Toxic Effects of Annonaceous Acetogenins from A*nnona cherimolia* (Magnoliales: Annonaceae) on *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract Plants belonging to the family Annonaceae have been commonly described in traditional medicine as remedies against head lice, and for their insecticidal properties. Characteristic constituents from a few genera of these plants are the annonaceous acetogenins. Fourteen annonaceous acetogenins have been isolated from our Argentine collection of the seeds of *A. cherimolia*. We report herein the antifeedant and insecticidal effects of nine of those acetogenins on *Spodoptera frugiperda* (J. E. Smith). The acetogenin squamocin, one of the major constituents of the extract, displayed toxic effects on early larval instars when incorporated to the larval diet at a dose of 50 μg per g of diet. The remaining annonaceous acetogenins tested, itrabin, asimicin, neoanonin, cherimolin-1, cherimolin-2, almuñequin, motrilin, and tucumanin produced pupal mortality and adult malformations leading to death, when incorporated to the larval diet at the same dose. The evaluation of indices of food consumption, growth, and food utilization indicated that squamocin was the only tested acetogenin to produce significant decrease in the growth rate and to reduce the efficiency with which larvae converted ingested food into biomass. All the acetogenins produced more than 80% pupal mortality with no dependence on the position of the THF rings or the number and location of the OH groups.

Key words Acetogenins · *Annona cherimolia* · nutritional indices · *Spodoptera frugiperda* · toxicity.

Introduction

Annonaceae is a large family of higher plants comprising more than 2000 species. Economically, the family is appreciated as a source of the edible fruits "pawpaw", "chirimoya", "sweetsop", "soursop", and "custard apple" among others (Heywood 1978). Oils from seeds of some plants are used for the production of edible oils (Ngiefu et al. 1976) while the wood of some Annonaceous trees has been employed for alcohol production (Savard and Espil 1951). Finally, many members of this family are currently being employed in folk medicine for various purposes.

The annonaceous acetogenins (ACG) are a large group of natural products isolated from a few number of genera of the Annonaceae family (*Annona*, *Asimina*, *Rollinia*, *Xilopia*, *Goniothalamus*, and *Uvaria*). The ACG are naturally occurring long-chain fatty acid derivatives possessing unique structures and powerful cytotoxic properties, with potential applications as insecticides, antiparasitics, and as a highly interesting new generation of antitumor drugs (Moeschler et al. 1986, 1987; Mikolajczak et al. 1988, 1989; Oberlies et al. 1995; Cavé et al. 1997; Alali et al. 1999; Guadaño et al. 2000; Raynaud et al. 2000). Their mode of action targets the mitochondrial electron transport with a specific action at NADH–ubiquinone oxidoreductase (NADH–dehydrogenase, also known as complex I).

Annona cherimolia is a 4-6 m tall tree distributed in tropical regions of South America, whose edible fruit is currently incorporated to commercial fruit juices. Previous studies on the chemical composition of the seeds from Spanish collections showed the presence of ACG (Cortes et al. 1991; 1993). Our own collection from the north-west of Argentina (Barrachina et al. 2004) yielded tucumanin, a β-hydroxy acetogenin only present in the Argentine collection, as well as 10 acetogenins previously described for the Spanish *A. cherimolia* (almuñequin, asimicin, cherimolin-1,

cherimolin-2, itrabin, laherradurin, molvizarin, motrilin, rolliniastatin-2, and squamocin). The acetogenins neoannonin, parviflorin, and squamocin-B also present in our collection had not been previously found in *A. cherimolia* from other countries. Previous reports (Guadaño et al. 2000) indicated that squamocin was toxic to *Leptinotarsa decemlineata* (Coleoptera) and *Myzus persicae* (Homoptera) adults. Additionally, parviflorin and asimicin resulted toxic to German cockroach (Alali et al. 1999) affecting nymphal development to some extent. Given the pesticidal potential of this class of compounds, the aim of the current study was to evaluate the antifeedant and toxic effects produced by the mentioned acetogenins on the polyphagous lepidopteran *Spodoptera frugiperda*.

Methods and Materials

Extraction and Purification of Acetogenins Ground seeds of *A. cherimolia* were percolated with methanol. Evaporation of the solvent yielded a crude MeOH extract which was further partitioned between $CHCl_3$ and H_2O. The solvent was then evaporated from the chloroform extract at reduced pressure and the residue was chromatographed on a silica gel column by using chloroform and increasing amounts of ethyl acetate (0-100%) and finally methanol as eluents. Chromatographic fractions were later processed on high-performance liquid chromatography (HPLC) by using a Beckman C 18 column (25 cm x 1 cm i.d., 5 μm particle size) and mixtures of acetonitrile and water as the mobile phase to yield pure acetogenins. Characterization of tested acetogenins was assessed by spectroscopic techniques (IR, ^{1}H-NMR, ^{13}C-NMR, and MS) as well as α_D determination, in comparison to previously reported data on the tested compounds.

