Davis) which was used for all experiments. However, the original of this colony was from a laboratory colony started from adult mosquitoes collected in Merced, CA, in the 1950s and maintained by Dr. Anthony Cornel in the Kearney Agricultural Center, University of California. The Davis colony has been reared at Davis under a photoperiod of 12:12 (L:D) h, 27 ± 1°C and 75% RH (Zeng et al. 2018, Ahmed and Vogel 2020). Because of 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 requested.

Chemicals

Flupyradifurone (99.5%) and sulfoxaflor (99.6%) were purchased from Chem Service, Inc. (West Chester, PA). Nitenpyram (99.9%), PBO (99%), CDM (99.8%), AMZ (96.8%) were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Acute Toxicity Bioassay

The acute toxicity bioassays were performed as described by Paul et al. (2006). In brief, 20 from fourth instar larvae of homogenous size were placed in 140-ml glass cups containing 99 ml of distilled water and 1 ml of pesticide (in acetone) solution. controls were only added by 1 ml of acetone. Furthermore, five concentrations (500, 50, 5, 0.5, and 0.05 ng/ml) were used for all assays for each pesticide, and each assay was held at 25°C. Three replicates were preceded for each concentration. Approximately 0.5 g dog food powder was added per replicate.

larvae were considered dead if they were not responding to the touching of a probe or if they could not reach the surface of the water. Because of the slow-acting response of these pesticides, mortality was determined after 24, 48, and 72 h of exposure due to the delay of the acute toxicity that needs to be effective.

Synergistic Action Bioassay

The synergistic action bioassays were assessed as reported by Ahmed and Matsumura (2012). Importantly, our previous study showed that PBO and OR agonists (CDM and AMZ) at a concentration of 20 µg/ml and 10 µg/ml, respectively, were the maximum concentrations that did not cause mortality during the 72-h posttreatment on fourth instar larvae of *Cx. quinquefasciatus*. Five different concentrations of each pesticide were used for all bioassays which were at least repeated twice. Percentage mortality was recorded after 24-, 48-, and 72-h posttreatment.

Analysis

Corrected mortality was regulated based on Abbott's formula (Abbott 1925). Data analysis, for instance LC_{50}, 95% CL values, slope, X_2, and *g*- values; were analyzed by using IBM SPSS Statistics Version 25 software (SPSS Inc., Chicago, IL). However, synergistic action was considered to be significant ($P \leq 0.05$) when the 95% CIs for the LC_{50} values for fourth instar larvae treated by the pesticide alone and did not overlap with those for larvae exposed to pesticide + synergist. Synergistic ratio (SR) was determined by dividing the LC_{50} value of the test pesticide by the LC_{50} that acquired for the combined effect of pesticide + synergist. However, toxicity index estimated as [(LC_{50} of the most toxic tested pesticide/LC_{50} of the tested pesticide) × 100].

Results

The toxicity data of flupyradifurone in comparison with sufloxaflor and nitenpyram on fourth instar larvae of *Cx. quinquefasciatus* after 24, 48, and 72 h of exposure are presented in Table 1. Flupyradifurone was the most potent pesticide among the tested pesticides (LC_{50} = 10.64, 2.45, and 0.87 ng/ml after 24, 48, and 72 h of exposure, respectively). However, nitenpyram was the least toxic one (LC_{50} = 302.54, 61.93, and 15.82 ng/ml after 24, 48, and 72 h of exposure, respectively).

The effects of the synergistic action of PBO on flupyradifurone are shown in Table 2. PBO synergized all selected pesticides especially flupyradifurone (SR = 3.69-, 4.02-, and 5.44-fold after 24, 48, and 72 h of exposure, respectively). Further, sulfoxaflor was the least synergized by PBO (SR = 3.18-, 3.82-, and 4.37-fold after 24, 48, and 72 h of exposure, respectively). In combination with the selected pesticides, CDM synergized all these pesticides. However, after 24 h of exposure, flupyradifurone was the most synergized pesticide followed by nitenpyram and sufloxaflor (SR = 10.23-, 9.27-, and 8.79-fold, respectively). The same trend was observed after 48 and 72 h of exposure (Table 3). The results of the synergistic action of AMZ with selected pesticides are demonstrated in Table 4. AMZ was synergized by all pesticides especially flupyradifurone (SR = 20.08-, 29.63-, and 31.07-fold after 24, 48, and 72 h of exposure, respectively). Further, sulfoxaflor was considered the minimal synergized pesticide among the selected pesticides (SR = 15.34-, 18.24-, and 23.06-fold after 24, 48, and 72 h of exposure, respectively).

