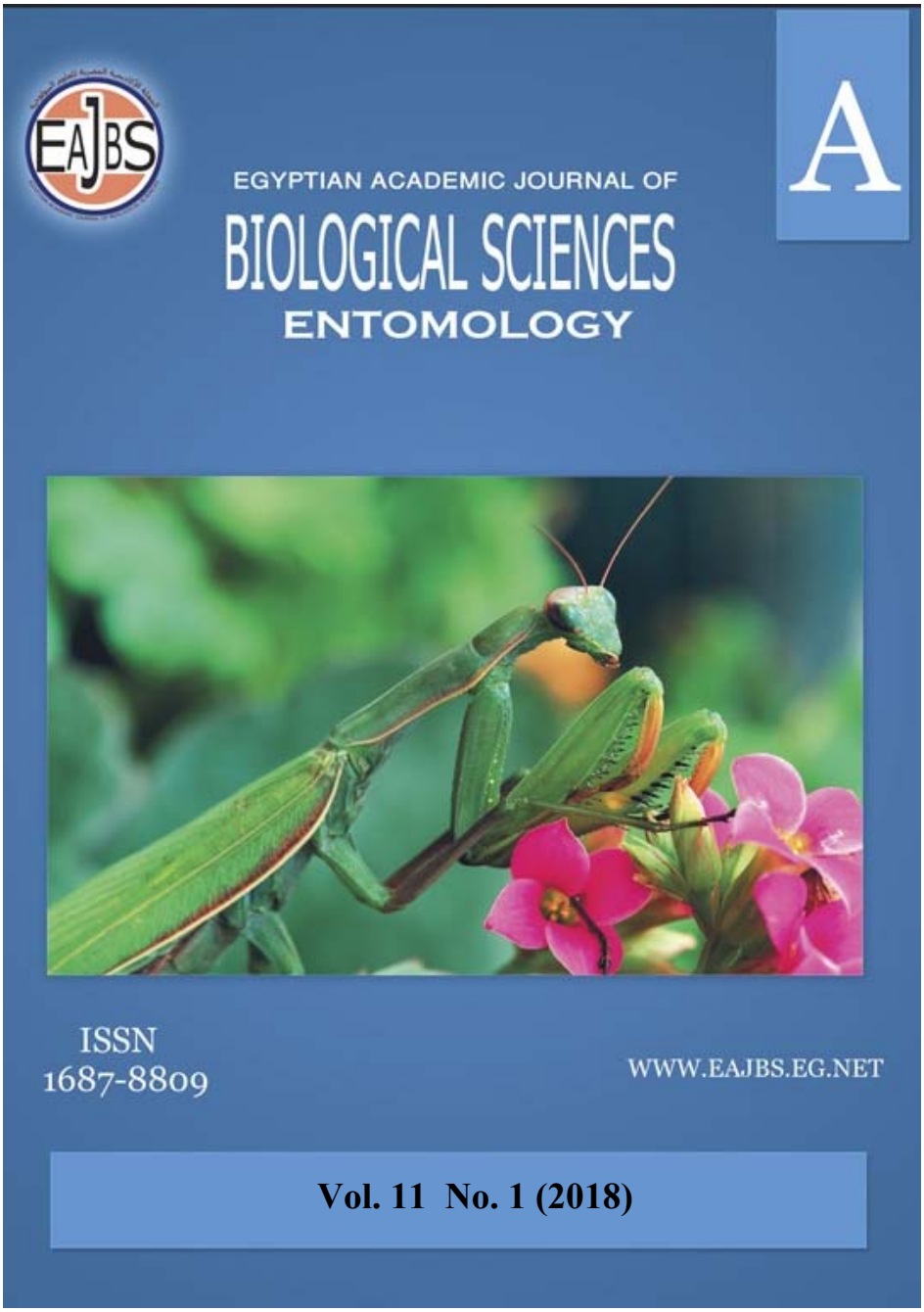

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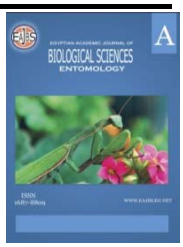

