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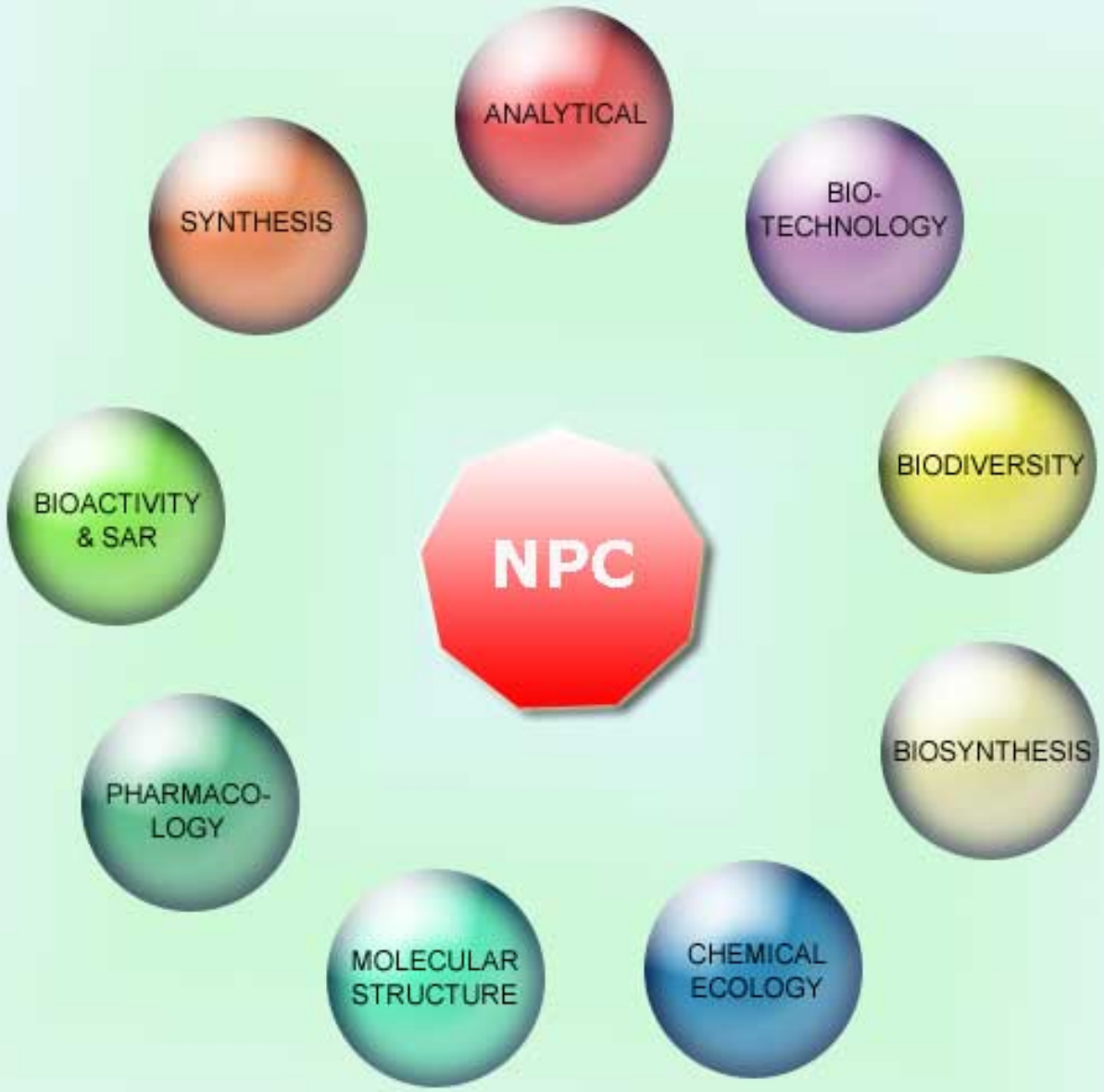

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Insecticidal, Mutagenic and Genotoxic Evaluation of Annonaceous Acetogenins

Olga Álvarez Colom[a], Analia Salvatore[b], Eduardo Willink[b], Roxana Ordóñez[a,c], María I. Isla[a,c], Adriana Neske[a*] and Alicia Bardón[a,c]

[a]*Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, Tucumán (4000), Argentina*

[b]*Estación Experimental Agroindustrial Obispo Colombres, Av. William Cross 3150, Las Talitas, Tucumán (4101), Argentina*

