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Impact of an tetramic acid derivatives compounds against cotton Lepidoptera pests' neonate larvae *Earias insulana*are (Boisd.)

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Abstract
Spirodiclofen (Envidor®), spiromesifen (Oberon®), and spiroetramat (Movento®) are an tetramic acid-based insecticide, pertaining to keto-enol pesticide group, which have a novel mode of action; it intervenes with lipid biosynthesis. It's recognized as activity against the sucking insects and mites. We measured the toxicity, developmental effect and enzyme activities of this group against cotton bollworm *Earias insulana*are. The result clearer that the 24 h-LC_{50} of spirodiclofen, spiromesifen and spirotetramat were 19.44, 16.88 and 12.05 ppm, respectively, and gave gradually decreased along three days. The compounds provided significant increased larval and pupal duration and causes malformed therefore affected on the Adult emergency and mortality of *E insulana*are. Furthermore, results achieved that the use of insecticides cause significantly modification on the activities of transaminases enzymes (AST and ALT), phenoloxidase and acetylcholinesterase, total protein and lipids have highest significant decrease. Results concluded that the tetramic acid-based insecticides toxicity against *Earias insulana*. Later, it possible used this novel group of insecticides as integrated spiny bollworm *E insulana* management.

Keywords: spiromesifen, spirodiclofen, spirotetramat, *Earias insulana*, enzymes

Introduction
Earias insulana (Boisd.) spiny bollworm (SBW) are responsible for significant yield losses in many important agricultural and horticultural cropping systems worldwide, especially considered a serious pest of cotton, okra, and other malvaceous plants in Egypt, and other countries of the Mediterranean basin, as well as in Asia and Africa (Abida *et al*., 2004) [2]. The larvae SBW feed on cotton pin square (terminal shoots), flowers, buds, and bolls, ruining fiber and ravaging seeds, and causing significant devastating in crops, especially cotton. Till now the traditional insecticides have been used to control *E. insulana* are (Boisd.) population in Egypt cotton field during the last three decades, the exhaustive use of these insecticides developed a resistance SBW, harmful effects on the natural enemies, non-target insects especially pollinators, and the risk for humans and the environment. So, it is important alternative modes of action insecticides into integrated pest management (IPM) programs designed to control SBW. Pesticides are pivotal pest management tools designed to reduce crop losses. On the world scale, means of chemical control (insecticides) the most widely implemented to suppressing harmful insects at sensible threshold (Casida and Durkin, 2013) [10]. However, illogical intens use of insecticide often induces uncontrolled effects on the environment and human health (Bolzonella *et al*., 2019) [6]. In this context, there is a global desire for new products with optimal activity, depress field application rates, more selectivity, convenient toxicological and environmental safety (Guedges *et al*., 2016; Jeschke, 2018) [19].
Tetramic acids Natural and synthetic heterocycles products has a high significant due to the wide range of biological activities against have antifungal (Sata *et al*.1999) [36], antiviral (Sun *et al.* 2015) [44], and anticancer agents (Matsunaga *et al.* 1991) [28]. as well, these molecules exhibit agricultural bioactivities, such as being fungicides (Li *et al.* 2000, and Latorse and Grosjean 2014) [48, 16], herbicidal (Graupner *et al.* 2003, Chen and Qiang 2017) [16, 12] and nematicidal (Lee *et al.* 2017) [24]. Reutericyclin, tenuazonic acid, streptolydigin, and equisetin are demonstrative samples of biologically active natural products, however the most renowned as commercial product is the insecticidal spirocyclic tetramic acid spirotetramat (Nauen *et al.* 2008, and Salazar *et al.* 2016) [30, 35].
Tetramic acids are new keto-enol insecticides mobility in both phloem and xylem that can be used in the production of many crops (Nauen *et al.* 2008) [30]. It is classified from the insecticide resistance mode action committee in Group 23 include spirodiclofen and spiromesifen (Nauen *et al.* 2008) [30]. It's performance as lipid biosynthesis inhibitors that decrease fertility and fecundity as soon as ingested orally by immature life stage of many insect pests. We aim from this work to shed floodlight on the efficacy of this insecticides group on the neonate larvae of *Earias insulana* (Boisd.) as lipid synthesis inhibitors, which has its importance as one of the

most destructive phytophagous lepidopterous pests in Egypt because it reasons diverse ravage plants like cotton, other field crops and vegetables. This work also involved the effects of the previous compounds on some physiological aspects and bio-chemical parameters of the insect.

