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Biochemical studies on some selected insecticides using SDS-PAGE and some esterase patterns on *Spodoptera littoralis* eggs and larvae

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Abstract
The ovicidal and larvicidal effects of the tested insecticides, Chlorantraniliprole (Coragen 20% SC) Indoxacarb (Steward 15% SC), Emamectin benzoate (Basha) and Pyridalyl (Pleo 50% EC) on the 4th larval instars and eggs of 1,2 and 3 days old of *Spodoptera littoralis* was studied by demonstrating total protein content, SDS-PAGE, α and β-esterase profile changes using polyacrylamide gel electrophoresis. All tested insecticides had different effects on total protein content according to the tested stages, 4th instar larvae, and eggs age. The total protein of 4th larval instars recorded highly significant reduction with all treated insecticides and the most reduced one was Pleo. No significant difference in total protein in the eggs of the same age and insecticides and highly significant difference between each insecticide and different egg age. The 3 days old eggs showed a non-significant reduction in all treatment, especially in the cases of Pleo and the lowest effect was Coragen treatments. While the protein of 2 days old eggs was increased with all treatment and the most increased was Coragen and Pleo in addition to the treatment with Coragen and Basha in case of one-day-old eggs. The electrophoretic pattern of proteinshowed significant effects on protein bands someof them are common among treated and untreated and others disappeared or created and specific for each insecticide in both eggs and larval samples. 40, 45, and 64 protein fractions detected in eggs of (1,2) and 3 days old with (four) and eight dominant bands, respectively. Proteins of the larvae detected 66protein bands with seven dominant bands. The α esterase bands of larvae separated into 33 bands with 3 dominant bands. While 3 days old eggs detected 27 with two dominant bands. In addition to β-esterase of larvae were 39 bands with five dominant bands, while 27 B-esterase bands of 3 days old eggs were detected with two dominant bands.

Keywords: Chlorantraniliprole, Indoxacarb, Emamectin benzoate, Pyridalyl, *S.littoralis*, ovicidal, larvicidal, protein

Introduction
The cotton leafworm, *Spodoptera littoralis* (Boisd) is one of the most important major pests of cotton plants. Larvae are polyphagous causing important economic losses in a lot of host plants on many field and vegetable crops. Existing insecticides are getting less effective due to insects developed resistant to them. These resistance problems are recently becoming more serious than before. So it requires that highly selective insecticides used for pest control, Sakamoto and Umeda (2003) [32]. Coragen is a member of the anthranilic diamide class of insecticides that acting on insect ryanodine receptors. Pyridalyl is an insecticide invented and developed by Sumitomo Chemical Co. and exerted excellent control against lepidopterous and thysanopterous pests on cotton, vegetable, and fruit. It is also effective on pests that have developed resistance to existing insecticides. Indoxacarb is a new class of oxidiazines insecticides (Wise *et al*., 2006) [41]. Emamection benzoate is a semi-synthetic avermectin derivative from the fermentation of soil microorganisms, (*Streptomyces avermititis*) family. There are a little information and studies recorded the ovicidal effect of insecticides on eggs of lepidopteran pests although a lot of studies were focused on larval control Mahmoudvand, *et al.,* (2011) [25]. The using of different insecticides groups against egg masses of *S. littorals* was very important to study the effectiveness of them to understand the ovicidal properties and their ability to use in controlling *S. littorals* eggs especially the egg masses are probably present in the same time with the larvae so we try to control them by trying to understand their mode of action.Many methods including gel electrophoresis were used to study protein expression profiles, α and β-esterasesin insect tissues can facilitate the identification of molecular targets that can be used for developing novel and environmentally benign controlling El-Sonbaty *et al.,* (2016) [13].

Materials and Methods
Test insect.
The field strain of cotton leafworm *Spodoptera littoralis* (Boisd.) obtained from Giza government and transformed into the laboratory of the cotton leafworm Department, Plant Protection Institute, Dokki, Giza. It maintained under laboratory conditions of 25 ± 2°C and60 ± 5% RH according to El Defrawi, *et al.,* (1964) [12] for two generations without contamination with insecticides. Egg stages (0-1 day, 2 days, and 3 days old) deposited ontafla leaves *Nerium oleander* and 4th instar larvae were used in the present study for estimating larvacidal activities of the four tested insecticides and their effect on the egg stages.

The insecticides.
The tested insecticides were: 1-Coragen 20% SC: 3-Bromo 4'-1-(3-chloro-2-pyridinyl)- 2'-methyl-6'-(methylcarbonyl)-pyrazole-5-carboxanilide. Chlorantraniliprole (rynaxypyr), Syngenta Agro, S.A.E Du Pont.
2-Steward15%EC: methyl 7-chloro-2, 5-dihydro-2[(methoxycarbonyl)[4-(trifluoromethoxy) pheny [amino [-carbonyl] indeno[1,2-*e*][1,3,4]oxadiazine- 4a-(3H)carboxylate. Indoxacarb, Du Pont.

3- Basha 1.9 % EC:Emamectin benzoate,(4R)-5-O-demethyl-4- deoxy-4-(methylamino) avermactin A1a+(4R)-5-Odemethyl-25-de(1-methylpropyl)-4- deoxy-4-(methylamino) -25-(1-methylethyl) avermactin A1a (1:9).Elhelb for Pesticides and Chemicals Co.
4- Pleo 50% EC:2, 6- dichloro -4 (3, 3-dichlorollyloxy) phenyl 3- [5- (trifluoromethyl)-2-pyridyloxy] Propyl ether.Pyridalyl invented and developed by Sumitomo Chemical Co., Ltd. Japan.

Bioassay
The toxicity of used insecticides Coragen, Steward, Basha, and Pleo, respectively was diluted by water to obtain stock solutions. Then - six successive diluted concentrations were prepared freshly before applied ranging from 0.00001 to 1 ml/ l for (0-24) hrs eggs ages and 4th larval instar of *S. littoralis* based on the preliminary experiments using dipping technique. Fresh caster bean leaves for larvae and egg patches on tafla leaves were dipped in different concentrations used for 20 sec. left them to dry at room temperature, then treated leaves offered to the starved 4th instar larvae to feed on. Control larvae were fed on untreated leaves, eggs patches dipped in water, and left to dry as control. Mortality was recorded and noticed their movement or paralysis using a brush for larvae, while the treated eggs check daily until all eggs hatched or died and corrected by Abbott formula (Abbott, 1925) [1]. The Lc_{50} values were obtained by probit analysis of mortality data post-treatment until complete hatching control eggs and post 48 for larvae according to Finney (1971) [14], consequently, LC_{50s} values, were used for calculating the toxicity index (Sun, 1950) which was used for comparing the relative potency of insecticides used.

