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PEST MANAGEMENT

Toxicity of *Porella chilensis* Sesqui- and Diterpenoids against Larvae of the Corn Pest *Spodoptera frugiperda* (J.E. Smith) (Lepidotera: Noctuidae)

FL Corzo[1,2], M Gilabert[1,3], MF Alcaide[4], A Bardón[1,3]

[1]Fac de Bioquímica, Química y Farmacia, Univ Nacional de Tucumán, Tucumán, Argentina
[2]Univ Nacional de Chilecito, Chilecito, Argentina
[3]INQUINOA-CONICET, Tucumán, Argentina
[4]Instituto de Morfología Animal, Fundación Miguel Lillo, Tucumán, Argentina

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Correspondence
A Bardón, Fac de Bioquímica, Química y Farmacia, Univ Nacional de Tucumán, Ayacucho 471, Tucumán 4000, Argentina; alisan@fbqf.unt.edu.ar

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Abstract
Porella, the largest genus of the family Porellaceae (Hepaticae) is widespread in the tropical and subtropical regions of South America. Most *Porella* species are rich sources of sesqui- and diterpenoids, many of which show interesting biological activities. Secondary metabolites produced by plants can interact with insects and act as antifeedants and growth regulators affecting hormone and nervous systems as well as stomach and muscle tissues. A previous chemical investigation of a Patagonian collection of *Porella chilensis* yielded sesqui- and diterpenoids that were now evaluated for their effects against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), a serious pest affecting corn crops mainly in the Americas. Four pinguisanes (1–4), three fusicoccanes (5–7), and one aromadendrane (8) from *P. chilensis* displayed larvicidal activity against *S. frugiperda* when incorporated to the larval diet at 100 and 200 μg/g of diet with a significant decrease in the larval growing rate. The observed effects were in part produced by severe alterations of the epithelial cells of the midgut as indicated by our histological studies.

Introduction

Studies on insect–plant interactions are relevant for the development of new plant-based pesticides. Interactions involve plant secondary metabolites that act as antifeedants and growth regulators affecting hormone and nervous systems as well as stomach and muscle tissues (Senthil-Nathan *et al* 2008).

The alimentary canal of insects consists of three zones, foregut, midgut, and hindgut. In Lepidoptera, the midgut epithelial tissue is made of columnar, goblet, endocrine, and regenerative cells which are involved in the processes of enzyme absorption and secretion, ionic homeostasis, and epithelium renewal (Sousa *et al* 2009). Epithelial cells of the midgut are protected from the contents of the gut by a delicate acellular sheath made of chitin called perithrophic membrane (PM). PM is secreted by digestive epithelial columnar cells surrounding the food eaten by the insect and can act as a physical and chemical barrier to the entry of pathogens. This membrane compartmentalizes digestion and protects microvilli of epithelial cells from direct contact with food particles, allowing the passage of digestive enzymes into the lumen, and consequently, the absorption of digestion products (Lehane 1997, Terra 2001). It is potentially a first line of defense reducing or eliminating the absorption of certain allelochemicals (Barbehenn 2001), therefore controlling detoxification processes.

Sousa *et al* (2009) reported that alterations in the midgut affect the growth and development of insects as result of physiological changes in which absorption and food transformation are involved. Many reports indicate

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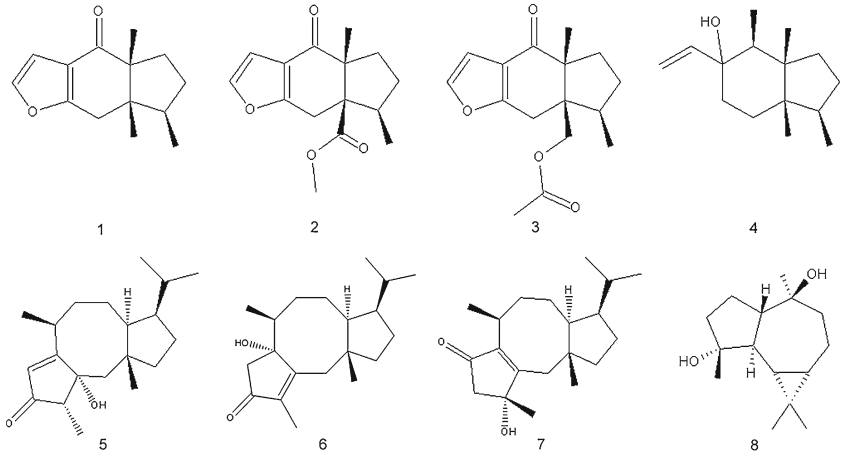