Materials and Methods

Insect used

The susceptible strain of spiny bollworm (SBW), *Earias insulana* used, the neonate *E. insulana*, larvae used in this experiment was acquired from laboratory strain rearing up in Bollworms Research Department, Plant Protection Research Institute, Agriculture Research Center, Egypt. Bringed up for several generations away from any infection with insecticides on the artificial diet that previously described by (Amer 2015) [3].

Chemicals

Commercial formulation of Spirodiclofen (Envidor® 24% SC), Spiromesifen (Oberon® 24% SC), and Spiroetramat (Movento® 10% SC), all produced by Bayer Crop Science, Germany were used in the present study and purchased from Egypt distributors. Other chemicals used were bought from Sigma-Aldrich Company.

Toxicity tests

To evaluate the toxicity of the spirodiclofen, spiromesifen, and spiroetramat compounds against neonate larvae of *E. insulana*, serial concentrations of the three tested compounds; (7.5,15,30,60,120, and 240 µg ml-1) for Spirodiclofen, (7.5,15,30,60, and 120 µg ml-1) for Spiromesifen, and (3.125,6.25,12.5,25,50, 100 and 200 µg ml-1) for Spiroetramat; were prepared. Three grams (3gm) of artificial diet was laid in a Petri-dish and each tested concentration was sprayed on the surface of the diet and left until dryness. Three replicates for each concentration. Each replicate 30 neonate larvae of the E. insulana were allowable to fodder on the treated diet and kept under constant conditions of 26 ± 1°C and 75±5 % RH. Similar number of larvae was transferred into untreated diet used as control at the same conditions. Mortality percentages were recorded after 24, 48 and 72 hours from all tested treatment to represent the acute toxicity of spirodiclolfen, spiromesifen, and spiroetramat compounds.

Biological assay

The corresponding value to the determined LC_{50} of each tested compound was sprayed on the surface of athee grams artificial diet in Petri dishes. The larvae of the SPW were feed on the treated diet for each compound. It kept under 26±1°C and 75±5 %RH (as a constant conditions) for three days. The survivors' larvae of each treatment were transferred individually to the diet tubes (2x7.5 cm) each containing about two grams of artificial diet by camel hair brush and anther group used as a control. The nozzle of tubes had hooded by cotton and kept under the preceding conditions in an incubator and inspected daily until pupation. Some biological aspects such as: larval mortality%, larval malformations, larval duration, pupal duration, and adult's emergence%, were estimated.

Biochemical assay

Samples larvae of *E. insulana* were collected after 12 days after 1st instar larvae treated, with three treatments and control for biochemical analyses. The tested larval samples were homogenized in distilled water. The homogenates were centrifuged at 5000 r. p. min. at 5 ^{0}C in refrigerated centrifuge. The supernatants were kept in deep freezer at -20 ^{0}C till use for biochemical assays. Larvae chemically analyzed for compounds and untreated check done in Physiological Department of Plant Protection Researches Institute, (P.P.R.I.). The methods used for analyses of Total soluble protein, lipids and determination of enzyme activity in total homogenate *E. insulana* larvae was carried out, as described by:

a. The total soluble protein estimated by the method of by Bradford (1976) [7].
b. Evaluation the total Lipids were estimated by the method of Knight *et al.* (1972) [22].
c. Each of Aspartate amino transferees (AST) and alanine aminotransferase (ALT) enzyme activities by the method of Reitman and Frankle (1957).
d. The reaction mixture of enzyme assay according to Ishaaya and Swirski (1976) [18].
e. Acetylcholinesterase was determined according to (Simpson *et al*.,1964) [40]

Statistical Analysis

The LC_{50}, chi square (χ^2), fiducial limits, and slope values were estimated according to probit analysis (Finney 1971) using Ldp line software according to Bakr (2000) [4]. The results were expressed as mean ± SE and statistically analyzed, using Costat Statistical Program Software (1990)

