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Electrophoretic and colorimetric pattern of protein and isozyme as reflex to diflubenzuron and chromafenozide treatments of *Spodoptera littoralis* (Boisd.)

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Abstract
The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), is a serious polyphagous pest of cotton and other cultivated crops in Egypt. Therefore, the LC_{50} of two insect growth regulators (IGRs), diflubenzuron and chromafenozide, were applied to the 2nd and 4th larval instars of *S. littoralis* to investigate their impacts on the total protein, carbohydrate and lipid contents of hemolymph and body homogenate of 6th instar larvae. Moreover, native protein, glycoprotein, lipoprotein, isozymes and fractional protein were detected using electrophoresis. Diflubenzuron produced non-significant effect ($p>0.05$) on the hemolymph total protein content, significant ($p<0.05$) and a highly significant ($p<0.01$) decrease in body homogenate total protein content when larvae treated as 2nd and 4th instars, respectively. Conversely, chromafenozide treatment showed non-significant difference in total protein contents of the whole-body homogenate of treated larvae. The application of the two IGRs produced a highly significant reduction ($P<0.01$) on the hemolymph total carbohydrate and lipids. Also, the two IGRs resulted in general reduction on the whole body homogenate total carbohydrate and lipid contents of the treated larvae. The present investigation recorded that there were differences between control and treated larvae in native protein, glycoprotein, lipoprotein and SDS-protein bands. Also, the patterns of alcohol dehydrogenase, aldehyde oxidase, α-esterase and β-esterase showed more number of bands in treated larvae as compared with control. These unique bands could be considered as protein markers to discriminate among the treated larvae with chosen IGRs. Data presented in this work help to understand a part how Diflubenzuron and chromafenozide be effective in controlling *S. littoralis.*

Keywords: Chromafenozide, diflubenzuron, spodoptera littoralis, proteins, isozymes

Introduction
The cotton leafworm, *S. littoralis* (Boisd.) is one of the major pests of cotton and several important crops in Egypt causing a considerable economic loss to farmers every year [1-2]. It has acquired resistance to different insecticides commonly used in Egypt throughout chemical control programs on various crops [3]. Recently, insect growth regulators (IGRs) have proven extremely effective as component of integrated management programs for control *S. littoralis* in Egypt [4-5]. IGRs are bio-rational compounds that disrupt the normal development of insects [6]. Moreover, IGRs have a selective mode of action as they act primarily on target species [7-8] so they fit the requirements for third generation pesticides.
From IGRs, Diflubenzuron is a safe, specific and environmentally friendly IGR [9] and considered a relatively safe tool for integrated pest control programmes [10]. Diflubenzuron has different mode of action against insects, contact and stomach poison [11] and chitin synthesis inhibitor [12]. Also, it has biochemical effects on proteins [13], carbohydrates [14] and lipids [15]. The second, Chromafenozide is non-steroidal ecdysteroid agonists compound act like 20-hydroxyecdysone by binding to ecdysteroid receptor complex and up- or down-regulate the expression of a series of genes which control the moulting progress in target insects. Interest in using chromafenozide as an insecticide is increasing [8-16]. Chromafenozide did not pose any hazards to consumers when applied in field conditions [17].
Quantitative and qualitative protein assays in insects hemolymph or tissues were of extensive rank for the understanding of different physiological processes. The most complex compounds and the most characteristic of living matter are proteins. They are present in all viable cells; nucleoproteins, are essential to the process of cell division, enzymes and hormones, control

many chemical reactions in the metabolism of cells. Thus, the separation and characterization of the insect proteins after treated with IGRs will be helpful in understanding the modulation of protein by IGRs which can be exploited for the management of pests [18]. So, the present study examined the native protein, glycoprotein, lipoprotein, isozymes and fractional protein as thumbprints illuminating the changes occurred in the hemolymph and body tissue homogenate of 6th instar larvae of the *S. littoralis* developed from 2nd and 4th instar larvae treated with LC_{50} of Diflubenzuron and chromafenozide.

2. Materials and Methods

2.1 Insect culture

A laboratory strain of *S. littoralis* was obtained in 2014 from Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza. Stock culture of the insect was reared according to El-Defrawy *et al*. [19] in Animal Research Laboratory, Department of Biological and Geological Sciences, Faculty of Education, Ain Shams Univ., Egypt. The culture was maintained in the laboratory at 27±2 °C and 70 ±5% rh.

2.2 Chemicals and Experimental design

Diflubenzuron (Dimilin®, suspension concentrate 480 g/l, Chemtura Co. Ltd. U.S.A.) and chromafenozide (Virtu®, suspension concentrate 50 g/l, Nippon Kayaku Co. Ltd., Japan) were used. The experiments were carried out on the 2nd and 4th larval instars with LC_{50} of Diflubenzuron and chromafenozide that have been estimated for each instar in our previously work [20]. Freshly castor oil beans leaves were dipped for 30 seconds in the estimated LC_{50}. Then, the treated leaves were left to dry for 30 minutes at room temperature. Sixty larvae of each instar were starved for 8 hours. The treated castor oil leaves were applied to the larval instars for 24 hours and then replaced by untreated leaves. Control experiments were done without any treatment. All treatments were incubated in above mentioned condition until the larvae reach 6th instar. There were four different treatments to be used in subsequent investigation, designed as D II, D IV, V II and V IV. The first and the second were the treatment with Diflubenzuron (LC_{50}=1.3 ppm) and (LC_{50}=3 ppm) when applied to larvae as 2nd instars and 4th instars, respectively. The other two were the treatment with (LC_{50}= 0.1ppm) of chromafenozide when applied to larvae as 2nd and 4th instars, respectively.