Toxicological studies on *S.littoralis* eggs (Ovicidal activity)and 4thlarval instars (Larvicidal activity).
The freshly laid egg-masses of *S. littoralis* of uniform age (0-1 day, 2 days and 3 days old) were collected and treated with the LC_{50s} values 0. 06 $x10^{-2}$, 0. 81 $x10^{-2}$,2.39$x10^{-2}$and 0.31 $x10^{-2}$ ml/ l of the tested insecticides, Coragen, Steward, Basha, and Pleo, respectively using dipping technique. Four replicates of egg masses each one contained egg batches about (200-300 eggs) each was dipped in LC_{50s} solutions of each insecticide for 20 sec. Other groups of eggs dipped in water to use as control and left to dry at the room temperature. Put them in clean Petri dishes for 24 hrs then collected and kept frozen at -20C°for analysis.
The same technique was applied for the 4th larval instars using caster bean leaves were treated with LC_{50s}0.0329, 0.157, 0.457, and 0.021 ml/ lof Coragen, Steward, Basha, and Pleo, respectively. The treated leaves offered to the starved larvae in a glass jar to feed for 48 hrs, untreated leaves (dipped in dist. water) offered to other groups as control and sample collected after 48hrs from treatment and kept frozen at -20C°till analysis. Total protein and protein fractionation for 4th instars, 1, 2, and 3-day old eggs, in addition to α- and β- esterase's were determined for the 4th larval instars and eggs of 3 day old.

Preparation of samples for analysis.
The treated and untreated eggsof 1, 2, and 3 days old and the 4th larval instars samples homogenized with 0.9% saline solution (1g/ 3ml) using a mortar and pestle then put in a clean eppendorf tube. Centrifuged at 5000 rpm for 10 min in the refrigerated centrifuge the supernatant of each sample (contain protein extract) was kept in a deep-freezer at -20C^0 until used for total protein and electrophoretic analysis.

Biochemical Studies.
Total protein: Total protein determined according to the kit method described by Bio Diagnostic Co., Gornal *et al*., (1949) [17].

Molecular Studies.
SDS-Polyacrylamide Gel Electrotrophoresis (SDS-PAGE).
The principle of protein electrophoresis is the movement of the charged molecules towards an electrode at the opposite charge through a supporting medium. Sodium Dodecyl Sulphate (SDS-PAGE) was used for the separation of protein subunits and the determination of their molecular weights (M.wt). SDS-PAGE was performed in10% for larvae and 12% for eggs acrylamide slab gels following the system of (Laemmli, 1970) [23], with some modification by (Sambrook *et al*., 1989) [33].

Isozymes
Non-denaturing polyacrylamide gel electrophoresis was conducted to identify isozymes variation. The utilized isozymes were α- and β- esterases were separated in 10 % native polyacrylamide gel electrophoresis as described by stagemann *et al*. (1985). For whole-body tissues of the 4th larval instars post 48 hrs of treatment and post 24 hrs of treatment for eggs of 3 days old of *S. littoralis*, also samples of untreated larvae or eggs were tested. In gels staining, protocols of Scandalios (1964) [34] were used for α- and β-Est thengels photographed.

Results and Discussion
1. **Toxicological studies on *S. littoralis* eggs (Ovicidal activity).**

The toxic effects of the tested insecticides of Coragen, Steward, Basha, and Pleo against *S. littoralis eggs* and 4th instar larvae were recorded after 24 hours of treatments for eggs and after 48 hrs for larvae. Data presented in Table (1) summarized the LC_{50s} values of them. The LC_{50s}values of one-day-old eggs, Coragen was the most effective insecticide that recorded (0. 061 $x10^{-2}$m/l) followed by (0.314 $x10^{-2}$ and 0. 81 $x10^{-2}$ m/l) for Pleo and Steward, respectively. Meanwhile, Basha appeared to be the least effective to record (2.39 $x10^{-2}$m/l) and Toxicity index (2.55).
Comparing the relative potency level for tested compounds, the obtained data show the relative potency values of Coragen, Steward, and Pleo, were 39.18, 2.95, and 7.61 times as toxic as the ovicidal action of Basha against the egg of one day old of *Spodoptera littoralis.*

Table 1: The LC_{50s} values of Coragen, Steward, Basha, and Pleo against *S.littoralis* eggs and 4th larval instars post- treatment.

Insecticide	One day old eggs					4th larval instar				
	LC_{50} ml/l	Slope ± SE	Confidence limit Lower- upper	Toxicity index	Relative Potency	LC_{50} ml/l	Slope ± SE	Confidential limit Lower- upper	Toxicity index	Relative Potency
Coragen	0.061x10^{-2}	0.32±0.05	(0.00028 0.0032)	100	39.18	0.0329	0.90±0.11	(0. 0212-0.0545)	63.83	13.89
Steward	0. 81 x10^{-2}	0.77±0.11	(0.0018-0.0233)	7.53	2.95	0.157	1.12±0.14	(0.079-0.612)	13.38	2.91
Basha	2.39 x10^{-2}	0.99±0.18	(0.0095-0.084)	2.55	1	0. 457	1.72±0.16	(0.3263-0.6181)	4.6	1
Pleo	0. 314 x10^{-2}	0.76±0.09	(0.00156-0.00612)	19.43	7.61	0. 021	1.51±0.27	(0.014--0.037)	100	21.76

Sun's toxicity index= LC_{50} or LC_{90} of the most toxic compound/ LC_{50} or LC_{90} of the tested compound x 100. Relative Potency = LC_{50} of the least toxic compound/ LC_{50} of the tested compound.

On the other hand, LC_{50s} values against 4th instar stated the most effective insecticide was Pleo (0. 021 m/l) followed by (0. 0329 and 0. 0157 m/l) for Coragen and Steward, respectively while the least effective was Basha 0.457 m/l.As shown in Table (1), the relative potency based on the LC_{50} values against the 4th instar, the larvicidal effectiveness values of Coragen, Steward, and Pleo were 13.89,2.91 and 21.76 folds, respectively as the larvicidal efficacy of Basha.

2-Biochemical studies

i-Determination of total protein in *S. littoralis* eggs.

The data in Table (2) observed no significant difference in total protein content of *S.littoralis* egg as a result of treatment with $LC_{50s}$of different tested insecticides Coragen, Steward, Basha, and Pleo among the eggs of the same age and highly significant difference between each insecticides and different egg age. The eggs of one day old treated with Coragen and Basha recorded a slight increase in total protein content by 3.06 and 6.11%, respectively. While Steward and Pleo recorded a slight decrease in total protein by -9.06 and -6.01% compared with untreated control of one day old (0.982 g/dl).

Total protein in eggs of 2 days old was no significant increased with all tested insecticides. It ranged between (5.6 and 21.14%) and the most increase insecticides were Coragen by 21.14 % followed by Pleo by 13.36 % then Steward 12.36 % while the least effective was Basha 5.6% compared with untreated control of 2 days old eggs (2.678 g/dl).