The most notable trend was that AMZ was most synergized by all selected pesticides followed by CDM and PBO. Further, flupyradifuron was the most synergized pesticide followed by sulfoxaflor and nitenpyram. Furthermore, the synergistic action was increased based on time-dependent changes as assessed 24, 48, and 72 h after pesticides initial exposure.

Toxicity index of flupyradifurone in comparison with sulfoxaflor and nitenpyram alone and plus PBO, CDM, and AMZ was presented in Fig. 1. After 24 h of exposure, flupyradifurone was more toxic than sulfoxaflor and nitenpyram by 8.31- and 28.41-fold, respectively (Fig. 1A). However, in combination with PBO, flupyradifurone was more potent than sulfoxaflor and nitenpyram by 9.64 and 29,85-fold, respectively (Fig. 1D). Further, flupyradifurone plus CDM, it was more toxic than sulfoxaflor and nitenpyram by 9.66- and 31.35-fold, respectively (Fig. 1G). Whereas, in combination with AMZ, flupyradifurone was more toxic than the rest of selected pesticides by 10.87- and 32.05-fold, respectively (Fig. 1J). The same pattern of toxicity was observed after 48 and 72 h of exposure (Fig. 1).

Table 1. Toxicity of flupyradifurone in comparison with selected pesticides on fourth instars of *Culex quinquefasciatus* after 24, 48, and 72 h of exposure

Compounds	n^a	After 24 h				After 48 h				After 72 h			
		$LC_{50}{}^b$ (95% CL)	Slope (± SE)	X^2 (df)[c]	*g*-value[d]	$LC_{50}{}^b$ (95% CL)	Slope (± SE)	X^2 (df)[c]	*g*-value[d]	$LC_{50}{}^b$ (95% CL)	Slope (± SE)	X^2 (df)[c]	*g*-value[d]
Flupyradifurone	300	10.64 (2.08–41.96)	4.69 (± 0.10)	1.72 (3)	0. 06	2.45 (0.45–8.62)	4.62 (± 0.11)	1.12 (3)	0.07	0.87 (0.19–2.50)	4.96 (± 0.13)	2.07 (3)	0.05
Sulfoxaflor	300	88.36 (26.42–210.51)	4.74 (± 0.11)	0.27 (3)	0.09	28.46 (8.14–132.23)	4.74 (± 0.11)	0.56 (3)	0.08	7.61 (1.77–30.15)	4.58 (± 0.10)	1.59 (3)	0.06
Nitenpyram	300	302.54 (74.81–432.42)	4.26 (± 0.12)	0.062 (3)	0.08	61.93 (14.74–71.55)	4.14 (± 0.10)	0.40 (3)	0.04	15.82 (4.27–67.05)	4.72 (± 0.11)	0.49 (3)	0.01

[a]*n* = number of larvae tested, excluding control.

[b]Concentration is expressed in ng/ml and the response determined after 24-, 48-, and 72-h exposure.

[c]df = degree of freedom.

[d]If *g*-value < 0.5, the data fit the probit model. Otherwise, the data do not fit the probit model and the analysis is invalid.

