

Toxic Effects of *Citrus aurantium* and *C. limon* Essential Oils on *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Citrus aurantium and *C. limon* were selected in the search for natural plant insecticides. The essential oils of *C. aurantium* and *C. limon* and ethanol extracts of the seeds, pulp, albedo, and peel of *C. aurantium* were incorporated into the larval diet of the lepidopteran pest *Spodoptera frugiperda*. Larval and pupal mortality were quantified and adult malformation was observed. *C. aurantium* essential oil had antifeedant action and the mixture of albedo ethanol extract and *C. aurantium* essential oil had toxic effects on *S. frugiperda* larvae at early stages, when they had not yet produced major damage to the crop. Our results indicated that a mixture of ethanol extract of albedo and *C. aurantium* essential oil (250 µg of extract mix per g of diet) deterred feeding by 46% and had the highest larval mortality (100%) of the materials tested. The peel extract (250 µg per g of diet) produced an increment in growth rate and diet consumption. However, 40% of the larval and 45% of the pupal populations died after 96 h of treatment. The blend of essential oil and *C. aurantium* albedo ethanol extract showed the lowest consumption and a poor nutrient conversion into biomass. Finally, the presence of D-limonene and nootkatone in the peel ethanol extract, and *C. limon* and *C. aurantium* essential oils, may be the cause of the response in the feeding behavior and toxic effects found on *S. frugiperda*.

Keywords: *Citrus aurantium*, *C. limon*, *Spodoptera frugiperda*, natural insecticide.

Plants synthesize a large variety of secondary metabolites that form part of adaptation and defense mechanisms against the pathogens and predators in their environment. The ability to generate these substances that may attract or repel other organisms arose as an evolutive strategy for survival. Thus, natural products constitute a desirable alternative for pest control [1-3]. Preliminary assays in our laboratory with natural products isolated from a series of extracts from aerial parts of plants and fruit seeds showed that they have toxic, antifeedant [1,3], cytotoxic [4], inhibitory [5,6], antibacterial [7] and deterrent properties [4,8].

The insecticidal action of the economically important Rutaceae family was approached in this work. We studied the *Citrus* genus, which includes species that have been cultivated for their edible fruits since time immemorial. The most popular are bitter orange (*Citrus aurantium*), sweet orange (*C. sinensis*), grapefruit (*C. maxima*), "toronjo" (*C. paradisi*), lemon (*C. limon*), tangerine (*C. reticulata*) and bergamot (*C. bergamia*).

Citrus (Rutaceae) have long been known in the literature for their varied biological activity. For example, *C. sinensis*, *C. limonia* [9] and *C. paradisi* [10] are larvicidal against *Aedes aegypti*. *C. aurantium* has an insecticidal

effect on *Bactrocera oleae* (Gmelin) and on adult flies that attack olives [11]. *C. limon* is toxic and affects the egg-laying capacity of *Ceratitidis capitata* [8]. *C. aurantium* peel contains citral, limonene and various flavonoids, including hesperidin, neohesperidin, naringin and rutin that could have cancer preventive [12] and antiviral properties [13]. *C. aurantium* essential oil contains linalool and limonene. The latter is one of many cyclic monoterpenes with known insecticidal properties [14].

Continuing with our search for natural insecticides from native South American plants, we evaluated the feeding behavior as well as the toxic effects produced by the blend of *C. aurantium* and *C. limon* essential oils and ethanol extracts of *C. aurantium* seed, pulp, albedo, and peel on *Spodoptera frugiperda*. Additionally, Consumption Index (CI), Growth (GR), and Efficiency in the Consumption Index (ECI) were evaluated. The "fall armyworm" (*Zea mays* L.) or "armyworm," *S. frugiperda* (Smith) (Lepidoptera: Noctuidae) is a widely distributed pest in America that causes serious economic damage to many crops.