2.3 Biochemical studies

For the biochemical analysis, hemolymph and larval body homogenate of 6th instar larvae of control and emerged from treated 2nd and 4th instar larvae with both IGRs were prepared. Larval hemolymph was collected according to Boctor [21]. Homogenates of the whole body of the larvae were prepared according to the method of Nasr *et al*. [22]. The collected hemolymph and the supernatant of larval body homogenate could be used directly or dispensed into 40 µl aliquots and stored at -5 °C until needed.

2.4 Assay of total protein, carbohydrate and lipid contents

Total protein content of the larval homogenate was assayed according to the method of Bradford [23]. The total carbohydrate content was estimated according to the method of Singh& Sinha [24]. For total lipid content, the method of Frings *et al*. [25] was adopted.

2.5 Proteins profiles by polyacrylamide gel electrophoresis

Electrophoretic pattern of native proteins, glycoproteins, lipoproteins, isozymes such as α- and β-estrases, alcohol hydrogenesis (ADH) and aldehyde oxidases (AO) of larvae were separated using non-denaturing discontinuous polyacrylamide gel electrophoresis (PAGE) according to the method of Davis [26]. Discontinuous polyacrylamide gel electrophoresis in the presence of the anionic detergent, sodium dodecyl sulphate (SDS-PAGE) was used for the separation of protein subunits and the determination of their molecular weights (mol. wt.) [27].

2.6 Statistical analysis

Results were expressed as mean ± standard deviation (SD.). The statistical significance of differences between means was determined by student ‘‘t-test” for paired observations by using Microsoft® Excel 2016.

3. Results

3.1 Total protein, carbohydrate and lipid contents

Table 1. Shows the effects of LC_{50} of Diflubenzuron and chromafenozide on *S. littoralis* larvae treated as second and fourth larval instar on total protein, carbohydrate and lipid contents. In hemolymph, IGRs treatment produced insignificant difference on the total protein content in the treated larvae in groups D II, D IV and V II from that of the control (C). Except group V IV, treatment produced a highly significant decrease (p<0.01) in the total protein content compared to that of the untreated group. Also, application of LC_{50} of Diflubenzuron and chromafenozide produced a highly significant reduction (P<0.01) on the hemolymph total carbohydrate and lipids contents of all treated groups compared to that of the untreated group.

In whole body homogenate, the application of LC_{50} of Diflubenzuron produced significant and a highly significant decrease in total protein content with group D II and D IV, respectively. Conversely, chromafenozide treatment showed insignificant difference in total protein contents of the whole-body homogenate of groups V II and V IV compared to that of the group C. Also, Application of the LC_{50} of the two IGRs resulted in general reduction on the whole body homogenate total carbohydrate and lipid contents of the treated larvae. The level of decrease was insignificant (p>0.05) in groups D IV and V II, significant (p<0.05) in group D II and a highly significant (p<0.01) in group V IV.

Table 1: Effect of three lethal concentrations of diflubenzuron and chromafenozide on total protein, carbohydrate and lipid contents of *S. littoralis* 3rd larval instar treated as second larval instar

Treatment	Group	Protein content (mg/ml)		Carbohydrate content (mg/ml)		Lipid content (mg/ml)	
		Hemolymph	Body Homogenate	Hemolymph	Body Homogenate	Hemolymph	Body Homogenate
Control	C	45.25±2.00	17.33±0.81	11.18±0.51	5.97±0.48	3.67±0.19	1.96±0.16
Diflubenzuron	D II	48.28±1.14 ns	11.38±1.05*	5.16±0.28***	4.39±0.10*	1.54±0.11***	1.31±0.02*
	D IV	48.63±2.14 ns	10.59±0.95**	7.05±0.08***	4.85±0.16 ns	2.25±0.03***	1.47±0.06 ns
Chromafenozide	V II	43.20±3.25 ns	19.23±2.1 ns	4.11±0.12***	4.92±0.16 ns	1.20±0.05***	1.46±0.06 ns
	V IV	20.35±1.66 ***	13.08±1.9 ns	6.38±0.12***	2.77±0.11**	2.00±0.05***	0.72±0.04 **

(1) Mean of four replicates.

Student *t*-test, levels of Significance: ns, not significant (p>0.05),*, significant (p<0.05), **, highly significant (p<0.01) as compared with control.

3.2 Native proteins, glycoproteins and lipoproteins patterns by PAGE

The native protein profiles for control and treated larvae was illustrated in Figure (1 a) and table 2 represented their computer analysis. The hemolymph protein profile comprised no monomorphic bands. Six polymorphic bands were scored at polymorphism percentage 54.55% and their Rf values are (0.18, 0.21, 0.24, 0.53, 0.58 and 0.61). Five unique bands were recorded at Rf values of 0.58 and 0.61 in control, 0.91 in D II, 0.64 in D IV and 0.71 in V IV. Also, the data revealed two monomorphic bands in native protein pattern of tissue homogenate, which were detected at approximately Rf values of 0.21 and 0.24. Eight polymorphic bands were scored with percentage of 53.33%. They were scored at rows 5, 6, 9, 11, 12, 13, 14 and 15. Five unique bands were recorded at Rf value 0.64 (C), 0.48 & 0.53 (D II) and 0.18 & 0.28 (D IV).

