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Natural and Derivatized Acetogenins Promising for the Control of *Spodoptera frugiperda* Smith

José Ruiz Hidalgo¹, Eduardo Alberto Parellada¹, Lilian Di Toto Blessing¹, Alicia Bardón¹,², Keshav Lalit Ameta³, Nancy Vera⁴, Adriana Neske¹

¹UNT-FBQF-Argentina, Institutode Química Orgánica, Tucumán, Argentina
²INQUINOA-CONICET, Tucumán, Argentina
³Department of Science, Mody University of Science and Technology, FASC, Lakshmangarh, India
⁴UNT-FBQF-Argentina, Instituto de Farmacia, Tucumán, Argentina
Email: klameta77@hotmail.com, aneske@fbqf.unt.edu.ar

Abstract

Annonaceous acetogenins (ACG), belonging to the family Annonaceae, represent a class of bioactive compounds whose toxic effects have been reported for several species of insects. Given their insecticidal properties, we first carried out the isolation of the ACG from a Brazilian collection of the seeds of *Annona squamosa* (Annonaceae) and prepared their methoxy methylated (MOM) and acetylated (OAc) derivatives by chemical methods. ACG analogues were semi-synthesized and characterized by spectroscopic techniques (¹H and ¹³C-NMR). We isolated ten natural acetogenins: squamocin, molvizarin, motrilin, rolliniastatin-2, almuñequin, cherimolin-1, cherimolin-2, annonacin, squamocin D and asiminecin. The main objective of this study is to report the antifeedant, toxic and nutritional effects of three of those natural acetogenins and their acetylated and methoxy methylated derivatives on *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae). The natural ACGs squamocin and molvizarin killed 100% of *S. frugiperda* larvae, followed by motrilin (80%). Acetylated derivatives had less toxic and nutritional effects that led to pupal mortality and adult fatal malformations. The addition of MOM derivatives to the larval diet has not toxic effects on larvae, but significantly reduces growth rate and efficiency in conversion of ingested food into biomass, affecting adult survival.

Keywords

Acetogenin, *Annona squamosa*, *Spodoptera frugiperda*, Acetylated Acetogenin, Methoxy Methylated Acetogenin

1. Introduction

Annona squamosa L. (Annonaceae), is a 6 - 8 m tall tree distributed in tropical and subtropical regions of America, and the Caribbean (Puerto Rico, Cuba, etc.), and is also common in El Salvador, Colombia, Peru, Venezuela, Brazil and eastern Bolivia, whose edible fruit is currently incorporated to commercial fruit juices.

Characteristic constituents of these plants are the annonaceous acetogenins that possess unique structures [1] and anticancer [2] [3], cytotoxic [4], insecticidal [5] [6], antiparasitic activities and as a highly interesting new generation of antitumor drugs [7] [8] [9] [10] [11]. Furthermore, acetogenin cytotoxicity would be strongly related to the conformation adopted in the membrane [12] [13] [14].

Regarding the insecticidal action of acetogenins, it has been reported that many of these compounds are toxic to *Myzus persicae* (Homoptera) adults and *Leptinotarsa decemlineata* (Coleoptera) [15], as well as to nymphs of the *German cockroach* [2].

Previous results from our laboratory indicated that some acetogenins from *A. cherimolia* and *A. montana* have larvicidal effects on the *Spodoptera frugiperda* (Lepidoptera) corn pest [16] [17], nymphal and adult mortality on the *Oncopeltus fasciatus* (Hemiptera) cotton pest [18], and insecticidal activity against *Ceratitis capitata* Wiedemann (Diptera) [19].

The aim of this work is the search of the most active structure of ACG with insecticide action on the polyphagous lepidopteran *S. frugiperda*. For this purpose, we first carried out the isolation of adjacent and no-adjacent, bis-THF ACGs with OH groups flanking the THF: squamocin (**1**), molvizarin (**2**), motrilin (**3**), rolliniastatin-2 (**4**), almuñequin (**5**), cherimolin-1 (**6**), cherimolin-2 (**7**), annonacin (**8**), squamocin D (**9**), asiminecin (**10**) (**Figure 1**) from *Annona squamosa.* Then, their acetylated (OAc) derivatives: squamocin (3 OAc) (11), molvizarin (3 OAc) (12), motrilin (3 OAc) (13) and methoxy methylated (MOM): squamocin (MOM) (14) and motrilin (MOM) (15), were prepared (**Figure 2**).