Table 2. Synergistic action of PBO on the toxicity of flupyradifurone in comparison with selected pesticides on fourth instars of *Culex quinquefasciatus* after 24, 48, and 72 h of exposure

Compounds + PBO[e]	n^a	After 24 h					After 48 h					After 72 h				
		$LC_{50}{}^b$ (95% CL)	Slope (± SE)	X^2 (df)[c]	*g*-value[d]	SR[f]	$LC_{50}{}^b$ (95% CL)	Slope (± SE)	X^2 (df)[c]	*g*-value[d]	SR[f]	$LC_{50}{}^b$ (95% CL)	Slope (± SE)	X^2 (df)[c]	*g*-value[d]	SR[f]
Flupyradifurone	300	2.88 (0.59–9.81)	4.75 (± 0.11)	1.24 (3)	0.07	3.69*	0.61 (0.15–2.75)	4.74 (± 0.12)	2.29 (3)	0.04	4.02*	0.16 (0.059–1.27)	4.59 (± 0.14)	0.99 (3)	0.09	5.44*
Sulfoxaflor	300	27.76 (8.24–120.70)	4.87 (± 0.11)	0.12 (3)	0.09	3.18*	7.45 (3.66–56.92)	4.71 (± 0.11)	0.57 (3)	0.03	3.82*	1.74 (0.57–11.46)	4.49 (± 0.11)	2.44 (3)	0.02	4.37*
Nitenpyram	300	85.86 (23.03–96.56)	4.47 (± 0.11)	0.20 (3)	0.07	3.52*	15.60 (4.02–79.42)	4.46 (± 0.10)	0.32 (3)	0.06	3.97*	3.39 (2.54–23.15)	5.44 (± 0.12)	0.92 (3)	0.02	4.67*

[a]*n* = number of larvae tested, excluding control.

[b]Concentration is expressed in ng/ml and the response determined after 24-, 48-, and 72-h exposure.

[c]df = degree of freedom.

[d]If *g*-value < 0.5, the data fit the probit model. Otherwise, the data do not fit the probit model and the analysis is invalid.

[e]Concentration of synergist was 20 µg/ml and larvae exposed to pesticide and synergist simultaneously.

[f]SR = synergistic ratio. Calculated by dividing the LC for pesticide by the LC of pesticide + PBO.

*SR significantly different from control without synergist (=1.0) at ($P \leq 0.05$).

Table 3. Synergistic action of CDM on the toxicity of flupyradifurone in comparison with selected pesticides on fourth instars of *Culex quinquefasciatus* after 24, 48, and 72 h of exposure

Compounds + CDM[e]	n[a]	After 24 h					After 48 h					After 72 h				
		LC_{50}[b] (95% CL)	Slope (± SE)	X^2 (df)[c]	g-value[d]	SR[f]	LC_{50}[b] (95% CL)	Slope (± SE)	X^2 (df)[c]	g-value[d]	SR[f]	LC_{50}[b] (95% CL)	Slope (± SE)	X^2 (df)[c]	g-value[d]	SR[f]
Flupyradifurone	300	1.04 (0.56–3.54)	4.60 (± 0.11)	0.49 (3)	0.09	10.23*	0.19 (0.080–1.45)	4.69 (± 0.14)	0.68 (3)	0.07	**12.89***	0.053 (0.023–0.67)	4.29 (± 0.16)	0.97 (3)	0.08	16.42*
Sulfoxaflor	300	10.05 (2.72–37.84)	4.85 (± 0.11)	1.01 (3)	0.06	8.79*	2.84 (1.56–19.43)	4.97 (± 0.11)	1.87 (3)	0.01	**10.02***	0.63 (0.26–4.63)	4.69 (± 0.12)	2.72 (3)	0.03	12.08*
Nitenpyram	300	32.64 (5.05–80.59)	4.73 (± 0.43)	0.045 (3)	0.05	9.27*	5.68 (1.88–28.79)	4.71 (± 0.11)	0.80 (3)	0.08	**10.90***	1.14 (1.02–10.92)	5.71 (± 0.13)	1.53 (3)	0.07	13.88*

[a]*n* = number of larvae tested, excluding control.
[b]Concentration is expressed in ng/ml and the response determined after 24-, 48-, and 72-h exposure.
[c]df = degree of freedom.
[d]If *g*-value < 0.5, the data fit the probit model. Otherwise, the data do not fit the probit model and the analysis is invalid.
[e]Concentration of synergist was 10 µg/ml and larvae exposed to pesticide and synergist simultaneously.
[f]SR = synergistic ratio. Calculated by dividing the LC for pesticide by the LC of pesticide + CDM.
*SR significantly different from control without synergist (=1.0) at ($P \leq 0.05$).