Assessment of New Ryanodine Receptor Activators Against The Cotton Leafworm, *Spodoptera littoralis* (Boisd.) at Semi-Field Conditions

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ABSTRACT

A semi-field experiment was designed in order to assess the insecticidal activity of three new ryanodine receptor activators against the cotton leafworm, *Spodoptera littoralis* (Boisd.) during 2016 cabbage growing season. Second and forth instar larvae were offered treated cabbage leaves treated with the recommended dose of the tested compounds at 24-h, 48-h, 72-h, five days, seven days and nine days post spray. Results revealed that general pattern was observed for the formulations, where the toxicity is increased with increasing the time lapsed after treatment. Absolute mortality percentage was obtained at 72-h post exposure for all tested compounds in both 2nd and 4th instar larvae. In addition, results revealed that the detoxification enzymes activity increased compared to control. In conclusion, the ryanodine receptor activators represent a novel mode of insecticide action, have the most favorable toxicological and ecotoxicological profiles, these profile make this chemicals useful tool in IPM of *S. littoralis*, however, the resistance risk of cotton leafworm on these insecticides should not be overlook.

INTRODUCTION

Cabbage, *Brassica oleracea* var. *capitata* L. (Family: Brassicaceae), has one of the longest developmental times among annual vegetable crops, with seeds planted indoors in March and harvest occurring in late Fall. During this time period there are numerous insect pests that feed on cabbage. The cabbage insect pest complex consists of sporadic pests such as root maggots, thrips, aphids, flea beetles, cabbage loopers, and annual pests such as the imported cabbage worm, cotton leafworm and diamondback moth. With the widespread insecticide resistance of the major conventional insecticide classes, it is necessary to develop some new insecticides with unique modes of action (Sabry *et al*., 2013). The newest major class of insecticides, the diamides, is rapidly replacing major uses of the earlier chlorinated hydrocarbons, organophosphates, methylcarbamates, pyrethroids, and neonicotinoids (Casida, 2009) because they are highly effective on major pests resistant to these

earlier chemo-types and appear to be safe for people and the environment (Hamaguchi and Hirooka, 2012). Lahm *et al*. (2005) stated that chlorantraniliprole activates the unregulated release of internal calcium stores, leading to Ca^{+2} depletion, feeding cessation, lethargy, and muscle paralysis, and finally insect death. Sattelle *et al*. (2008) reported that chlorantraniliprole, the first commercialized ryanodine receptor insecticide from the anthranilic diamide class, has exceptional insecticidal activity on a range of lepidopteran pests and on other orders such as Coleoptera, Diptera, Isoptera and Hemiptera. There are currently two types of diamide insecticides, i.e., the anthranilic diamides with the important compounds chlorantraniliprole (Lahm *et al*., 2012) and cyantraniliprole (Lahm *et al*., 2012) and the phthalic diamides exemplified by flubendiamide (Hamaguchi and Hirooka, 2012). The current study aimed to evaluate the insecticidal activity of three ryanodine receptor activator compounds against the 2nd and 4th instar larvae of the cotton leafworm, *Spodoptera littoralis* (Boisduval) under semi-field conditions in cabbage field and assessing their biochemical effect against treated larvae.

MATERIALS AND METHODS

Rearing insects:

A laboratory susceptible strain of the cotton leaf worm *Spodopter littoralis* (Boisd.) (Lepidoptera: Noctuidae), reared for more than 10 generations was obtained from the Research Division of the cotton leaf worm, Plant Protection Research Institute. Insects were reared under controlled conditions in an incubator at 26 ± 2º C, of 65 ± 10% R. H., and 8:16 L: D photoperiod at the Plant Protection Research Institute, Dokki-Giza, Egypt.