2. Materials and Methods

2.1. Acetogenins

Grinded seeds of *A. squamosa* were percolated with methanol. Solvent was evaporated to produce a crude MeOH extract which was then partitioned between $CHCl_3$ and H_2O. The residue produced by further evaporation of the chloroformic phase was chromatographed on a silica gel column by using chloroform, ethyl and finally methanol as eluents. The isolation of ACG from the subextract was guided by thin layer chromatography (TLC) with the Kedde's reagent. Chromatographic fractions were processed on high-performance liquid chromatography (HPLC) by using RI detection, a Beckman C 18 column (5 μm particle size, 25 cm × 1 cm i.d.) and mixtures of methanol and water (90:10) as the mobile phase to yield pure ACG. Characterization of tested natural ACG was assessed by spectroscopic techniques (IR, ^{1}H-NMR, ^{13}C-NMR, and MS) as well as a_D determination, in comparison to previously reported data.
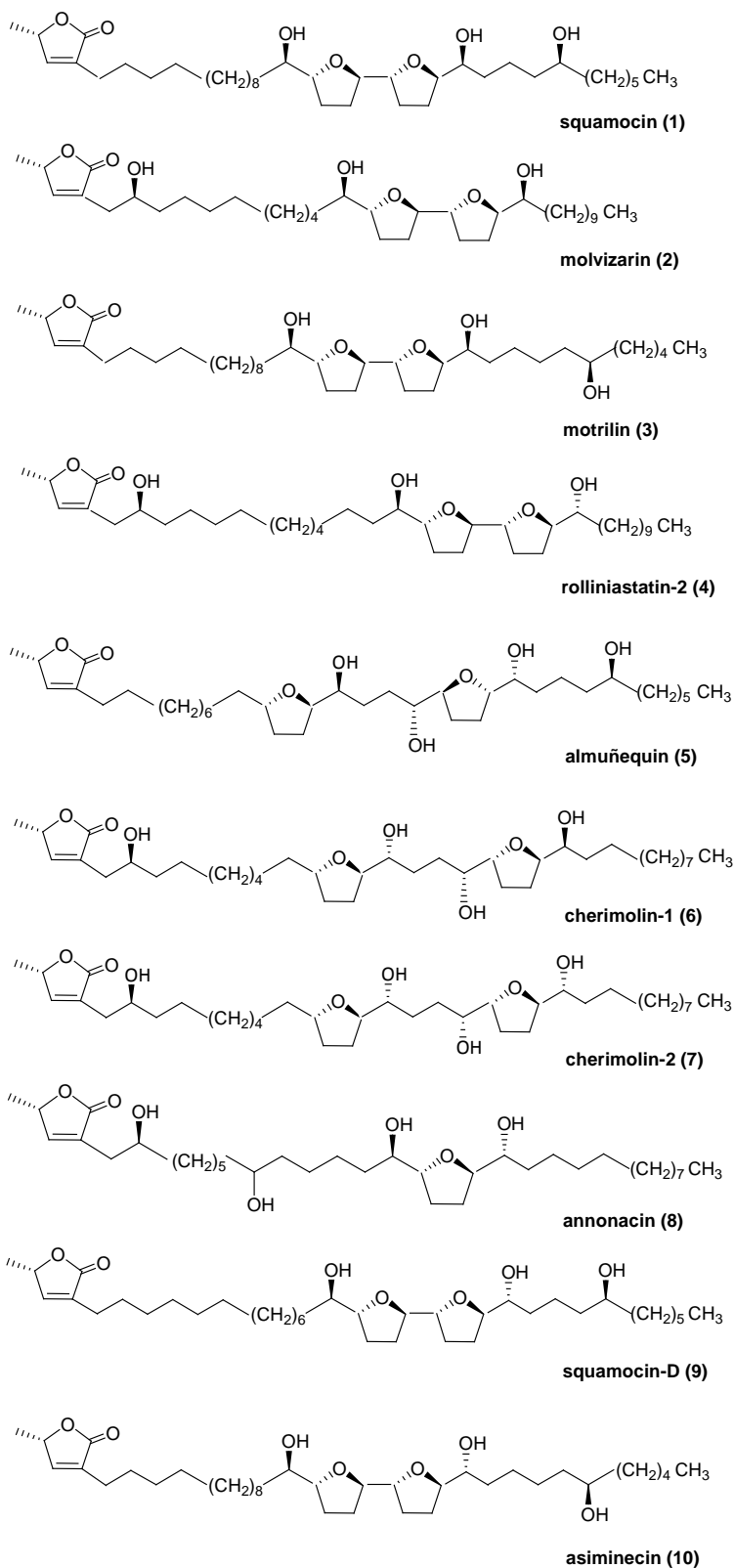
Figure 1. Natural ACG from *A. squamosa* methanolic seed extract evaluated for their nutritional alterations and toxic effects against *S. frugiperda.*
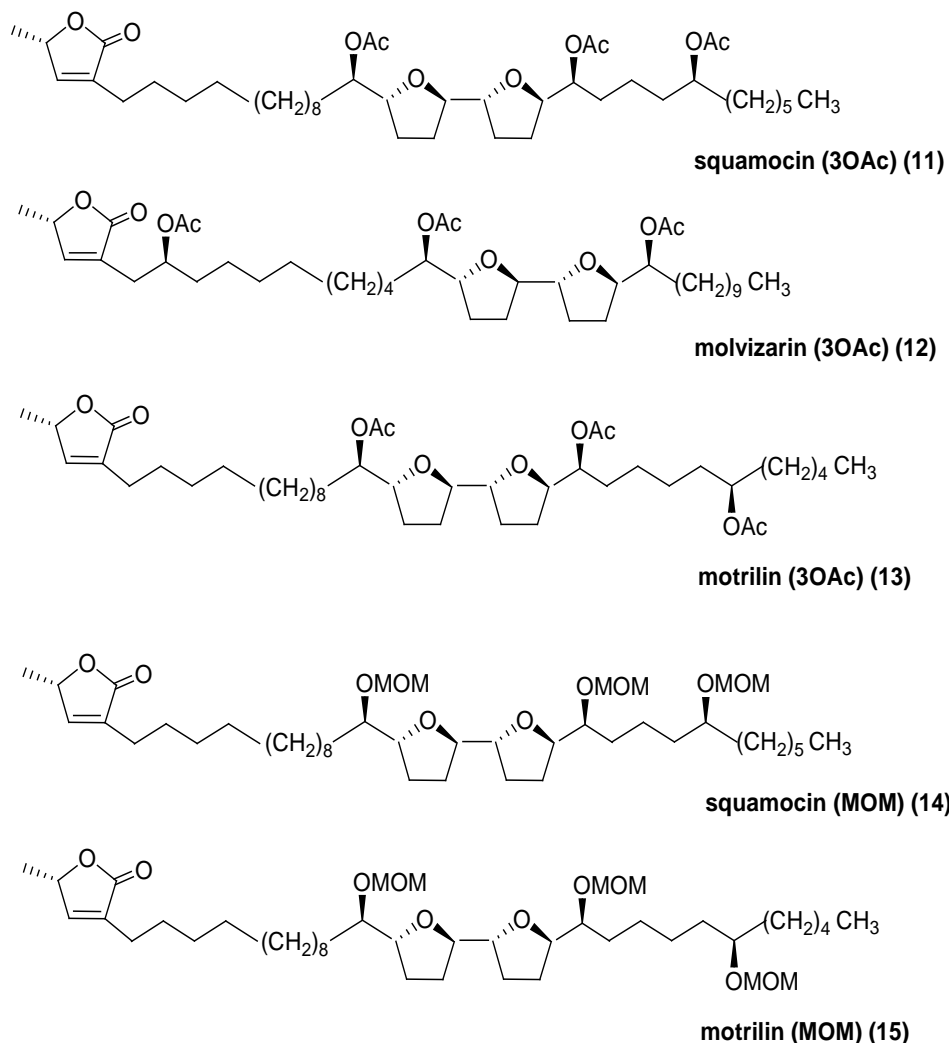
Figure 2. Structurally modified ACG: acetylated and methoxy methylated derivative evaluated for their nutritional alterations and toxic effects against *S. frugiperda.*

