Bioresource Technology 100 (2009) 3669–3673

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Antifeedant activity of ethanolic extract from *Flourensia oolepis* and isolation of pinocembrin as its active principle compound

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ARTICLE INFO

Article history:
Received 10 October 2008
Received in revised form 9 February 2009
Accepted 9 February 2009
Available online 1 April 2009

Keywords:
Flourensia oolepis
Asteraceae
Antifeedant
Flavonoids
Pinocembrin

ABSTRACT

The ethanolic extract from *Flourensia oolepis* aerial parts showed strong antifeedant activity against the pest larvae, *Epilachna paenulata*, with an antifeedant index (AI%) of 99.1% at 100 μg/cm^2. Based on chromatographic fractionation of the extract, guided by bioassays on *E. paenulata*, the flavanone pinocembrin (**1**) was isolated as the most active principle. In a choice assay, compound **1** showed strong antifeedant activity against *E. paenulata*, *Xanthogaleruca luteola* and *Spodoptera frugiperda* with an AI% of 90, 94 and 91% ($p < 0.01$) respectively, at 50 μg/cm^2. The dosages necessary for 50% feeding inhibition of the insects (ED_{50}) were 7.98, 6.13 and 8.86 μg/cm^2, respectively. The feeding inhibitory activity of **1** against *E. paenulata* was compared with the activity of other structurally related flavonoids like naringenin, which was inactive up to 100 μg/cm^2, catechin which was nearly 6 times less active than **1**, and quercetin which was equally active as **1**. The effect of these on the feeding behavior of *E. paenulata* was also studied.

1. Introduction

In a previous screen for natural pesticides based on 27 native plants from the Central Region of Argentina (Palacios et al., 2007), *Flourensia oolepis* (Asteraceae) was found to possess the greatest anti-insect, germination inhibition and bactericidal activity.

The genus *Flourensia* comprises 25 species distributed throughout America (Uriburu et al., 2004); *F. oolepis* S.F. Blake (common name: chilca) is a ligneous and resinous bush widely distributed in the hilly area of the provinces of Córdoba and San Luis (central Argentina). Previous chemical studies of the genus *Flourensia* reported the isolation of flavonoids from *Flourensia cernua* (Dillon et al., 1976; Dillon and Mabry, 1977), chromenes, benzofurans and sesquiterpenoids from both *F. cernua* (Kingston et al., 1975) and *Flourensia heterolepis* (Bohlmann and Jakupovic, 1979), and *p*-coumaric acid derivatives from *Flourensia thurifera* (Faini et al., 1997).

Essential oil from *F. oolepis* collected in San Luis province was mainly composed of γ-gurjunene, δ-cadinene, 2-methylene-4,8,8-trimethyl-4-vinyl-bicyclo[5.2.0]nonane, santolinetriene and τ-muurolene (García et al., 2007). Plants collected in Córdoba province contained the same group of compounds but with a higher percentage of oxygenated terpenes; τ-cadinol, β-selinene, linalool, β-eudesmol, τ-muurolol and α-thujene as the principal components (Priotti et al., 1997). Other than these data, there is little information about the composition of plant extracts. Petrol extracts from plants collected in San Luis afforded euparin, whereas the ethyl acetate extract gave ilicic acid, 2′,4′-dihydroxychalcone, 7-hydroxyflavanone and the sesquiterpene alcohol 4α-H-eudesm-11(13)-en-4,12-diol (ilicol) (Guerreiro et al., 1979). In addition, little is known about the bioactivity of *F. oolepis*. The essential oil from this plant showed a repellent effect against *Tribolium castaneum* Herbst adults in an oil dose ranging from 192–750 μg/cm^2 (García et al., 2007) by choice bioassay. The oil also exhibited 83% feeding inhibition for the Colorado Potato beetle (*Leptinotarsa decemlineata* Say) at 100 μg/cm^2. The settling behavior of the aphid *Myzus persicae* Sulzer was affected by the essential oil, but no activity was found against other aphid, *Rhopalosiphum padi* L., at 100 μg/cm^2 (García et al., 2007).

