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Insecticidal Activity of *Zygophyllum album* Constituents on *Spodoptera littoralis* (Boisd.) Larvae

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Abstract: The aerial parts of *Zygophyllum album* L., Zygophyllaceae were collected, air dried, pulverized and extracted with different solvent systems. The six separated fractions were screened for their lethal and sub-lethal effects against the 4th instar of the cotton leaf-worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Methanol originated fractions (I, II and III) were more effective than both acetone soluble fraction (IV) and the ethylacetate originated fraction (V). On contrary, fractions IV and V were more effective in reducing the larval weight. Several morphogenetic effects on larvae, pupae and adults are revealed. Lethal and sub-lethal effects were evaluated through LC_{50}, LT_{50} and EC_{50} values. GC-MS analysis proved the most lethal active fraction is mostly rich in glycosides of quinovic acid and flavanolone aglycones.

Key wards: *Zygophyllum album* · Insecticidal activity · *Spodoptera littoralis* · Quinovic acid · Flavanolone glycosides

INTRODUCTION

The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is a destructive pest affecting the cotton wealth. It has been controlling with low specificity neurotoxic synthetic compounds, which negatively affect the environmental phases and non-target biota. Public concerns over biological control as a replacement of chemicals have been growing [1]. Several plant extracts affected this pest killing larvae and inhibiting pupation and adult emergence percentages as soosan, *Pancratium maritimum* [2], *Commiphora molmol* (myrrh), *Peganum harmala* L. [3,4]. Anti-feeding activities against this insect were referred to plants originated natural compounds as clerodane diterpenoids in *Ajuga pseudoiva* and the acridone alkaloid xanthoxoline in *Fagara macrophylla* [5,6] or trans-beta-methylthioacrylate in *Balantiopsis cancellata* [7] and a beyerane diterpenoid, ent-3beta-(3-methyl-2-butenoyl)oxy-15-beyeren-19-oic acid in *Plectranthus saccatus* Benth [8].

At the same time, Zygophyllaceae is biologically active family. *Zygophyllum gaetulum* exhibited antiinflammatory effect on mice and rats [9]. *Z. coccineum* extract showed marked larval mortality with inhibiting pupation and adult emergence of *Culex pipiens* [10]. The compound 3-beta-(3,4-Dihydroxycinnamoyl)-erythrodiol was isolated as the cytotoxic constituent of *Z. geslini* [11]. *Z. album* produced a significant antidiarrhoeal activity in rats and relaxation of rabbit's duodenal smooth muscle with calcium channel blocking effect [12]. It showed antiviral activity against Herpes simplex virus type 1 (HSV-1) [13]. *Z. fabago* was effective against *Candida albicans* and *Escherichia coli* [14]. Inhibition effects on acetylcholinesterase, butyrylcholinesterase and lipoxygenase enzymes by *Z. eurypterum* are owed to pterocarpans, atricarpan A, B, C and D [15]. This manuscript studies the lethal and sub-lethal insecticidal efficiencies of *Z. album* L., Zygophyllaceae contents against the 4th instar of the cotton leaf-worm, *S. littoralis* larvae. The most active fraction contents were identified.

MATERIALS AND METHODS

Separation of the Plant Constituents: The tested perennial plant, *Z. album* (Tarteer, Orfass, Bahsanan and Hamd in arabic) was collected from Wadi El-Netron,