Tested compounds:

Insecticides used in the studies were the commercial formulation of flubendiamide (20% WDG) with the trade name Takumi® from Samtrade Company, Chlorantrianliprole (20% SC) with the trade name Coragen® from Dupont, Egypt, and Cyantraniliprole (10% SC) with the trade name Exirel® from Dupont, Egypt. All tested compounds were supplied from Plant Protection Research Institute (PPRI), Dokki, Giza, Egypt. All tested compounds were applied at the recommended concentrations according to the recommendation of Agricultural Pesticide Committee (APC), Ministry of Agriculture and Land Reclamation, Egypt.

Semi-field application:

In order to evaluate the insecticidal activity of the tested compounds against *S. littoralis* 2nd and 4th instar larvae, a semi-field experiment was designated. The study was carried out throughout 2016 cabbage season at El-Ramla village, Benha Center, Qaluibia Governorate. The field area was cultivated with cabbage on December 1st 2016. The standard agricultural practices were applied. The experimental area was divided into plates of 1/16 feddan (262.5 m^2). The treatment was arranged in randomized complete blocks design (RCBD) with three replicates each. Application of tested compounds was on February 1st. Temperature degrees in the experimental area were 20-22 ± 2° C and the relative humidity was 0-5 ± 10%.The cabbage plants were sprayed using a backpack sprayer and collected 24-h, 48-h, 72-h, five days, seven days and nine days post spray. Treated and untreated leaves were then supplied to separate sets of 2nd and 4th instar larvae of the cotton leaf worm. Mortalities were recorded daily 24 hrs after the treated cabbage leaves were offered to larvae.

Biochemical assay:

1.Sample preparation:

Larvae were treated as 4th instar with tested compound at (concentration). After 24h treatment, one gram of treated larvae was weighed. Larvae were then homogenized on ice in ice-cold 100 homogenization buffer (0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU and 20% glycerol). The homogenate was centrifuged at 4ºC, 10,000 g for 30 min using sigma 3K 30 rotors NO. 12158, sigma laboratories centrifuge 3K30, the solid debris and cellular material were discarded. The supernatant was transferred into a clean Eppendorf tube, placed on ice and used immediately for mixed function oxidase (MFO), glutathione S-transferase (GST), aspartate transaminase (AST) and alanine aminotransferase (ALT) enzyme activities.

2,Enzyme assays:

2.1.Mixed function oxidase (MFO):

The assay of MFO was conducted using the procedures developed by Rose *et al*. (1995) with slight modification (Letelier *et al*., 2009). A hundred µL of 2 mM ρ-Na solution and 90 µL enzymes were added to a clean Eppendorf tubes. The mixture was incubated for 3 min at 27º C and the reaction initiated by the addition of 10 µL of 9.6 mM NADPH. All mixtures were incubated for 20 min at 37º C. Reactions were stopped by addition of 100 µl TCA 15% and samples were then centrifuged at 5000 rpm for 20 min using IEC-CRU 5000 cooling centrifuge and supernatants were collected. To develop the colorimetric reaction, 100 µl of supernatant is mixed with 100 µl of 1 M NaOH in each well of transparent 96-well Costar microplate (Corning Life Sciences, Lowell, MA). The absorbance of the reaction product, ρ-NP, was measured at 405 nm by using an ELISA plate reader (STAT FAX-2100). An assay mixture with denaturizing enzyme instead of live enzyme was used as the blank wells and was subtracted as background. Specific activities for the ρ-NA substrate was calculated based on the ρ-NP standard curve which was carried out by determining the color absorbance for a series of ρ-NP concentrations ranged from 0.4 nanomoles to 50 nanomoles in 100 ml 30% ethanol. It was fitted using the least squares method (K =0.0485 nm ρ-NP/ well), and expressed in nm ρ-NP/min.mg protein. All reaction readings were conducted in triplicate.