Results

Toxicological studies

Toxicity of Tetramic acids to cotton bollworm *E. insulana*:

Toxicity of spirodiclofen, spiromesifen, and spirotetramat against a susceptible strain of spiny cotton bollworm *Earias insulana* neonates under laboratory conditions after different exposure times were presented in Table (1). Spirotetramat was proved to be the most effective insecticide against *E. insulana* neonates after exposure periods

for 24, 48, and 72 h with lethal concentration (LC_{50}) values of 12.05, 8.48, and 6.47 ppm, respectively. Spirodiclofen was the less effective insecticide against *E. insulana* neonates after exposure period 24 h with LC_{50} value 19.44 ppm, while it came in second followed spirotetramat after exposure periods 48 and 72 h with LC_{50} values 10.82 and 8.32 ppm. Spiromesifen was the less effective insecticide against *E. insulana* neonates after exposure periods 48 and 72 h with LC_{50} values 13.36 and 11.67 ppm, but it was in the second rank after spirotetramat with LC_{50} value 16.88 ppm after the exposure period of 24h.

Larval and pupal durations

The results in Table 2 show that the larval and pupal durations for *E. insulana* treated as neonatal larvae with the Tetramic acids pesticides; spirodiclofen, spiromesifen, and spirotetramat; were significantly different from the untreated check.

The time wished for fulfillments of the larval development stage increased to 18.7, 21.2, and 19 days in the three Tetramic acids insecticides, respectively, compared with the untreated check (14.1 days).

Despite the increase in the number of days wished for fulfillments of the larval stage of *E. insulana* treated by the three insecticides; spirodiclofen, spiromesifen, and spirotetramat; it caused a significant increase in the rate of larval malformations (6.3%, 8%, and 6%) respectively, compared with the untreated check (1.33%). The pupal duration from newly hatched larvae treated with the three insecticides was significantly different compared to the untreated check.

spirodiclofen was highly significantly different (10.2 days), followed by spirotetramat (8.5 days) and spiromesifen (8.4 days) with the untreated check (6.8 days) as in Table (2). A highly significant difference was shown in the total duration of the immature stages (larvae and/or pupae) when newly hatched larvae were treated with spiromesifen, spirodiclofen, and spirotetramat. the duration of the immature stages increased to 30.03, 28.07 and 27.05 days respectively, compared with the untreated check (20.9 days). Also, the data in Table (2) show that the percentage of adult emergence in *E. insulana* after the treatment of neonatal larvae with the three Tetramic acids (spiromesifen, spirodiclofen, and spirotetramat) was significantly altered compared with the untreated check (88%). The percentage of adult emergence was highly significantly decreased to 51.7 % with spiromesifen, followed by spirodiclofen (71%) and spirotetramat (73.3%).

Biochemical impacts

Aspartate amino transferees (AST) and alanine aminotransferase (ALT) enzyme activities

Data in Table (3) shows the transaminase enzymes activity on resulted from neonatal larvae of *E. insulana* treated with tested insecticides; the levels of AST were increased to 9332 IU/L in the treated of spirodiclofen was significantly altered, while AST levels were decreased significantly to (1409 and 1153 IU/L) in the treated of spiromesifen and spirotetramat, respectively, compared with the untreated check (1524 IU/L). Also, the levels of ALT gave highly significant increases on the neonatal larvae of *E. insulana* treated with spiromesifen (1592.33 IU/L) followed by spirodiclofen and spirotetramat (1093 and 1019 IU/L) compared with the untreated check (698 IU/L).

Phenoloxidase enzyme activity

Tested the phenoloxidase activity of neonatal larvae *E. insulana* treated with the tested insecticides (spirodiclofen, spiromesifen, and spirotetramat). The results, produced by laboratory selection, had the highest significance of phenoloxidase activity (27 O.D.units/g.b.wt) in spiromesifen followed by spirotetramat with phenoloxidase activity (23 O.D.units/g.b.wt) compared with the untreated check (13.9 O.D.units/g.b.wt), conversely, spirodiclofen had no significant difference compared with the untreated check (11.9O.D. units/g.b.wt) as shown in Table (3).