This work describes the insecticidal activity of an ethanolic extract from *F. oolepis* and the isolation of its active principle, the activity of which is compared to that of other flavonoids and known natural insecticides. The bioassays were made on pest insects such as *Epilachna paenulata* (Coccinelidae, Chrysomelidae), which affects species from the family Cucurbitaceae, *Xanthogaleruca luteola* (Coleoptera, Chrysomelidae), an elm tree predator, and *Spodoptera frugiperda* (Lepidoptera, Noctuidae), the fall armyworm that affects many crops but is especially important on corn and soybeans.

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doi:10.1016/j.biortech.2009.02.050

2. Methods

2.1. Plant material

Aerial parts of *F. oolepis* were collected in Traslasierra Valley, Córdoba, Argentina in March 2006. A voucher specimen (UCCOR 135) has been deposited at the Herbarium Marcelino Sayago of the Faculty of Agricultural Science, Catholic University of Córdoba and was identified by the agronomist, Gustavo Ruiz.

2.2. General experimental procedures and apparatus

^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Bruker NMR with a Bruker AC 200 console (Bruker, Germany). Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H and ^{13}C NMR spectra measured in DMSO-d_6. Electron impact mass spectra (EI-MS) were obtained at 70 eV by GC–MS on a Hewlett–Packard 5970 Series mass spectrometer interfaced with a Hewlett–Packard 5890 gas chromatograph fitted with a column (HP-5MS, 15 m × 0.25 mm i.d., temperature from 100–200 °C, 10 °C/min). UV spectra were recorded in EtOH on a Hewlett Packard 8452 A diode array spectrophotometer. Optical rotation angle was measured in a JASCO DIP-370 spectropolarimeter (JASCO Co., Tokyo, Japan).

2.3. Chemicals and chromatographic absorbents

Azadirachtin, catechin and quercetin were purchased from Sigma Chemical Co., Inc. (St. Louis, MO) and naringenin was a gift from Dr. Mónica Nazareno (Universidad Nacional de Santiago del Estero, Argentina). Analytical TLC was performed on silica gel 60 F_{254} Merck plates (Darmstadt, Germany). Silica gel grade 200–400 mesh, 60 Å, for column chromatography and DMSO-d_6 were purchased from Sigma Chemical Co., Inc. (St. Louis, MO). All other solvents were purchased from Merck (Darmstadt, Germany) and Fischer Scientific (New Jersey, NJ).

2.4. Extraction and isolation of the active compounds

Essential oil of *F. oolepis* was obtained by steam-distillation (95 °C) of dry leaves (200 g, 0.08% yield). In order to isolate the compound responsible for the antifeedant activity of *F. oolepis*, bio-guided fractionation of the ethanolic extract was carried out. Air-dried aerial parts of *F. oolepis* (97 g) were extracted with ethanol for 24 h at room temperature. After removal of the solvent (reduced pressure), extract was obtained (7.8 g, 8.04% yield). This extract had strong antifeedant activity against larvae III of *E. paenulata*, used as reference insect. The extract (1.8 g) was fractionated by silica gel column chromatography and 13 fractions were eluted with a gradient of hexane/Et_2O and MeOH. The active fractions 7–9 (AI% = 97–100% at 100 μg/cm^2), which were eluted with hexane/Et_2O 50:50, were then rechromatographed on silica gel with a gradient of hexane/Et_2O from 100% hexane to 100% Et_2O. A crystalline solid was isolated (258 mg, 1.14% yield respect to plant material) and identified as pinocembrin (**1**) by ^{1}H NMR, ^{13}C NMR and MS techniques.