Brine Shrimp Test Chromatographic fractions of the chloroform extract of *A.*

cherimolia seeds were tested in three concentrations, 100, 10 and 1 ppm (three replicates for each) in the brine shrimp test (BST) following the usual procedure (Mc Laughlin et al. 1993).

Test Insects and Diet *S. frugiperda* larvae were obtained from our laboratory population. The larval diet consisted of a mixture of yeast (3 g), bean boiled and milled (250 g), wheat germ (12.5 g), agar agar (12.5 g), ascorbic acid (1.5 g), methyl *p*-hydroxybenzoate (1.5 g), formaldehyde (4 mL of a 38 % water solution), and water (500 mL).

Toxicity Bioassay A portion of the artificial diet was impregnated with acetone and, after solvent removal, this portion was employed as control diet. Another portion was impregnated with an acetone solution of pure acetogenin in order to leave 50 μg of each compound per g of diet. After evaporation of the solvent, control and treated diets were placed in test tubes (10 replicates for treated and 10 for control diets). Two 2nd instar larvae were placed in each tube and were kept at 27°C until emergency of the 1st generation of adults. Mortality was registered by counting the emerged adults.

Determination of Consumption (RCR), Growth (GR), and Food Utilization (UR) Indices Two 2nd instar larvae of homogeneous size were placed in a test tube at the beginning of the experiment, and the larval weighted was accurately determined. Test and control diets were also weighted and offered to larvae in each tube. Ten replicates for control and ten for each treatment were employed. Tubes were kept at 27°C. Every five days the larval weight was determined and every addition of diet with the corresponding weight was recorded. For a ten days period, starting with 2nd instar larvae, measurements were made of: relative consumption rate (RCR), the average of consumed diet per day corrected for initial body weight; growth rate (GR), the average of larval weigh increment per day corrected for initial body weight; and food utilization

rate (UR), the consumption rate corrected for final body weight **(**Waldbauer 1968; Shea and Romeo 1991).

RCR = D/Bt

GR = (A – B)/Bt

UR = D/At

D = *Food eaten during the experiment period,* B =*Initial larval weight,* A = *Final larval weight,* t = *Experiment period*

Statistical Analysis The results are reported as mean ± SD . The differences in the mean values were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pair wise multiple comparisons of groups. In all statistical analysis, $P > 0.05$ were considered not significant (Statistix 7.1 2000).

Results

Annonaceous acetogenins The isolation of annonaceous acetogenins from the chloroform extract was guided by the brine shrimp lethality test. The most toxic fractions ($LC_{50} < 1$ ppm) were those containing acetogenins. Repeated chromatographic processes permitted the separation of the annonaceous acetogenins (Fig. 1) neoannonin (Kawazu et al. 1989) (**1**), itrabin (Cortes et al. 1991) (**2**), almuñequin (Fujimoto et al. 1994) (**3**), asimicin (Rupretch et al. 1990) (**4**), squamocin (Kawazu et al. 1989) (**5**), motrilin (Cortes et al. 1991) (**6**), cherimolin-1 (Cortes et al. 1993) (**7**), cherimolin-2 (Cortes et al. 1993) (**8**) and tucumanin (Barrachina et al. 2004) (**9**), that were exhaustively purified by reversed phase HPLC to be employed in the bioassays. Acetogenins represent around 0.07% of the seed weight.

Toxicity of annonaceous acetogenins At the dose tested (50 μg/g of diet) all the compounds produced high mortality rates (over 80%) during the pupal stage. The most

toxic were squamocin, itrabin, cherimolin-1, neoannonin and asimicin, as shown in Table 1. Only squamocin killed 100% of the population during the larval stage. Noteworthy, every surviving adult was malformed in abdomen and wings as shown in Fig. 2 A and B. Significant increments in the duration of the larval stage where observed for cherimolin-2 (38%), itrabin (46%), asimisin (35%), motrilin (45%) and almuñequin (51%) related to control (Table 1). Pupal mortality is apparently related to the observed malformations as shown in Fig. 3 A and B.