2,2.Glutathione S-transferase (GST):

GST activity was measured according to the method of Asaoka and Takahashi (1983) using ethanolic solution of DNB as a substrate with slight modification as done with El-Shahawi and Al Rajhi (2000). The standard assay mixture (1ml) contained 1.5 mM GSH, 100 mM phosphate buffer pH7, 50 µl of enzyme source, and the reaction was started by the addition of 0.5 mM DNB. After incubation at 37º C for 20 min, the reaction was terminated by the addition of 0.1 ml acetic anhydride. The reaction was left for 5 min at room temperature, and then mixed with 1 ml of 1% (W/V) sulfanilamide in 20% (W/V) HCl followed by 1 ml of 0.02% (W/V) N-(1-naphthyl) ethylenediamine dihydrochloride. After diazo-coupling for 20 min at room temperature, 0.1 ml of 1% (W/V) ammonium sulfamate (freshly prepared) was added to the mixture. The mixture was left for 5 min, and then the absorbance at 540 nm was recorded using Sequoia-Turner Model 340 Spectrophotometer. An assay mixture without enzyme was used as the blank.

2.3.Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT):

Activity of both enzymes AST and ALT was measured according to the method of Reitman and Frankel (1957), using Diamond Diagnostic kit (Diamond Co.

Egypt). In this method, 100 µl of enzyme source was added to 500 µl of 100 mM phosphate buffer of pH=7.2 containing 80 mM L-aspartate as a substrate for AST or 80 mM D-L-alanine as a substrate for ALT, and 4 mM α-ketoglutarate. This mixture was incubated for 30 min at 37˚C. After that, 500 µl of developing color reagent (4 mM 2, 4-dinitrophenylhydrazine) was added and the solution was incubated for 20 min at room temperature. Lastly, 5 ml of 0.4 N NaOH was added then mixed and left at room temperature for five min. An assay mixture without enzyme source was used as the blank and the absorption was measured at the wave length of 546 nm using Sequoia-Turner Model 340 Spectrophotometer. An assay mixture without enzyme was used as the blank. AST and ALT specific activities were determined as IU/mg protein/hr and calculated as a percentage of control.

Statistical analysis:

Data were statistically analyzed by ANOVA procedure at $P<0.05$ using SPSS statistics 17.0 release 17.0.0 software. When the ANOVA statistics were significant at ($P \leq 0.01$), means were compared by the Duncan's multiple range test using SPSS 17.0 software. Reduction percentage in 2nd and 4th instar larvae over the control was calculated according to Henderson and Tilton (1955). The detoxification enzymes activity data were subjected to an ANOVA analysis followed by t-test, treatment means were compared by least significant difference (LSD) at 5% level. The standard deviation (SD) of four replications was calculated. Means were compared with each other using Student-Newman Keuls (SNK) test (LSD at $P < 0.05$).

RESULTS AND DISCUSSION

Efficiency of tested compounds against 2nd and 4th instar larvae of *S. littorlais* under semi-field conditions:

Data presented in table (1) showed the efficiency of the recommended dose of tested compounds against the 2nd instar larvae of *S. littoralis* under semi-field application. General pattern was observed for the formulations, where the toxicity is increased with increasing the time lapsed after treatment, and then, gradual decrease was observed. Results also revealed that absolute mortality percentage was obtained on 72-h post exposure treatments for all tested compounds. In addition, a gradual decrease in mortality percentage was observed on the 5th day till the 9th day.

Results obtained in table (2) showed the efficiency of the recommended dose of tested compounds against the 4th instar larvae of *S. littoralis* under semi-field application. Generally, results revealed that the toxicity was increased with increasing the time lapsed after treatment. Results also revealed that absolute mortality percentage was obtained on 72-h post exposure treatments for all tested compounds. In addition, a gradual decrease in mortality percentage was observed on the 5th day till the 9th day.

These results were agreement with those obtained by Jia *et al*. (2011), Barrania (2013), Barrania *et al*. (2013), and Khalifa *et al*. (2015) they found that chlorantraniliprole has a potent insecticidal activity against several important foliage feeding insect pests. Obtained results agreed also with Jameel and Jamal (2017) as they treated *S. litura* with flubendiamide. In addition, chlorantraniliprole causes feeding cessation, lethargy, muscle paralysis and ultimately death by activating the ryanodine receptor (Cao *et al*., 2010). Temple *et al.* (2009) stated that Rynaxypyr® (chlorantraniliprole) demonstrated very good activity at relatively low rates against all three tested major caterpillar pests of cotton in their study, including tobacco budworm, bollworm, and fall armyworm.