Table 4. Synergistic action of AMZ on the toxicity of flupyradifurone in comparison with selected pesticides on fourth instars of *Culex quinquefasciatus* after 24, 48, and 72 h of exposure

Compounds + AMZ[e]	n[a]	After 24 h					After 48 h					After 72 h				
		LC_{50}[b] (95% CL)	Slope (± SE)	X^2 (df)[c]	g-value[d]	SR[f]	LC_{50}[b] (95% CL)	Slope (± SE)	X^2 (df)[c]	g-value[d]	SR[f]	LC_{50}[b] (95% CL)	Slope (± SE)	X^2 (df)[c]	g-value[d]	SR[f]
Flupyradifurone	300	0.53 (0.088–1.63)	4.68 (± 0.13)	0.79 (3)	0.05	20.08*	0.092 (0.039–0.63)	4.25 (± 0.19)	1.45 (3)	0.06	26.63*	0.028 (0.0090–0.37)	3.78 (± 0.21)	0.78 (3)	0.04	31.07*
Sulfoxaflor	300	5.76 (0.99–10.15)	5.23 (± 0.12)	2.78 (3)	0.02	15.34*	1.56 (0.51–6.91)	4.91 (± 0.11)	2.79 (3)	0.02	18.24*	0.33 (0.12–2.35)	4.65 (± 0.12)	1.31 (3)	0.02	23.06*
Nitenpyram	300	16.99 (1.33–17.09)	4.98 (± 0.11)	0.039 (3)	0.08	17.81*	3.11 (1.29–11.38)	5.43 (± 0.12)	2.29 (3)	0.05	19.91*	0.57 (0.37–4.05)	5.33 (± 0.13)	1.04 (3)	0.07	27.75*

[a]*n* = number of larvae tested, excluding control.
[b]Concentration is expressed in ng/ml and the response determined after 24-, 48-, and 72-h exposure.
[c]df = degree of freedom.
[d]If *g*-value < 0.5, the data fit the probit model. Otherwise, the data do not fit the probit model and the analysis is invalid.
[e]Concentration of synergist was 10 µg/ml and larvae exposed to pesticide and synergist simultaneously.
[f]SR = synergistic ratio. Calculated by dividing the LC for pesticide by the LC of pesticide + AMZ.
*SR significantly different from control without synergist (=1.0) at ($P \leq 0.05$).

