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Original Article

Activity of grindelanes against important maize pest *Spodoptera frugiperda* and their selectivity of action on non-target environmental bacteria

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Abstract

In the search for new plant-derived products with antifeedant and insecticidal potential against economically important insects, 11 grindelanes (two as natural acids, and nine as methylated derivatives) purified from the foliar tissue of *Grindelia chiloensis* (Cornel.) Cabrera (Asteraceae) were evaluated against the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae). Larvae of this moth are considered to be the main pest of maize, that causes severe losses on local agricultural production. Remarkable alterations in feeding behavior, larval weight, larval and pupal stage lengths, and adult fertility, as well as malformations and mortality in specimens during their life cycle were noticed in the feeding preference tests and no-choice diet assays. Antifeedant indexes (AI%) calculated in food preference bioassays reached up to 79% for the lipophilic grindelane 3b, the most potent antifeedant agent. Grindelane 7b showed a better insecticidal activity (median lethal dose, LD_{50} = 26.49 μg g^{-1}) and 8b was potentially neurotoxic due to acetylcholinesterase inhibition. Ultrastructure of the middle portion of the digestive tract of larvae fed with small amounts of natural grindelane 9a supplemented to the diet revealed cell damage, and thickening of the peritrophic membrane and a striking number of mitochondria, as evident signs of defense against toxic substances ingested by the larvae. Besides, this study demonstrated that these effective insecticides did not have inhibitory effects on beneficial plant and soil bacteria which would allow ecological pest management.

Abbreviated abstract

Natural grindelanes and derivatives showed antifeedant and insecticide potential against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Remarkable alterations in feeding behavior, larval weight, larval and pupal duration, and adult fertility were observed when grindelanes were added to the larval diet. Ultrastructure of the middle portion of the digestive tract of larvae revealed cell damage, thickening of the peritrophic membrane, and a striking number of mitochondria, as signs of defense against grindelanes. However, labdane insecticides had no inhibitory effects on non-target environmental bacteria.

Graphic for Table of Contents

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Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is a polyphagous migratory species, native to tropical and subtropical regions of the Americas (Andrews, 1980). It was established in western Africa in 2016, rapidly followed by detections throughout sub-Saharan Africa, India, and most recently southeastern Asia (Jeger et al., 2017; Nagoshi et al., 2020; Nboyine et al., 2020). Almost 200 hosts have been described for *S. frugiperda*, including cultivated (maize, rice, and cotton) and non-cultivated species (Casmuz et al., 2010). Two morphologically identical strains of FAW are recognized, commonly referred to as the maize strain and the rice strain, due to host preferences. There is a high level of genetic differentiation between the strains as well as differences in diurnal mating patterns and female sex pheromones. This pest categorization follows the taxonomy of Pogue and considers *S. frugiperda* as a single species with two strains (Diaz Napal et al., 2015; Jeger et al., 2017). Some biological characteristics, such as polyphagy, a high reproductive capacity, adult dispersion, and multiple generations per year, favor high infestation rates of this pest on maize throughout the year (Horikoshi et al., 2016).

Larvae affect grain productivity and cause important economic losses in Argentina and elsewhere in South America. In Brazil, it is considered to be the most important pest of maize, estimated to cause more than 400 million USD damage annually (EFSA, 2017) and in northeastern Argentina, FAW cause losses that fluctuate between 17 to 72% (Murúa et al., 2015). The FAO estimates that Brazil alone spends 600 million USD annually controlling infestations of *S. frugiperda* (Wild, 2017). A 2018 farmer survey in Ghana and Zambia reported yield losses due to FAW in maize to be 26–40 and 35–50%, respectively; estimated mean yield loss due to FAW in Ghana was 470 200 ton with a monetary value of 1.773 million USD (Nboyine et al., 2020).

Pest populations are currently being controlled using several methods that include insecticides, biological control, pheromones, host plant resistance, mechanical control, and genetically modified crops (Nair et al., 2019). Agricultural pests, such as *S. frugiperda*, have traditionally been controlled by the application of chemical insecticides. However, chemical control has many disadvantages, such as low selectivity, high toxicity toward non-target organisms such as mammals and beneficial microorganisms, low degradation (bioaccumulation), and a negative impact on the environment and human health (Goławska et al., 2014). Moreover, *S. frugiperda* has developed resistance against at least 24 active substances, including some *Bacillus thuringiensis* Berliner insecticidal proteins used in transgenic maize crops (Aguirre et al., 2016;

Abrahams et al., 2017). Consequently, more appropriate tools for rational *Spodoptera* control and sustainable grain production should be explored.

Among the natural alternatives for pest control, plants are known as main sources of allelochemicals, generated by fascinating plant-insect interactions. Today, natural products that act as molecular messengers (or chemical signals) can serve as prototypes that inspire the synthesis of new insecticide structures (Kubo, 2006; Moreno et al., 2017).

Grindelia chiloensis (Cornel.) Cabrera (Asteraceae) 'gold button' is a shrub indigenous to Patagonia, Argentina, and, like most of the species of the genus *Grindelia*, produces copious amounts of a resinous exudate through specialized ducts and glandular trichomes that accumulate on the surface of leaves, stems, and involucres of flowers (Ahmed et al., 2001). The major components of *Grindelia* resin are numerous bicyclic diterpene acids of the labdane type, called grindelic acid and its grindelane derivatives, found in dry weight percentage of 0.04–0.24% in some species (Bohlmann et al., 1982; Mahmoud et al., 2000; Ybarra et al.; 2005; Mesurado et al., 2017). Most diterpenoids represent specialized metabolites that are often species-specific and play critical roles in the ecological interactions of plants (Keeling & Bohlmann, 2006; Gershenzon & Dudareva, 2007; Hanson, 2011; Zerbe et al., 2015). Indeed, labdane-type diterpenoids isolated from foliar tissues (leaves, flowers, or herbaceous stems), and resinous plant exudates are bioactive towards different target organisms such as *Tenebrio molitor* (L.), *Leptinotarsa decemlineata* (Say), pathogenic fungi, and bacteria (Rose, 1980; Singh et al., 1999; Jassbi et al., 2002; Hernández et al., 2005; Mesurado et al., 2017). Hence, these structures are currently targets for total synthesis (Lin & Chein, 2017).