The glycoprotein patterns (Table 3 and Fig. 1 b) showed that hemolymph comprises two monomorphic bands, which were scored at Rf (0.029 & 0.56). No polymorphic nor unique bands were detected at the glycoprotein pattern. While the resulted profile in tissue homogenate comprises only one monomorphic band (Rf = 0.056), no unique bands and only one polymorphic band was scored at row 1 (Rf= 0.029) at all samples except V IV.

The lipoprotein patterns (Table 4 and Fig. 1c) of the hemolymph revealed the presence of three monomorphic bands, (75%) which were detected at Rf values (0.084, 0.093 and 0.11). But there was one band at Rf (0.13) which considered unique band and scored after chromafenozide application to the 4th instar larvae (V IV). Also, there was not any monomorphic band. Lipoprotein analysis of tissue homogenate comprised only one monomorphic band, which was detected at Rf (0.11) whereas no unique bands were recorded. On the other hand, four polymorphic bands (80%) were scored at row1 (control & D II at Rf: 0.084), row2 (D IV, V II and V IV at Rf: 0.093), row4 (control, D II, D IV, & V IV at Rf: 0.12) and row5 (control, D IV& V II at Rf: 0.13).

Table 2: Relative fragmentation (Rf) and amount of native protein bands detected in electropherograms of 6th larval instar of *S. littoralis* hemolymph and tissue homogenate after diflubenzuron and chromafenozide treatment

Rows	Rf	Amount % in Hemolymph					Amount % in Homogenate				
		(C)	(D II)	(D IV)	(V II)	(V IV)	(C)	(D II)	(D IV)	(V II)	(V IV)
1	0.18	9.01	6.54		49.04	31.97			15.38		
2	0.21	5.36	11.78	15.04			21.60	12.21	14.75	19.08	16.42
3	0.24			17.94	50.83	27.25	17.38	10.74	13.35	12.95	16.77
4	0.28								7.43		
5	0.34							7.06	9.82		
6	0.41							8.53	6.88		
7	0.48							9.59			
8	0.53							9.18			
9	0.58	17.96								18.84	19.40
10	0.61	18.47									
11	0.64			30.04			21.60				
12	0.71					40.70	23.05	11.71			
13	0.74	23.54	21.26	21.65					10.16	16.93	18.25
14	0.82	25.51	13.64					15.85	12.16	15.86	12.63
15	0.86		19.39	15.44							
16	0.88						16.33			16.28	16.39
17	0.91		27.24					15.15	10.18		
Total number of bands		6	6	5	2	3	5	9	9	6	6

Table 3: Relative fragmentation (Rf) and amount of glycoprotein bands detected in electropherograms of 6th larval instar of *S. littoralis* hemolymph and tissue homogenate After treated with diflubenzuron and chromafenozide

Rows	RF	Amount % In Hemolymph					Amount % in Homogenate				
		(C)	(D II)	(D IV)	(V II)	(V IV)	(C)	(D II)	(D IV)	(V II)	(V IV)
1	0.029	30.58	32.17	29.03	25.59	20.97	19.88	41.81	22.28	13.09	
2	0.056	69.42	67.83	70.97	74.41	79.03	80.12	58.19	77.72	86.91	100
Total number of bands		2	2	2	2	2	2	2	2	2	1

Table 4: Relative fragmentation (Rf) and amount of lipoprotein bands detected in electropherograms of 6th larval instar of *S. littoralis* hemolymph and tissue homogenate as control and treated larvae with diflubenzuron and chromafenozide

Rows	RF	Amount % In Hemolymph					Amount % in Homogenate				
		(C)	(D II)	(D IV)	(V II)	(V IV)	(C)	(D II)	(D IV)	(V II)	(V IV)
1	0.084	25.98	18.50	19.24	19.40	24.60	22.04	23.53			
2	0.093	36.52	41.98	39.07	39.63	23.94			23.51	30.35	31.77
3	0.11	37.50	39.52	41.69	40.97	24.44	35.08	53.16	34.82	45.29	40.65
4	0.12						23.90	23.31	21.31		27.58
5	0.13					27.02	18.98		20.36	24.35	
Total number of bands		3	3	3	3	4	4	3	4	3	3