Fig 1 Compounds evaluated: *1* norpinguisone, *2* norpinguisone methyl ester, *3* norpinguisone acetate, *4* pinguisenol, *5* andadensin, *6* fusicoauritone, *7* 3α-hydroxyfusicocc-2(6)-en-5-one, and *8* ent-4β,10α-dihydroxyaromadendrane.

that natural products of plants can produce histological alterations in the alimentary canal of insects when incorporated to the larval diet. Epithelial midgut cells of larvae of the lepidopteran *Plodia interpunctella* (Hübner), which fed on diets treated with the natural products harmaline (at 250 µg/g) and 20-hydroxyecdysone (at 50 µg/g) exhibited severe damage (Rharrabe *et al* 2007, 2009), while *Spodoptera frugiperda* (J.E. Smith) larvae that fed on 60 µg/g neem oil showed columnar, regenerative, and caliciform cells necrosis (Roel *et al* 2010). PM was damaged and it mixed with epithelial cells of the lepidopteran *Spodoptera exigua* (Hübner) after treatment with the natural product goniothalamin at 10 µg/g (Senthil-Nathan *et al* 2008). Based on the aforementioned results and due to *S. frugiperda* larvae causes severe damage by consuming foliage of more than 80 species of plants, including many of agricultural importance (e.g., corn, cotton, sorghum, and diverse pasture grasses) (Murúa & Virla 2004), we decided to evaluate the toxicity and effects on the alimentary canal produced by the terpenoids 1–8 (Fig 1), previously isolated from an Argentine collection of *Porella chilensis* (Gilabert *et al* 2011), to larvae of *S. frugiperda*. The aim of this study was to assess the effects of *P. chilensis* terpenoids on the development and survival of *S. frugiperda* larvae by assessing histological alterations caused in the midgut.

Material and Methods

Test insects and diet

Larvae were obtained from our laboratory colonies, originally collected from corn plants (*Zea mays*) and maintained on artificial diet during four generations. Colonies had not been previously exposed to insecticides, and were maintained at controlled conditions (25±1°C; 60–70% RH; 12 h photophase) on a bean-based diet prepared as described elsewhere (Murúa *et al* 2003).

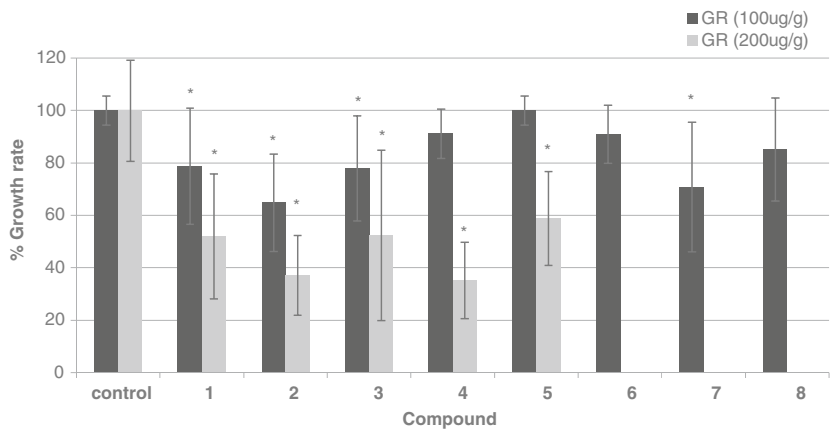


Fig 2 Growth rate (percentage) of treated larvae of *Spodoptera frugiperda* as compared to control larvae (considered as 100%) after 14 days of exposure to one of several terpenoids (n=20). *Asterisk* For treatments at 100 µg/g, compounds *1*, *2*, *3*, and *7* show significant differences in comparison with the control group (Dunnet's test, F=7.27, df=126, P<0.001). For treatments at 200 µg/g, all compounds show significant differences in comparison with the control group (Dunnet's test, F=18.4, df=81, P<0.001).