Antifeedant activity was evaluated on the basis of consumption of diet treated for diet control, through the choice of bioassay with larvae of the second stage of a

laboratory colony of *S. frugiperda*. *C. aurantium* essential oil (100 and 250 ppm) caused the most antifeedant activity among all the natural products assayed under the conditions of the experiment, producing a significant decrease in larval growth. Toxicity was determined by evaluating *S. frugiperda* larval and pupal mortality. They were fed an artificial diet treated with different essential oils and extracts at a final concentration of 100 and 250 mg essential oil / g of diet; an untreated diet was used as control. The mixture of 125 ppm albedo ethanolic extract and 125 ppm *C. aurantium* essential oil proved to be the deadliest of all products evaluated as it caused 100% larval mortality under the conditions of the experiment.

Nutritional indices that take into account diet consumption (CI), larval growth (GR) and diet consumption efficiency (ECI) were also evaluated.

The mixture of essential oil and ethanol extract of *C. aurantium* albedo showed the lowest consumption and a poor nutrient conversion into biomass. This results in a significant decrease of larval growth and the subsequent 100% mortality of larvae in the early stages of their life cycle.

Extraction: For the present study, the essential oils of *C. aurantium* and *C. limon*, and the ethanol extracts from seeds, pulp, albedo, and peel of *C. aurantium* were obtained.

Choice test: As shown in Table 1, the incorporation of 100 and 250 ppm of essential oil of *C. aurantium* into the artificial diet of *S. frugiperda* displayed the strongest antifeedant effects ($FR_{50} = 0.43$ and 0.37 , respectively). *C. aurantium* albedo, pulp and seed ethanolic extracts (100 and 250 ppm), *C. limon* essential oil (100 and 250 ppm) and the blend of *C. aurantium* and *C. limon* (100 and 250 ppm) essential oils did not present a significant antifeedant action under the conditions of the experiment. Grated peel extract (250 ppm), a mixture of 125 ppm of albedo and 150 ppm of *C. limon* essential oil and the combination of 150 ppm of albedo and 150 ppm of *C. aurantium* essential oil had a slight anti-feedant effect on *S. frugiperda*. Among all the natural products assayed, *C. aurantium* essential oil (100 and 250) had the highest antifeedant activity as it caused a marked decrease in larval growth under the conditions of the experiment.

Extract toxicity: As shown in Table 1, 100 and 250 ppm of *C. aurantium* grated peel, pulp and seed ethanolic extracts did not have a significant toxic effect under the experimental conditions. The mixture of *C. aurantium* and *C. limon* essential oils (100 and 250 ppm) had a slight toxic effect on *S. frugiperda* with a larval mortality of 50% to 60%. However, the combination of albedo ethanolic extract of *C. aurantium* (100 and 250 ppm), *C. aurantium* and *C. limon* essential oils (250 ppm), plus the mixture of albedo ethanolic extract and *C. limon* essential oil

Table 1: Antifeedant action and toxic effects on *S. frugiperda* larvae.

Compounds	(% Larval mortality		(% Pupal mortality		FR ₅₀	
	100	250	100	250	100	250
Dose (ppm)	100	250	100	250	100	250
<i>C. limon</i> essential oil	73	94	17	6	0.87±0.24	0.52±0.21
<i>C. aurantium</i> essential oil	78	90	22	10	0.43±0.27	0.37±0.16
Mix <i>C. limon</i> and <i>C. aurantium</i> essential oil	53	67	20	7	0.70±0.38	0.85±0.45
<i>C. aurantium</i> albedo extract	83	94	5	6	1.16±0.40	0.94±0.30
<i>C. aurantium</i> seed extract	44	33	17	22	0.84±0.31	0.77±0.20
<i>C. aurantium</i> pulp extract	28	39	11	22	0.66±0.26	0.60±0.26
<i>C. aurantium</i> peel extract	44	56	11	22	1.20±0.62	0.44±0.29
Mix <i>C. aurantium</i> albedo extract and <i>C. limon</i> essential oil	ND	80	ND	20	ND	0.46±0.14
Mix <i>C. aurantium</i> albedo extract and essential oil	ND	100	ND	0	ND	0.46±0.19

Numbers represent Mean ± SD, $n=3$. *Means within a row followed by the same letter are not significantly different ($P>0.05$, Tukey multiple range test). ND: No determined.

Table 2: Nutritional indices.