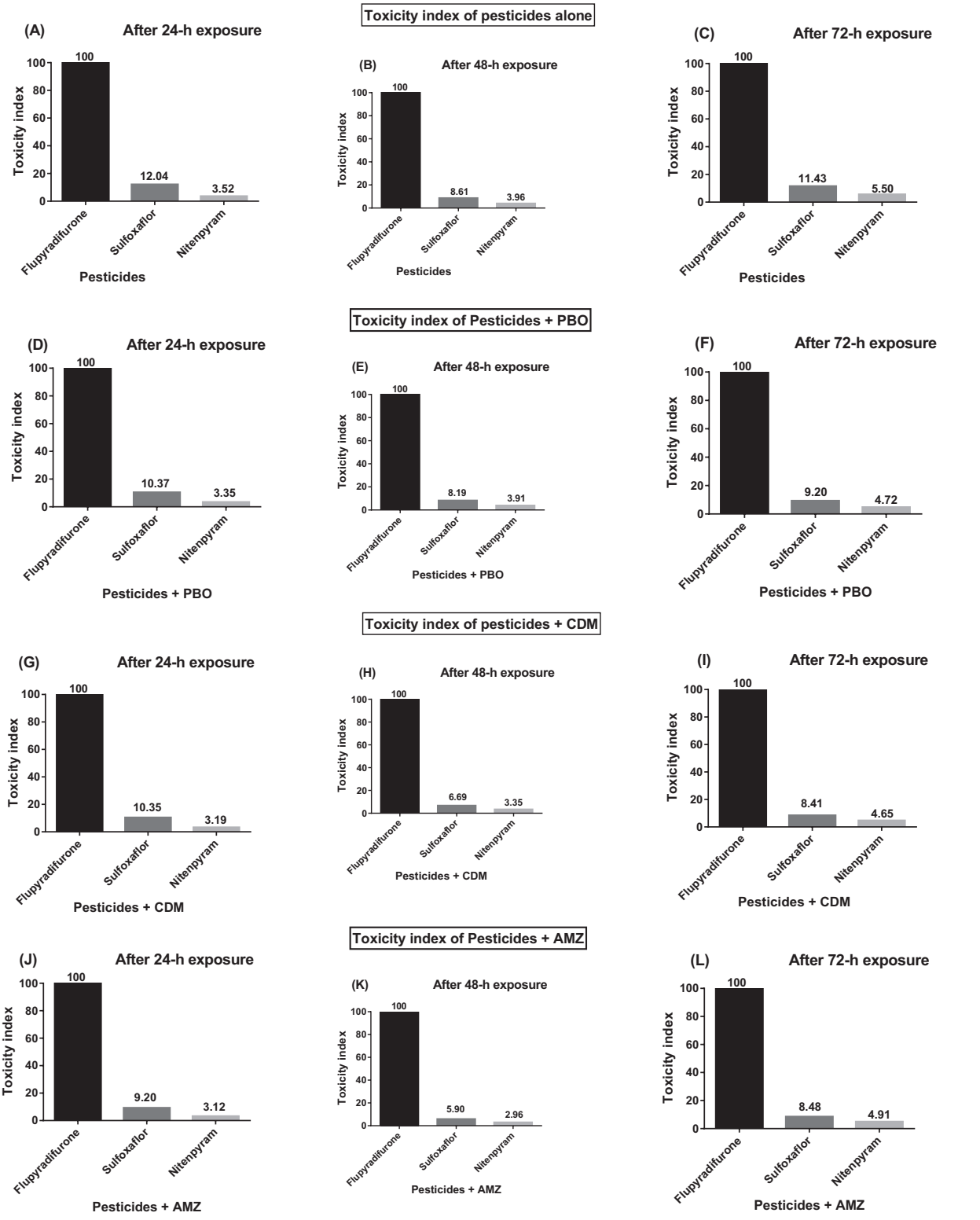

Fig. 1. Toxicity index of flupyradifurone in comparison with sulfoxaflor and nitenpyram alone (A, B, and C), plus PBO (D, E, and F), CDM (G, H, and I), and AMZ (J, K, and L) on fourth instar larvae of *Culex quinquefasciatus* after 24, 48, and 72 h of exposure. Toxicity index = [(LC_{50} of the most toxic tested pesticide/LC_{50} of the tested pesticide) × 100].

Discussion

Pesticide resistance has been a long-time issue in mosquito control. However, facilitating new strategies will be paramount. In this regard, the discovery of new pesticides that have distinctive chemical and physical properties as well as unique mode of action is considered substantial. In this interim, flupyradifurone is considered a novel pesticide that acts on nAChRs in insect's nervous system.

In our study, flupyradifurone revealed a significant toxicity against fourth instar larvae of *Cx. quinquefasciatus* in comparison with the selected pesticides.

To date, there are no reliable data focusing on the efficacy of flupyradifurone on *Cx. quinquefasciatus*. However, flupyradifurone's potent toxicity has been shown toward several different insect pests, particularly, the sap-feeding pests which could be due to its physical and chemical properties. Ma et al. (2019) found that flupyradifurone was the most toxic pesticide among the tested pesticides and the LC_{50} values which was 1.88 and 201.91 mg/l for the SS and SulR strains, respectively, on *A. gossypii* adults. In another study on the same insect pest, Liang et al. (2019) demonstrated that the time of development of the fourth instar and the preadult as well as the total prereproductive period of *A. gossypii* adults were significantly prolonged, plus, their fecundity was significantly decreased after treated with LC_{25} of flupyradifurone after 48 h. Further, Tang et al. (2019) reported that flupyradifurone was very toxic on *M. persicae* adults with a 48-h LC_{50} of 8.49 mg/l. Smith et al. (2016) found that flupyradifurone was the most potent pesticide and LC_{50} values from field populations of *Bemisia tabaci* adults ranged from 0.01 to 1.47 ppm for flupyradifurone, 0.04 to 3.35 ppm for dinotefuran, 0.90 to 24.95 ppm for imidacloprid, and 0.97 to 24.43 ppm for thiamethoxam.