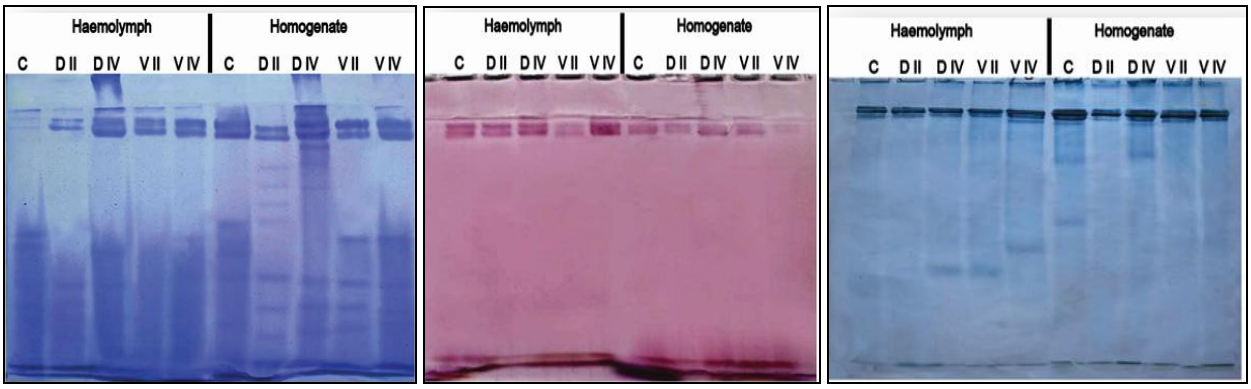

Fig 1a **Fig 1b** **Fig 1c**

Fig 1: Electrophoretograms of native protein pattern (Fig. 1a), glycoproteins (Fig. 1b) and lipoproteins (Fig. 1c) of hemolymph and body homogenate of *S. littoralis* as control and treated with diflubenzuron and chromafenozide

3.3 Isozymes patterns

Table 5 and Fig. 2a showed ADH profile in the hemolymph and tissue homogenate of control and treated samples. In hemolymph and the body homogenate, a newly formed band was observed in all treated groups DII, DIV, VII and VIV with Rf value 0.084. Table 6 and Fig. 2b demonstrated aldehyde oxidase (AO) banding pattern of the studied hemolymph and tissue homogenate samples. AO was observed in the hemolymph of control as one subunit of enzyme with Rf value 0.034. While, in treated groups AO pattern consisted of two subunits of enzyme with Rf =0.034 and 0.046. In the body homogenate there is only one band of AO in all groups except group V II had two bands of AO.

Fig. 2c and Table 7 demonstrated α-Est banding pattern of the studied hemolymph and tissue homogenate samples, respectively. α-Est electrophoretic banding patterns of all tested samples exhibited two bands with Rf 0.38 and 0.54. In hemolymph, band in row 1 with Rf value of 0.04 was common band appeared in control group and all treated groups. While the band in row 2 with Rf value of 0.15 was detected only in group V IV. In body homogenate, two bands were appeared in all groups. The electrophoretic pattern of esterases detected by β-NA as substrate (Table 8 and Fig. 2d) was obvious through 6 electrophoretic bands with Rf value ranged from 0.28 to 0.91 as shown in Bands in rows number 4 and 5 with Rf value of 0.61 and 0.74 were common bands appeared in control group and all treated groups. In addition, the band in row3 with Rf of 0.48 was not detected in esterase pattern of control larvae.

Table 5: Relative fragmentation (Rf) and amount of ADH bands detected in electropherograms of 6th larval instar of *S. littoralis* hemolymph and tissue homogenates control and treated larvae with diflubenzuron and chromafenozide

Rows	RF	Amount % in Hemolymph					Amount % in Homogenate				
		(C)	(D II)	(D IV)	(V II)	(V IV)	(C)	(D II)	(D IV)	(V II)	(V IV)
1	0.057	100	66.89	68.20	24.57	75.89	63.51	72.66	66.50	58.77	68.61
2	0.084		33.11	31.80	75.43	24.11	36.49	27.34	33.50	41.23	20.99
Total number of bands		1	2	2	2	2	2	2	2	2	2

Table 6: Relative fragmentation (Rf) and amount of Ao bands detected in electropherograms of 6th larval instar of *S. littoralis* hemolymph and tissue homogenates control and treated larvae with diflubenzuron and chromafenozide

Rows	RF	Amount % in Hemolymph					Amount % in Homogenate				
		(C)	(D II)	(D IV)	(V II)	(V IV)	(C)	(D II)	(D IV)	(V II)	(V IV)
1	0.034	100		5.47	36.52	32.90	100	100	100	15.04	100
2	0.068		100	94.53	63.48	67.10				84.96	
Total number of bands		1	1	2	2	2	1	1	1	2	1

Table 7: Relative fragmentation (Rf) and number of α-Est bands detected in electropherograms of 6th larval instar of *S. littoralis* hemolymph and tissue homogenate as control and treated larvae with diflubenzuron and chromafenozide

Rows	RF	Amount % in Hemolymph					Amount % in Homogenate				
		(C)	(D II)	(D IV)	(V II)	(V IV)	(C)	(D II)	(D IV)	(V II)	(V IV)
1	0.21	100	100	100	23.59	100	70.05	28.50	32.99	23.91	36.88
2	0.41				76.41		29.95	71.50	67.01	76.09	63.12
Total number of bands		1	1	1	2	1	2	2	2	2	2

Table 8: Relative fragmentation (Rf) and number of β-Est bands detected in electropherograms of control and treated 6th instar larvae with diflubenzuron and chromafenozide of *S. littoralis* hemolymph and tissue homogenate

Rows	RF	Amount % in Hemolymph			Amount % in Homogenate						
		(C)	(D II)	(D IV)	(V II)	(V IV)	(C)	(D II)	(D IV)	(V II)	(V IV)
1	0.28					13.98	5.57				
2	0.41					13.61	16.14	13.71	16.93	14.41	9.46
3	0.48		35.41	19.30	31.25	12.02	7.40	9.60	10.98	12.04	11.42
4	0.61	28.41	35.00	11.06	24.32	22.39	8.54	12.56	10.67	19.09	20.18
5	0.74	71.59	29.59	57.18	44.43	28.02	30.88	36.00	33.87	35.11	38.52
6	0.91			12.46			30.56	28.14	27.55	19.35	20.33
Total number of bands		2	3	4	3	5	6	5	5	5	5