Table 2: Total protein in *S. littoralis* eggs post 24 hrs of treatment with LC_{50s} values of Coragen, Steward, Basha, and Pleo.

Egg old	Total protein g/dl						F-value	L-S-D
	1 –day old		2-days old		3-days old			
Insecticide	Mean ± SE	Change %	Mean ± SE	Change %	Mean ± SE	Change %		
Coragen	1.01^{b}± 0.03	+3.06	3.24^{a}± 0.46	+21.14	3.13^{a}± 0.24	-1.82	17.487**	1.03918
Steward	0.89^{b}±0.05	-9.06	3.01^{a}± 0.44	+12.36	2.83^{a}± 0.39	-11.22	11.668**	1.1878
Basha	1.04^{b}± 0.06	+6.11	2.83^{a}± 0.24	+5.6	2.65^{a}±0.3	-16.78	45.457***	0.5049
Pleo	0.92^{b}± 0.03	-6.01	3.04^{b}± 0.26	+13.36	2.44^{a}± 0.26	-23.31	26.835**	0.72789
Control	0.98^{b}± 0.10	-	2.68^{ab} ± 0.29	-	3.18^{a}±0.91	-	4.338***	1.91529
F-value	0.9951^{ns}		0.3756^{ns}		0.4475^{ns}			
L-S-D	0.1936		1.10802		1.4798			

Means with the same letter are not significantly different at p <.0001.

On the other hand, the eggs of 3 days old recorded a non-significant reduction in the total protein content which ranged between (-1.82 and -23.31%). The most reduced insecticide was Pleo by -23.31% followed by Basha -16.78% then Steward -11.22% and the lowest effect was Coragen by -1.82% compared with untreated control of 3 days old eggs with all tested insecticides.

ii-**Determination of total protein in *S. littoralis* larvae.**

Total protein in the 4th larval instars of *S. littoralis* post 48 hrs of treatment (Table 3) showed a highly significant reduction with all tested insecticides. This reduction ranged between (-48.73 and -69.36) and the most reduced insecticide was Pleo by -69.36 % followed by Basha by -64.21% then Steward-53.53% while the least reduced one was Coragen-48.73% compared with untreated control.

Table 3: Total protein in the 4th larval instars of *S. littoralis* post 48 hrs of treatment with LC_{50s} values of Coragen, Steward, Basha and Pleo.

Insecticide	Total protein g/dl				
	Coragen	Steward	Basha	Pleo	Control
Total protein Mean ± SE	14.54^{b}±1.11	13.18^{b}±0.55	10.15^{c}±0.56	8.69^{c}±0.74	28.36^{a}±1.27
Change %	-48.73	-53.53	-64.21	-69.36	
F-value	76.852***				
L-S-D	1.9863				

Means with the same letter are not significantly different at p <.0001

There are few studies regarding the effect of the insecticides on lepidopterous eggs and focused on the control of larval stage. The insecticides used in this study were effective on the eggs and 4th larval instar *S. littoralis*. The LC_{50s} obtained for ovicidal effect are higher than those for the larvicidal bioassay. This fact agrees with that observed by Liu *et al*. (2002) [24]; Boiteau and Noronha (2007) [9] and Mahmoudvand, *et al.*, (2011) [25] on *T. ni*, *O. nubilalis*, and *P. Xylostella*. The treated eggs with different insecticides observed three states larvae died before hatching inside the

egg, died after ingestion a small portion of the membrane inside the treated egg, or died before completely exiting from the egg. The ovicidal effect indicated contact toxicity of the tested insecticides to the best of our knowledge where the eggs treated with Coragen dried and became like ash that may be due to the dehydration effect. Where some eggs could be failed to hatch although the head of the neonate larvae was seen as in Basha. That may refer to its mode of action ordue to the high active ingredient as in Pyridalyl which has contact toxicity causing the highest (97.72%) egg mortality compare with Indoxacarb by reducing the hatchability (Mahmoud vand *et al.*, 2011) [25]. Also, Coragen had contact and ingestion effect by its effect on the cuticle (Abdel-Aziz *et al.*, 2020) [10]. Abdel-Aziz and Sayed (2014) [11] rynaxypyr and Indoxacarb exhibit an excellent (100%) inhibitory activity in suppressing the number of egg deposited followed by Pyridalyl and Emamectin benzoate by 97.8 and 97.0 %.The chorion is formed from a number of chemically distinct layers which are tanned protein layeron the outside, fibrous protein layer, and lipoprotein layer (wigglesworth and Beament, 1950) [39]. The maturation of insect eggs is dependent basically on the materials taken up from the surrounding and materials synthesized by the ovary Indrasith *et al.*, (1988) [21]. These materials include lipids, proteins, and carbohydrates which necessary for embryogenesis (Kanost *et al.*, 1990) [8]. That means the ovicidal activity of tested insecticides is due to adsorption of them into the chorion and subsequent oral uptake as the neonate chews through the chorion to hatch so the larvae ingest a dose of them is sufficient to affect the larva to die within the egg. That agrees with Pineda *et al.*, (2007) [30] where the penetration of insecticide into the eggs prevents hatching by interfering with embryonic cuticle synthesis; so the new hatch probably can't use its muscle to free itself from the egg. So any changes in them may be lead to failure in maturation and hatching. Generally, changes in protein content probably reflect the balance between synthesis, storage, transport, and degradation of structural as well as response to particular physiological conditions. Wilkinson, (1976) [40] reported that protein helps to synthesize microsomal detoxifying enzyme which helps in detoxification.

Data showed a different effect on the total protein contents of both eggs and larval stages of the different insecticides, Coragen, Steward, Basha, and Pleo. Total protein content had no significant effect between all tested insecticides and eggs of the same age. On the other hand, a highly significant difference between each insecticide with the different eggs age 1, 2, and 3 days old; where Basha recorded a highly significant difference while Coragen, Steward and Pleo recorded significant effect. The most effective insecticides were Coragen and Pleo by + 21.36 and -23.31 compare with control. Total protein content in egg stages increases as egg development in control samples, while the treatment caused fluctuation in the case of one day old. Increasing in 2 days old and reduction in 3 days old. The most effective one was Coragen on 2 days old and was Pleo on 3 days old. Our results agree with Hassan, (2009) three-day-old eggs are more affected than that of one or two days old in the case of Indoxacarb while the reverse in Methoxyfenozide.The increase in protein content with different insecticides treatments may be attributed to the increased activity of protein biosynthesis by its tool (amino acids) or may be a kind of detoxification mechanism also, the compensatory physiological mechanism in response to some insecticides (Neoliya, *et al.*, 2005) [29].