Interestingly, PBO, CDM, and AMZ enhanced the toxicity of the selected pesticides. In the presence of the synergists, PBO, CDM, and AMZ, toxicity increased dramatically, especially after 48 and 72 h of exposure. However, many studies focused on the synergistic action of PBO on pesticides that disrupts the insect nAChR. Paul et al. (2006) found that PBO increased the toxicity of imidacloprid by 7.8-fold after treatment of 72 h on the fourth instars of *Aedes aegypti* and by >2,000-fold on adult female of *Ae. aegypti* after 48 h of exposure. Moreover, Ahmed and Vogel (2016a) evaluated the synergistic action of PBO on seven selected neonicotinoid pesticides (imidacloprid, dinotefuran, thiamethoxam, thiacloprid, acetamiprid, clothianidin, and nitenpyram) on *Ae. aegypti* adults after 72 h of exposure and found that the SRs were 363-, 813-, 941-, 607-, 504-, 517-, and 815-fold, respectively. Further, in a different study, Ahmed and Vogel (2016b) demonstrated that PBO enhanced the toxicity of sulfoxaflor pesticide on *Ae. aegypti* fourth instar larvae and adults after 72-h exposure by 10.3- and 8.3-fold, respectively. Ahmed and Othman (2020) revealed that PBO significantly synergized imidacloprid and its nanoformulations. The most synergistic effects were found with IMD03 (nanoform) and the lowest was imidacloprid itself on fourth instar larvae of *Cx. pipiens*. In our study, the effect of PBO on the toxicity of selected pesticides was considerably different in terms of the degree of synergism. The synergistic effects could be affected by the different expression of the cytochrome P450 enzymes that are involved in the metabolism of these pesticides.

Importantly, CDM and AMZ showed significantly synergistic action toward the tested pesticides particularly flupyradifurone. Ahmed and Matsumura (2012) stated that the synergistic action that occurs via the OR agonists was greatest with AMZ on selected neonicotinoid pesticides (SR ranged from 1.6- to 11-fold) and was most apparent after 72 h of exposure on fourth instar larvae of *Ae. aegypti*. Ahmed and Vogel (2016b) found that AMZ plus sulfoxaflor had the greatest synergism, especially on fourth instar larvae and adults after 72 h of treatment (SR = 16.3- and 29.6-fold, respectively). The synergistic effects of the OR agonists by the selected pesticides are likely based on the suppression of detoxification enzymes, enhancing the penetration or uptake, and/or depression related to the activities of the nervous system (Ahmed and Matsumura 2012; Ahmed and Vogel 2016a,b). However, other physiological processes affected by the OR agonists could be responsible for the synergism that occurred. For instance, OR agonists may interact with endogenous hormones such as neurotransmitters, neuromodulators, and/or neurohormones which are known to regulate diverse physiological and behavioral processes in insect pests (Roeder 2005). Further, Ismail and Matsumura (1992) and Ahmed et al. (2015) explained that the synergistic action of OR agonists is likely due to its effects on the elevation of blood-sugar levels which leads to strong excitation that causes anorexia in insect pests and results in acceleration of the process of energy exhaustion in insects treated with the selected pesticides.

In summary, we demonstrated that flupyradifurone represents an effective pesticide in controlling *Cx. quinquefasciatus*. Flupyradifurone mediate its effects differently from that of other neonicotinoid and sulfoximine pesticides in that it reversibly binds to nAChRs and lacks metabolization by cytochrome P450 enzymes in sucking insect pests that resistant to neonicotinoid pesticides. Thus, it demonstrates unique and fast action against a broad spectrum of sucking pests especially that exhibits resist to neonicotinoid pesticides. Therefore, efforts should continue to understand the biochemical and molecular mode of action and the toxicological effects of flupyradifurone on *Cx. quinquefasciatus* mosquitoes.

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