[c]*INQUINOA – CONICET, Ayacucho 471, Tucumán (4000), Argentina*

aneske@fbqf.unt.edu.ar

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Annonaceous acetogenins represent a class of bioactive compounds whose primary mode of action is the inhibition of NADH-ubiquinone oxidoreductase (Mitochondrial Complex I). Given the potential pesticidal use of these compounds, we evaluated the effects of seven acetogenins: squamocin (**1**), molvizarin (**2**), itrabin (**3**), almuñequin (**4**), cherimolin-1 (**5**), cherimolin-2 (**6**), and tucumanin (**7**) isolated from *Annona cherimolia* Mill. against *Ceratitis capitata* Wiedemann (Tephritidae). These acetogenins did not display insecticidal action at 250 µg of treatment per g of adult diet. However, the oviposition capacity of *C. capitata* females was significantly altered by some of the acetogenins at this concentration. The most potent compounds were itrabin, molvizarin and squamocin. Moreover, significant differences were detected in the preference of oviposition sites when itrabin and squamocin were spread on the surface of artificial fruits at doses of 30 µg/cm^2. Additionally, we investigated the mutagenic effects displayed by itrabin, as well as the phytotoxic and genotoxic action of squamocin and itrabin. Both compounds displayed slight phytotoxic and genotoxic effects on roots of *Allium cepa* at 2.5 µg/mL though no mutagenic effects were detected at 0.25, 0.5 and 2.5 µg/mL on *Salmonella typhimurium* strains TA98 and TA100.

Keywords: *Ceratitis capitata*, plant metabolites, *Salmonella typhimurium*, *Allium cepa*.

The Annonaceae, a large family of tropical plants, has been intensely investigated over the last 20 years, largely because of the discovery of the annonaceous acetogenins (ACG), a group of C32/C34 fatty-acid derived natural products. Most of the acetogenins are compounds possessing 1-3 THF rings, a γ-lactone (either saturated or unsaturated), and a long unbranched aliphatic chain. Currently, these natural products are regarded as the most potent antitumor compounds of recent years [1], acting as potent inhibitors of complex I (NADH: ubiquinone-oxidoreductase) in the mammalian mitochondrial electron transport system [2a].

Regarding the insecticidal action of acetogenins, it has been reported that many of these compounds are toxic to *Leptinotarsa decemlineata* (Coleoptera) and *Myzus persicae* (Homoptera) adults [2b], as well as to nymphs of the German cockroach [1]. Previous results from our laboratory indicated that the acetogenins from *Annona cherimolia* and *A. montana* produce larvicidal effects [2c,2d].

The Mediterranean fruit fly, *Ceratitis capitata*, attacks a wide variety of hosts in subtropical and temperate regions of Argentina, causing serious economic damage [2e]. Continuing with our search for natural insecticides from native South Americans plants, we evaluated the adult mortality, the oviposition capacity of females, the egg viability, and the larval mortality of F1, as well as the alterations on the ovipositional behavior produced by seven ACGs of *A. cherimolia* seeds, squamocin (**1**), molvizarin (**2**), itrabin (**3**), almuñequin (**4**), cherimolin-1 (**5**), cherimolin-2 (**6**) and tucumanin (**7**) on *C. capitata.* Additionally, the phytotoxic, genotoxic and mutagenic effects displayed by itrabin, as well as the phytotoxic and genotoxic action of squamocin, the two most active ACGs on *C. capitata*, were investigated.

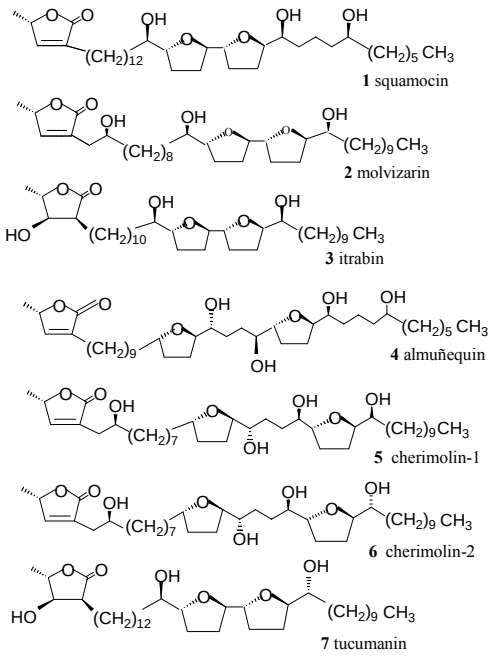

Figure 1: Annonaceous acetogenins employed in the experiment.