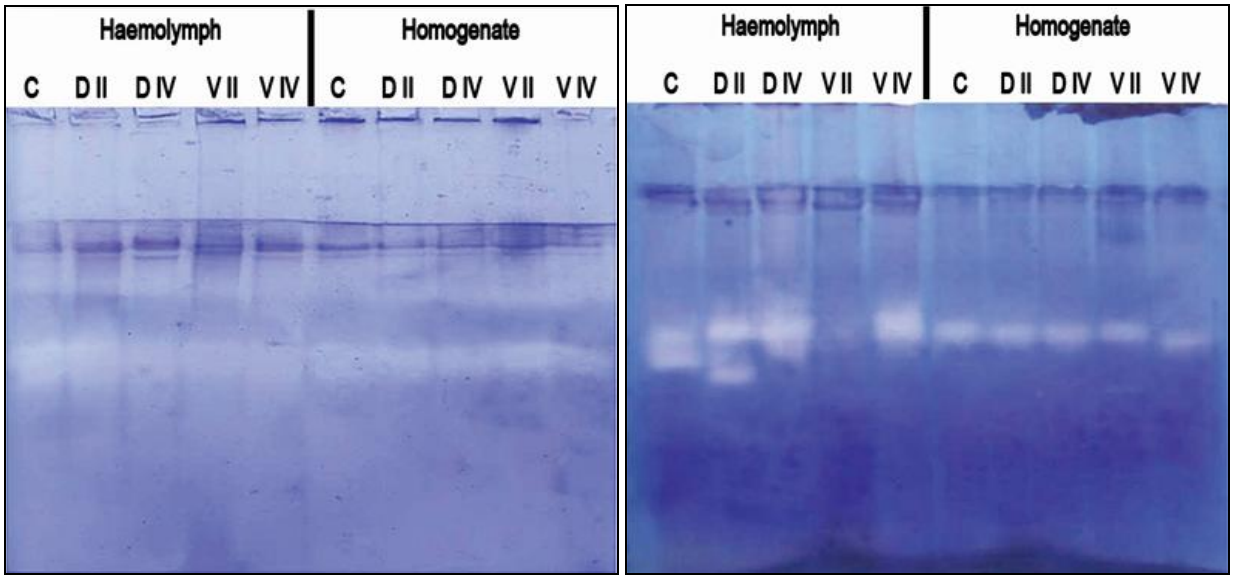

Fig 2a **Fig 2 b**

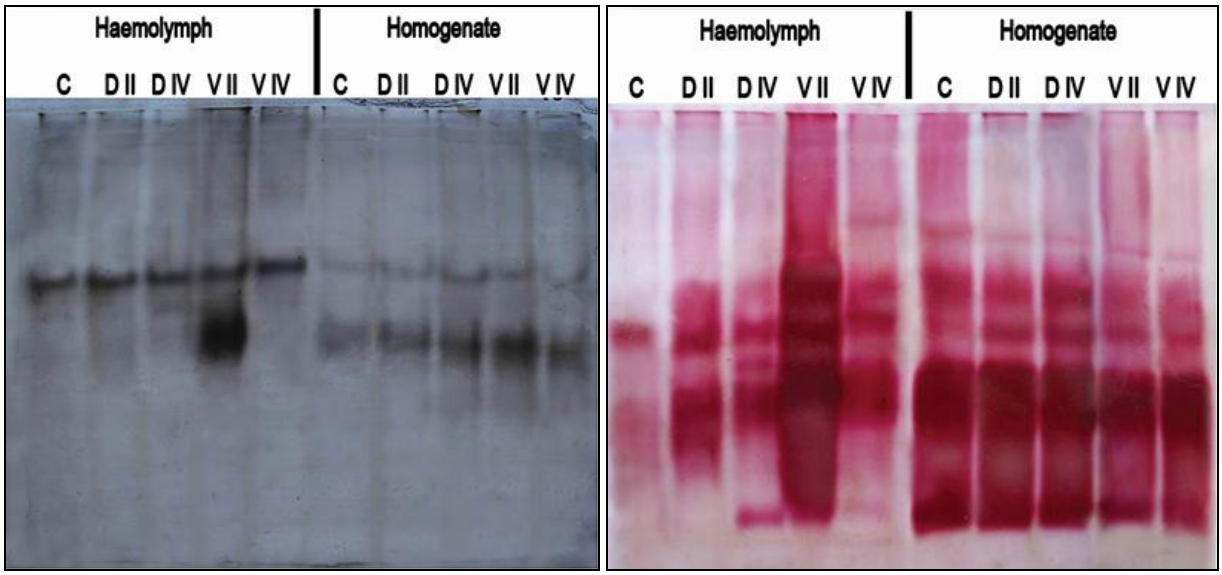

Fig 2c **Fig 2d**

Fig 2: Electrophoretograms of isozymes patterns, alcohol dehydrogenesis (Fig. 2a), aldehyde oxidases (Fig. 2b), α-esterases (Fig. 2c) and β-estrases (Fig. 2d) of hemolymph and body homogenate of *S. littoralis* as control and treated with diflubenzuron and chromafenozide