In the current study, the effects and mode of action of two natural acids and nine methylated grindelanes of *G. chiloensis* on the feeding behavior, survival, and development of *S. frugiperda* were determined. For this purpose, light and electron microscopy, quantitative analysis of larval feeding behavior, and an anti-cholinesterase activity assay were performed. The morphological alterations in the insect gut were detected through transmission electron microscopy (TEM), which was useful to evaluate the ultrastructural changes (Dequech et al., 2007) that took place after the ingestion of a diet supplemented with a bioactive grindelane over the period between the second and sixth larval stages.

Many plant- and soil-associated bacteria are involved in key biogeochemical processes and beneficial interactions with plants (Sukumar et al., 2013; de Souza Vandenberghe et al., 2017), and the grindelanes' impact on these non-target microorganisms has been evaluated. Available

insecticides 9a (acid grindelane), and methylated derivative 8b were assessed concerning their in vitro biodegradability and selectivity of action, using two indigenous non-pathogenic bacteria from soil and plants: *Lactobacillus plantarum* and *Bacillus subtilis*. In this work, we also demonstrated that these bacteria survive the treatment by releasing surface-active substances that are essential in the biodegradation processes of lipophilic substances such as grindelanes. Microbial biosurfactants/bioemulsifiers reduce surface and interface tension between liquid and solid substances that increase the bioavailability of hydrophobic substances (organic pollutants), and thus enhance biodegradation (Whang et al., 2008; Lee et al., 2018).

Materials and methods

Natural labdanes and their derivatives

Isolation and characterization of natural labdanes known as grindelanes (3a and 9a) and methylated grindelanes (1b−9b) obtained from aerial parts of a Patagonian collection of *G. chiloensis* (LIL no. 607200) used in this study were previously described by Mesurado et al. (2017). Structural identification of the compounds was performed by high resolution spectroscopic and spectrometric methods and comparison with previously reported data (Figure 1).

Pest insect

Larvae of *S. frugiperda* were collected from maize, *Zea mays* L. (Poaceae), of Tucumán cultivars (Argentina) and brought to the laboratory for rearing on an artificial diet. *Spodoptera frugiperda* individuals were maintained in a rearing chamber at 26 ± 2 °C, 60 ± 3% r.h., and L10:D14 photoperiod, and periodically (every 3 months) renewed with field specimens. The larval diet consisted of a mixture of yeast (3 g), bean boiled and milled (250 g), wheat germ (12.5 g), 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, approximately 60%).

Larval feeding behavior study

Larval food choice assay. A portion of the artificial diet was mixed with acetone, and after solvent removal in vacuo, this portion was employed as a control diet. Another portion was homogeneously mixed with an acetone solution of each compound (treatment) to leave 50 and 100 µg of each compound per g of wet diet after volatilization of acetone (0.005 and 0.01%, respectively, lower concentrations than those found in fresh leaves, see Discussion). Then, 100 mg

of control and the same amount of treated diet was placed in a glass tube (150 × 15 mm). Between the two diet portions, a second instar of similar size was introduced into the tube. The larva could choose the diet, and, when 50% of the control diet had been eaten (approximately 48 h after starting the assay), the remaining diets (control and treated) were weighed. The experiment was carried out in 20 replicates, and maintained in a rearing chamber at 26 ± 2 °C, 60 ± 3% r.h., and L10:D14 photoperiod. Results of the choice test were then reported by AI (antifeedant index) = [1 – (T/C)] 100, where T and C represent the amounts eaten by insects of treated and control diets, respectively (Alva et al., 2012). Hence, if the control diet (C) was consumed twice as much as the treated diet (T), the value of the antifeedant index (AI) would be 50.

No-choice test. Second instars of similar size were weighed and individually placed in glass tubes (150 × 15 mm). Treated and control diets, prepared as described for food choice assay, were also weighed and offered to larvae in each tube (20 replicates for control and 20 for each treatment). Larvae were allowed to eat, and, when 50% of the control diet had been eaten (approximately 48 h after starting the bioassay), the remaining diets (control and treated) were weighed. The experiment was carried out in 20 replicates and maintained in a rearing chamber at 26 ± 2 °C, 60 ± 3% r.h., and L10:D14 photoperiod. To evaluate the feeding behavior under 'no-choice' conditions, the antifeedant index AI was also calculated.

Toxicity assay and calculation of nutritional indexes. A portion of the artificial diet was impregnated with acetone and, after solvent removal in a vacuum desiccator, this portion was employed as a control diet. Another portion was homogeneously mixed with an acetone solution of each grindelane to leave 50 and 100 μg of compound per g of wet diet after volatilization of acetone, then control and treated diets were placed in test tubes (20 replicates for treated and 20 for control) with second instars (one per tube) of homogeneous size and accurately weighted and kept at 26 ± 2 °C, 60 ± 3% r.h., and L10:D14 photoperiod until the emergence of the first generation of adults. Ten days after the beginning of the experiment, the larval weight and diet consumed were recorded. Average diet consumption (CI), growth rate (GR), and efficiency of conversion of ingested food index (ECI) were calculated as follows: CI = D/t, where D is the weight of food eaten during the experimental period; the larval weight increment per day, GR = (A – B)/t, where A is the final larval weight, B is the initial larval weight, and t is the period of evaluation (t = 10 days); and ECI = [(A − B)/D]100 (Ramirez et al., 2010). From these indexes,

percent reductions were determined as follows: RCI = [(1 − $CI_{Treated}/CI_{Control}$) × 100], RGR = [(1 − $GR_{Treated}/GR_{Control}$) × 100], and RECI = [(1 − $ECI_{Treated}/ECI_{Control}$) × 100]. Lufenuron (Syngenta, Basel, Switzerland), an effective insecticide against *S. frugiperda*, was employed as a positive control at 100 µg g^{-1}. For median lethal dose (LD_{50}) determination, only the major bioactive compounds were tested in individual experiments with grindelane solutions at 12.5, 25, 50, 75, and 100 µg g^{-1} added to the diet (20 replicates for treated and 20 for control) and the larval mortality was registered. The LD_{50} values were calculated using MINITAB release 14 statistical software for Windows (Minitab, State College, PA, USA).