Nutritional Indices In order to assess the mechanism of action leading to mortality produced by the treatments we observed the nutritional effects produced by the incorporation of the mentioned acetogenins to 2nd instar larval diet of *S. frugiperda* (Table 2). As shown in Table 2, the only tested acetogenin to produce significant decrease in the larval growth rate (GR = 0.16) was squamocin ($F = 2.10$; $df = 9$; $P < 0.04$). Additionally, the values of the UR index indicated that squamocin (UR = 1.48) was the only compound that altered the capacity of converting food into biomass ($F = 31.05$; $df = 9$; $P < 0.001$).

Discussion

Insecticidal and toxic effects of annonaceous acetogenins have been reported on several insect species (Alali et al. 1999). A previous report (He et al. 1997) indicated that acetogenins possessing adjacent bis-THF rings, with three hydroxyl groups, displayed more potent insecticidal effects against the yellow fever mosquito larvae, in a contact assay. Furthermore, squamocin (**5**), a bis-THF-trihydroxy acetogenin, has been described as having ovicidal and larvicidal activity on species of *Drosophila* (Kawasu et al. 1989), and insecticidal effects on *Leptinotarsa decemlineata* and *Myzus persicae* adults. Additionally, squamocin affects negatively the reproduction of *Myzus persicae*

(Guadaño et al. 2000).

The present is the first report on the antifeedant and toxic effects of acetogenins of *A. cherimolia* on *S. frugiperda* larvae. As shown in Fig. 1, all of the evaluated acetogenins carry two THF rings and, in six of them, the THF are adjacent. They have also two, three or four OH groups. Our results indicated that only squamocin killed 100% of *S. frugiperda* larvae at the dose tested, while the remaining adjacent bis-THF-trihydroxy acetogenins, had little or no effect. The most important toxic action was observed on pupae. In fact, all the acetogenins produced more than 80% pupal mortality with no dependence on the position of the THF rings or the number and location of the OH groups.

The evaluation of food consumption (RCR) indicated that the tested acetogenins are not antifeedant agents because no significant differences have been observed in the RCR values related to control. The high value of UR for squamocin (1.48) is due to the very low larval weight even when larvae consume the diet regularly, suggesting that squamocin reduces of the efficiency to convert food into biomass.

Acetogenins show a tremendous potential for development of new natural pesticides. Based on our results, the methanol extract of *A. cherimolia* seeds, which can be easily prepared, could be employed as an effective, economical and environmentally friendly pesticide to control *S. frugiperda.*

Acknowledgments

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References

Alali F, Liu, X and Mc Laughlin J (1999) Annonaceous acetogenins: Recent Progress. J. Nat. Prod 62:504-540

Barrachina I, Neske A, Granell S, Bermejo A, Chahaboune N, El Aoued N, Álvarez Colom O, Bardón A, Zafra Polo M (2004) Tucumanin, a β- hydroxy γ-lactone bistetrahydrofuranic acetogenin from *Annona cherimolia*, potent inhibitor of mitochondrial complex I. Planta Médica 70:866-868

Cavé A, Figadére B, Laurens A, Cortes D (1997) Acetogenins from Annonaceae. In: Herz, W., Kirby, G. W., Moore, R.E., Steglich, W. and Tamm, Ch. (Eds.), Progress in the Chemistry of Organic Natural Products, Springer-Verlag: New York 70:81-288

Cortes D, Myint S, Hocquemiller R (1991) Molvizarin and Motrilin: two novel cytotoxic bis-tetrahydro-furanic γ-lactone acetogenins from *Annona cherimolia*. Tetrahedron 47:8195-8202

Cortes D, Myint S, Dupont B, Davoust D (1993) Bioactives acetogenins from seeds of *Annona cherimolia*. Phytochemistry 32:1475-1482

Fujimoto Y, Murasaki C, Kakinuma K, Eguchi T, Ikekawa N, Furuya M, Hirayama K, Ikekawa T, Sahai M, Gupta YK, Ray AB Tetrahedron letters 31, 535

Guadaño A, Gutierrez C, De La Peña E, Cortes D, Gonzalez-Coloma A (2000) Insecticidal and Mutagenic Evaluation of Two Annonaceous Acetogenins. J. Nat. Prod 63:773-776

He K, Zeng L, Ye Q, Shi G, Oberlies N, Zhao G, Njoku C, Mc Laughlin J (1997) Comparative SAR Evaluations of Annonaceous Acetogenins for Pesticidal Activity. Pestic. Sci 49:372-378

Heywood, VH(Cortes, D. 1991) (1978) Flowering Plants of the world. University Press,