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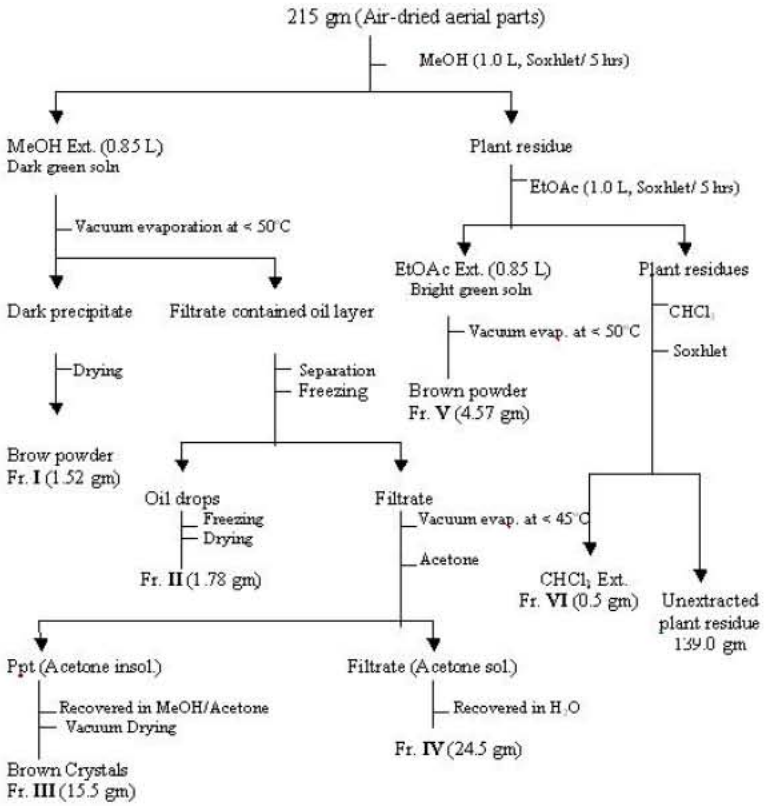


Fig. 1: Extraction and separation of *Z. album* plant constituents

Behira Governerate, Egypt. The collected aerial parts were cleaned from soil and the old stems were removed. Soxhlet extraction of 215 gm for 5 hours successively with 1.0 liter of methanol, ethyl acetate and chloroform was carried out. The dark green methanolic solution (0.85 L) was concentrated to 0.25 L under vacuum at < 50°C. A dark precipitated was filtered through a glass filter and dried to brown powder as fraction I (1.52 gm). An oily layer was separated from the filtrate, frozen for 48 hours and dried to fraction II as a creamy powder (1.78 gm). The remained filtrate was further concentrated and re-extracted twice with acetone (100 ml in each). The acetone insoluble precipitate was recovered in 25% acetone in methanol, concentrated in vacuum and completely dried to fraction III (15.5 gm) as a crystalline powder. However after removal of organic solvents from the acetone soluble fraction, it was recovered in water. It weighs 24.5 gm (fraction IV). The bright green ethylacetate solution (0.9 L) was concentrated to 0.1 liter producing a precipitate that was dried to fraction V (4.57 gm) as a brown powder. The chloroform extract was concentrated under reduced pressure to 0.5 gm of oily constitute (fraction VI). The percentages of methanol, ethylacetate and chloroform extracts as well as the un-extracted plant residues were 19.7, 2.12, 0.23 and 64.7 with 86.7% recovery of the un-extracted sample Figure 1.

Insecticidal Effects: The mother colony of cotton leaf worm, *S. littoralis* was reared on a semi artificial growing medium [16,17] at 27 ±3.0°C and 60±5 % RH. Insecticidal activity of the separated fractions was studied on the 4th larval instar. They were dissolved in dimethylsulfoxide (DMSO) and introduced to the freshly prepared semi artificial used medium at 10, 100, 200, 500 and 1000 µg/gm. Control was concurrently carried out under the same conditions. The dimethylsulfoxide was at a concentration as high as 1 %. After medium solidification, ten newly moulted 4th instar larvae were placed on 4 gm of the medium in a plastic pot as a replicate. Three replicates were used for each treatment. After 4 days feeding on the poisoned medium, the treated larvae were transferred to non-poisoned medium. The alive larvae were counted daily and mortality percents were calculated [18]. Effect of exposure time on mortality was shown. Sub-lethal morphogenetic effects on reduction in larval weight, developmental growth of the treated larvae and malformations of the produced pupae and emerged adults were examined and monitored.

Statistical Analysis: Mortality and larval weight reduction percents were analyzed using the analysis of variance (ANOVA) and Student-Newman-Kules Test. LC_{50}, LT_{50} and EC_{50} values were determined using probit analysis [19].