Compounds	GR _T /GR _C		CI _T /CI _C		ECI _T /ECI _C	
	(%)		(%)		(%)	
Dose (ppm)	100	250	100	250	100	250
<i>C. limon</i> essential oil	22.7	19.7	122.5	83.4	19.0	23.8
<i>C. aurantium</i> essential oil	12.6	8.6	18.4	19.5	68.2	45.4
Mix <i>C. limon</i> and <i>C. aurantium</i> essential oil	31.2	17.5	70.6	97.2	41.0	50.0
<i>C. aurantium</i> albedo extract	16.4	22.9	75.4	104.9	20.3	20.3
<i>C. aurantium</i> seeds extract	153.7	161.2	115.0	87.2	130.4	182.6
<i>C. aurantium</i> pulp extract	55.4	73.3	91.9	96.6	60.0	77.1
<i>C. aurantium</i> peel extract	94.9	44.4	96.8	49.5	95.2	81.4
Mix <i>C. aurantium</i> albedo extract and <i>C. limon</i> essential oil	ND	16.5	ND	30.3	ND	54.8
Mix <i>C. aurantium</i> albedo extract and essential oil	ND	8.5	ND	30.5	ND	21.8

For comparison purposes, rates are expressed as a relationship between treatment and control.

(250 ppm) produced a significant toxic effect on *S. frugiperda* with 83% to 94% mortality. The blend of 125 ppm albedo ethanolic extract and 125 ppm *C. aurantium* essential oil turned out to be the deadliest of all since it caused 100% larval mortality under the experimental conditions. Total larval period could not be measured as mortality occurred at an early stage.

Nutritional indices: In order to assess the mechanism of action leading to treatment induced mortality we analyzed the nutritional effects produced by the addition of the above mentioned extracts to the 2nd instar larval diet of *S. frugiperda*. As shown in Table 2, larvae fed with ethanolic extracts of *C. aurantium* grated peel (100 ppm), pulp (100 and 250 ppm) and seed (100 and 250 ppm) presented physiological indices similar to those of control larvae. The addition of a blend of *C. aurantium* and *C. limon* essential oils (100 and 250) to the larval diet caused no significant changes to the nutritional indices in connection with larvae fed the control diet. The nutritional index values obtained for larvae fed with albedo ethanolic extract (100 and 250 ppm), grated peel (100 ppm), *C. aurantium* (100 and 250 ppm) and *C. limon* (100 and 250 ppm) essential oils revealed the presence of toxic compounds when compared with the control. The

differences observed became much more significant when a mixture of albedo ethanolic extract of *C. aurantium* and *C. limon* essential oil was added to the diet and with a mixture of *C. aurantium* essential oil and albedo ethanolic extract. The latter was the blend that had the lowest intake percentage and the poorest nutrient absorbed conversion. This resulted in an important larval growth decrease and subsequent 100% larval mortality at very early stages in their life cycle.

Identification and quantification of ethanol extract constituents by GC-MS: GC-MS analyses of *C. aurantium* essential oil showed the presence of 14 components identified by comparison of their mass spectra with Wiley library data. The major components were: α -thujene (3.3%), α -pinene (1.0%), sabinene (4.7%), D-limonene (83.3%), allo-ocimene (0.1%), α -terpineol (0.5%), verbenol (0.1%), 3-carene (0.8%), geranial (0.2%), δ -elemene (0.2%), linalyl acetate (0.2%), caryophyllene (0.4%), humulene (0.1%) and nootkatone (0.2%).

The components of the essential oil of *C. limon* and the ethanol extracts of the seeds, pulp, albedo, and peel of *C. aurantium* analysed by GC-MS were identified by comparison of their mass spectra with Wiley library data. The main compounds found are listed in the additional information table. Our results reveal the presence of toxic compounds, such nootkatone, one of the most important and expensive aromatic compounds of *Citrus*. In previous reports we showed that nootkatone encapsulated with lignin in a spray was as an effective repellent against deer ticks that carry Lyme disease. Similarly, D-limonene has been attributed with a defensive function against herbivores. The presence of D-limonene and nootkatone in the peel ethanol extract, and *C. limon* and *C. aurantium* essential oils, but only nootkatone in the albedo ethanol extract may be the cause of the response in the feeding behavior and toxic effects found on *S. frugiperda*. These compounds are not present in extracts of seeds and pulp of *C. aurantium*.