Acetyl cholinesterase enzyme activity (AchE)

Exposed the neonatal larvae of *E. insulana* to insecticides; the AchE activity gave significantly decreased (126 ug AchBr/min/g.b.wt) with spiromesifen to decreased by 10.24% of the untreated check, spirotetramat recorded (131 ug AchBr/min/g.b.wt) decreasing by 6.19% of the untreated check, while spirodiclofen treatment (143 ug AchBr/min/g.b.wt) has no significant difference compared with the untreated check (140 ug AchBr/min/g.b.wt).

Total protein and lipid levels

Data in Table (3) show that total protein significant differences among all tested insecticides were observed as compared with the untreated check, the treatment of the neonatal larvae of *E. insulana* caused a significant decrease in total proteins, the highest decrease (10 mg/g) recorded in spiromesifen by 56.14% less than untreated check, although spirotetramat was ranked second impact (12 mg/g) to be 44.55% less than untreated check followed by spirodiclofen15 mg/g decreased by 33.5% than untreated check (22 mg/g).

Also, the data in Table (3) demonstrate that total lipid were provided significantly decreased when exposed the neonatal larvae of *E. insulana* to the three tested insecticides (spiromesifen, spirodiclofen, and spirotetramat ranged (31, 37 and 38 mg/g) respectively, compared with untreated check (54 mg/g).

Table 1: Comparative toxicity of three tetramic acid-based insecticides to the susceptible strain of spiny cotton bollworm *Earias insulana* neonates under laboratory conditions

Treatment	Time (hour)	LC_{50} (µg ml^{-1}) (Fiducial Limits)	Slope ± SE	χ^2 (df)	Regression Equation	R^2
Spirodiclofen	24	19.443 (17.662 – 22.629)	2.463 ± 0.192	3.996 (3)	Y = 2.463 X + 1.824	0.992
	48	10.822 (9.443 -12.889)	2.041 ± 0.214	3.065 (3)	Y = 2.041 X + 2.89	0.985
	72	8.316 (7.385 -9.805)	2.443 ± 0.226	5.716 (2)	Y = 2.443 X + 2.755	0.976
Spiromesifen	24	16.882 (13.473 – 21.762)	1.65 ± 0.113	2.903 (3)	Y = 1.165 X + 3.569	0.989
	48	13.358 (11.253 -16.849)	1.513 ± 0.123	5.226 (3)	Y = 1.513 X + 3.299	0.982
	72	11.669 (9.907 -14.671)	1.653 ± 0.167	3.633 (2)	Y = 1.653 X + 3.236	0.985
Spirotetramat	24	12.045 (9.886 – 14.792)	1.456± 0.122	0.811 (3)	Y = 1.456 X + 3.426	0.998
	48	8.481 (7.043 -10.155)	1.788 ± 0.179	3.879 (2)	Y = 1.788 X + 3.339	0.988
	72	6.466 (5.122 -7.837)	1.578 ± 0.178	2.856 (2)	Y = 1.578 X + 3.72	0.989

LC_{50}: The median Lethal Concentration (Concentration till death 50%), (X^2) chi square, (df) degrees of freedom, (SE) Standard error, and (R^2) the Coefficient of Determination.

Table 2: Biological aspects of neonate larvae of *E. insulana* treated by three tetramic acid-based insecticides.

Treatments	Larval duration days (Mean ± SE)	Malformed (%)	Pupal duration days (Mean ± SE)	Time mature Larval & Pupa days (Mean ± SE)	Adult Emergency (%)
Spirodiclofen	18.7 ± 0.88^a	6.3 ± 0.88^a	10.2 ± 0.30^a	28.7 ± 0.85^a	71 ± 4.16^b
Spiromesifen	21.2 ± 0.94^a	8 ± 2.08^a	8.4 ± 0.42^b	30.1 ± 0.79^a	51.7 ± 6.01^c
Spirotetramat	19 ± 0.52^a	6 ± 1.53^a	8.5 ± 0.47^b	27.5 ± 0.81^a	73.3 ± 3.38^b
Untreated Check	14.1 ± 0.35^b	1.3 ± 0.33^b	6.8 ± 0.23^c	20.9 ± 4.56^b	88 ± 3.61^a
$LSD_{0.05}$	2.340	4.48	1.190	2.44	14.39
Significant	***	*	**	***	**

a, b and c statistically significant at $p < 0.05$ with reference to control. Means followed by the same letters are not significantly different according to the $LSD_{0.05}$.