GC–MS Analysis: GC-EIMS analysis of the most active fraction was performed on a GCMS-QP 2010, GC Hewlett-Packard HP 6890A, Column HP5 (coated with 5% diphenyl/95% dimethyl-poly-siloxane (30 m × 0.25µm film thickness × 0.32mm id) was used as the stationary phase. Helium served as mobile phase with a constant pressure of 160.0 kPai. Direct injection of the sample in acetone (1µl of the standard solution, concentration 0.5 mg/ml) was performed in a splitless mode with an inlet temperature of 220 °C. Column Oven Temp.: 80.0°C. Column Flow: 3.20 ml/min with linear velocity: 63.8 cm/sec. The applied oven temperature program included an initial step for 1 min at 80°C, temperature shifted up to 250°C with 10°C/min, followed by 70 min at 250°C. Mass conditions continued to 88 min with m/z range of 45.00- 800.00.

RESULTS AND DISCUSSION

Insect Mortality: As recorded in Table (1), the tested fractions killed the treated larvae in a concentration and time dependent effect. Methanol originated fractions (Fractions I, II and III) were more effective than both acetone and ethylacetate originated fractions (fraction IV and V). Fractions IV and V caused their effects with lethal concentration caused 50% (LC_{50}) of the treated population of 459.1 and 271.3 µg/gm, respectively after 12 days exposure. Mortality was increased by fraction III with LC_{50} value of 115.7 µg/gm after the same time. Fractions I and II appeared more effective with LC_{50} values of 667.8

Table 1-a: LC_{50} values of the separated fractions against *S. littoralis* larvae

	After 8 days					After 12 days				
Frac.	LC_{50}	95% C.L.	Slope ± SE	Chi² (χ^2)	P	LC_{50}	95% C.L.	Slope ± SE	Chi² (χ^2)	P
I	667.8	379.4–1194	$0.61 \pm 8.7 \times 10^{-3}$	0.37	0.93	7.54	(1.3– 38.2)	$0.333 \pm 7.0 \times 10^{-3}$	1.56	0.68
II	526.7	325.9 – 860	$0.66 \pm 8.9 \times 10^{-3}$	0.19	0.97	2.65	0.2-32.8	$0.279 \pm 7.1 \times 10^{-3}$	5.02	0.21
III	> 1000					115.7	(62.1–213)	$0.347 \pm 6.9 \times 10^{-3}$	0.69	0.86
IV	> 1000					459.1	(224 –964)	$0.519 \pm 7.4 \times 10^{-3}$	1.97	0.52
V	> 1000					271.3	152.3–488.3	$0.465 \pm 7.34 \times 10^{-3}$	0.77	0.83

LC_{50} values were calculated in µg/gm.

Table 1-b: LT_{50} values of the separated fractions against *S. littoralis* larvae

		Tested Concentrations (µg/gm)				
Frac.		0	100	200	500	1000
I	LT_{50}	11.19	10.11	9.31	8.53	7.0
	(95% C. L.)	(10.62 – 11.8)	(9.65 – 10.59)	(8.13 – 8.94)	(8.13 – 8.94)	(6.49 – 7.56)
	Slope ± SE	6.09 ± 0.395	5.58 ± 0.265	5.11 ± 0.1998	4.995 ±0.172	3.19 ± 0.112
	Chi² (χ^2)	5.14	4.67	5.95	10.58	0.89
	P	0.240	0.395	0.29	0.094	0.974
II	LT_{50}	11.0	10.13	9.07	8.49	7.85
	(95% C. L.)	(10.5 – 11.48)	(9.62 – 10.66)	(8.65 – 9.52)	(8.1- 8.93)	(7.52 – 8.22)
	Slope ± SE	7.35 ± 0.547	5.04 ± 0.225	4.9 ± 0.182	4.75 ± 0.162	5.6 ± 0.179
	Chi² (χ^2)	0.912	4.44	9.321	14.06	12.66
	P	0.961	0.432	0.088	0.0519	0.117
III	LT_{50}	> 12 days	> 12 days	11.21	10.08	8.82
	(95% C. L.)			(10.34 – 12.2)	(9.48 – 10.73)	(6.2 – 12.5)
	Slope ± SE			3.69 ± 0.171	4.12 ± 0.169	4.5 ± 0.16
	Chi² (χ^2)			1.53	2.91	2.795
	P			0.895	0.689	0.834
IV	LT_{50}	> 12 days	> 12 days	> 12 days	> 12 days	10.2
	(95% C. L.)					(8.32 – 12.48)
	Slope ± SE					4.35 ± 0.184
	Chi² (χ^2)					4.11
	P					-
V	LT_{50}	> 12 days	> 12 days	> 12 days	11.30	8.77
	(95% C. L.)				(10.2 –12.58)	(7.82 – 9.84)
	Slope ± SE				2.81 ± 0.133	1.92 ± 0.103
	Chi² (χ^2)				4.6	0.6
	P				0.402	0.988