Annonaceous acetogenins: Squamocin (**1**), molvizarin (**2**), itrabin (**3**), almuñequin (**4**), cherimolin-1 (**5**), cherimolin-2 (**6**), and tucumanin (**7**) (Figure 1) were obtained and purified from seeds of *A. cherimolia* [2f]. Their structures were determined by comparison of their spectroscopic features with data previously reported in the literature.

Table 1: Oviposition-deterrent activity of ACGs on *C. capitata*.

Compounds (30 µg/cm^2)	Nº eggs laid on the treated substrate (T) [a]	Nº eggs laid on the nontreated substrate (C) [a]	%OI
Molvizarin	297.0 ± 2.8a	294.0 ± 11.3a	1.0
Itrabin	163.0 ± 9.8b	281.5 ± 3.5a	-42.1
Almuñequin	183.0 ± 4.2a	190.0 ± 9.9a	-3.9
Cherimolin-1	259.0 ± 2.8a	240.0 ± 14.1a	-4.6
Cherimolin-2	226.7 ± 4.3a	213.7 ± 19.6a	6.1
Squamocin	203.5 ± 9.2b	248.3 ± 11.3a	-18.0

Numbers represent Mean ± SD, n=3. [a]Means within a row followed by the same letter are not significantly different (P>0.05, Tukey multiple range test).

Oviposition-deterrent activity: To facilitate data interpretation, an oviposition index was defined as %OI = 100 T/C-100, where T is the number of eggs laid in the treated substrate, and C is the number of eggs deposited in the nontreated substrate. This index takes negative values for oviposition deterrents, and positive values for oviposition attractants. As shown in Table 1, the compounds that significantly deterred oviposition of *C. capitata* were itrabin and squamocin (%OI = -42.1 and -18.0, respectively) at 30 µg/cm^2.

Oviposition capacity and effects on the offspring: Oviposition capacity was significantly affected by the treated diets. The bioassay demonstrated that females from the first generation that fed on 250 ppm of treated diets with molvizarin, itrabin and squamocin oviposited around one-fourth (0.15 ± 0.01, 0.15 ± 0.01 and 0.20 ± 0.01, respectively) the number of eggs of the control females (Table 2). The remaining ACGs, almuñequin, tucumanin, cherimolin-1 and cherimolin-2, reduced the oviposition capacity to around half the number of eggs of control females (0.34 ± 0.05, 0.33 ± 0.06, 0.33 ± 0.06 and 0.40 ± 0.10, respectively). Eggs viability was not affected by the treatments and, therefore, no significant differences were registered in the hatching percentage of treatments in relation to control (Table 2). Treatments with molvizarin, itrabin, squamocin, and cherimolin-2 produced low but significant larval mortalities of 13.9 ± 1.3, 13.5 ± 3.6, 18.2 ± 1.3, 12.9 ± 2.7 %, respectively, as shown in Table 2.

Table 2: Oviposition capacity and effects on the offspring of *C. capitata* produced by ACG.

Compounds	Volume of eggs[a] (mL)	(%) Hatch[a]	Larval Mortality[a] (%)
Control	0.63 ± 0.06a	97.0 ± 1.0a	7.6 ± 0.8a
Molvizarin	0.15 ± 0.01b	97.0 ± 1.7a	13.9 ± 1.3b
Itrabin	0.15 ± 0.01b	96.0 ± 0.6a	13.5 ± 3.6b
Squamocin	0.20 ± 0.01b	98.0 ± 1.2a	18.2 ± 1.3c
Almuñequin	0.34 ± 0.05c	97.0 ± 1.0a	7.0 ± 1.0a
Tucumanin	0.33 ± 0.06c	97.0 ± 1.0a	11.8 ± 2.1a
Cherimolin-1	0.33 ± 0.06c	97.0 ± 1.5a	12.9 ± 2.7b
Cherimolin-2	0.40 ± 0.10c	98.0 ± 0.6a	11.2 ± 3.4a

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

Table 3: Phytotoxic and genotoxic action of squamocin and itrabin on *Allium cepa* roots.