The 4th larval instar of *S. littoralis* treated with Coragen showed slow motion looks like paralysis and small in size as 2ndinstars that may be due to temporary mouth parts paralysis lead to stop feeding and shrinking of larvae may be due to dehydration (lose water) or /and disturbance in cuticle formation that because of its absorbed through the cuticle with its contact action. That agrees with many workers on *S. littoralis*many insecticides decreased feeding efficiency and protein amount of an insect´s body as Indoxacarb which act as antifeedant, due to the destructive effect of some of the cerebral neurosecretotry cells in the brain which responses for protein secretion in treated larvae or mechanical lipoprotein formation using for repairing the damaged organs, tissues or cells (Hassan, 2009, Hamouda and Dahi, 2008) [10], Coragen had contact and ingestion effect caused a disturbance in cuticle and midgut structure (Abdel-Aziz *et al.*, 2020) [10]. Abdel -Aziz 2014 [11]; Abdel-Aziz *et al.*, 2013 [11]; 2017; 2020 and Hassan *et al.*, 2014 Highly significant reduction in total protein in larvae treated with Indoxacarb, Pyridalyl, and others and attributing the great disturbance which may have several reasons, enzyme activities, as a significant decrease in total carbohydrates, lipids, chitinase, and protein synthesis as a result of insecticidal treatment to the synthesis of the proteinases needed for insecticide detoxification. Protein leakage during intoxication may arise from reduced body weight, conversion of protein to amino acids, degradation of protein to release energy, or the direct effect of the tested insecticides on the amino acids transport of the cell *(*Rawi *et al.*, 1995). Other worker on the 3rd larvae of *E. cautella* total protein significant decreased in Coragen and Pleo by (-40.9 and -19.2%), respectively (Fouad *et al.*, 2019) [15].

iii-SDS electrophoretic protein pattern of *S. littoralis* eggs of one day old.

SDS electrophoretic protein pattern of *S. littoralis* eggs post-treatment with Lc_{50s} (0. 061 $x10^{-2}$, 0. 81 $x10^{-2}$, 2.39 $x10^{-2}$and 0.314 $x10^{-2}$ m/l) of Coragen, Steward, Basha, and Pleo, respectively recorded in Table (4) and Figs (1) represented the lanes (1-5), (6-10) and (11-15) for 1, 2 and 3 days old egg samples, respectively. Electrophoretic pattern of protein of the one-day-old eggs was obvious approximately (40 protein fractions) separated and distributed as 9, 8, 7, 6 and 10 bands for untreated, Coragen, Steward, Basha and Pleo samples, respectively with molecular weight (M.wt.) in range (27 -176 kDa). Four of them, band numbers 2, 3, 7, and 9 were dominant for untreated and treated eggs with M.wt176, 128, 69,and 56 kDa, respectively and with different amounts %. Band number 9 has a max. amount % 31.54, 10.38, and 10.06 observed in Basha, Pleo,and Steward treatment, respectively compared with 9.3 for untreated. Band number 2 has a max amount of 9.56 for Basha and 10.74 for Coragen in the band no 3 while band no. 7 had max. amount 5.16 for Stewerd compare with 3.79, 7.82 and 7.35 for untreated, respectively. While the minimum amount was 3.61% of the band no 7 for Basha treatment.

The appearance or disappearance of bands may be due to treatment and characteristics for tested insecticide. The band no.10 of M.wt. 47 kDa. is a characteristic band for treated samples of Coragen, Steward, and Pleo with amount %10.31,11.61, and 5.97%, respectively. Five bands no's 4,

5, 6, 11 and 12 were disappeared in treated samples, where bands no 4 and 11 of M.wt. 108 and 30 kDa disappeared in Coragen samples. The bands no 5, 6, and 12 of M.wt. 91, 73, and 27 kDa disappeared in Steward samples. The bands no 5,11 &12 of M.wt.91, 30 and 27 kDa disappeared in Basha samples compare with untreated.

Table 4: Molecular weight (M.wt) and amount % of SDS protein of *S. littoralis* eggs one and two day old post- treatment with Lc_{50s} of tested insecticides.

				One day old eggs					Two days old eggs				
Lane	**Marker**		**M.wt**	**control 1**	**Coragen 2**	**Steward 3**	**Basha 4**	**Pleo 5**	**control 6**	**Coragen 7**	**Steward 8**	**Basha 9**	**Pleo 10**
Band no.	M.wt	%		%	%	%	%	%	%	%	%	%	%
1	245	2.11											1.96
2	180	2.53	176	3.79	9.04	7.55	9.56	6.48	4.59	5.24	9.88	5.32	10.69
3	135	3.25	128	7.82	10.74	6.63	7.17	9.32	7.14	9.57		8.23	10.31
4	100	4.72	108	5.83		7.87	4.7	5.9		5.15	2.98		12.73
5			91	6.46	3.41			3.66		1.84	3.78		9.42
6	75	4.95	73	4.93	4.38		4.44	5.56	4.27	4.72	3.82	3.41	3.13
7			69	7.35	4.43	5.16	3.61	3.91	8.52	4.23	6.26	5.6	4.01
8	63	4.15	63									8.60	5.74
9			56	9.30	4.08	10.06	31.54	10.38	4.09	8.5	4.71	7.55	0.84
10	48	5.25	47		10.31	11.61		5.98	5.64	12.6		3.13	4.97
11			30	2.34		15.14		4.14	19.39		22.22	7.14	
12	25	5.67	27	21	7.1			7.77		20.86		4.44	
13	20	7.74	20									6.44	3.91
14			11										7.9
Tota bands				9	8	7	6	10	7	9	7	10	12

M. wt: molecular weight %: band amount

iv-SDS electrophoretic protein pattern of *S. littoralis* eggs of 2 days old.

Proteins of *S. littoralis* eggs of 2 days old were electrophoretically separated in Table (4) and Fig (1) represented the lanes (6-10) for untreated and 4 treated egg samples. A total of forty-five protein bands were distinguished, with M.wt. ranging from (30-176) and (19-288) KDa for untreated and treated, respectively which distributed as 7, 9, 7, 10, and 12 bands for untreated, Coragen, Steward, Basha, and Pleo samples, respectively. Among these four bands no's 2, 6, 7, and 9 were dominant in both untreated and treated eggs with M.wt.176, 73, 69, and 56 KDa, respectively. However, their amount % was fluctuated 10.69, 4.72, 8.52, and 8.49%, respectively.Also bands no. 3 and 10 of M.wt. 128 and 47 KDa with max. amount 10.31 and 12.26 were dominant in both untreated and treated eggs except in Steward treatment was disappeared. The other protein fractions are often related to the insecticide used so they are considered to be treatment.Bands 4 and 5 of M.wt. 108 and 91 KDa were characteristic for Coragen, Steward, and Pleo with max. amount 12.73 and 9.42 of Pleo, respectively. Bands 8 and 13 created only in Basha and Pleo treated eggs with M.wt 63 and 24 KDa. with max. amount 8.6 and 6.43 for Basha. Band 12 with M.wt 27KDa created only in Coragen and Basha which may specific for them with max. amount 20.86 for Coragen, in addition to only two bands no.1 and 14 of M.wt. 288 and 19 KDa appear in Pleo. While band no. 11 of M.wt 30 KDa disappeared only in the case of Coragen and Pleo.

v-SDS electrophoretic protein pattern of *S. littoralis* eggs of 3 days old.