LT_{50} values were calculated in days. Degree of freedom (DF) was 5

Table 2: Reduction in larval weight by the separated fractions, shown as EC_{50} values

Frac.	* EC_{50} (µg/gm)	95% Conf. Limit	Slope ± SE	Chi^2 (χ^2)	P	DF
I	121.3	86.7 – 169.3	$0.82 \pm 8.41 \times 10^{-3}$	1.29	0.711	3
II	176	139.7 – 221.8	$1.23 \pm 12.4 \times 10^{-3}$	0.74	0.847	3
III	98.95	72.7 – 134.3	$0.942 \pm 8.93 \times 10^{-3}$	2.94	0.373	3
IV	111.6	77.94 – 159.2	$0.772 \pm 8.13 \times 10^{-3}$	4.51	0.155	3
V	103.93	76.4 – 141.1	$0.933 \pm 8.91 \times 10^{-3}$	4.77	0.146	3

* EC_{50}, Effective concentration caused 50 % larval weight reduction
Data were recorded after 7 days exposure

Fig. 2: Comparison between the treated and un-treated *S. littoralis* larvae
Upper: Normal size of the un-treated larva (control)
Middle: Treated with fraction I (500 µg/gm), Fractions II, III, IV and V (200 µg/gm).
Lower: Treated with fractions I and II (1000 µg/gm), fractions III, IV and V (500 µg/gm).
Treated larvae were unable to shed off moulted cuticle with a reduction of weight

and 526.7 µg/gm, respectively after 8 days exposure in comparison to > 1000 µg/gm in case of fractions III, IV and V. Their toxicity was enhanced with LC_{50} values of 7.54 and 2.65 µg/gm by increasing the exposure time to 12 days. The exposure time affected the larval mortality as LT_{50} was decreased at higher concentration. Fractions I and II were the most obedient of this effect. While fraction IV was the less active fraction with LT_{50} of >12 days at all tested concentrations, its LT_{50} was 10.2 days (8.32–12.48) at 1000 µg/gm. Fraction V achieved 11.30 and 8.77 LT_{50} values at 500 and 1000 µg/gm, respectively. Fraction III exceeded their effects (Table 1). It is important to notice that using these fractions at low concentrations for long time is more useful than at high concentration for short time. Fraction I for example exhibited 56.7% mortality at 100 µg/gm after 11 days exposure (1100 CT units). The same effect (56.7% mortality) was caused at 200 µg/gm after 10 days (2000 CT unit) and at 500 µg/gm after 9 days (4500 CT unit). Similar behavior was noticed in case of fraction II achieving 60% mortality at 200 µg/gm after 11 days (2200 CT unit) and at 1000 µg/gm after 9 days (9000 CT unit).