Table (1): Reduction percentage in 2nd instar larvae of *Spodoptera littoralis* over control treated with tested compounds under semi-field conditions

Compound	Dose	% Reduction in 2nd instar larvae over Control post exposure						
		24h	48h	72h	5days	7days	9days	Total mean
Takumi® 20% WGD	100 gm/Fed	7.70	99.0	100.00	90.89	77.50	59.07	70.59
Coragen® 20% SC	60 cm/Fed	12.70	100.0	100.00	98.6	88.10	78.50	75.5
Exirel® 20% SC	75 cm/20 L	11.70	100.0	100.00	95.88	84.90	66.50	74.16
Untreated	Water	--	--	--	--	--	--	--

Table (2): Reduction percentage in 4th instar larvae of *Spodoptera littoralis* over control treated with tested compounds under semi-field conditions

Compound	Dose	% Reduction in 4th instar larvae over Control post exposure						
		24h	48h	72h	5days	7days	9days	Total mean
Takumi® 20% WGD	100 gm/Fed	5.71	97.50	100.00	85.74	74.80	50.45	70.14
Coragen® 20% SC	60 cm/Fed	10.88	98.90	100.00	92.93	80.10	71.49	76.71
Exirel® 20% SC	75 cm/20 L	9.88	97.88	100.00	91.00	79.90	63.71	74.73
Untreated	Water	--	--	--	--	--	--	--

Effect of tested compounds on enzyme activity:

1.Detoxification enzymes:

1.1.Mixed function oxidase (MFO):

The Mixed Function Oxidase (MFO) enzyme activity was measured in treated 4th instar larvae 48-h post treatment with tested compounds (Table 3). Data obtained showed the activity of MFO was significantly increased due to treatment with tested compounds compared to untreated. The highest enzyme activity was obtained when treated the 4th instar larvae with Exirel and Takumi (0.783±0.003 and 0.681±0.003 nm/min/mg protein, respectively).

1.2.Glutathione s-transferase (GST):

Data presented in table (3) showed the effect of tested compounds on the glutathione S-transferase enzyme activity in 4th instar larvae 48-h post treatment. Results showed that the GST activity was decreased compared to control. The lowest enzyme activity was obtained when Takumi was used (0.011±0.00 $O.D_{540}$ nm/min/mg protein).

The detoxification enzymes; MFO and GST, are involved in the detoxification of many groups of insecticides. They were reported previously as immune factor in insects. This was concurrent to **Cao *et al.* (2010)** who reported increased activity of GST in *Helicoverpa armigera* larvae. Furthermore, results agreed with **Lai *et al.* (2011)** who aforementioned that the MFO and GST activity was increased in *S. exigua* larvae treated with chlorantraniliprole. In addition, **Su *et al.* (2012)** aforesaid increased in the detoxification enzyme in treated larvae of *S. litura* with chlorantraniliprole.

1.3.Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT):

Results in table (4) revealed that treatment of 4th instar larvae with tested compounds for 48-h increased the enzyme activity of aspartate aminotransferase (AST) compared to control. The highest enzyme activity of AST was observed in

Coragen treatment (979.00±2.40 IU/mg protein/hr) while the lowest AST activity was obtained in Takumi treatment. Furthermore, results in table (4) showed that the activity of alanine aminotransferase (ALT) increased due to treatment for 48-h compared to control. Coragen treatment showed the highest ALT activity (625.30±4.09 IU/mg protein/hr).

The determined changes in the AST and ALT activity levels in the 4th instar larvae of *S. littoralis* following exposure to tested compounds suggested that *S. littoralis* exhibited adaptive elevation in the activity levels of both the aminotransferase enzymes, thereby probably aiding gluconeogenesis through transamination of glucogenic amino acids to meet the energy demand under the toxicity of tested compounds. Radwan *et al.* (1992) reported that the possible mechanism involved in the elevation of AST and ALT levels may be due to the tissue damage, as a result of the increased synthesis and/or the decreased metabolism of both enzymes.