Pinocembrin (**1**): white powder; mp 194–195 °C; $[\alpha]_D^{20} = -22.81$ (*c* 0.86, EtOH); EI-MS: *m/z* (relative intensity,%) 256 (100 M$^+$), 179 (82), 152 (67), 124(52), 96 (31), 69 (34); ^{1}H NMR (DMSO-d_6), 20.7 °C, δ 2.77 (1H, dd, *J* = 17.2, 3.2 Hz, H-3a), 3.06 (1H, dd, *J* = 12.8, 17.2 Hz, H-3b), 5.44 (1H, dd, *J* = 3.2, 12.8 Hz, H-2), 5.52 (1H, d, *J* = 2.2 Hz, H-6), 6.01 (1H, d, *J* = 2.2 Hz, H-8), 7.41 (5H, m, H-2′-6′); ^{13}C NMR (DMSO-d_6) 25 °C, δ 40.45 (C-3), 80.17 (C-2), 95.94 (C-8), 96.84 (C-6), 102.69 (C-10), 127.47 (C-2′/6′), 129.39 (C-4′) 129.46 (C-3′/5′), 139.59 (C-1′), 163.59 (C-9), 164.41 (C-5), 167.62 (C-7), 196.75 (C-4). The spectral data were identical to previously published data of pinocembrin (Bick et al., 1972; Neacsu et al., 2007; Adelmann et al., 2007).

2.5. Insects

E. paenulata (Coccinelidae, Chrysomelidae) larvae were obtained from a laboratory colony, reared on a natural diet of *Curcubita maxima* leaves and maintained in a growth chamber at 24 ± 1 °C and 70–75% relative humidity, with a photoperiod of 16/8 h light–dark cycle, and periodically renewed with field specimens. *S. frugiperda* (Lepidoptera, Noctuidae) larvae and *X. luteola* (Coleoptera, Chrysomelidae) adults were collected in the experimental field of the Catholic University of Córdoba, and they were fed on pumpkin and elm leaves, respectively, for two or three days before using them in bioassays.

2.6. Insect bioassay

The antifeedant experiments of crude extract, fractions and compounds **1**–**5** (Fig. 1) were carried out by leaf-disk choice test (Carpinella et al., 2003). Two cotyledon leaves from a *C. maxima* seedling were placed in a Petri dish, and a glass disk with two 1 cm^2 diameter holes was placed on top. A third-instar *E. paenulata* or *S. frugiperda* or a *X. luteola* adult was placed equidistant from both a treated and an untreated (solvent control) leaf-disk and allowed to feed for 24 h, except for the assay against *S. frugiperda* larvae which was stopped when 50% of the food offered was consumed (3–4 h). The dosages applied were 100, 50, 10, 5 and 1 μg/cm^2 dissolved in 10 μl of ethanol. Control leaves received only 10 μl of ethanol. Ten replicates were run for each treatment. In the case of the choice test carried out with the third-instar *S. frugiperda* and *X. luteola* adults, cotyledons of *C. maxima* seedlings and elm leaves (*Ulmus procera* Salisb.), respectively, were used as food. The relative amounts (recorded in percentages from 0 to 100) of the treated and untreated substrate area eaten in each feeding choice test were estimated visually by dividing the food area in imaginary quarters. The measurements were always done by the same operator. The antifeedant index (AI%) was calculated as $[1-(T/C)] \times 100$, where *T* and *C* represent the consumption of treated and untreated foods, respectively (Carpinella et al., 2003). For Fig. 2, the Feeding Index (FI), calculated as $[(C-T)/(C+T)] \times 100$, was used instead of AI% because the former gave equal scales at positive and negative values, whereas AI% gave an enhanced scale at negative values. Data were compared by using the Wilcoxon signed paired rank test, $\alpha = 0.05$. After calculation of the AI, the relative potency (ED_{50} values: the effective dosage for 50% feeding reduction) of **1**–**5** was determined by Probit analysis for each insect species.

3. Results and discussion

In order to find new, safe, effective insecticides to be used mainly in organic agriculture, the activity of extract from the native Argentinian plant *F. oolepis* was studied. Essential oil from leaves of *F. oolepis* assayed against *E. paenulata* showed no or poor antifeedant effect up to 100 μg/cm^2 (data not shown). However, the ethanolic extract of the aerial parts of this plant showed strong antifeedant activity against *E. paenulata* larvae with an AI% = 99.1% at 100 μg/cm^2. Through a bio-guided chromatographic fractionation of the extract, based on bioassays with *E. paenulata*, compound **1** (Fig. 1) was isolated as the most active principle. The structure of **1** was deduced by ^{1}H and ^{13}C NMR and MS spectral experiments and the compound was identified as pinocembrin.