SDS electrophoretic protein pattern of *S. littoralis* eggs of 3 days old recorded in Table (5) and Figs (1) represented the lanes (11-15) for untreated and 4 treated egg samples. The protein pattern was approximately (64 protein fractions) detected and distributed as 14, 11, 13,14, and 12 bands for untreated, Coragen, Steward, Basha, and Pleo samples, respectively with M.wt. in range (19-288 kDa). Eight dominant bands no's 2, 3, 6, 9, 10, 12, 17, and 18 with M.wt. 194, 158, 114, 78, 72, 62, 24, and 19 KDa, respectively between untreated and treated eggs with different max. amount 10.2, 9.72, 4.75, 5.23, 4.38, 7.15, 18.56 and 9.52, respectively.

Some bands created and others disappeared; band 14 created only in untreated eggs of M.wt.52 KDa and disappeared in treated with max. amount of 4.18. Five bands (4,7),8, 11, and 15 were in untreated and disappeared in some treatment with M.wt. (139,107), 100, 67 and 50 KDa, disappeared in (Coragen & Pleo), (Steward), (Coragen, Steward, & Basha) and (Basha & Pleo), respectively. On the other hand, Bands 1, 5, 13, and 16 with M.wt. 288, 126, 58, and 37KDa were created only in (Basha & Pleo), (Coragen, Steward, & Pleo), (Basha) and (Steward & Basha), respectively.

Table 5: Molecular weight (M.wt.) and amount % of SDS protein of *S. littoralis* eggs 3 days old post- treatment with Lc_{50S} of tested insecticides.

				Three-day old eggs				
Lane	**Marker**			**Control 11**	**Coragen 12**	**Steward 13**	**Basha 14**	**Pleo 15**
Band no.	M.wt.	%	M.wt.	%	%	%	%	%
1	245	2.11	288				3.49	1.52
2	180	2.53	194	7.27	10.2	8.42	5.96	7.61
3			158	5.84	4.47	2.8	3.02	9.72
4	135	3.25	139	13.41		3.85	11.02	
5			126		11.61	13.99		3.54
6			114	1.54	4.75	2.82	4.36	2.47
7			107	3.75		5.70	3.50	
8	100	4.72	100	2.83	3.58		3.82	2.45
9	75	4.95	78	4.83	3.71	3.78	3.28	5.23
10			72	3.44	4.38	2.93	4.15	3.39
11			67	1.66				3.58
12	63	4.15	62	4.58	6.04	6.21	7.15	6.03
13			58				5.56	
14			52	4.18				
15	48	5.25	50	10.12	11.71	2.88		
16			37			7.95	7.39	
17	25	5.67	24	6.67	6.56	4.86	7. 9	18.56
18	20	7.74	19	7.94	6.93	9.52	7.24	8.13
Total bands				14	11	13	14	12

M. wt: molecular weight %: band amount

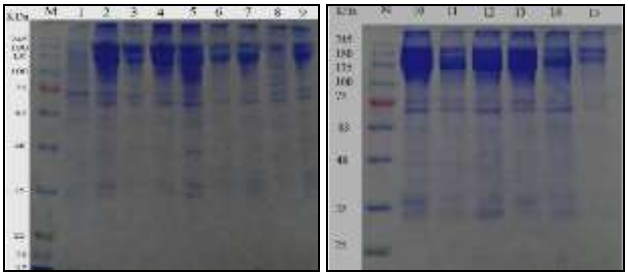

Fig 1: SDS-Polyacrylamide Gel Electrotrophoresis protein patterns of *S. littoralis* eggs post 24 hrs of treatment with LC_{50s} values of tested insecticides Where, M: marker, lanes (1-5), (6-10) and (11-15) represented eggs of 1, 2 and 3 days old, respectively as untreated, Coragen Steward, Basha and Pleo, respectively with each day.

vi-SDS-Polyacrylamide Gel Electrotrophoresis (SDS-PAGE) of the 4th larval instars of *S. littoralis*.

The SDS-PAGE analysis of the protein profiles of whole body tissues of the 4th larval instar of *S. littoralis* recorded in Table (6) and Fig (2) showed 66 proteins, bands, with M.wt.ranging from 22-130 KDa. These bands distributed as11,10,16,14 and 15 for untreated, Coragen, Steward, Basha, and Pleo, respectively. Seven dominant bands, no's 12,13,14,15, 16, 17, and 18 in both treated and untreated larvae withM.wt. 41, 38, 34, 29, 25, 23, and 22 KDa with max. amount 9.7, 9.44, 8.19, 7.48, 14.13, 12.64 and 5.84, respectively.

Results also showed that six bands no's 2, 5, 9, and 10 of M.wt.125, 77, 48, and 46 KDa were created by treatment Steward, Basha, and Pleo with max. amount (5.58,21.85, 3.87) in Pleo and 6.44 in Basha, respectively. Bands no's 3 and 4 of M.wt. 93 and 86 KDa created by (Coragen& Steward) and (Steward & Pleo) with the max amount (19.44 and 33.58), respectively. In addition to only one band no 11 of M.wt. 43 KDa was created by larvae treated with 4 tested insecticides, its max.amount 4. 6 in Basha.Also, only oneband no 6 of M.wt. 64 KDa with max.amount 4.82 was present in untreated control and disappeared from all tested samples and maybe characteristic it. On the other hand, three bands no's 6 and (7 and 8) of M.wt. 64 and (52 and 50 KDa) disappeared in (Steward, Basha & Pleo) and Coragen.

The treatment with different insecticides used in this study disturbs the protein of *S. littoralis* eggs, treated with Coragen, Steward and Pleo, creates only one specific band (no. 10) in eggs of one day old; the two days old eggs treatment with Coragen creates three specific bands (4,5 and 12); treatment with Steward creates two bands (4 and 5); treatment with Basha creates three specific bands (8, 12 and 13); while treatment with Pleo results in six specific bands (1, 4, 5, 8, 13 and 14). In case of three-day-old eggs the treatment with Coragen, creates only one specific band (no. 5); treatment with Steward, creates two bands (5 and 16); Basha creates three specific bands (1, 13 and 16); Pleo, creates two specific bands (1and 5) and the disappearing of others bands compare with the control that as a result of treatment may be considered as insect defense. The protein bands of treated samples were completely

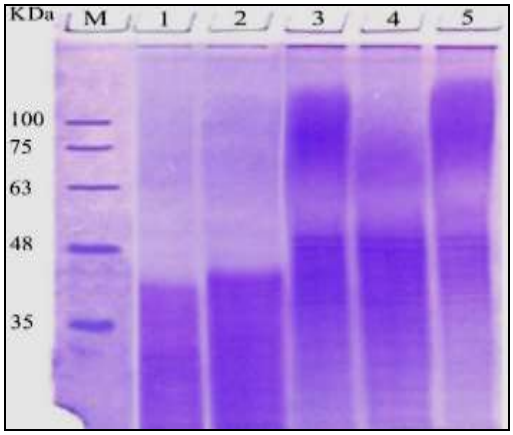

Fig 2: SDS-Polyacrylamide Gel Electrotrophoresis protein patterns of *S.littoralis* of th 4th instar larvae post 48 hrs of treatment with LC_{50s} values of tested insecticides Where, M: marker, lanes (1-5) represented untreated, Coragen, Steward, Basha and Pleo, respectively.