Additional observations were performed on sublethal effects of grindelanes throughout the fall armyworm's life cycle (up to 45 days). Larval, pupal, and adult deformities, larval and pupal stage duration, and adult emergence were recorded. Finally, the mortality of larvae and pupae was determined.

Microplate assay for AChE inhibitory activity. The assay for cholinesterase was performed according to Ellman et al. (1961) with some modifications (López et al., 2002). Fifty µl of AChE in phosphate buffer (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, pH 7.6) and 50 µl of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 min at room temperature before adding 100 µl of the substrate solution (0.1 M Na_2HPO_4, 0.5 M DTNB, and 0.6 mM ATCI in Millipore water, pH 7.5). The absorbance was read in a Thermo Scientific Multiskan FC microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 405 nm after 5 min. The enzyme inhibitory activity was calculated as a percentage compared to an assay using a buffer without any inhibitor. The enzyme inhibitory data were analyzed with the software package Prism (GraphPad, San Diego, CA, USA). The 3a and 9a, 1b–4b, 6b–7b, and 8b concentrations to calculate the IC_{50} values were 15, 30, 60, 120, 160, and 200 µM in AChE assay. The IC_{50} values are the means ± SD of three separate determinations, each performed in triplicate.

Electron microscopic studies. Morphological analysis of *S. frugiperda* larvae (from treatment and control experiments) was performed using a Zeiss EM109 (Carl Zeiss NTS, Oberkochen, Germany) transmission electron microscope (CIME-CONICET, Tucumán, Argentina). The natural compound 9a was chosen as a representative labdane for the diet treatment.

For histological studies, midgut samples (T and C) were fixed in Karnovsky's solution

(formaldehyde 2.66%, glutaraldehyde 1.66%, and sodium phosphate buffer 0.1 M, pH 7.4) and incubated overnight at 4 ºC. The fixed samples were washed 3× with the sodium phosphate buffer for 10 min each time and then fixed overnight with a 50/50 solution of sodium phosphate buffer/2% osmium tetroxide (OsO_4). The samples were washed 3× with distilled water for 1 min each time, and a 50/50 solution of phosphate buffer/uranyl acetate was added. After 30 min incubation in the dark, the solution was discarded and the samples were rapidly washed with ethanol 70, 90, and 100% for 1 min, and then dehydrated 3× with ethanol 100%, 20 min each time, and 3× in acetone for 10 min each time. Then the samples were incubated for 30 min in a 50/50 solution of acetone/Spurr resin, followed by inclusion of the specimens in Spurr resin and heated at 60 ºC for 24 h. Ultra-thin sections were made with an ultramicrotome, mounted on copper grids, and contrasted with uranyl acetate and lead citrate.

Action of plant-derived insecticides on non-pathogenic environmental bacteria

Bacterial strains and media. In the present research, *L. plantarum* CE358 and *B. subtilis* INBF1 isolated from indigenous plants and soil were screened. The culture medium employed was PTYG (15 g l^{-1} peptone, 10 g l^{-1} tryptone, 10 g l^{-1} yeast extract, and 5 g l^{-1} glucose, pH 6.0). These bacteria were isolated and identified by Dr. SN González (CONICET, Argentina).

Determination of surface activity. In total 10 µl of each compound (8b and 9a) was incorporated into the culture medium at a final concentration of 100 µg ml^{-1}. Then, the liquid medium was inoculated with the assayed bacterium, according to CLSI (2006). After 24 h incubation at 30 °C, bacterial growth was measured at 600 nm using a microtiter plate reader (Power Wave XS2; Biotek, Winooski, VT, USA), and viable cells were counted by plating onto an agar plate. Bacterial ability to produce surface-active substances, useful to biodegradation of lipophilic substances, was explored in the following step.

Each bacterial strain was statically cultured in 10 ml of PTYG supplemented with each bioinsecticide (8b and 9a at 100 µg ml^{-1}, separately) for 7 or 14 days at 30 °C in duplicate in 10-ml borosilicate glass flasks. The inoculum was adjusted to 10^5 UFC ml^{-1}. Besides, control experiments were conducted without the assayed grindelane compounds. After incubation, the bacterial cells were removed by centrifugation for 5 min. A portion of each supernatant was subjected to an oil spreading assay to evaluate its surface activity.

The oil spreading assay is a rapid and highly sensitive method for surface-active substance

detection (Sambanthamoorthy et al., 2014). For the bioassay, 20 µl of mineral oil was placed on a crystallizer (180 mm in diameter) with demineralized water (200 ml). Then, 10 µl of each supernatant was separately put in the center of the oil film. If biosurfactant is present in the supernatant, the oil is displaced, and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. For pure biosurfactant, a linear correlation between the quantity of surfactant and clearing zone diameter is given (Walter et al., 2013). The diameters of clear halos (mm) visualized under visible light were measured in quintuplicate concerning the control supernatant. Tween 80 (Polysorbate 80; Merck, Darmstadt, Germany) was employed as a non-ionic surfactant and emulsifier (positive control). The negative controls were the culture medium (without bacteria) and solutions of each compound assayed.

Thin-layer chromatography. Chloroform extracts obtained of each supernatant were chromatographed by TLC (Silica gel 60 GF_{254}, Merck) using the Godin reagent to detect grindelanes and standards of compounds 8b and 9a. The chloroform: ethyl acetate (50:50) system was employed as an optimum mobile phase.

Partition coefficient prediction. The typical quantitative descriptor of lipophilicity, *n*-octanol/water partition coefficient, was calculated using the Chem 3D Ultra program after minimizing grindelane energy by the semi-empirical MM2 program (Cambridge Scientific Computing, Cambridge, UK) (Mesurado et al., 2017).