Morphogenetic Effects

Body Weight: As presented in Table (2), the average weight of the treated larvae was reduced by the tested fractions in a systematic arrangement with concentration. On contrary to mortality, fractions IV and V were more effective in reducing the larval weight with effective concentration of 50% (EC_{50}) values of 111.6 and 103.9 µg/gm, respectively. Fractions I, II and III reduced the larval weight with lesser degrees with EC_{50} of 121.1, 176.0 and 98.95 µg/gm, respectively, although they were lethally more effective. In general, it was noticed that larvae with reduced weight are more tolerant to the tested fractions that may reflects presence of some constituents in these fractions effective in interfering some biological processes inhibiting their growth. This reduction was shown as comparison of the treated and untreated larval weight in Figure (2).

Developmental Growth: The duration of *S. littoralis* larvae was significantly affected with the tested fractions as treated larvae required longer time to reach the next stadium in comparison to control. This effect was a fraction and concentration dependent. The untreated larvae appeared so healthy and normally developed completely to the pupal stage after 11 days. 30% and 100% of the resulted pupae were turned to the adult stage after 14 and 18 days, respectively. All treatments stopped the development of the treated larvae at the larval stage (100% larvae) till 11 days at 10µg/gm. After 14 days fraction I allowed pupation of 34, 24 and 13 % of the treated larvae at 10, 100 and 200 µg/gm comparing with 20, 0 and 0% in case of fraction II and 18, 11 and 8% in case of fraction III, respectively. Both fractions IV and V appeared nearly similar in their effects as the pupation percentages were 23, 10 and 7% and 15, 12 and 7%, respectively. No adults appeared in all treatments even after 18 days comparing with 100% adult in the untreated larvae control. The different effect, at 10µg/gm is shown in Figure (3).

Several pupal malformation forms were noticed in the produced pupae from population treated with fractions II, III and IV at 10µg/gm after 12 days. A type of