The influence of chemical agents and their toxic effects on insects and their feeding behavior can be used in the development of environment friendly pest control agents. This is the first report regarding the effects of *C. aurantium* and *C. limon* essential oils and *C. aurantium* seed, pulp, albedo, and peel ethanol extracts on *S. frugiperda*. The high mortality produced by treatments with 100 and 250 ppm of *C. aurantium* essential oil is a promising result for the design of a natural pest control formulation with the cited extracts.

Experimental

Extraction: The plant material (seeds, pulp, albedo, and peel) was dried at room temperature and ground. Thirty g of ground plant material was placed in a preweighed flask with enough solvent to cover it completely. Maceration was carried out at room temperature with occasional

agitation. The extracts obtained were concentrated in a rotary evaporator at temperatures not exceeding 45°C to avoid disrupting potentially active principles.

Essential oil isolation: The oil was obtained from fresh material (*C. aurantium* and *C. limon*) by steam distillation in a Clevenger-type trap (Figmay). Two fractions were obtained: an insoluble distillate separated by decantation and a soluble one that was extracted with diethyl ether. The oil was dried over anhydrous sodium sulfate and stored at 4°C.

Test insects and diet: *S. frugiperda* larvae were obtained from our laboratory population. The larval diet consisted of a mixture of yeast (3 g), boiled and ground beans (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).

Choice test: A portion of artificial diet (10 g) was impregnated with acetone and, after solvent removal, was employed as a larval control diet. Another portion was impregnated with an acetone solution of each pure extract in order to leave 100/250 μ g of compound per g of diet (treated). After solvent evaporation, control and treated diets were placed in test tubes (20 replicates) in which 2nd instar larvae were placed between both portions of diet kept at 27°C and 60 \pm 15% relative humidity. When 50% of the control diet had been eaten, control and treated diets were removed from the tubes and weighed accurately. Results of the choice test were then reported by the feeding ratio $FR_{50} = T/C$ [15], where T and C are the weights of diets consumed in the treated and control experiments, respectively.

Toxicity test: Control and treated diets were placed in different test tubes (10 replicates for both treated and control experiments) in which 2nd instar larvae were placed and kept at 27°C and 60 \pm 15% relative humidity until emergence of the 1st generation of adults. Larval developmental periods, as well as mortality rates, were recorded for treatments with all the extracts (100 and 250 ppm) and control experiments. Photographic records of abnormal larvae and pupae were obtained during the experiment.

Determination of Consumption (CI), Growth (GR), and Efficiency in the Consumption Index (ECI) Indices: Two 2nd instar larvae of homogeneous size were placed in a test tube at the beginning of the experiment, and larval weight was accurately determined. Test and control diets were also weighed and offered to larvae in each tube. Ten replicates for control and ten for each treatment were employed. Tubes were kept at 27°C. Larval weight and diet additions with the corresponding weight were recorded. For a 10 day period, starting with 2nd instar larvae, measurements were made of average diet consumption (CI), growth rate (GR), and efficiency in the consumption index (ECI).

$$CI = D / t$$

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

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

D = Food eaten during the experimental period; B = Initial larval weight; A = Final larval weight; t = Experiment period; GR: Average daily weight gain during the experiment. CI: Average daily diet consumption during the experiment.

GR, CI and ECI values were calculated for the experiment and control treatments. For comparison purposes, rates are expressed as a relationship between treatment and control; the latter are considered 100%. Values are expressed as (GR_T/ GR_C) 100% (CI_T/ CI_C) 100% and (ECI_T/ ECI_C) 100% in the tables.

Identification and quantification of ethanol extract constituents by GC-MS: GC-MS analysis was carried out using a Trace GC Ultra (GC System) and Polaris Q Mass selective detector equipped with a DB 5MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). The carrier gas was He (1 mL/min); splitless mode (9:1). The

injector temperature was 300°C. The initial temperature of the column was 50°C, held for 5 minutes, then 100°C at a rate of 10°C/min. This was held at 100°C for 1 min, reaching 250°C at a rate of 7 °C/min. It was kept at 250°C for 2 mins and then taken to 280°C at a rate of 10°C/min. The temperature remained at 280°C for 15 mins. The transfer line was 300°C, ion source temperature 220°C, electron impact 70 eV, mass defect 200 amu, max ion time 25 µs.