Toxicity bioassay

A portion of the artificial diet was impregnated with acetone and, after solvent evaporation, this portion was employed as control. Another portion of diet was impregnated and thoroughly mixed with a solution (in acetone) of pure compounds in order to leave 100 and 200 µg of each one per gram of diet. After evaporation of the solvent, control and treated diets were placed in test tubes (15×1.5 cm, 20 replicates for control and 20 for treated). Accurately weighed larvae of homogeneous size (second instar) were individually placed in each tube and kept under the earlier mentioned rearing conditions. Test tubes were covered with a small piece of moistened cotton to prevent diet desiccation. Larvae were allowed to feed on weighed diet (50 mg per day) until emergency of the first generation of adults. Fourteen days after the beginning of the experiment, the larval weight was determined again, in order to record the larval growth rate [GR=$(A-B)/t$], which gives the average of larval weight increment per day [A=final larval weight, B=initial larval weight, and t=14 days (period of the evaluation)]. The amount of diet consumed by the larva was also calculated for the same period (14 days) in order to determine the consumption index [CR=D/t, is the average of the larval diet consumed per day where D is the total weight of food consumed during the 14 days of experiment]. In addition, adult malformations were also evaluated (Ramírez *et al* 2010).

For the histological analysis, larvae (from treatment and control experiments) were fixed in Bouin's fixative (Carson 1997) for 24 h. After dehydration in a graded ethanol series (70%, 90%, and 100%), larvae were incorporated in pure *n*-butanol for 24 h to soften the cuticle. Larvae were placed in a mixture of *n*-butanol-histowax (1/1) at 60°C for 24 h, and then in pure histowax at 60°C for 24 h. Blocks were cut into 5–7 µm thick sections in a Minot microtome (LEICA RM 2035) and stained with hematoxylin and eosin. Histological analysis was performed in a LEICA DM 1000 microscope and photographs were taken in a SONY DSC-W 100 microcamera.

Table 1 Mortality rates (percentage ± SEM) of *Spodoptera frugiperda* larvae fed on a bean-based artificial diet amended with 100 or 200 µg/g of compounds 1–8 for 14 days (n=20).

Compound	Larval mortality (%)	
	100 µg/g	200 µg/g
Control	5±1.1	5±1.1
1	30±2.3	50±2.5
2	20±2.0	45±2.5
3	20±2.0	30±2.3
4	25±2.2	40±2.4
5	5±1.1	10±1.5
6	15±1.8	ND[a]
7	20±2.0	ND[a]
8	30±2.3	ND[a]

[a] Not determined.

Statistical analysis

Differences in the mean values were evaluated by analysis of variance for one-way classification followed by a post hoc analysis using Dunnet's test (P<0.05) by using the SigmaPlot Version 12.0.