2.2. Structural Modification of ACG

Acetylated analogues, squamocin (3 OAc) (**11**), molvizarin (3 OAc) (**12**) and motrilin (3 OAc) (**13**) were obtained by chemical acetylation with acetic anhydride and addition of triethylamine (25˚C, N_2 atmosphere) followed by solvent evaporation and purification on silica gel column. The compounds of interest were identified by high resolution spectroscopic methods (^{1}H and ^{13}C-NMR) [20].

Methoxy methylated derivatives, squamocin (MOM) (**14**) and motrilin (MOM) (**15**) were obtained by adding *N,N*-diisopropylethylamine and methoxymethyl chloride to a dichloromethanic solution of natural ACG under a nitrogen atmosphere. The reaction was followed by TLC until the formation of products. MOM derivatives were purified by flash column chromatography. The identification of the bis-THF ACG methoxy methylated, were achieved by comparison of spectroscopic data ^{1}H-NMR and ^{13}C-NMR with the sample of the original ACG.

2.3. Test Insect and Diet

Spodoptera frugiperda's natural life cycle is between 26 and 31 days. Females lay between 200 and 300 eggs for 5 to 6 days. Larvae emerge between 48 and 72 h and remain in this state between 12 and 15 days. Then, they pass to pupal stage where they remain for 7 days, after which the adults emerge. The larval diet of *S. frugiperda* consists in a mixture of yeast (3 g), boiled and milled bean (250 g), wheat germ (12.5 g), ascorbic acid (1.5 g), agar agar (12.5 g), methyl p-hydroxybenzoate (1.5 g), formaldehyde (4 mL of a 38 % water solution), and water (500 mL).

2.3.1. Treatment

Natural ACG and their semisynthetic analogues solutions were prepared at 100 μg/mL, all under the same conditions.

2.3.2. No Choice Test

Control larval diet is prepared by adding acetone to a portion of artificial diet (10 g). Another portion was impregnated with an acetonic solution of each ACG in order to leave 100 μg of compound per g of diet (treated). After evaporation of the solvent, treated diets and control diet were placed in test tubes (20 replicates) in which second instar larvae were kept at 27°C and 60% ± 15% relative humidity. When 50% of control diet had been eaten, control and treated diets were removed from the tubes and weighted accurately. Results of the no choice test were then reported by the feeding ratio FR50 = T/C [6], where T and C are the weights of diets consumed in the treated and control experiments, respectively.

2.3.3. Toxicity Test

Treated and control diets were placed in different test tubes (20 replicates for treated and 20 replicates for control experiments) in which second instar larvae were placed to be maintained at 27°C and 60% ± 15% relative humidity until emergency of the first generation of adults. Larval developmental periods as well as mortality rates were recorded for treatments with all the ACGs (100 ppm) and control experiments [21].

2.3.4. Nutritional Indices

Similar second instar larvae were placed in several test tube at the beginning of the experiment and larval weight was measured. Test and control diets were also weighed and provided to larvae in each tube. Twenty replicates for control and twenty for each treatment were employed, tubes were kept at 27°C. Weight variations produced by larval growth and diet addition were recorded. Average diet consumption (CI), growth rate (GR) and efficiency in the consumption index (ECI) were measured for a ten day period, starting with second instar larvae.

$$CI = D/t$$

$$GR = (A - B)/t$$

$$ECI = (A - B)/D$$

D = Food eaten during the experiment period.

A = Final larval weight.

B = Initial larval weight.

t = Experiment period.

CI: Average daily diet consumption during the experiment.

GR: Average daily weight gain during the experiment.

CI, GR and ECI values were calculated for the experiment and control treatments. For comparison purposes, treatment-control ratio was calculated. Control ratio is considered 100%. Values are expressed as (CIT/CIC) 100%; (GRT/GRC) 100% and (ECIT/ECIC) 100% in the tables [21].

2.4. Statistical Analysis

The results are reported as mean values ± SD. The differences between the mean values were evaluated by analysis of variance (one way ANOVA). The Tukey test was used for all pair wise multiple comparisons of groups. $P > 0.05$ were considered not significant difference between treatments [22].

3. Results and Discussion

3.1. Annonaceous Acetogenins

Chromatographic processes permitted the separation of 10 known ACG: squamocin (**1**) [23], molvizarin (**2**) [24], motrilin (**3**) [24], rolliniastatin-2 (**4**) [25], almuñequin (**5**) [26], cherimolin-1 (**6**) [4], cherimolin-2 (**7**) [4], annonacin (**8**) [27], squamocin D (**9**) [28] and asiminecin (**10**) [29] (**Figure 1**), that were exhaustively purified by reversed phase HPLC to be employed in the bioassays. The ACGs **4**, 6, 7 and **10** had not been previously isolated from *A. squamosa.*Acetogenins represent around 0.07% of the seed weight.