Statistical analysis

Differences in the mean values were evaluated by ANOVA for one-way classification followed by Tukey's multiple range test by using STATISTIX 10 (Analytical Software, Tallahassee, FL, USA). In all analyses, $\alpha = 0.05$.

Results

Antifeedant activity

None of the tested compounds stimulated the feeding behavior of *S. frugiperda* (Table 1). The incorporation of 100 µg of 3b and 9b per g to the larval diet produced 79 and 67% of deterrence of feeding, respectively, compared to control. Diets treated with the grindelanes 1b, 2b, 3a, 4b, 5b,

6b, 7b, 8b, and 9a also produced 32–65% of feeding deterrence. Similar effects were observed at 50 μg g^{-1}, with 3b and 9b also the most active at this concentration (64 and 58%, respectively; Table 1). In contrast, lufenuron had a mean (± SD) antifeedant index of 23.47 ± 2.49 with the high concentration assayed (Table 1).

Toxic and sub-lethal effects on *Spodoptera-frugiperda*

Toxicity assays allowed us to evaluate significant inhibitory effects on growth and larval feeding behavior exerted by grindelanes relative to the control group (Table 2). Strong reductions of >90% in larval growth (Figure 2) were triggered by the ingestion of a diet containing 2b, 3a, 7b, 8b, or 9a at 100 μg g^{-1}; these effects were closely related to a significant reduction of the consumed diet (RCI% = 67−72).

Grindelanes 2b, 3a, 5b, 6b, 8b, and 9a added to the larval diet of *S. frugiperda* at 100 μg g^{-1} produced 100% larval mortality 45 days after starting feeding. At half-maximum concentration, mortality diminished from 30 to 45% (Table 3), except for the most potent grindelane 7b which caused 100% larval mortality at 21 days, for both concentrations. Comparatively, lufenuron at 100 μg g^{-1} of diet brought about 100% larval mortality on *S. frugiperda* only 7 days after administration.

At the highest concentration, some grindelanes produced sub-lethal effects during all life cycle of *S. frugiperda*, including larval and pupal malformations (Figures 3A,B), decreases in mobility, and extension of the duration of the larval stage in relation to the untreated control group. Pupal mortality was 15−30% (Table 3). All compounds at 50 μg g^{-1} prolonged larval and pupal periods, decreased pupal size, produced pupal mortality of 5−20%, and the few surviving adults were unable to lay fertile eggs.

Mortality was mainly triggered by grindelanes that altered the molting process, resulting in larvae with exuvia retention (Figure 3A), pupae with retention of larval characteristics (Figure 3B), and adults with pupal characteristics (Figure 3C). Emerged adults showed 100% oviposition inhibition caused by atrophy of the wings and legs that did not allow copulation (Figure 3D), unlike the control group (Figure 3E).

Anti-cholinesterase activity of insecticide grindelanes

In the anti-cholinesterase activity test, compound 8b inhibited acetylcholinesterase significantly with an IC_{50} = 57.04 ± 1.20 μM, exerting a probable neurotoxic mode of action. Acid compounds

3a and 9a and methylated grindelanes 1b−4b and 6b−7b had IC_{50} >200 μM; hence, they were not active against acetylcholine esterase enzyme. The positive control galantamine gave an IC_{50} = 0.61 ± 0.05 μM.

Microscopic findings into insect-tissues

To evaluate chronic post-ingestive effects of natural grindelanes, gut morphology studies were carried out using sixth instars fed from the second instar on an artificial diet treated with acid grindelane 9a, and larvae control. Larvae fed on a diet supplemented with 100 μg g^{-1} of bioactive compound 9a showed obvious signs of dehydration and the collapse of the abdominal segments associated with the consumption of the treated diet (Figure 2).

The electron microscopy technique made it possible to visualize ultrastructural alterations in the *S. frugiperda* larva gut caused by the incorporation of compound 9a into the diet. The most vulnerable part of the insect gut to foreign substances included in the diet was the midgut (mesenteron), and notable changes in the gut cells were observed.

The microphotograph of a midgut portion of the control shows typical microvilli, a peritrophic membrane, and firm epithelial tissue with ordered cells (Figure 4A). In contrast, the treated larva exhibits midgut alterations caused by grindelane 9a, such as a thickening of the peritrophic membrane (Figure 4B). The cell deterioration can be observed at a magnification of 4 800×, as seen in Figure 4B.

Also, abundant mitochondria in cells close to the intestinal lumen were observed (Figure 4C). These microscopic findings were not observed in any of the control samples.

Impact of plant-derived insecticides on non-target plant and soil bacteria

Acid and methylated grindelanes 8b and 9a did not exert growth inhibitory effects on non-pathogenic environmental bacteria at 100 μg ml^{-1} (Figure 5). Chromatographic profile analyses of the supernatants obtained by incubation of environmental bacteria in the presence of *G. chiloensis* insecticides revealed the absence of 8b (100 μg ml^{-1}) in 1 week for both strains, whereas the 9a biodegradation took place in 3 weeks under the same conditions.

Lipophilic grindelanes 8b and 9a increased the surface-active substance biosynthesis which was inferred by the oil spreading halo measurements of the supernatants obtained from the treated cultures in comparison with the control supernatant [mean (± SD) halo diameter of 117.5 ± 10 mm with an increment of 161%, and 150 ± 0 mm with an increment of 233%, respectively vs. 45 ± 7

mm for *B. subtilis*]. Milder results were observed with the *L. plantarum* supernatants with regards to the control supernatant (150 ± 0 mm from the culture supplemented with methylated grindelane 8b vs. 145 ± 4 mm from the culture supplemented with acid grindelane 9a (with increments of 11 and 7%, respectively), and from *L. plantarum* supernatant without grindelanes (135 ± 1 mm). The bacterial supernatants from treated and untreated cultures were more potent than Tween 80 (46.7 ± 2.9 mm; Figure 5).

It is important to note that 8b, 9a, and the solvent system of the compounds previously added to the culture medium did not exert any surface activity by themselves.