Results and Discussion

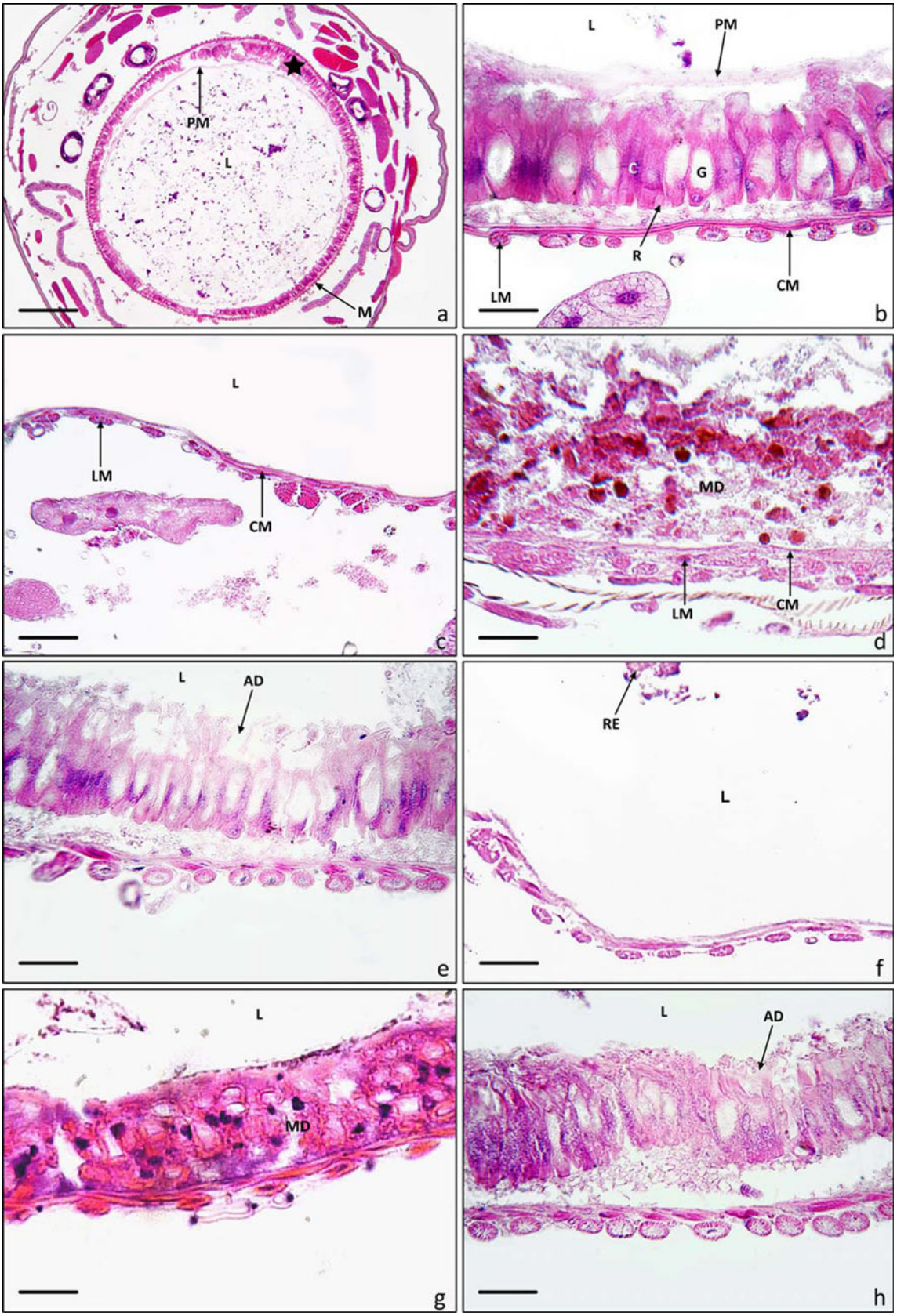
Purification of terpenoids 1–8

Compounds 1–8 (Fig 1) had been previously isolated from an Argentine collection of the liverwort *P. chilensis* (Gilabert *et al* 2011). Purity and chemical structures were assessed employing HPLC (single peaks in the chromatogram) and spectroscopic data which were compared with those previously reported.

Toxic effects on S. frugiperda larvae

Compounds 1–8 were incorporated to the larval diet of *S. frugiperda* at 100 µg per gram of diet while only 1–5 were added at 200 µg/g (compounds 6–8 underwent partial decomposition when incorporated to the bioassay at 200 µg/g).

No significant differences (P>0.05) in the consumption rates for control and treatments were detected at the doses tested. However, the GR of second instars for 14 days was significantly decreased when larvae were fed on diets with 200 µg/g of compounds 1–5 (Dunnet's test, df=81, F=18.4, P<0.001) by 48%, 63%, 48%, 65%, and 41% in the larval growth (measured by larval weight) respectively, compared to control (Fig 2). In addition, at 200 µg/g, the treatment with compounds 1–5 produced 50%, 45%, 30%, 40%, and 10% mortality. At 100 µg/g, only compounds 1, 2, 3, and 7 significantly reduced the larval growth by 21%, 35%, 22%, and 29%, respectively, compared to control, indicating a dose dependent effect (Dunnet's test, F=7.27, df=126, P<0.001). At the same dose, compounds 1–8 killed from 5% to 30% of the larval population (Table 1). Mortality values were not adjusted for natural mortality observed in control group (5%). Treatments produced neither pupal nor adult mortality or malformations.