ACG acetylated (OAc) derivatives: squamocin (3 OAc) (**11**), molvizarin (3 OAc) (**12**) and motrilin (3 OAc) (**13**), and their methoxy methylated (MOM) derivatives: squamocin (MOM) (**14**) and motrilin (MOM) (**15**) (**Figure 2**) were synthesized and purified, and then characterized by spectroscopic techniques (**Table 1** and **Table 2**).

3.2. No Choice Test

As indicated in **Table 1**, the incorporation of 100 μg/g of diet of ACG squamocin into the artificial diet of *S. frugiperda* displayed the highest antifeedant effects (FR_{50}) followed by squamocin (3 OAc) (0.66 ± 0.17 and 0.77 ± 0.14; respectively), and caused a marked decrease in larval growth under the conditions of the experiment. The ACGs motrilin, motrilin (3 OAc), molvizarin and molvizarin (OAc) did not present a significant antifeedant action under the conditions of the experiment (**Table 3**).

3.3. Toxicity of Annonaceous Acetogenins

The results obtained in the tests of toxicity for each ACG show that all natural ACG, possesses a lethal effect on the *S. frugiperda* larvae in the early stages (**Table 3**) with

Table 1. ^{1}H-NMR data of 11, 12, 13, 14 and 15.

Proton	[1]H (shift in ppm)				
	11	12	13	14	15
4	-	5.11 (*m* 1H)	-	-	-
13	-	4.94 (*m* 1H)	-	-	-
15	4.80 (*dt* 1H)	-	3.90 (*dt* 1H)	3.90 (*dt* 1H)	3.39 (*dt* 1H)
22	-	4.85 (*m* 1H)	-	-	-
24	4.90 (*m* 1H)	-	4.01 (*m* 1H)	3.90 (*m* 1H)	3.88 (*m* 1H)
28	4.80 (*m* 1H)	-	-	3.40 (*m* 1H)	-
29	-	-	3.99 (*m* 1H)	-	3.60 (*m* 1H)
4-(OAc)	-	2.09 (*s* 3H)	-	-	-
13-(OAc)	-	2.19 (*s* 3H)	-	-	-
15-(OAc)	2.10 (*s* 3H)	-	2.04 (*s* 3H)	-	-
22-(OAc)	-	2.19 (*s* 3H)	-	-	-
24-(OAc)	2.10 (*s* 3H)	-	2.09 (*s* 3H)	-	-
28-(OAc)	2.10 (*s* 3H)	-	-	-	-
29-(OAc)	-	-	2.06 (*s* 3H)	-	-
15-(MOM)	-	-	-	4.70 (*s* 2H) 3.50 (*s* 3H)	4.65 (*s* 2H) 3.37 (*s* 3H)
24-(MOM)	-	-	-	4.70 (*s* 2H) 3.50 (*s* 3H)	4.69 (*s* 2H) 3.37 (*s* 3H)
28-(MOM)	-	-	-	4.70 (*s* 2H) 3.50 (*s* 3H)	-
29-(MOM)	-	-	-	-	4.74 (*s* 2H) 3.39 (*s* 3H)

Table 2. ^{13}C-NMR data of 11, 12, 13, 14 and 15.

Carbon	[13]C (shift in ppm)				
	11	12	13	14	15
4	-	72.00	-	-	-
13	-	75.45	-	-	-
15	73.50	-	75.19	74.08	74.08
22	-	75.28	-	-	-
24	75.00	-	75.40	71.23	71.23
28	73.50	-	-	71.43	-
29	-	-	71.92	-	71.43
4-(OAc)	-	21.23a - 170.69c	-	-	-
13-(OAc)	-	31.00a - 170.73c	-	-	-
15-(OAc)	21.50a - 170.76c	-	21.20a - 170.68c	-	-
22-(OAc)	-	31.00a - 170.96c	-	-	-
24-(OAc)	21.50a - 170.76c	-	21.22a - 170.73c	-	-
28-(OAc)	21.50a - 170.80c	-	-	-	-
29-(OAc)	-	-	21.11a - 170.81c	-	-
15-(MOM)	-	-	-	55.71a - 96.82b	55.33a - 96.79b
24-(MOM)	-	-	-	56.67a - 96.73b	55.33a - 96.70b
28-(MOM)	-	-	-	55.73a - 96.13b	-
29-(MOM)	-	-	-	-	55.73a - 96.17b

[a]Methyl: CH_3; [b]Methylene: CH_2; [c]Carbonyl: C=O.

Table 3. Antifeedant and toxic effects of natural and modified ACG on *S. frugiperda*.