Discussion

Plants have been successful in colonizing most environments and their success is due in part to their ability to resist or tolerate herbivore attack. Interactions between plants and insect herbivores are important determinants of plant productivity in managed and natural vegetation. In response to insect attack, plants have evolved a range of defenses to reduce the threat of injury and loss of productivity (Mitchell et al., 2016). In the search for new strategies in crop protection, plant insecticides and plant-derived semiochemicals are being considered as good candidates for agricultural pest control and more sustainable alternatives (Bullangpoti et al., 2012). In this context, we have explored for the first time the antifeedant and insecticidal properties of pure grindelanes from the Argentinean endemic species *G. chiloensis*. Remarkable alterations in feeding behavior, larval weight, larval and pupal stage lengths, and adult fertility, as well as malformations and mortality exerted by labdanes against *S. frugiperda* (strain maize), that cause important losses of local agricultural production, were demonstrated in the present study. Methylated derivative 7b (LD_{50} = 26.49 μg g^{-1}) was the most active grindelane and only 8b was potentially neurotoxic due to acetylcholinesterase inhibition.

Antifeedant indexes (AI%) calculated in food preference assays fluctuated from 20 to 79% for all substances at 100 μg g^{-1} and higher than those obtained by non-choice tests. The results in both bioassays were coherent and stronger than those found with lufenuron at 100 μg g^{-1}. Hassanali & Bentley (1987) consider that the deterrent effect is strong when AI values exceed 75%; hence, 3b at 100 μg g^{-1} had a high antifeedant effect under choice test conditions.

It is important to note that the lipophilic properties of 3b and 9b were higher than those of their acids as previously determined by Mesurado et al. (2017). Thus, methylated derivatives could be most persistent in the tissues and sensorial organs, and for this reason, *S. frugiperda* larvae

would show greater rejection of the treated diet with these lipophilic grindelanes. According to Lakshmanan et al. (2012), these antifeedant substances can be described as allomones, which inhibit feeding and do not kill the insect pests directly, but rather limit their developmental potential considerably and act as a phagodeterrent or phagorepellent (Marcinkevicius et al., 2017). None of the assayed grindelanes had a phagostimulant activity.

All assayed botanical insecticides and their derivatives were more effective as antifeedants than agrochemical lufenuron at 100 μg g^{-1}. In the toxicity test, 7b was the most potent with an LD_{50} of 26.49 (33.55−19.43) μg g^{-1}. This compound produces a strong growth reduction (96%) and a significant reduction in food consumption (64%) even at the lowest concentration assayed, leading to 100% larval mortality between 4 and 21 days after starting the bioassay, whereas lufenuron (100 μg g^{-1}) produces total larval mortality only after 7 days.

On the other hand, a decrease in the ECI values indicates that more ingested food is metabolized to obtain energy, and a lower amount is converted into biomass. Diversion of energy to other metabolic pathways, such as those involved in detoxification of allelochemicals, may be the cause of the decrease in efficiencies (Koul & Isman, 1991). Hence, 7b would produce a chronic post-ingestive toxic effect due to a strong drop in the ECI value (RECI 90%). Similar results were also seen with *Trichilia americana* (Sessé & Mociño) TD Pennington extract on *S. litura* (Wheeler & Isman, 2001).

An interesting structure-activity relationship to consider is that the presence of two ester functions in molecule 7b increases insecticidal activity 5× in relation to the monoester derivative 4b (less active). This result could be due to a decrease of lipophilic properties (7b has a theoretical *n*-octanol/water partition coefficient of 4.81, and 4b one of 2.44).

All grindelanes altered the molting process, resulting in larvae with exuvia retention, pupae with retention of larval characteristics, and adults with pupal characteristics evidencing an incomplete metamorphosis. The results suggest that diterpenoid grindelanes affected hormonal processes, such as those carried out by ecdysone and the juvenile hormone. Many natural steroids and terpenoids were used for disrupting the insect life cycle. Ecdysone agonists are highly selective against lepidopteran larvae, without affecting non-target organisms. Moreover, their narrow spectrum of activity, positive ecotoxicological profile, and short persistence in the environment make these compounds promising against many economically important agriculture and forest pests (Zarate et al., 2011; Sláma, 2013).

It is also important to investigate the anticholinesterase action of active metabolites against

the *S. frugiperda* model, to propose a probable mode of insecticidal action. Cholinesterase (or acetylcholinesterase) is an essential enzyme for normal functioning of the nervous system of insects. Acetylcholinesterase inactivates the chemical messenger acetylcholine, which is normally active at the junctions between nerves and muscles, between many nerves and glands, and at the synapses between certain nerves in the central nervous system. When cholinesterase levels are low because of excessive inhibition, the nervous system can malfunction, which can lead to death. Certain chemical families of pesticides, such as organophosphates and carbamates primarily work (with a few exceptions) against arthropod pests by interfering with or inhibiting cholinesterase (Fishel, 2015). Our results demonstrated that only 8b significantly inhibited acetylcholinesterase (IC_{50} = 57.04 ± 1.20 µM). Likewise, Ortiz et al. (2016) reported that alkaloids (montanine and homolycorine types) isolated from the bulbs of *Hippeastrum argentinum* (Pax) Hunz. showed a moderate cholinesterase inhibitory activity. However, 17-hydroxycativic diterpenic acid isolated from *Grindelia ventanensis* A Bartoli & RD Tortosa displayed a better inhibitory activity (IC_{50} = 21 µM) (Alza et al., 2014).

Dequech et al. (2007) consider morphological studies an important tool to understand the mode of action of natural products. From this point of view, the findings found at the level of the intestinal ultrastructure would indicate that one of the modes of action of the grindelanes would be stress. Our results show that amphoteric compounds, such as grindelane 9a that contain a polar head (COOH) and a lipophilic skeleton, alter the perithrophic membrane (PM) of the phytophagous insect *S. frugiperda*. Thickening of the PM by ingestion of the treated diet indicates a clear insect defensive response. The PM is an acellular sheath made of chitin that protects midgut epithelial cells acting like a physicochemical barrier against the entry of pathogens and toxic substances. This is considered as a potential line of defense that reduces or eliminates the absorption of certain allelochemicals, thus controlling detoxification processes (Barbehenn, 2001; Corzo et al., 2012). Certain sesquiterpenoids, diterpenoids, and neem oil induced histo-physiological alterations in the peritrophic matrix (Roel et al., 2010; Corzo et al., 2012). Moreover, the abundant number of mitochondria in the treated samples would be a defense signal against toxic substances ingested by the larvae. These organelles are essential to generate the development of the first line of physical defense of the immune system that protects the midgut.