Table 3: Biochemical and transaminase enzymes activity on resulted from neonatal larvae of *E. insulana* treated with three tetramic acid-based insecticides

Treatments	AST (IU/L)	ALT (IU/L)	Phenoloxidase (O.D. units/ g. b. wt)	AchE	Protein (mg/g)	Lipids (mg/g)
Spirodiclofen	9332 ± 152^a	1093 ± 44.9^b	12 ± 0.94^c	143 ± 5.04^a	15 ± 0.52^b	37 ± 1.11^b
Spiromesifen	1409 ± 10.7^{bc}	1592 ± 30.0^a	27 ± 0.89^a	126 ± 2.60^b	10 ± 0.26^d	31 ± 0.53^b
Spiroteramat	1153 ± 38.8^c	1019 ± 7.21^b	23 ± 1.55^b	133 ± 2.30^{ab}	12 ± 0.40^c	38 ± 4.85^b
Untreated Check	1542 ± 73.8^b	698 ± 21.0^c	$14 \pm ??^c$	140 ± 2.89^a	22 ± 0.66^a	54 ± 2.10^a
$LSD_{0.05}$	283.39	95.22	3.35	12.129	1.57	10.70
Significant	***	***	***	*	***	*

a, b and c statistically significant at $p < 0.05$ with reference to control. Means followed by the same letters are not significantly different according to the $LSD_{0.05}$.

Discussion

Our results showed that tetramic acids insecticides spirodiclofen (Envidor®), spiromesifen (Oberon®), and spirotetramat (Movento®) have increasing toxicity against neonatal larvae of *E. insulana*, the LC_{50} ppm of the tested insecticides gradually decreased along three days and effort slowly and had decent residual activity. Agree with Youhui *et al.* (2016) [52] indicated that spirotetramat works slowly but with high efficacy for the control of immature cotton aphids. Also, Nauen *et al.* (2008) [30] reported that spirotetramat is known to cause the death of immature stages of the aphids and whiteflies from 2 to 10 days following application and it has good residual activity. Similar results were observed by Kay and Herron (2010) [21], spirotetramat acted on larval *Frankliniella occidentalis* slowly, with a reduction in larval numbers not clearly apparent until days 9, 10, or 13 in various trials. Bruck *et al.* (2009) [9] definite that the quickness of action of spirotetramat is variable, and depends on the life stage of the target insect and on external parameters. Our results clearly that the larval and pupal duration of *E. insulana* had suffered effect by tested tetramic acids insecticides were increased, in addition to the presence of malformed and longevity were increased, so that the present adult emergency reduced.

The AST and ALT are transaminases known to be crucial links between carbohydrate and protein metabolism. The activity of these enzymes alters during different physiological or pathological conditions (Martin *et al.* 1981) [27]. The AST takes part in the conversion of aspartate and α-ketoglutarate to oxaloacetate (and vice versa) during the citric acid cycle and transamination (Ramzi and Zibaee 2014) [33]. The ALT catalyzes the transfer of the amino group from L-alanine to α-ketoglutarate. The products of this reaction are pyruvate and L-glutamate (Wang *et al.* 2012) [47]. This enzyme can be an indicator of digestion efficiency and transportation of nutrients

between midgut, hemolymph, and fat bodies (Ramzi and Zibaee 2014; Senthil *et al.* 2006; Zibaee *et al.* 2011) [33, 39, 53]. Increased activity of AST and ALT in *E. insulana* hemolymph may indicate that these enzymes were released and/or were un-blocked by tetramic acids tested insecticides (spirodiclofen, spiromesifen, and spirotetramat), The determined changes in the AST and ALT activity levels Corresponds to (Abdel Aziz *et al.* 2018) [1] explained that the 4th instar larvae of *S. littoralis* exposure to tested compounds 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) [32] 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.