◀ Fig 3 Light microscopy of the larval midgut of *Spodoptera frugiperda* (stained with hematoxylin and eosin). **a** Section of larva where midgut is clearly observed (×10; *scale bar* 400 μm). **b** Midgut epithelium of control larva (×100; *scale bar* 40 μm). **c** Midgut fragment of larva treated with 100 μg/g of compound 1—note the absence of the epithelial layer (×100; *scale bar* 40 μm). **d** Fragment of midgut of larva treated with 100 μg/g of compound 2 with destruction of the cell membrane (×100; *scale bar* 40 μm). **e** Midgut fragment of larva treated with 100 μg/g of compound 3, epithelial cells with apical destruction (×100; *scale bar* 40 μm). **f** Fragment of midgut of larva treated with 100 μg/g of compound 4 with epithelial layer absent (×100; *scale bar* 40 μm). **g** Fragment of midgut of larva treated with 100 μg/g of compound 7 with destruction of the cell membrane (×100; *scale bar* 40 μm). **h** Fragment of midgut of larva treated with 100 μg/g of compound 8 where epithelial cells show apical destruction (×100; *scale bar* 40 μm). *PM* Peritrophic membrane, *M* muscle tissue (*star*) epithelial layer, *L* lumen, *C* columnar cell, *G* goblet cell, *R* regenerative cell, *CM* circular muscle, *LM* longitudinal muscle, *AD* apical cell destruction, *RE* remaining epithelium, *MD* destruction of the cell membrane.

Compounds 1 and 4 induced the most important alterations in the midgut epithelium, 1 being the terpenoid to produce the highest mortality rate (Fig 3). The PM and the epithelial layer were absent, leaving only the muscle tissue and the remaining epithelium spread in the lumen (Fig 3c, f). Treatment with compound 2 produced a partial lysis of the epithelial tissue (Fig 3d), while compounds 3 (Fig 3e) and 8 (Fig 3h) induced a deterioration of the apical region of the gut epithelial cells. Compound 7 produced partial destruction of epithelial cells and alter their nuclei in which a dense chromatin is observed (Fig 3g). In addition, all the mentioned compounds destroyed the PM of the midgut which was not detected in the histological analysis of the treated larvae. Treatments with compounds 5 and 6 at 100 μg/g did not produce histological alterations of the midgut. As earlier mentioned, compounds 5 and 6 did not inhibit larval growth at the mentioned dose.

Histological alterations are responsible, in part, for the decrease of the growing rates and mortality of the larvae. The compounds we tested produced histomorphological alterations similar to those reported by Roel *et al* (2010) when neem oil was incorporated to the larval diet of *S. frugiperda* at 400 ppm. It is important to point out that the active compound of neem oil is the nortetraterpenoid azadirachtin. Compared to the natural products goniothalamin (Senthil-Nathan *et al* 2008) and 20-hydroxyecdysone (Rharrabe *et al* 2009), previously evaluated for their toxicity to lepidopteran larvae, the terpenoids reported herein produce the same effects at higher concentrations. However, our products are more toxic (produce the same effects at lower doses) than harmaline (Rharrabe *et al* 2007), a natural compound reported to generate histological damage on larvae of the lepidopteran *P.interpunctella.*

This is the first report on the toxic effects and histological damages produced by terpenoids of *P. chilensis* on the larval midgut epithelium of *S. frugiperda.* Liverwort terpenoids, that are in many cases enantiomers of those found in higher plants, have been rarely investigated for their potential to control insects, although a few compounds displayed promising activities. Plagiochilin A, a sesquiterpenoid from many species of the large genus *Plagiochila*, and fusicogigantone A (a diterpenoid), widespread in liverworts, produced 55% and 75% *S. frugiperda* larval mortality, respectively, at 100 ppm (Ramírez *et al* 2010). The exploration of the insecticidal potential of terpenoids from *P. chilensis* indicates that more studies are necessary to further characterize the full range of lethal and sublethal effects they may induce. Although the terpenoids of *P. chilensis* are not as toxic as that of other natural products as azadirachtin (Martinez & van Emden 2001) or the sterols from *Myrtillocactus geometrizans* (Céspedes *et al* 2005), their structures are a lot simpler, and are, therefore, easier to be obtained by synthetic procedures.

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