Itrabin (µg/mL)	Root elongation[a] (cm)	Mitotic index[a] (%)	Anaphasic aberration[a]	Micronucl ei number[a]
0	2.6±0.2	59±9a	1±1a	-
0.25	2.2±0.3	57±4a	ND	-
0.5	2.3±0.1	52 ±3a	2±1a	-
2.5	1.8±0.2	44 ±5b	3±1a	1±1a
Squamocin (µg/mL)				
0.25	2.2±0.2	41.4 ±5b	3±1a	-
0.5	1.9±0.1	34.2 ±7c	5±2b	-
2.5	1.7±0.1	30.7 ±3c	7±2c	2±1a

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

Allium cepa test: Macroscopic analysis of the treated roots revealed neither modification in consistency and shape nor tumors, hooked or twisted roots. The rate of inhibition of root elongation was around 30% for the maximum concentration (2.5 µg/mL) of itrabin and squamocin. The mitotic index (MI) is a parameter that estimates the frequency of cellular division. Its reduction could be due to either the inhibition of DNA synthesis or a blocking in the G_2 phase of the cell cycle, preventing the cell from entering mitosis. Squamocin produced a decrease between 30 and 50% of the MI values at the doses tested (0.25-2.5 µg/mL) (Table 3). Moreover, itrabin and squamocin induced anaphasic aberrations, as shown in Table 3.

Salmonella typhimurium test: Itrabin was tested for its mutagenic potency at 0.25, 0.5 and 2.5 µg/plate with and without S9 mix on both *S. typhimurium* TA98 and

Table 4: Mutagenic effects displayed by itrabin on *Salmonella typhimurium* strains TA98 and TA100.

Dose (µg/plate)	Mutagenicity relation (MR)			
	-S9		+S9	
	TA 98	TA 100	TA 98	TA 100
0.25	1.2	0.98	0.95	1.10
0.5	1.07	0.82	0.98	0.97
2.5	1.06	0.90	1.10	1.15
DMSO control	1.10	0.90	0.78	1.20
Positive control	2.26	2.91	2.89	3.01

MR: Revertants number in ACG plate/ Revertants number in control plate. Results are expressed as the mean number of revertants (standard error of triplicate plates).

TA100 strains. Non significant differences in the revertants number were detected for the treated experiments compared with control, as shown in Table 4. Our results are in agreement with the earlier studies reported about the mutagenicity of ACGs [2b].

The influence of chemical agents on the ovipositional behavior of insects can be used to control pests as well as vector insects. Some natural products display oviposition-deterrent activity against different insects. Factors that affect the oviposition behavior of some tephritid fruit flies include host-plant odor as well as the physical and chemical characteristics of the oviposition substrate, such as size, shape, color, and presence of deterrent chemicals. *C. capitata* has a wide geographical distribution and is considered one of the most important pests in the world affecting many species of commercially valuable fruits, including citrus. Therefore, substances that affect their behavior can be used in the development of pest-control agents.

This is the first report on the effects of acetogenins of *A. cherimolia* on *C. capitata*. The alterations in the capacity and behavior of oviposition of females produced by treatments with 250 ppm of itrabin, molvizarin and squamocin, together with the low genotoxicity and no mutagenicity, are promising results for the design of a natural pest control formulation employing the cited ACGs.

Experimental

Purification and identification of acetogenins: Dried and powdered seeds of *A. cherimolia* were percolated with methanol. The methanol extract was further partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ extract was processed by chromatographic techniques, including silica gel columns and high performance liquid chromatograph employing the methodology previously reported [2c]. Structural determination of acetogenins was assessed by spectroscopic data in comparison with previously reported data [3a-3e, 4a].

Test insects and diet: A colony of *C. capitata* was initiated with pupae obtained from infested oranges from the northwest of Argentine. Adults were fed on artificial diets made of water and a mixture of sugar and yeast hydrolysate (3:1). They were maintained in a rearing room with a photoperiod of 12L:12D, at 24±2°C and 60±10% relative humidity.

Oviposition-deterrent activity: Artificial fruits (oviposition substrates) were prepared by boiling a mixture of peach juice (500 mL), agar (15 g), and sodium benzoate (one teaspoonful, as preservative) and pouring the preparation into cylinders wrapped in plastic (Rolopac). After cooling, the surface of the wrapped cylinders was pricked with a needle and 30 µg of the test compound was deposited per cm^2. Control cylinders were impregnated only with the solvent that was then removed by evaporation. Three groups of *C. capitata* adults were selected from the laboratory colony. Each group, consisting of 7 male-female pairs, was placed in a small cage and covered with voile (a light, almost transparent cloth made of silk). Two agar cylinders (sample and control) were placed over the voile, and females oviposited on either one or the other according to their preference. After 4 days, eggs were gently rinsed from the agar and counted. All assays were conducted in triplicate [4b].