It is well know that the Phenoloxidase system is a major defense system in many invertebrates which ultimately leads to melanization of pathogens and damaged tissues and convert phenols to quinones, which subsequently polymerize to form melanin. The process of melanization depends on the activation of the enzyme phenoloxidase (PO). in many insect species, the functioning of PO in hemolymph determines the host resistance to parasites (Liu *et al.* 1976, Brivio *et al.* 2002) [8], parasitoids (Stoltz and Cook 1983) [42], some microorganisms (Wilson *et al.* 2002, Kong *et. al.* 2013) [23] and wound healing and hemolymph coagulation (Kanost *et al.*, 2004) [20]. Also, González-Santoyo and Córdoba-Aguilar (2012) [15] indicate that PO is a costly trait, whose production and maintenance have fitness costs for hosts. Phenoloxidase does not seem to be an indicator of resistance but rather of host condition. These data point out that the activity of PO up-impressed when *E. insulana* was treated by tetramic acids insecticides (spiromesifen, and spirotetramat) due to the role of PO in insects, which provided by Tang (2009) [45] where he reported that PO refers to an enzyme with the tyrosinase-like activity that catalyzes the oxidation of monophenols to diphenols and quinones in the insect. Also, Wang *et al.* (2020) [50] informed that PO plays an important role in the growth and development of insects and is a key enzyme for melanin synthesis. Likewise plays a role in insect immune processes and PO activity is regarded as an important marker of host immunity (Cerenius and Söderhäll 2004, Nappi and Christensen 2005) [11, 29]. Hence, PO activity assays and activation methods are necessary tools to probe the role and function of PPO in insect melanization (Wu *et al.* 2020) [50].

Acetyl choline is thought to be an excitatory neurotransmitter at synapses in the insect central nervous system (CNS) (Gerschenfeld 1973, Pichon 1974) [14, 31], Furthermost of the present generation of insecticides are inhibitors of the enzyme acetylcholinesterase which hydrolyses acetylcholine, terminating its synaptic actions (Corbett 1974, Sattelle 1980) [13, 37]. The study of developmental neurotoxicity appeared to decrease in retention was observed in the memory phase of the water maze for females at all doses (Stuart 2000, EPA 2005) [43]. Aim study point-out that a reduced level of AchE its means inhibits the cholinesterase enzyme from breaking down Ach, increasing both the level and duration of the neurotransmitter action.

Proteins are biological molecules imparting essential roles in insect growth and metabolism. In addition to their role as enzymes, structural, and regulatory proteins are also crucial to complete the life cycle of insects, so the highly significant reduction in total proteins in these work referred to treated tetramic acids insecticides (spirodiclofen, spiromesifen, and spirotetramat) against neonatal larvae of *E. insulana* has problem fateful to complete life cycle due to decreased of total proteins.

Lipids are the primary storage molecules and an essential source of energy in insects during reproduction, prolonged periods of flight, starvation, and diapause. The coordination center for insect lipid metabolism is the fat body; therefore, the tested insecticides suffer effects on these biological processes as effeteness on total lipid. These agree with Yin *et al.* (2014) [51] assessed that the sub-lethal doses of spirotetramat caused oxidative stress and lipid peroxidation. Zhang *et al* (2018) reported that spirotetramat has teratogenic effects on zebrafish embryos development, the 96 h-LC_{50} value is 4.108 mg/L, which is equivalent to our results 5.94 mg/L).

Conclusion

The three tested tetramic acid-based insecticides caused high mortality in *Earias insulana* (Boisd.) at the different concentrations used. The LC_{50} concentration of the three insecticides prolonged larval and pupal duration and reduced its present emergence, also, changed the level of lipid, protein, AST, ALT, phenoloxidase, and Acetyl cholinesterase activities. This specified that, the tetramic acid-based insecticides toxicity against *Earias insulana*. Hence, it is possible to use this novel group of insecticides as integrated spiny bollworm *E insulana* (Boisd.) management programs. In addition, this group needs a lot of studies against lepidopteran pests.

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