Statistical analysis: The results are reported as mean ± SEM. The differences in the mean values were evaluated by variance analysis (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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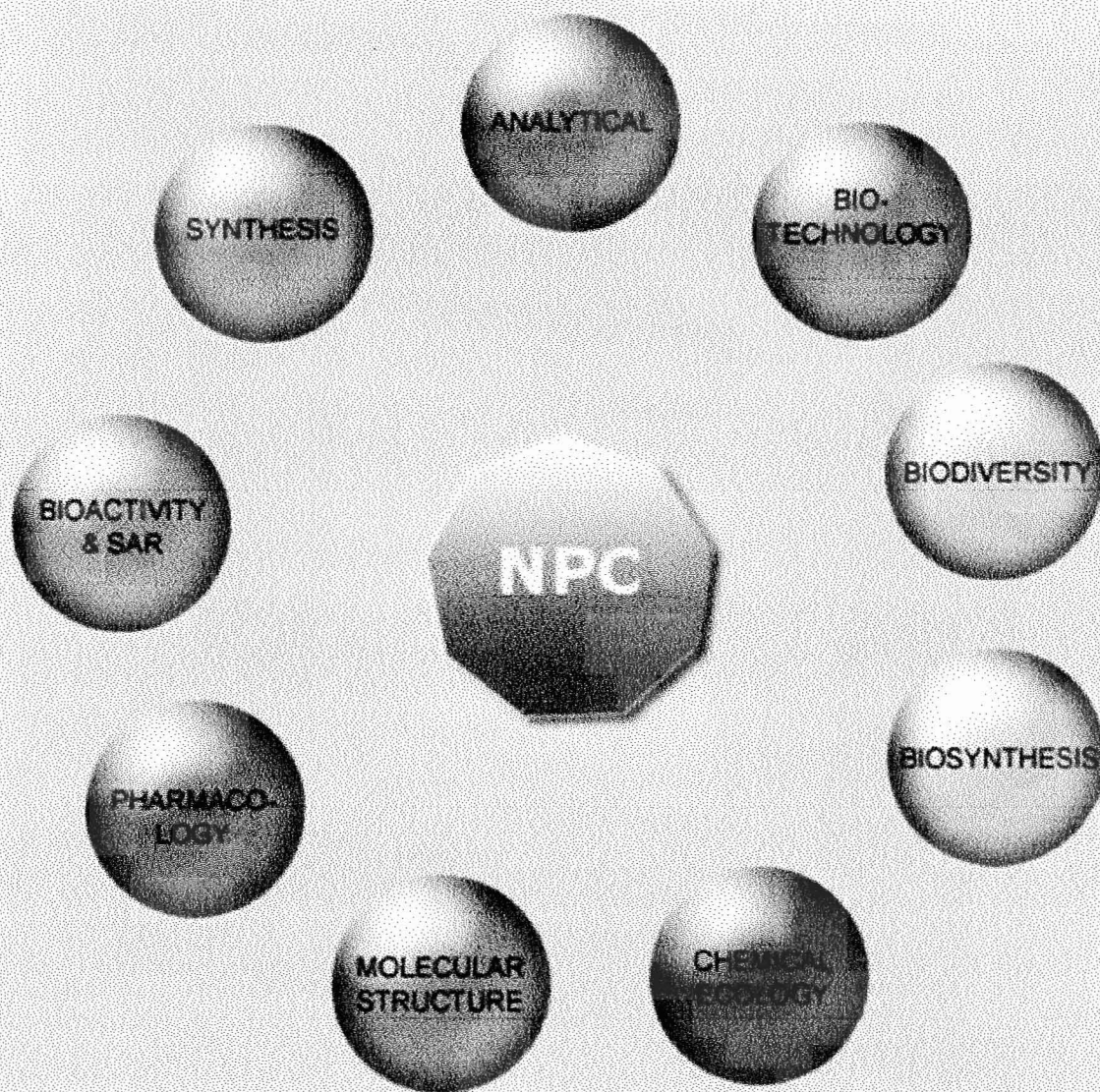
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