Table 6: Molecular weight (M.wt.) and amount % of SDS protein of *S. littoralis* 4th instar larvae post 48 hrs of treatment with LC_{50s} values of tested insecticides.

Lanes		Marker	M.wt.	control 1	Coragen 2	Steward 3	Basha 4	Pleo 5
Band no.	M. wt.	%		%	%	%	%	%
1			130	1.69	2.52			
2			125			1.35	2.13	5.58
3	100	3.61	93		4.03	19.44		
4			86			1.74		33.58
5	75	3.76	77			11.52	16.11	21.85
6	63	7.22	64	4.82				
7			52	3.34		7.71	11.02	6.12
8			50	3.33		3.21	3.71	3.87
9	48	10.25	48			3.22	3.71	3.87
10			46			2.05	6.44	3.6
11			43		1.56	2.32	4.6	2.64
12			41	9.7	5.89	4.3	5.1	2.45
13			38	3.84	5.75	6.16	9.44	4.90
14	35	11.87	34	8.19	4.97	5.32	3.29	4.53
15			29	7.48	2.76	5.84	4.99	1.36
16			25	5.93	14.13	5.97	8.42	1.42
17			23	12.64	8.19	4.19	6.92	1.44
18			22	3.11	5.84	3.62	1.9	1.55
Total bands				11	10	16	14	15

M. wt: molecular weight %: band amount

different from those of the control, so these may be differences in biological, biochemical activities or as defensive proteins and synthesized as an immune reaction. The protein fractions of treated 4th larval instars, the treatment with Coragen, creates two specific bands (no. 3 and 11); Steward creates 7 specific bands (2,3,4, 5, 9,10 and 11); Basha creates five specific bands (2, 5, 9,10 and 11); while treatment with Pleo creates six specific bands (2,4,5,9,10 and 11) with different amount%. That agree with Hassan, *et al.,* (2014) there were 31 and 27 bands with M.wt. in range (9.63-144.57) and (10.76-144.57) KDa was detected in untreated and Indoxacarb treatment, respectively, disappearing of 7 bands, in addition to one unique band with M.wt. 16.3 KDa and an amount % 1.64 was detected. The appearance of new protein bands upon treatment may be due to increase in protein synthesis or to the liberation of free radicals which affect nitrogenous compounds directly and leads to a breakdown of the peptide linkage, causing fragmentation of protein molecules (Megahed,1996; Gehad and Shaurub, 1997and El-Bermawy and Abdel Fattah 2000) [11]. Protein pattern of 4th, 2ndand 6th instars treated with Pyridalylcreates three, two, and one characteristic bands, respectively (Dahi, *et al.*, 2011) [10]. In addition to disappearing other bands compare with the control that agrees with Hassan and Abdel Hafez (2009) [20] the treatment with Lc_{50}s of Spinosad and radiant causing missing bands or expressed with less density of protein of 4th instar of *S. littoralis*. These results may add some interpretations of the changes associated with the differences in the insecticidal activity of tested compounds, which confirmed by the results obtained by Shoukry *et al.*, (2003) [36] forty-eight different protein bands were distinguished, with M.wt. ranging from 18-232 KDa. Beckage and Kanost (1993) [22], the proteins of 55.3-kDa, significantly decreased in concentration in the treated larvae and the level of some subunits of 240, 75,78, 22 kDa, were slightly changed while insecticyanin (19 kDa) was detected at higher levels in larvae. Proteins between 75 and 55.3 kDa may include storage proteins, such as hexamerins (Nakamatsu and Tanaka, 2003) [28]. The relative abundance of the 229-, 75-, 41.2-, 39.4-, 37.2-, 35.6-, 34.7-, 33.9-, 32.1-, 29.6-, 23.1-, and 19.0-kDa proteins. In addition to 24.6 and 19 kDa protein bands increased significantly. These bands may be defensive proteins, such as insectacyanins, which are synthesized as an immune reaction Altuntas, *et al* (2010) [7]. Protein profile detected a dominant group of protein bands ranging in M.wt. from 72 to 84 kDa (Shoukry *et al.*, 2003) [36]. These are thought to be storage proteins. Storage proteins are used as an amino acid reserve for the production of adult proteins (also known as 81/82, 76 and 74 K) Miller and Silhacek (1982) [27].

ii-Isozymes analysis

a-α- esterase electrophoretic pattern of the 4th larval instars and 3 days old eggs of *S. littoralis*.

α- and β- esterase electrophoretic patterns of whole body tissues of the 4th larval instars of *S. littoralis* recorded in Table (7) and Fig (3) and represented the lanes (1-5) for untreated and 4 treated larvae samples post-treatment for 48 hrs with LC_{50} (0.0329, 0.157, 0. 457 and 0. 021 ml/l) of Coragen, Steward, Basha, and Pleo, respectively.

The maximum number of α esterase band of larval whole-body tissues is separated into 33 bands which were not necessarily present in all samples and distributed as 7, 6, 8, 6, and 6 bands. Whereas the maximum bands number were detected in Steward samples 8 bands with approximately Rf ranging between (0.16 - 0.89). On the other hand, the minimum number 6 bands were detected by Coragen and (Basha and Pleo) treatments with different Rf range (0.16-0.89) and (0.16- 0.79), respectively. There are 11 comprises polymorphic bands were observed 3 of them no's 2, 3, and 10 dominant between treated and untreated samples with Rf 0.16 and (0.24 and 0.64) with max. amount 15.1, (4.86 and 16.25) for Pleo and Basha, respectively compare with (12.26, 2.68, and 14) in untreated. One unique band no. 4 was detected and characteristic for untreated larvae at Rf value 0.29 and max. amount 3.09 and absent in all treated samples. In addition to three bands, no's 6 and (8 and 12) present in untreated with Rf 0.38 and (0.57 and 0.89) and absent in (Steward, Basha & Pleo) and (Basha & Pleo) and

the max. amount was no 8. There were 3 bands no's 5, 7, and 11 with Rf 0.34, 0.5, and 0.79 max. amount in range (7.73 - 13.75), (13.32 - 22.46), and (19.18 - 13.36), respectively detected only with Steward, Basha, and Pleo. These bands may be due to the treatment and considered as characteristic for them.