Oxford

Kawasu K, Alcántara J, Kobayashi A (1989) Isolation and structure of neoannonin, a novel insecticidal compound from the seeds of Annona squamosa. Agric. Biol. Chem 53:2719-2722

Mc Laughlin JL, Chang ChJ, Smith DL (1993) Simple Bench-Top Bioassays (Brine Shrimp and Potato Discs) for the Discovery of Plant Antitumor Compounds. In: Kinghorn, D. and Balandrin, M.F. (Eds.), Human Medicinal Agents from Plants, Amer. Chem. Soc. Symp. Series, 534, Amer. Chem. Soc. Washington DC 112-137

Mikolajczak K, Mc Laughlin J, Rupprecht J (1988) Control of pests with Annonaceous acetogenins. US patent 4721727

Mikolajczak K, Mc Laughlin J, Rupprecht J (1989) Control of pests with Annonaceous acetogenins. US patent 4855319

Moeschler H, Pfluger W and Wendish D (1986) Annonin insecticide. German Patent DE 3438763 A1

Moeschler H, Pfluger W and Wendish D (1987) Pure annonin and process for the preparation. US Patent 4689232

Ngiefu, CK, Paquot,C and Vieux A (1976) Oleagineus 31, 545

Oberlies N, Jones J, Corbett T, Fotopoulos S and Mc Laughlin J (1995) Tumor cell growth inhibition by several Annonaceous acetogenins in an *in-vitro* disk diffusion assay. Cancer Lett 96:55-62

Raynaud S, Fourneau Ch, Laurens A, Hocquemiller R, Loiseau Ph and Bories Ch (2000) Squamocin and benzyl benzoate, acaricidal components of *Uvaria*. Planta Medica 66:173-175

Rupretch J, Hui Y and McLaughlin J. (1990) Annonaceous acetogenins: A Review. J.

Nat. Prod. 53: 237- 278

Savard J and Espil L (1951) Centre Tech. Forestier Trop. Nogent sur Marne Publ. 3:7

Shea ChS and Romeo JT (1991) Nutritional Indices: do they explain toxicity of *Calliandra* amino acids. Florida Entomologist 74:10-17

Statistix 7.1 (1985-2000) Analytical Software.

Waldbauer GP (1968) The consumption and utilization of food by insects. Adv. Insect Physiol 5:229-288

Fig. 1. Annonaceous acetogenins from *A. cherimolia* seeds evaluated for antifeedant and toxic effects on *S. frugiperda.*

Fig. 2. Adults of *S. frugiperda.* (A) Ventral view of untreated adult. (B) Treated adult showing malformation of abdomen and wings.

Fig. 3. Pupae of *S. frugiperda*. (A) Ventral view of normal pupae. (B) Deformed pupae showing deficient melanization and malformation in wings, legs and antenna cover.

Table 1. Effects of annonaceous acetogenins on the life cycle of *S. frugiperda*

Table 2. Nutritional alterations produced by annonaceous acetogenins on larvae of *S. frugiperda*.

Table 1

Compounds	Larval duration[a] (d)	(%) Larval mortality	(%) Pupal mortality	(%) Malformed adults
Control	12.1 ± 2.6 a	10	20	0
neoannonin	15.5 ± 1.6 a	10	100	0
itrabin	18.8 ± 2.6 b	30	100	0
almuñequin	19.7 ± 1.5 b	30	90	10
asimicin	17.3 ± 1.5 b	30	100	0
squamocin	Mortality at early instars	100	100	0
motrilin	18.0 ± 2.7 b	20	90	10
cherimolin-1	14.0 ± 0.0 a	0	100	0
cherimolin-2	17.7 ± 3.6 b	10	90	10
tucumanin	14.7 ± 3.3 a	20	80	20

[a] Mean ± SD. Means followed by the same letter are not significantly different ($P > 0.05$, Tukey multiple range test).