3.4 SDS-PAGE

Fig. 3 demonstrated the SDS-PAGE protein profiles for control and treated larvae, while Tables 9 & 10 represented their computer analysis. In hemolymph, the total number of bands represented in the control and treated larvae were 61 bands. The maximum number of bands was 13, observed in samples D II and V IV, whereas the minimum number was 11, which recorded in sample V II. The highest molecular weight of the observed protein bands was 301.88 KDa (V IV), while the lowest one was 11.56 KDa (D IV). The present investigation revealed 5 common rows appeared between control and treated samples. These monomorphic bands were approximately 207.626 (row 3), 130.01 (row 4), 102.02 (row 5), 62.073 (row 8) and 16.16 KDa (row 20). Ten bands were comprised polymorphic bands at rows 7, 9, 12, 13, 14, 15, 16, 17, 19 and 21. Five unique bands were recorded at C: 54.29, D II: 21.08, D IV: 11.56 (the lowest molecular weight) and V IV: 45.86 & 301.88 (the highest molecular weight) (Table 9).

Body homogenate recorded a maximum number of 42 bands but all bands were detected at approximately molecular weights ranging between 68.77 and 10.29 KDa (Table 10). The maximum number of bands was 7 which observed in samples D IV and V IV, whereas the lowest number was 5 and recorded in samples D II and V II. The highest molecular weight of the observed protein bands was 68.77 KDa and scored in the control sample, while the lowest one was 10.29 KDa and recorded in sample V II. There is only one monomorphic band, which was detected at approximately molecular weights of 35.19 KDa. Eight polymorphic bands were detected at rows 8, 9, 10, 11, 13, 15, 16 and 17 in the protein pattern. In addition, three unique bands were scored at molecular weights 68.774 kDa (C), 62.10 & 27.32 kDa (V IV).

Table 9: Molecular weight (Mol. Wt.) and number (Amo.) of SDS-PAGE protein bands detected in electropherograms of 6th larval instar of *S. littoralis* hemolymph as control and treated larvae with Dimilin® and Virtu

Lanes	Marker		Lane 1 (C)		Lane 2 (D II)		Lane 3 (D IV)		Lane 4 (V II)		Lane 5 (V IV)	
Rows	Mol. Wt	Amount	Mol. Wt	Amount	Mol. Wt	Amount	Mol. Wt	Amount	Mol. Wt	Amount	Mol. Wt	Amount
1											301.88	2.19
2	245	2.39										
3	180	2.87	212.5	2.37	196.25	2.72	201.67	3.58	207.08	3.00	220.63	3.49
4	135	3.63	128.27	2.74	130.29	2.81	128.94	4.51	130.96	3.56	131.63	4.04
5	100	3.85	100	3.28	100	3.08	103.37	4.67	102.69	3.79	104.04	4.01
6	75	6.93										
7			70.55	4.97	70.778	2.99			72.55	3.88	72.55	3.80
8	63	5.89	59.61	4.56	61.54	3.14	63.44	3.37	62.75	7.42	63	8.27
9					58.161	4.122	58.88	4.15				
10			54.29	2.53								
11	48	6.99									45.86	3.37
12			43.34	3.04	44.11	3.36	41.79	5.19	44.50	3.32		
13					37.91	3.15	38.68	2.64	38.29	4.74	40.23	5.47
14	35	6.84	37.13	3.20	34.85	2.53			35.19	2.48	36.35	2.40
15			33.67	1.83			31.32	5.79				
16			28.38	6.48					29.55	6.36	29.85	7.66
17	25	8.19	25.14	4.63							26.02	3.44
18	20	5.21			21.08	4.59						
19			18.43	6.49			17.81	9.84	18.15	4.45	18.63	4.05
20	17	7.77	15.98	3.97	16.09	12.16	15.64	2.78	16.20	4.42	16.88	4.47
21					13.83	2.76	12.81	3.31				
22	11	5.11					11.56	2.67				
Total number of bands	12		13		12		12		11		13	

Table 10: Molecular weight (Mol. Wt.) and number (Amo.) of SDS-PAGE protein bands detected in electropherograms of 6th larval instar of *S. littoralis* tissue homogenate as control and treated larvae with diflubenzuron and chromafenozide

Lanes:	Marker		Lane 1 (C)		Lane 2 (D II)		Lane 3 (D IV)		Lane 4 (V II)		Lane 5 (V IV)	
Rows	Mol. Wt.	Amount	Mol. Wt.	Amount	Mol. Wt.	Amount	Mol. Wt.	Amount	Mol. Wt.	Amount	Mol. Wt.	Amount
1	245	2.15										
2	180	2.38										
3	135	3.72										
4	100	3.92										
5	75	7.58										
6			68.77	3.65								
7	63	5.87									62.10	3.43
8							50.43	4.71			51.84	6.07
9	48	7.25	48.25	5.32	47.27	5.45	47.38	8.90				
10			41.70	5.20							43.66	8.08
11					36.75	2.76	36.754	6.88	39.12	5.12		
12	35	6.78	35.82	5.69	36.23	4.81	34.68	10.68	33.4	4.70	35.825	8.56
13			30.84	5.18	28.28	4.61	28.92	6.39	29.24	5.12		
14	25	8.65									27.32	5.02
15							19.028	6.42			18.52	8.21
16	17	5.88	15.02	5.16	15.87	5.56	16.718	3.73	16.43	7.10		
17	11	8.51							10.29	5.13	12.48	5.64
18	4.294	6.47										
Total number of bands	12		6		5		7		5		7	