Pinocembrin is a dihydroxyflavanone, widely distributed in nature, and has been isolated from different species of plants such as *F.*

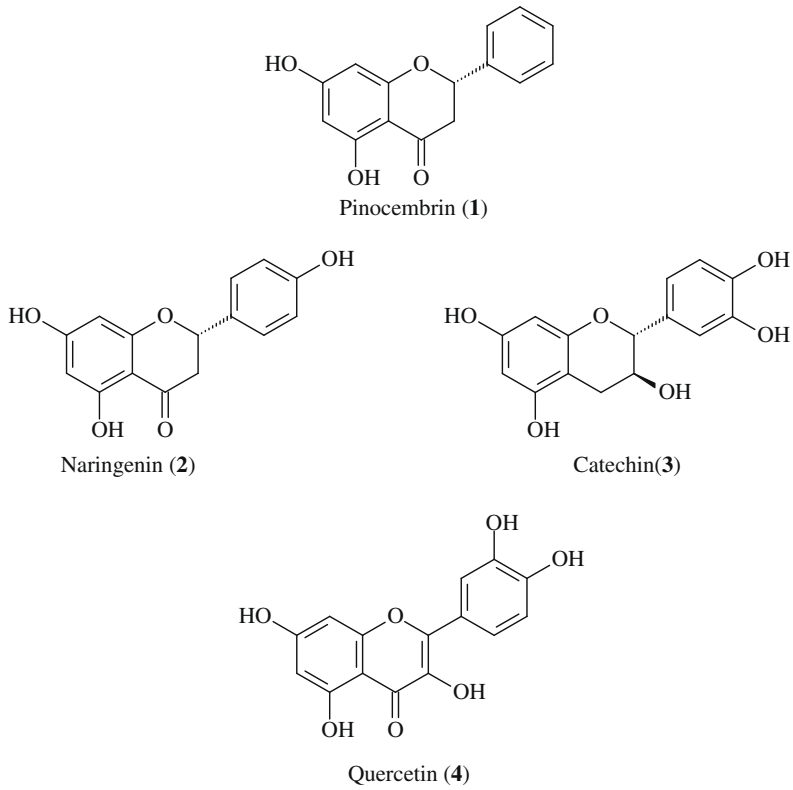

Fig. 1. Chemical structure of compounds **1–4**.

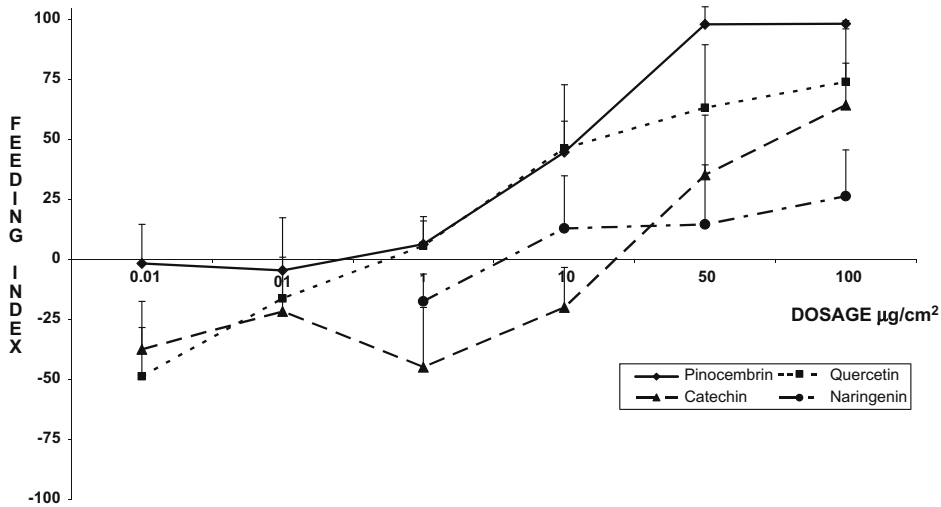

Fig. 2. Effect of **1**, **2**, **3**, and **4** on the feeding behavior of *Epilachna paenulata*. Feeding index = $[(C-T)/(C+T)] \times 100$. A negative value indicates a phagostimulant and a positive value an antifeedant effect.