Treatment with tested insecticides caused the disappearing of one band of Rf 0.29.in addition to bands no (6), (8 and 12) were disappeared as results of treatment with (Steward, Basha & Pleo) and (Basha & Pleo). In addition to three bands no's 5, 7, and 11 created by Steward, Basha, and Pleo. Disappearing of α esterase bands in treated larvae may be due to the toxicant effect of insecticide or due to defense or resistance mechanism of the larvae to insecticide and its mode of action of the insecticides. Coragen has little changes in a number of α esterase bands but affects on the band amount compare with untreated.

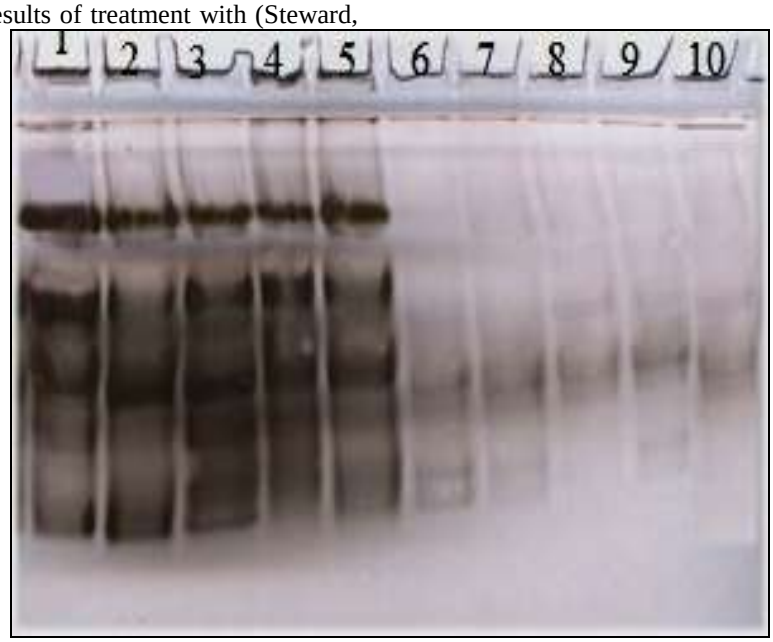

Fig 3: α esterase electrophoretic pattern of *S. littoralis*,where lanes (1-5) represented the 4th instars untreated, Coragen, Steward, Basha and Pleo, respectively and (6-10) represented eggs of 3 days old, untreated, Coragen Steward, Basha and Pleo, respectively

Table 7: Relative fragmentation Rf and amount % of α- esterases 4th instar larvae and eggs of *S. littoralis* post- treatment with tested insecticides.

		4th instar larvae					three days old eggs				
Lanes		**Control 1**	**Coragen 2**	**Steward 3**	**Basha 4**	**Pleo 5**	**Control 6**	**Coragen 7**	**Steward 8**	**Basha 9**	**Pleo 10**
Rows	R.f	%	%	%	%	%	%	%	%	%	%
1	0.11										5
2	0.16	12.26	11. 5	9.95	11.23	15.1	6.39	10.88	10.45	7.42	3.43
3	0.24	2.68	3.2	3.69	4.86	3.18	11.19	7.32	6.47	4.29	3.26
4	0.29	3.09									
5	0.34			12.79	13.32	19.18				8.53	
6	0.38	20.55	11.54				11.00		11.87		12.29
7	0.5			7.73	16.55	19.23			28.9	47.60	28
8	0.57	21.01	24.30	18.44			23.38	38.46	24.24		25.02
9	0.6						14.64	15.65			
10	0.64	14	10.55	16.22	16.25	13.2					
11	0.79			13.75	22.46	23.36	15.14	12.1		11.25	
12	0.89	16.21	24.07	9.62							
Total bands		7	6	8	6	6	6	5	5	5	6

%: band amount

α esterase electrophoretic pattern of 3 days old eggs samples was collected post 24 hrs post-treatment with LC_{50}s ($0.061x10^{-2}$, 0. 81 $x10^{-2}$, 2.39 $x10^{-2}$ and 0. 314 $x10^{-2}$ml/l) of Coragen, Steward, Basha, and Pleo, respectively and represented in Table (7) and Fig(3) for untreated and 4 treated samples, respectively in lanes (6-10). Total band number was 27 bands distributed as 6, 5, 5, 5, and 6 bands with Rf ranging between (0.16 and 0.79) and (0.11 and 0.79) foruntreated and treated, respectively. The band no's 2 and 3 with Rf values, 0.16 and 0.24 were dominant bands between untreated and treated egg samples with max. amount 10.88 and 11.19 for Coragen and untreated.

Some bands created and others disappeared, bands, no's 1,5 and 7 with Rf 0.11, 0.34, and 0.5 created with Pleo, Basha, and (Steward, Basha & Pleo), respectively with max. amount 5, 8.53, and 47.6. Four bands no's, 6, 8, 9 and 11 with Rf 0.38, 0.57, 0.6 and 0.79 with the max. amount ranged (11-38.46) were disappeared in some treated samplesas, (Coragen & Basha), Basha, (Steward, Basha & Pleo) and (Steward & Pleo), respectively.So these bands may be (related) or used as an indicator for treatment with these insecticides and produced as a result of treatment.

ii-β -esterase electrophoretic pattern of4th instar larvae and 3 days old eggs of *S. littoralis.*

β esterase electrophoretic pattern of whole body tissues of the 4th larval instars of *S. littoralis* recorded in Table (8) and Fig (4) and represented the lanes (1-5) as untreated and 4 treated larval samples post-treatment for 48 hrs with LC_{50s} (0.0329, 0.157, 0. 457 and 0. 021 ml/l) of Coragen, Steward, Basha, and Pleo, respectively.

Total band numbers of β-esterase were 39 bands and distributed as 7,7, 8,8 and 9 bands with Rf ranging between 0.04 and 0.89 and theamount % ranged between (1.79 and 26.58). Five of them, no's 4, 6, 8, 10, and 11 with Rf values,0.17, 0.24, 0.4,0.59 and0.68, respectively were dominant between untreated and treated larvae with max. amount26.58, 8.18, 20.02, 25.61 and15.53 for untreated, Basha, Pleo, untreated, and Steward, respectively. Some bands created and others disappeared, bands, no's.(2 and12) and 9 with Rf values, (0.08 and 0.77) and 0.53 created in some treatment and may be specific for the treatment (Basha & Pleo) and (Steward, Basha & Pleo), respectively and the max. amount was12.99with Basha. On the other hand, two bands no's (1 and 13) with Rf (0.4 and 0.89) were disappeared in Basha while band no. 1 disappeared only in Pleo.