In vitro full degradation of bioactive grindelanes (8b and 9a) mediated by two indigenous environmental bacteria that synthesize surface-active substances was demonstrated for the first time in this study. Indeed, 8b and 9a, and the solvent system of these compounds previously added

to the culture medium, did not exert any surface activity by themselves. Therefore, the increase in *Lactobacillus* supernatant surface activity would be due to an increase in surface-active substances by an induction to the biosynthesis thereof in the bacterial cultures (Verni et al., 2020). The degradation process for 8b took place during the 1st week, whereas for 9a it took 2 weeks under the same conditions. This difference could be explained by the fact that compound 9a is more lipophilic and could remain longer in lipid membranes. Indeed, natural grindelane 9a has a theoretical partition coefficient of 4.63, and 8b one of 3.97.

It is important to note that surface-active substances optimize degradation processes of lipophilic substances or toxins for bacteria and their biosynthesis occurs as an effective adaptive stress response (Whang et al., 2008; Fracchia et al., 2010; Luque Castellane et al., 2017). Bioactive grindelanes displayed a selective activity because they were active against *S. frugiperda*, but they did not exert growth inhibitory effects on non-target environmental bacteria such as *B. subtilis* and *L. plantarum*. These species are involved in biogeochemical processes and beneficial plant-microbial interactions and are therefore considered as plant probiotic microorganisms in agriculture and forestry (Sukumar et al., 2013; de Souza Vandenberghe et al., 2017).

In conclusion, our results contribute to the knowledge of *G. chiloensis* as a rich resource of botanical insecticides against *S. frugiperda*. The isolated labdane diterpenoids from a few grams of the aerial parts of this species displayed significant insecticidal effects and alterations in the feeding behavior of *S. frugiperda* larvae. Total grindelane content in dry aerial tissues of *G. chiloensis* was 0.13% (Mesurado et al., 2017), and 0.08% in fresh plant material, 8× higher than that used in the bioassays (0.01%). This fact suggests that grindelanes may play a strategic role in plant-insect interactions as antifeedants and insecticides. Besides, these lipophilic diterpenoids would produce synergistic effects as previously observed by Gershenzon & Dudareva (2007) in other terpenoids. This leads us to assume that lipophilic grindelanes exert an important role in plant defense in nature.

The malformations observed during the FAW life cycle and adult infertility favor insect population control. Also, the plant-derived insecticides did not exert a growth-inhibitory action on non-target bacteria. These results led us to promote the growth and conservation of *G. chiloensis*, and its metabolites could be used as models for the development of new biorational insecticides for the control of this important American maize pest.

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Figure legends

Figure 1 Chemical structures of grindelanes 1–9.

Figure 2 *Spodoptera frugiperda* larva: control (left) and treated with 9a at 100 μg g^{-1} (right).

Figure 3 (A–D) Malformations on *Spodoptera frugiperda* life cycle stages and (E) control adult. (A) Larva deformed by treatment with 1b at 100 μg g^{-1}. (B) Control pupa (left) and pupa with larval characteristics (right) by treatment with 9b at 100 μg g^{-1}. (C) Pupal-adult intermediate retaining the pupal characters by treatment with 9a at 100 μg g^{-1}. (D) Adult with deformed wings, legs, and antennae by treatment with 3b at 100 μg g^{-1}.

Figure 4 Microphotographs (4 800×) of (A) midgut epithelial cells of control *Spodoptera frugiperda* larva with microvilli (MV) and vacuoles with intestinal content (V), and (B) fragment of midgut of larva treated with 100 μg g^{-1} of compound 11a showing thickened peritrophric membrane (PM), epithelial cell damage (CD) around a microvillous, and a regenerative cell (RC).

(C) Abundant mitochondria in the midgut of the treated larva (7 500×).

Figure 5 (A) Mean (± SD) relative bacterial growth (%) of non-pathogenic environmental bacteria – *Bacillus subtilis* (Bs) and *Lactobacillus plantarum* (Lp) –, without (control) or incubated with solutions of 8b and 9a at 100 µg ml^{-1}, measured at 600 nm using a microtiter plate reader. (B) Surface activity of supernatants [mean (± SD) diameter of halos, mm]. Means within a panel capped with the same letter are not significantly different (Tukey's test: $P>0.05$).

Table 1 Effects of grindelanes 1–9 (see Figure 1) from *Grindelia chiloensis* aerial parts at 50 and 100 µg g^{-1} on the feeding behavior of *Spodoptera frugiperda* larvae: mean (± SD; n = 20) antifeedant index (AI) = [1 – (T/C)] × 100, where C and T represent the amount of control and treated diets, respectively, consumed during choice or no-choice tests. AI >75% indicates a highly deterrent activity, whereas AI between 75 and 50% indicates a moderate activity

	Choice		No-choice	
	50 µg g^{-1}	100 µg g^{-1}	50 µg g^{-1} [1]	100 µg g^{-1}
1b	45.09 ± 4.01a	51.89 ± 4.97a	17.55 ± 1.21a	22.24 ± 1.80ab
2b	28.81 ± 3.69bcd	44.92 ± 5.20b	13.58 ± 2.21bc	20.92 ± 1.71ab
3a	16.30 ± 0.77e	32.26 ± 3.16c	15.60 ± 1.26abc	22.11 ± 3.43ab
3b	63.73 ± 3.17f	79.22 ± 2.81d	27.42 ± 2.95d	30.16 ± 2.89c
4b	28.87 ± 0.99bcd	64.93 ± 3.03e	14.47 ± 2.77bc	27.74 ± 4.72cde
5b	36.12 ± 4.96g	49.70 ± 6.41ab	16.49 ± 1.66ab	23.37 ± 1.51abe
6b	25.48 ± 2.50h	43.96 ± 4.22b	16.12 ± 1.97ab	25.36 ± 5.10ade
7b	30.27 ± 2.99bc	42.85 ± 5.36b	27.61 ± 3.27d	37.71 ± 4.23f
8b	26.77 ± 3.74ch	46.14 ± 6.33ab	9.14 ± 0.83e	19.55 ± 3.43b
9a	19.34 ± 2.75e	47.96 ± 4.67ab	12.96 ± 1.72b	28.27 ± 3.48cd
9b	58.50 ± 4.69f	67.07 ± 1.33e	23.12 ± 3.23f	29.44± 3.72cd
Lufenuron (100 µg/g)	ND	23.47 ± 2.49f	ND	13.07 ± 1.61g