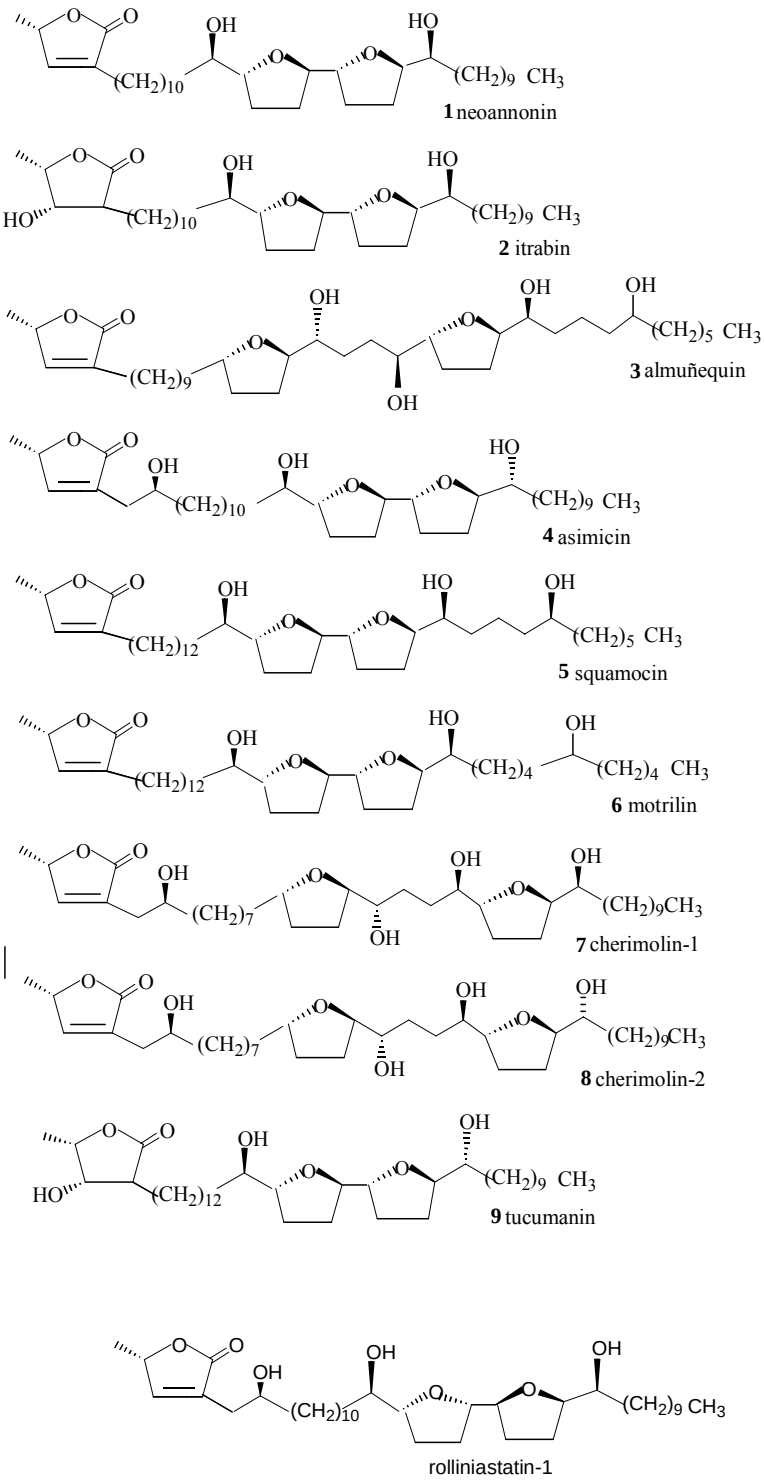
Table 2

Products	GR[a]	UR[a]	RCR[a]
Control	1.02 ± 0.40a	0.23 ± 0.07a	3.91 ± 1.63a
neoannonin	0.90 ± 0.15a	0.26 ± 0.11a	3.23 ± 1.09a
itrabin	0.59 ± 0.11a	0.26 ± 0.06a	3.01 ± 0.40a
almuñequin	1.10 ± 0.44a	0.19 ± 0.06a	3.50 ± 0.10a
asimicin	0.77 ± 0.17a	0.21 ± 0.08a	2.85 ± 1.05a
squamocin	0.16 ± 0.05b	1.48 ± 0.62b	3.95 ± 2.23a
motrilin	1.19 ± 0.88a	0.22 ± 0.15a	3.50 ± 1.02a
cherimolin-1	0.91 ± 0.24a	0.31 ± 0.05a	3.82 ± 1.16a
cherimolin-2	0.97 ± 0.41a	0.23 ± 0.12a	3.69 ± 1.77a
tucumanin	0.81 ± 0.32a	0.21 ± 0.07a	2.52 ± 0.59a

RCR (Consumption Index); GR (Growth Index); UR (Food Utilization Index). Means within a column followed by the same letter are not significantly different ($P > 0.05$, Tukey multiple range test).

[a]Numbers in columns represent mean ± SD.

Fig. 1


1 neoannonin
2 itrabin
3 almuñequin
4 asimicin
5 squamocin
6 motrilin
7 cherimolin-1
8 cherimolin-2
9 tucumanin
rolliniastatin-1