Compounds	FR50a	(%) Larval mortality	(%) Pupal mortality	(%) Emergency adults
Control	-	10	0	90
Squamocin	0.66 ± 0.17	100	0	0
Molvizarin	1.00 ± 0.15	100	0	0
Motrilin	1.09 ± 0.15	80	15	5
Squamocin (3 OAc)	0.77 ± 0.14	45	30	25
Molvizarin (3 OAc)	1.21 ± 0.21	60	30	10
Motrilin (3 OAc)	0.96 ± 0.15	80	0	20
Squamocin (MOM)	0.95 ±0.15	15	0	85
Motrilin (MOM)	0.98 ± 0.19	5	5	90

[a]Mean ± SD.

high mortality effect at 100 μg/g of diet over than 80%. The ACGs structurally transformed by acetylation showed less toxicity than natural ACGs (Table 3). The pupal mortality of squamocin (OAc), molvizarin (OAc) and motrilin were 30%, 30% and 15%, respectively. It is apparently related to the observed malformations as shown in Figure 3(A) and Figure 3(B). The surviving adults showed malformations and decrease in size relative to the control (Figure 4(A) and Figure 4(B)), that led them to death before laying eggs. Significant increments in the duration of the larval stage where observed for all ACGs tested.

3.4. Nutritional Indices

According to results obtained on food consumption and its utilization, it has been observed that motrilin, squamocin (OAc) and molvizarin (OAc) at 100 μg/g of diet, influenced GR and ECI of instar larvae. A drop in ECI indicated that more food has been metabolized for energy and less has been converted to insect biomass (*i.e.*, growth). These, resulted in an important larval growth decrease and subsequent significant larval mortality at very early stages in their life cycle and would reveal the presence of toxic compounds when compared with control ($P < 0.05$, Tukey multiple range test). Motrilin (3 OAc) had the lowest consumption percentage and the lowest conversion of absorbed nutrient (Table 4). Although, the addition of 100 μg/g of MOM derivatives produced no significant toxic effects (Table 3) and changes in nutritional index values (Table 4) all emerged adults died within 24 h.

The most important toxic action was observed with natural ACG against *S. frugiperda* larvae at the dose tested, following acetylated and MOM derivatives. These results are in agreement with a previous report from our team for other ACGs isolated from different species of Annonaceae [14].

Figure 3. Pupae of *S. frugiperda*. (A) View of normal pupae; (B) Deformed pupae showing deficient melanization and malformation in wings, legs and antenna cover (molvizarin-3 OAc).
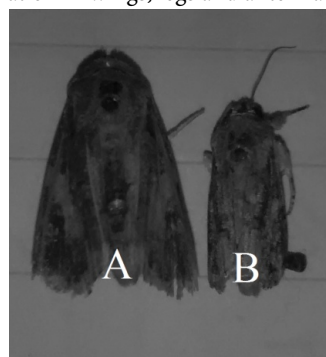

Figure 4. Adults of *S. frugiperda*. (A) View of untreated adult; (B) Treated adult (squamocin-3 OAc) showing malformation of wings and legs.

Table 4. Nutritional effects of natural and modified ACG on *S. frugiperda*.

Compounds	CIT/CIC [a,b] (%)	GRT/GRC[a,b] (%)	ECIT/ECIC[a,b] (%)
Squamocin	N/D	N/D	N/D
Molvizarin	N/D	N/D	N/D
Motrilin	77 ± 2^a	12 ± 2^a	16 ± 2^a
Squamocin (3 OAc)	97 ± 7^d	33 ± 3^a	34 ± 16^a
Molvizarin (3 OAc)	$83 \pm 9^{b,c}$	47 ± 10^b	56 ± 12^b
Motrilin (3 OAc)	$92 \pm 4^{c,d}$	85 ± 16^c	92 ± 17^c
Squamocin (MOM)	75 ± 6^a	51 ± 8^b	68 ± 9^b
Motrilin (MOM)	$78 \pm 5^{a,b}$	53 ± 7^b	69 ± 7^b

[a]Numbers in columns represent mean ± SD. [b]Means within a column followed by the same letter are not significantly different ($P > 0.05$, Tukey multiple range test). N/D: Not determined (Mortality at early instars).

Since hydroxyls groups flanking the THF have been blocked in MOM and acetylated derivatives a decrease in insecticidal activity is observed. This highlights the importance of the hydroxyl group to the insecticidal activity of natural ACG.

Acknowledgement*s*

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