Means within a column followed by the same letter are not significantly different (Tukey's test: P>0.05).

ND, not determined.

Table 2 Mean (± SD; n = 20) nutritional reductions (%) produced by grindelanes from *Grindelia chiloensis* aerial parts at 50 and 100 µg g^{-1} on *Spodoptera frugiperda* larvae, based on three nutritional indices

	50 µg g^{-1}			100 µg g^{-1}		
	RCI	RGR	RECI	RCR	RGR	RECI
1b	16.96 ± 2.30a	29.03 ± 4.15ab	14.05 ± 1.61ab	32.92 ± 5.17ab	53.48 ± 4.89a	32.68 ± 4.38a
2b	15.72 ± 2.86a	28.73 ± 3.32ab	15.55 ± 2.53a	71.75 ± 4.70c	95.15 ± 1.92b	79.02 ± 7.34b
3a	36.92 ± 7.29b	62.25 ± 7.32c	46.78 ± 4.22c	72.44 ± 6.58c	95.73 ± 1.86b	83.16 ± 2.37b
3b	30.74 ± 4.27c	35.40 ± 6.80d	7.11 ± 0.99de	34.91 ± 5.92a	41.54 ± 6.65c	10.55 ± 1.79c
4b	9.18 ± 0.95d	20.23 ± 3.54e	14.82 ± 3.85a	35.60 ± 3.10a	34.77 ± 5.45d	3.14± 0.30d
5b	31.17 ± 4.72c	37.04 ± 3.52d	8.84 ± 0.22de	72.88 ± 3.73c	86.09 ± 3.95e	48.94 ± 7.93e
6b	23.62 ± 3.82e	33.23 ± 3.14ad	12.62 ± 2.67ab	52.88 ± 6.42d	75.18 ± 8.23bf	49.05 ± 5.46e
7b	64.27 ± 2.94f	96.42 ± 0.84f	90.00 ± 2.26f	66.78 ± 3.03c	96.82 ± 0.88b	90.45 ± 2.66f
8b	24.39 ± 3.69e	31.77 ± 5.01ad	10.34 ± 1.6bd	72.50 ± 5.49c	93.76 ± 2.14b	77.13 ± 7.88b
9a	31.07 ± 4.96c	50.25 ± 5.96g	28.87 ± 6.35g	71.55 ± 6.90c	95.39 ± 1.71b	82.56 ± 3.69b
9b	31.68 ± 4.94c	32.38 ± 5.56ad	6.59 ± 0.97de	33.88 ± 4.20ab	35.47 ± 6.67cd	7.55 ± 1.09g
Lufenuron (100 µg/g)	ND	ND	ND	–	–	–

$RCI = [(1 - CI_{Treated}/CI_{Control}) \times 100]$, $RGR = [(1 - GR_{Treated}/GR_{Control}) \times 100]$, $RECI = [(1 - ECI_{Treated}/ECI_{Control}) \times 100]$, where 'R' indicates reduction, CI (average diet consumption) = D/t (t = 10 days, and D = weight of food eaten during 10 days), GR (growth rate) = (A – B)/t, (A = final larval weight, B = initial larval weight), and ECI (efficiency of conversion of ingested food) = $[(A - B)/D] \times 100$.

ND, not determined; –, dead larvae.

Means within a column followed by the same letter are not significantly different (Tukey's test: $P>0.05$).

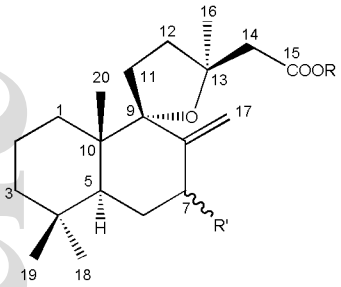
Table 3 Median lethal dose (LD_{50}, with the 95% confidence intervals in parentheses) and mean (± SD; n = 20) sublethal effects of grindelanes from *Grindelia chiloensis* aerial parts at 50 and 100 µg g^{-1} throughout the life cycle of *Spodoptera frugiperda*: larval and pupal period duration (days), larval and pupal mortality (%), and deformation of emerging adults (%)

	LD_{50}	Larval period (days)		% larval mortality		Pupal period (days)		% pupal mortality		% deformed emerging adults	
Control		16.40 ± 0.94a		-		8.74 ± 0.80a		-		-	-
		50 µg g^{-1}	100 µg g^{-1}	50	100	50	100	50	100	50	100
1b	ND	19.00 ± 1.69b	22.50 ± 3.50b	25	35	8.75 ± 1.06a	8.45 ± 1.43a	20	25	10	15
2b	69.01 (61.32-76.70)a	18.33 ± 1.18b	ILC	30	100	10.23 ± 1.30b	-	20	-	15	-
3a	58.33 (51.46-65.20)a	16.38 ± 0.96a	ILC	45	100	11.85 ± 0.80bc	-	20	-	15	-
3b	ND	18.25 ± 3.30b	17.43 ± 0.76c	20	30	9.36 ± 1.15bd	9.67 ± 0.71b	10	30	10	10
4b	ND	11.89 ± 2.20c	19.76 ± 1.75d	30	40	10.00 ± 0.74b	18.43 ± 1.55c	10	15	5	15
5b	69.01 (61.32-76.70)a	22.06 ± 2.59d	ILC	30	100	16.46 ± 1.05e	-	20	-	15	-
6b	63.31 (53.21-73.42)a	18.36 ± 1.22b	ILC	40	100	11.14 ± 0.95bc	-	10	-	5	-
7b	26.49 (33.55-19.43)b	ILC	ILC	100	100	-	-	-	-	-	-
8b	65.89 (56.95-74.84)a	18.83 ± 1.19b	ILC	35	100	10.60 ± 2.03b	-	20	-	10	-
9a	63.18 (56.02-70.33)a	17.94 ± 1.91ab	ILC	40	100	13.50 ± 1.26f	-	20	-	20	-
9b	ND	16.88 ± 0.72a	18.69 ± 2.39de	35	25	8.93 ± 0.73a	8.91 ± 0.83a	5	15	10	10
Lufenuron (100 µg/g)	ND	ND	ILC	ND	100	–	–	ND	–	ND	–