Evaluation of the oviposition capacity and effects on the offspring: Newly emerged adults (1:1, male:female) of *C. capitata* were sexed and placed in a cage of wooden frames and canvas lined with a mosquito net in order to mate and lay eggs. Insects were fed on diets treated with 250 µg of each pure ACG per g of diet. An identical cage was set for a control experiment in which the diet did not contain the treatment. The number of adults was registered daily. Oviposition substrates were provided in the cages. After 5 days the eggs laid by females in the treated substrate (T) and nontreated substrate (C) were collected, the volume of eggs determined and the eggs placed in Petri dishes with larval diet. Dishes were placed in a rearing chamber under controlled temperature and humidity (25°C and 80% RH). After 3 days the number of emerged larvae was determined and, after 18 days, the number of pupae was quantified.

Plant genotoxicity test (Allium cepa test): For the *A. cepa* (onion) root anaphase aberration assay and the *A. cepa* root micronuclei assay [4c], equal sized young bulbs of common onions were used. All onion bulbs were kept in commercial mineral water for 48 h until root emergency. Six onion bulbs were exposed to 0.25, 0.5, and 2.5 µg/mL of each ACG (suspended in a 0.1:10 DMSO-water mixture) for 24 h in the dark. Then the roots of 3 bulbs were cut up and fixed for 24 h in a 1:3 acetic acid-ethanol solution. The remaining 3 bulbs were kept in tap water for another 24 h (recovery time), and then the roots were fixed, as indicated previously.

Finally, all roots were stored in 70% ethanol. In the negative control experiment, 3 onion bulbs were exposed only to a 0.1:10 DMSO-water solution and they were submitted to the procedures previously described for treated bulbs. Three onion bulbs were exposed to a 10^{-4} M aqueous solution of methyl methanesulfonate in a positive control experiment. Macroscopic parameters such as length of roots, number of tumors, and hooked and twisted roots were registered and compared with control experiments to determine the phytotoxic action of ACG. Mitotic index (1000 cells per slide) was used to evaluate cellular division rate. Anaphasic aberrations (bridges, laggard chromosomes, and fragments; 800 anaphasic cells per sample) and micronuclei formation (5 slides, 1000 cells per slide) were the microscopic parameters assessed as indicators of DNA damage. For 40X microscopic observations, chromosomes were stained by the following procedure. Tips were cut from roots to be further hydrolyzed in 1 M HCl at 60°C for 10 min before staining in Schiff's reagent for 15 min. A portion of stained tip was immersed in a drop of 45% acetic acid, placed on a clean slide, and covered with a coverglass in order to have a single layer of cells.

Mutagenicity assay: The mutagenic effects of ACG were assayed according to the Ames test using *Salmonella typhimurium* strains TA98 and TA100 [4d], with and without metabolic activation (500 µL of S9 mix fraction). The tested strains were cultured in Oxoid Nutrient Broth for 12 h. Different concentrations of ACGs (0.25 - 0.5 µg/plate) in DMSO solution, were added to 2 mL of soft agar containing L-hystidin (0.05 mM), D-biotin (0.5 mM), and 0.1 mL of bacterial culture. Then, this mixture was poured onto a plate containing minimum medium (Oxoid Nº2). The plates were incubated at 37°C for 48 h, and the His^+ revertant colonies were manually counted. S9 fraction was obtained from liver of Sprague-Dawley rats pretreated with a mixture of polychlorinated biphenyls (Araclor 1254). All experiments were analyzed in triplicate. A sample was considered to be mutagenic when the number of revertant colonies was at least twice the negative control yield and showed a significant response in analysis of variance. For the positive control experiment, the direct-acting mutagen 4-nitro-*o*-phenylenediamine (NPD, 5 µg/plate) and the indirect-acting mutagen isoquinoline (IQ, 0.1 µg/plate for TA98 and 0.5 µg /plate for TA 100) were incorporated.

Statistical analysis: The results are reported as mean ± SEM. 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 analyses, values of $P > 0.05$ were considered not significant (Statistix 7.1 2000).

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