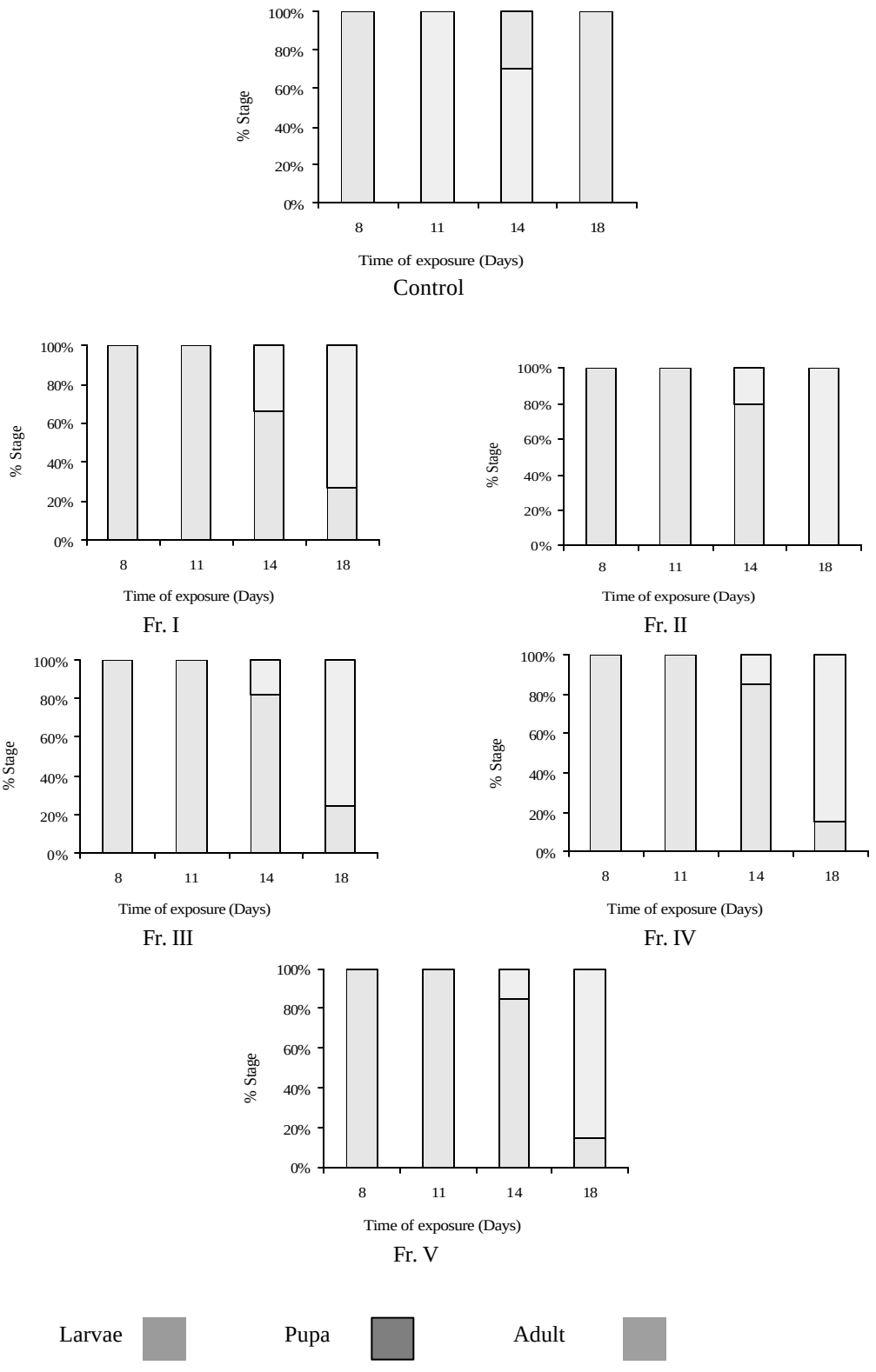


Fig. 3: Effect of the tested fractions on *S. littoralis* larvae developmental growth at 10 µg/gm

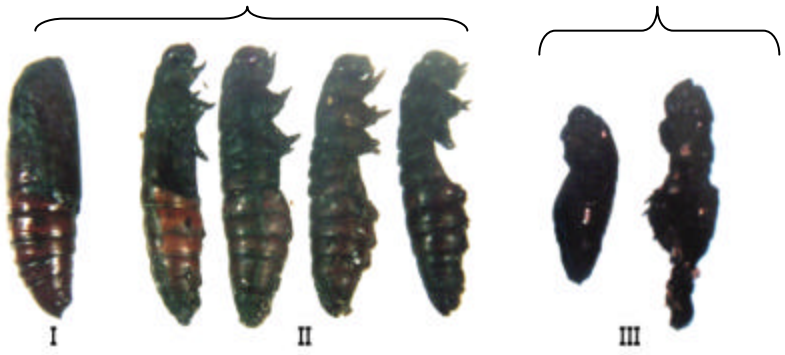


Fig. 4: Malformations in the produced pupae comparing with control
I Normal pupa (control); II, juvenilized larval-pupal intermediates; III, abnormal pupae failed to shed the larval cuticle

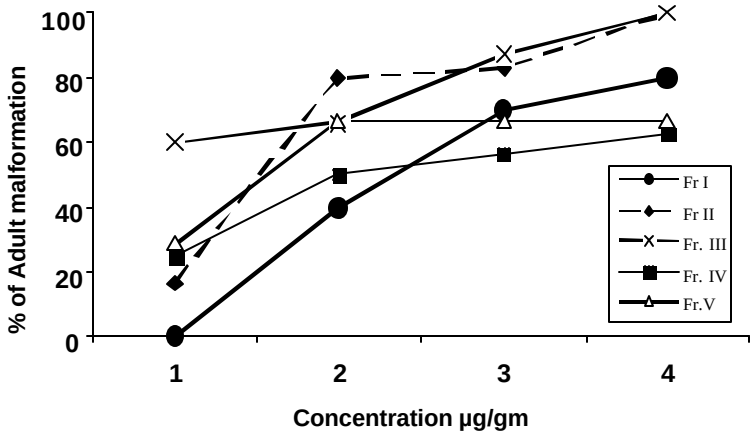


Fig. 5: Effect of the tested fractions on *S. littoralis* adult (produced from the treated larvae) 1,10; 2,100; 3,200; 4,500 µg/gm