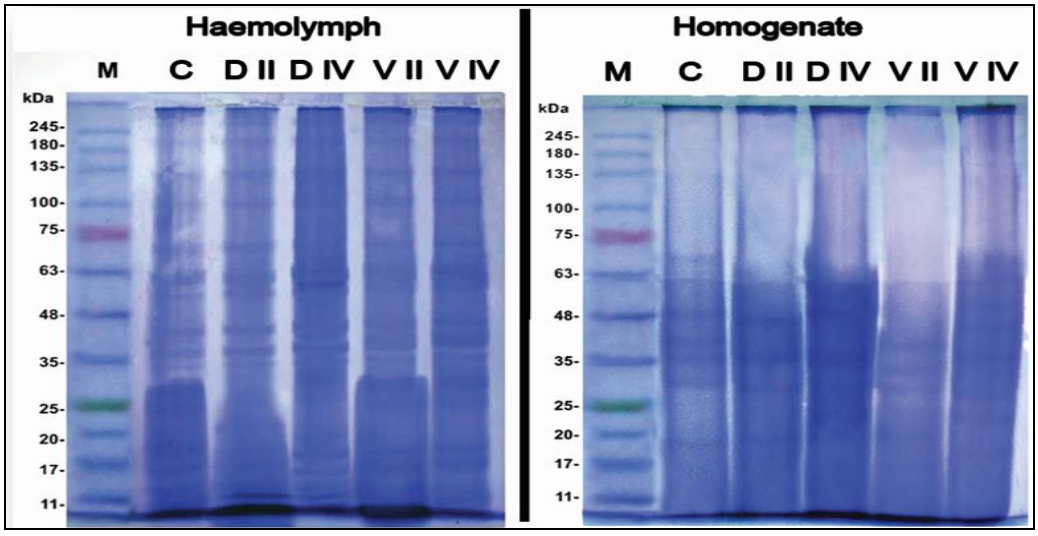

Fig 3: Electrophoretograms of SDS- fraction ptotein pattern of hemolymph and body homogenate of *S. littoralis* as control and treated with diflubenzuron and chromafenozide

4. Discussion

Proteins, carbohydrates and lipids are major biochemical components necessary for an organism development, growth and performance of its vital activities. Protein, carbohydrate and lipid contents increased, sometimes decreased, in some insect species as a response to the action of different IGRs. These contradictory findings may be due to differences in species sensitivity, the potency of the IGRs, or the developmental stage [28]. IGRs were described as agents whose primary action is on insect metabolism. Ultimately, they interfere and disrupt the process of growth, development and metamorphosis of the target insects, particularly when applied during the sensitive period of insect development [29].

Present results revealed that the treatment of *S. littoralis* larvae with LC_{50} of Diflubenzuron and chromafenozide produced reduction in total protein. The decrease in total proteins may be resulting from the inhibition of DNA synthesis and the decrease in the activity of various enzymes [30]. Also, the lower protein concentration may be resulted from DNA damaged causing switch off some essential genes responsible for production of this protein [31]. Our observations are in contradict with the findings of Linvy *et al*. [18] they reported a significant increase in the total protein contents of the hemolymph of *Spodoptera mauritia* larvae after application of IGR.

Carbohydrates were stored in insect body as glycogen and trehalose, which converted into glucose. Various amounts of glycoproteins may serve as an amino-sugar reserve for chitin synthesis. Also, they are important group as a major energy source and as a vital component of the insect exoskeleton [32]. They remain necessary for normal function of male and female reproductive systems. Carbohydrates involved in accumulation of yolk granuoles in developing oocyte and necessary for normal embryo development. These may be converted to amino acids and to fats for storage [33]. In the present study, the significant and highly significant reduction of hemolymph and body homogenate total carbohydrate contents of the treated larvae (2^{nd} and 4^{th} instar) could be attributed to starvation, damage of the alimentary canal by the tested IGRs, and/or lowers carbohydrate levels by enhancing carbohydrate uptake by tissues and cells.

Lipids are structural components of the cell membrane and cuticle providing a rich source of metabolic energy for periods of sustained energy demands and facilitate water conservation bath by the formation of an impermeable cuticular barrier and by yielding metabolic water upon oxidation [34]. They are considered as basic components in both hormones and pheromones [35]. In the present findings, the total lipid content in hemolymph and tissue homogenate of *S. littoralis* 2^{nd} and 4^{th} instar larvae treated with Diflubenzuron and chromafenozide recorded degrees of reduction in the total lipid contents. The disturbances in total lipid content of hemolymph and whole body homogenate of treated larvae could be due to the juveno-mimic effect of the chromafenozide supported by the findings of Hill and Izatt [36] who discussed that lipid accumulation is related directly to a lack of juvenile hormone. Furthermore, these effects in lipid content might be understood by the ability of tested IGRs to modify the synthesis of certain metabolites and disrupt the function of the organism [37]. The treated larvae may be utilize lipids as a source of energy after the reduction of carbohydrate content [38].