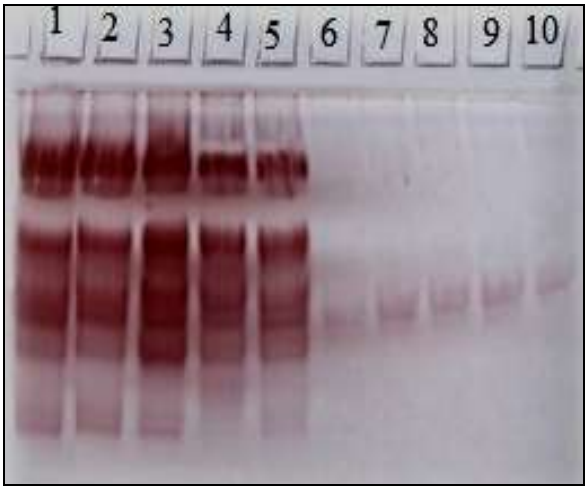

Fig 4: β esterase electrophoretic pattern of *S. littoralis*, where lanes (1-5) represented the 4th instars untreated, Coragen, Steward, Basha and Pleo, respectively and (6-10) represented eggs of 3 days old, untreated, Coragen Steward, Basha and Pleo, respectively

B-esterase electrophoretic pattern of 3 days old eggs samples was collected post 24 hrs post-treatment with LC_{50}s ($0.061x10^{-2}$, 0. 81 $x10^{-2}$, 2.39 $x10^{-2}$ and 0. 314 $x10^{-2}$ml/l) of Coragen, Steward, Basha, and Pleo, respectively and represented in Table (8) and Fig(4) as untreated and 4 treated samples, respectively in lanes (6-10). Total band numbers were 27 bands and distributed as 6, 5, 5, 5, and 6 bands with Rf ranging between 0.04 and 0.77. Two bands, no's, 1 and 9 with Rf values, 0.04 and 0.53, respectively were dominant between untreated and treated egg with max. amount 4.1and 21.75 in Pleo and Basha. Some bands created and others disappeared, bands no 3, 5, 7, and 8 with Rf values, 0.11,0.21,0.33 and 0.4 created in (Steward, Basha & Pleo), (Coragen & Steward), (Basha & Pleo) and Steward, respectively with max. amount 8.35 in Steward. On the other hand, two bands no's, (6, 10) and 12 with Rf values, (0.24, 0.59) and 0.77 were disappeared in (Basha & Pleo) and (Steward, Basha & Pleo), respectively compared with untreated.

Table 8: Relative fragmentation Rf and amount % of β-esterase's of 4th instar larvae and eggs of *S. littoralis* post-treatment with tested insecticides.

Lanes		4th instar larvae					three days old eggs				
		control 1	Coragen 2	Steward 3	Basha 4	Pleo 5	control 6	Coragen 7	Steward 8	Basha 9	Pleo 10
Rows	R.f	%	%	%	%	%	%	%	%	%	%
1	0.04	1.93	1.9	2.42			3.73	3.86	3.8	3.33	4.1
2	0.08				4.83	3.13					
3	0.11								4.1	5.41	5.03
4	0.17	26.58	25.67	22.27	14.8	17.98					
5	0.21							4.86	8.35		
6	0.24	5.52	5.46	5.89	8.18	6.03	15.02	6.71	4.69		
7	0.33									4.47	5.02
8	0.4	17.31	17.74	17.2	13.75	20.02			6.26		
9	0.53			12.09	12.99	6.47	13.84	4.3	15.77	21.75	17.79
10	0.59	25.61	25.14	9.13	7.81	8.54	27.26	18.18	8.03		
11	0.68	6.03	7.30	15.53	8.47	7.2					
12	0.77				2.61	1.79	4.78	6.06			
13	0.89	8.08	5.6	4.49		2.23					
Total bands		7	7	8	8	9	5	6	7	4	4

%: band amount

α and β- esterase for both eggs and 4th of *S. littoralis* recorded high effect due to the treatment with tested insecticides. The difference in migration of esterase bands may be attributed to the difference in M.wt as the difference in the net charge of constituent proteins. These results are agreement with many authors Sharaawi, *et al*., (2002) [35] the β-easterase pattern detects 14 bands two of them were dominant for 4th larval instar with Rf 0.065 and 0.26 and different denisty 38.4 and 20.7%. the major type of esterases was cholinesterases in dominant concentration. In the same trend twenty-eight and 33 esterase bands of α and β-naphthylacetate in *S. littoralis* larvae, detected by using four inhibitors observed by El-Bermawy, (2000) [35]. Effect on the pattern of enzymes activity, quite a fluctuation differing from the normal behavior the changes of α –GPDH and AO pattern in treated *S. littoralis*, stages may be referred to depression or mutations of regulating gene which responsible for the biosynthesis of polypeptide chain building these enzymes (Hassan, 2009). The esterase pattern of the 6th instar of *S. littoralis* detected different bands than the normal as a result of treating with Lc_{50}s of radiant and spinosad Hassan and Abdel Hafez (2009) [20]. The enzyme activity of α and β- esterase and the patterns of 4th of *S. littoralis*, treated with Pyridalyl showed differences in treated than control (Dahi, *et al*., 2011) [10]. Hassan, *et al*., 2013 detected great differences in a number of zones α –naphthylacetate 36 esterase bands with Rf ranging between 0.01 to 0.28 while in case of β –naphthylacetate 39 esterase bands with Rf ranging between 0.01 to 0.92 in control and treated larvae post-treatment with compounds derived from rice straw. Indoxacarb, Emamectin benzoate, and Pyridalyl had inhibited all the enzymes of 4th larval instar of *S. littoralis*, Indoxacarb was the most effective by reduced α-esterase while Pyridalyl was had highly significant stimulation β- esterase (Abdel-Aziz, 2014). The increasing activity of several detoxification enzymes added stress on enzyme expression system to synthesize new and higher amounts of detoxification as esterases which showed to protect the insect from the poisoning of insecticide as a part of a defense mechanism by decreasing the body weight defense against insecticide stress.

So, the physiological response to insecticides in *S.littoralis*is associated with the changes in the expression of stress-related proteins and esterases. The secretion of protein and /or easrterase in high concentration in treated insects to minimize the effect of toxic material and believed that they may be specific for the detoxification of certain insecticides or due to defense or resistance mechanism. So further investigations are required to identify these proteins and esterases and their activity using different assays; Also due to a little information about the effect of insecticides residues on eggs so, time applications to the most susceptible insect stage should typically at egg lay, egg hatch and/or newly hatched larvae, that prevent the establishment and growth of populations of pest before reaching the damaging levels at low using rates. That needs more studies and research to known the gene response.

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