Fig. 6: Different abnormal forms of the produced adults comparing with control
I, Normal adult (control); II, abnormal adults failed to shed the pupal cuticle; III, adult with dwarf wings

Table 3: GC-MS identification of the active fraction constituents

Compound	Area %	Rt (min.)	Ms spectral data (m/z and relative abundance)
1	5.64	14.8	316 (31.90), 301 (3.59), 287 (27.10), 273 (94.14), 183 (19.89), 143 (15.32), 227 (4.01), 213 (24.68), 129 (85.80), 117 (97.29) and 99 (60.3)
2	3.44	17.4	503 (2.11), 475 (1.25), 429 (9.91), 401 (2.62), 371 (12.03), 355 (33.32), 341 (11.86), 281 (30.88), 237 (11.17), 227 (22.97), 207 (16.94), 163 (4.08), 147 (24.25), 127 (12.93), 99 (33.63) and 73 (100)
3	5.01	18.0	504 (6.99), 503 (12.55), 475 (0.84), 429 (25.31), 401(2.67), 355 (31.76), 341 (8.99), 295 (9.68), 281 (34.51), 221 (40.75), 207 (19.99), 183 (3.63), 147 (39.73), 127 (11.79), 99 (21.38) and 73 (100)
4	2.75	18.6	503 (3.14), 475 (1.60), 429 (16.94), 401 (4.31), 371 (13.98), 355 (39.77), 341 (12.22), 281 (35.64), 237 (21.25), 223 (17.77), 207 (19.95), 163 (5.52), 147 (29.99), 127 (15.05), 99 (35.58) and 73 (100)
5	64.96	19.3	582 (0.17), 504 (0.47), 503 (0.94),429 (2.14), 355 (4.39), 296 (0.36), 281 (3.31), 277 (21.72), 167 (39.3), 149 (100), 132 (2.13) and 113 (12.43)
6	4.07	21.0	582 (1.39), 539 (1.29), 504 (3.00), 503 (8.80), 475 (1.05), 429 (27.74), 401 (3.69), 355 (43.17), 341 (9.73), 295 (10.69), 281 (29.99), 221 (43.52), 207 (24.15), 183 (4.97), 147 (42.64), 127 (13.05), 99 (36.07) and 73 (100)

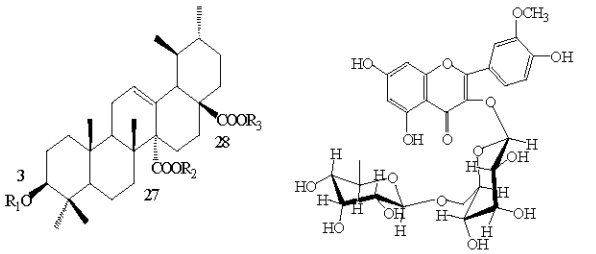
juvenilization as larval-pupal intermediates was observed where the posterior of the body only transformed to the pupal shape while the anterior portion had the larval head capsule and thoracic legs. These intermediates are shown in Figure (4). This further indicates that treatment induced an effect typical to juvenile hormone excess.

Effect on the Produced Adults: The effect of the tested fractions exceeded to the adult stage produced from treated larvae after 25 days. Fractions affected the resulted adult systematically with the tested concentration. Juvenilized adults failed to shed the pupal cuticle preventing their getting out pupal skin, *i.e* produced imperfect alive adults. This effect was differed according to the tested fraction. Fractions I, II and III produced imperfect adults ranged from 0.0-80%, 16.6–100% and 60-100%, respectively at the concentration range of 10-1000 µg/gm comparing with the untreated adults that were normal. Fractions IV and V were less effective with 25-62.5% and 28.4 – 66.7 % of imperfect adults, respectively. So, it could be concluded that methanol originated fractions (I, II and III) were more effective than IV and V. Fraction III was the most effective followed by fractions II and I. In brief, treatments had delayed effects and expressed as developmental abnormalities in the adult stage e.g. imperfect adults, adults with eclosion problems and dead adults before emergence Figures (5 & 6).