In conclusion, this study indicated that the ryanodine receptor activators had high toxicity on larvae of *S. littoralis*; the toxicities resulted mainly from immediate lethality. The ryanodine receptor activators represent a novel mode of insecticide action, have the most favorable toxicological and ecotoxicological profiles, these profile make this chemicals useful tool in IPM of *S. littoralis*, however, the resistance risk of cotton leafworm on these insecticides should not be overlook.

Table (3): Effect of tested compounds on MFO and GST activities in 4th instar larvae of *Spodoptera littoralis*

Tested compounds	Enzyme activity (Mean ± S. E.)	
	GST ($O.D_{540}$nm/min/mg protein)	MFO (nm/min/mg protein)
Takumi® 20% WGD	0.011±0.00[b]	0.681±0.003[a]
Coragen® 20% SC	0.016±0.00[a]	0.455±0.003[b]
Exirel® 20% SC	0.017±0.00[a]	0.783±0.003[a]
Untreated	0.024±0.00	0.273±0.003

Numbers within the same column followed by the same letter are not significantly different according to Student-Newman Keuls (SNK) test (LSD0.05).

Table (4): Effect of tested compounds on AST and ALT activities in 4th instar larvae of *spodoptera littoralis*

Tested compounds	Enzyme activity (Mean ± S. E.) (IU/mg protein/hr)	
	AST	ALT
Takumi® 20% WGD	886.00±1.00[a]	594.00±4.36[a]
Coragen® 20% SC	979.00±2.40[a]	625.30±4.09[a]
Exirel® 20% SC	888.00±10.0[a]	562.70±6.52[a]
Untreated	862.00±2.6	464.90±0.62

Numbers within the same column followed by the same letter are not significantly different according to Student-Newman Keuls (SNK) test (LSD0.05).

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ARABIC SUMMARY

تقييم فاعلية منشطات مستقبلات الريانودين الجديدة ضد يرقات دودة ورق القطن فى ظروف شبه حقلية

محمد فتحي عبد العزيز، مروة محمد محمود الصباغ، وسارة محمد إبراهيم عبد الكريم

قسم بحوث دودة ورق القطن- معهد بحوث وقاية النباتات- مركز البحوث الزراعية- الدقي- مصر

هدفت الدراسة الحالية إلى تصميم تجربة شبه حقلية لتقييم فاعلية ثلاثة مركبات من منشطات مستقبلات الريانودين ضد يرقات دودة ورق القطن، خلال موسم زراعة الكرنب عام ٢٠١٦.تم معاملة يرقات العمر الثاني والرابع حيث تم تغذية اليرقات بأوراق الكرنب المعاملة مسبقاً بالجرعة الموصى بها من المركبات التي تم اختبارها وتقديمها لليرقات في المعمل بعد ٢٤ ساعة، ٤٨ ساعة، ٧٢ ساعة، خمسة أيام، سبعة أيام وتسعة أيام بعد الرش. وكشفت النتائج زيادة سمية المركبات محل الدراسة مع زيادة الوقت بعد المعاملة. وتم تسجيل أعلى نسبة موت عند التعرض لمدة ٧٢ ساعة لجميع المركبات المختبرة في كل من يرقات العمرالثاني والرابع. وبالإضافة إلى ذلك، كشفت النتائج إلي زيادة فى نشاط إنزيمات إزالة السموم مقارنةً باليرقات غير المعاملة. وختاماً، فإن منشطات مستقبلات الريانودين تعد مركبات ذات طريقة فعل جديدة في مبيدات الحشرات، مما يجعلها طريقه فعالة في نظام المكافحة المتكاملة لدودة ورق القطن، ومع ذلك، لا ينبغي التغاضي عن خطر حدوث مقاومة لدودة ورق القطن لهذه المبيدات الحشرية