The native protein patterns of hemolymph and whole-body homogenate of control *S. littoralis* 6^{th} instar larvae varied greatly according to the sensitivity to the tested IGRs. Each protein reflected the activity of specific gene through the production of enzyme responsible for a specific biological character [39]. Thus, changes in the protein patterns may reflect specialization and adaptation in the organism [40].

In the present study, new protein bands appeared in treated samples after utilization of tested IGRs. These observations referred to the formation of immune protein as a defense action to the presence of foreign molecules, as IGRs, in the larval bodies [41]. However, many types of proteins produced to detoxify or might be responsible for the toxic effects of IGRs on larval mortality, inhibition of pupation, decreased pupal weight and fecundity. This is also being suggested after usage of precocenes (I and II) on *Eyprepocnemis plorans* [42] and Cascade and Match against *S. littoralis* [43]. All the new protein bands probably contain immune proteins [44].

Most of protein backbones are modified by associated carbohydrate structure. This modification illustrated a vital physiological function for protein folding, stability and subcellular location, as well as protein-protein interactions, recognition and signaling. The structure and size of the carbohydrate chain can be very diverse and can alter the physicochemical characteristics of a protein. Moreover, glycoproteins elaborated in other physiological processes such as embryonic development, growth, circadian rhythms, cell attachment as well as maintenance of organ structure, immunity and fertility [45]. Addition of oligosaccharide molecule to polypeptide chain affected structure, solubility, charge or sensitivity of protein to proteolysis [46].

IGRs interfere with the synthesis of lipids and their mobilization as promoted to convert into other metabolites or fatty acids [47]. On the other hand, Tanani *et al*. [48] discussed the odd case of lipid increasing by conversion of accumulated carbohydrates to lipids as a reverse material. The noticed lipoprotein reduction in the present work may be explained by a destructive effect of IGRs on some of the cerebral neurosecretory cells of the brain [49] responsible for secretion of the proteins of the treated larvae consequently DNA synthesis is inhibited [50-51].

Alcohol dehydrogenases (ADH) are a group of dehydrogenase enzymes that facilitate the inter-conversion between alcohols in larval feeding sites and aldehydes or ketones with reduction of nicotinamide adenine dinucleotide (NAD + to NADH). As they serve to break down the toxic effect of alcohols, they also participate in generation of useful aldehyde, ketone, or alcohol groups during biosynthesis of various metabolites [52]. During insect development, dehydrogenases are very vital tools for the investigation activities of insect metabolism. Insecticides disturb dehydrogenases activity [53-54].

Aldehyde oxidase (AO), in many organisms, functions as catalyst in the transformation and oxidation of aldehydes into carboxylic acid. In the present study, the recorded pattern of AO in treated groups differed than the control group and this may be due to the higher metabolic rate in tested samples. Garcin *et al*. [55] denoted the capacity of AO in *D. melanogaster* to detoxify acetaldehyde and use it for energy production. Moreover, Barnes [56] stated that the inversion-allozyme polymorphism of AO is directly involved in the adaptation to a specific environmental component. AO-isozyme patterns in adult *Hypera postica* can be influenced by developmental age and environmental conditions [57].

Our study of esterase isozymes patterns revealed that there were differences in bands number between control and treated larvae. IGRs caused a considerable change in all band numbers and amount percentages compared to the control

ones. Because of their proteinaceous nature, esterases were defined by their ability to catalyze the hydrolysis of ester bonds within lipophilic compounds [58]. Therefore, esterases played important roles in the insecticide resistance of the pest [59]. They represented one of the major enzyme families involved in the detoxification of insecticides as upregulation of insecticide resistant mechanisms in insects [60]. On the same run, Anwar and Abd El-Mageed [61] reviewed that Diflubenzuron could increase β-Est and decrease in α-Est activities. Also, Hamdy and Azab [62] reported that the enzyme activity of α-esterase in *S. littoralis* after treatment was increased with chlorfluazuron and hexaflumuron while levels of β-esterase enzyme were decreased with all tested compounds. The interaction network between enzyme and insecticide elucidated that estrases are enzymes potentially required for multiple resistance phenotype [63].

The present study showed different activities of α-esterase, β-esterase, AO and ADH in treated larvae as compared with control. These differences in enzymes activities may be play role as insecticides detoxification leading to increase the susceptibility of the insect towards insecticides [64]. Insecticides may add stress on enzyme expression system to synthesize new and higher amount of detoxification enzymes [65]. These could be the possible reasons for the arrested growth and mortality [66].

Finally we recorded that there were differences between control and treated larvae in SDS-protein bands. There were five monomorphic bands in hemolymph and one in tissue homogenate while polymorphic bands recorded ten & eight in hemolymph and tissue homogenate, respectively. Moreover, hemolymph scored five unique bands and tissue homogenate showed three bands. Alteration in protein profile is reported for methoxyfenozide treated *Bomboxi mori* [67] and Spodoptera mauritia larvae [18]. Linvy *et al*. [18] reported that the increase in hemolymph storage protein appears to be a more general effect of the IGR mimic exposure to the larvae.

5. Conclusion

In the present study, Diflubenzuron and chromafenozide treatments produced different changes on the biochemical parameters in the hemolymph and body homogenate of the treated 2nd and 4th instars larvae of *S. littoralis*. These biochemical changes could be considered as biochemical markers to discriminate among the treated larval instar with chosen IGRs.

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