GC-MS Identification of the Most Active Fraction: From obtained spectra, the most active fraction is mostly rich in quinovic acid and flavanolone glycosides as major components. fragment ions of Quinovic acid aglycone appeared at m/z 442 (2.5), 429 (28.3), 398 (2.1), 207 (26.5), 190 (4.5) and 189 (23.0) due to decarboxylation or loss 3-OH group. In sugar, fragments at 147 and 163 (characteristic for rhamnopyranosyl and glucopyranosyl moieties) are produced through glucosidic bond cleavage either between the quinovic acid and the sugar moiety or between the two sugar moieties. Sugar fragment ions at m\z 371, 281, 237, 222, 221, 127 and 99 are referred to the common carbohydrate fragmentation pathways by loss of water molecules or cross ring fission. Compounds 2 and 4 eluted at 17.4 and 18.6 minutes retention time appeared identical in their spectra with nearly same pattern explaining the structure of quinovic acid glucopyranosyl-glucopyranoside ester with different retention time that may refer to different glycosidation position on the quinovic acid [20]. Fragment ion at m/z 475 indicated loss of the two glucose units. In compounds 3 and 6, absence of fragment at m\z 237 proved the absence of glucose moiety. They were identified as quinovic acid quinovopyranosides with different molecular size (number of sugar units). It was revealed that this may be the reason of their different retention times as well as compound 3 can be considered as a fragment of compound 6 [21]. Compound 5 was characterized with its produced fragments at m/z 149, 132 and 113, which reflect the presence of xylose moiety. Fragment at m\z 296 proved two linked hexoses moiety (rhamnose-xylose) besides produced fragments through hexose ring fission. Regarding the flavonolone glycoside (1), fragments at m/z 316, 301 and 287 characterized the aglycone and its M-CH_3 and M-CH_3O moieties. Fragments at m/z 129, 145, 273 and 117, produced through cleavage of sugar moieties.

Based on comparison of the obtained spectra and reviewed literature [20-24], the contained compounds were isorhamneutin-3-*O*-rutinoside (1), quinovic acid 28-*O*-β-D-glucopyranosyl(2-1)β-D-glucopyranoside ester) or 27-*O*-β-D-glucopyranosyl(2-1)β-D-glucopyranoside ester (2

and 4), quinovic acid-3β-*O*-β-D-quinovo pyranoside (3), quinovic acid-3β-*O*-[β-D-quinovopyranosyl(3-7)-β-D-xylopyranoside (5). and 3-β-*O*-β-D-quinovopyranosyl-quinovic acid (28-1) quinovopyranosyl ester (6) (Table 3). These findings support the fact describing quinovic acid glycosides as a chemotaxonomic significance in the genus *Zygophyllum*. The results indicated the positive relation between insecticidal effects and quinovic acid glycosides content. Although there was no clear data regarding the insecticidal activity against, *S. littoralis*, the biological activities of *Z. fabago* was referred to triterpenoidal saponins in its aerial parts [25]. It is proved that *Z. eichwaldii* is used as an antiseptic, antieczemic and antidiabetic medicine for stomach and liver diseases due to its biologically active triterpene glycosides [26]. Using of *Z. album* for treatment of diabetes, dermatitis, spasms, dysmenorrhoea and *Z. geslini* against diabetes as some other *Zygophyllum* species was owed to main constituents as zygophyllin, quinovic acid and glycosides, which have been demonstrated to have anti-inflammatory and antipyretic activity [27]. This study proved the insecticidal activity of *Z. album* against the Egyptian cotton leaf worm, *S. littoralis* Boisd.



1

	R_1	R_2	R_3
2	H	Glc (2→1)Glc	H
3	D-quinovose	H	H
4	H	H	Glc (2→1)Glc
5	D-quinovose(3→7)β-D-xylose	H	H
6	D-quinovose	H	D-quinovose

Structures of compounds 1-6

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