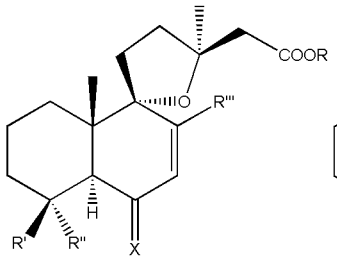
Means within a column followed by the same letter are not significantly different (Tukey's test: P>0.05).

Lufenuron at 100 µg g^{-1} exerted 100% larval mortality after 7 days.

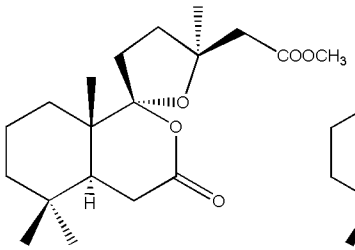
ILC, incomplete larval stage (dead larvae); ND, not determined; –, without individuals (pupae).



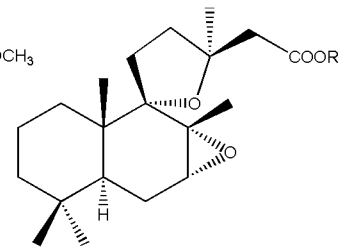
1b R = Me, R' = *B*-OH
2b R = Me, R' = *a*-OH



3a R = H, R', R'', R''' = Me, X = O
3b R, R', R'', R''' = Me, X = O
4b R, R'', R''' = Me, R' = OH, X = O
5b R, R'', R''' = Me, R' = CH_2OH, X = H_2
6b R, R', R''' = Me, R'' = CH_2OH, X = H_2
7b R, R', R''' = Me, R'' = COOH, X = H_2



8b



9a R = H
9b R = Me

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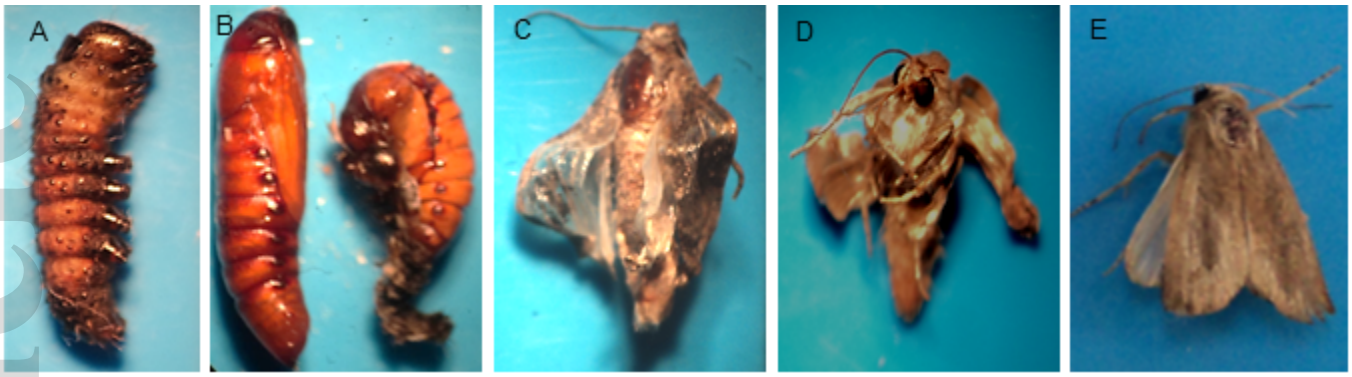
eea_13067_f2.tif



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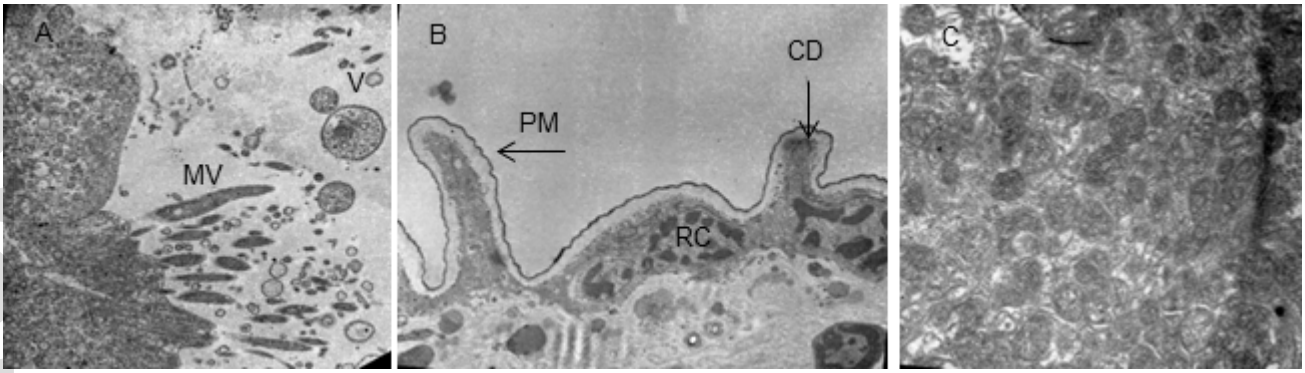

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