ilicifolia (Dillon and Mabry, 1977), *Eucalyptus sieberi* (Bick et al., 1972), *Boesenbergia pandurata* (Tewtrakul et al., 2003), *Pinus strobus* (Hanawa et al., 2001), *Pinus banksiana* (Neacsu et al., 2007), *Populus deltoides* (Adelmann et al., 2007), *Lippia graveolens* (Lin et al., 2007), *Piper hostmannianum* (Lago et al., 2004), *Glycyrrhiza uralensis* (Hayashi et al., 2003) and *Dalbergia louvelii* (Beldjoudi et al., 2003) among others. Pinocembrin has been also reported as one of the principal components of propolis from different geographical origins such as Brazil (Salomão et al., 2004), Uruguay (Kumazawa et al., 2004), Argentina (Nieva Moreno et al., 2005), Bulgaria (Salomão et al., 2004) and China (Usia et al., 2002).

Pinocembrin (**1**) has previously been reported to have broad spectrum antibacterial (Bremner and Meyer, 1998), antifungal (Lago et al., 2004), antiviral (Tewtrakul et al., 2003), antioxidant (Bremner and Meyer, 1998; Santos et al., 1998), anti-inflammatory (Sala et al., 2003), and vasorelaxant (Zhu et al., 2007) activities, and to improve rat cognitive impairments induced by chronic cerebral hypoperfusion (Guang and Du, 2006).

Only one report concerning the effect of flavonoids, including **1** and catechins, on the reduction of growth of the corn borer insect *Ostrinia nubilalis* Hubner (Abou-Zaid et al., 1993), has been published to our knowledge.

In the choice test assay, compound **1** was evaluated against *E. paenulata*, *X. luteola* and *S. frugiperda*. These insects ate significantly ($p < 0.05$) less food when they were fed leaves treated with **1** at 10, 50, 100 μg/cm^2. Pinocembrin, at 50 μg/cm^2, demonstrated significant activity against all three species as indicated by the 90, 94 and 91% ($p < 0.01$) rejection of feeding by *E. paenulata*, *X. luteola* and *S. frugiperda*, respectively (Table 1). The dosage necessary for 50% feeding inhibition (ED_{50}) was calculated as 7.98, 6.13 and 8.86 μg/cm^2, respectively (Table 2). This result adds to and is consistent with the well-established knowledge that flavonoids contribute to the defense of plants against herbivory (Harborne and Williams, 2000).

The ED_{50} values indicate that the two coleopteran specialists were as susceptible as the lepidopteran generalist. Polyphagous insects usually are better provided with detoxification enzymes that allow them to adapt to different food sources. This mechanism results in the insect having a higher tolerance to secondary metabolites (Schoonhoven et al., 1998; Krishnan and Kodrík, 2006). However, in this case, larvae of the generalist *S. frugiperda* were very sensitive to the presence of **1** with a relatively low ED_{50}.

We compared the antifeedant activity of **1** with other structurally related flavonoids such as naringenin (**2**), catechin (**3**), quercetin (**4**) (Fig. 1) and with the limonoid azadirachtin (**5**), a well known natural antifeedant from the neem tree (Kraus, 1995). In the choice assay against *E. paenulata*, **2** was weakly active, showing an $ED_{50} > 100$ μg/cm^2; catechin **3**, with an ED_{50} of 47.6 μg/cm^2, was nearly 6 times less active than **1**. Quercetin **4** was of the same order of activity of **1** with an ED_{50} of 9.26 μg/cm^2 (Table 2).

Comparing the activity of **1** and **4**, it can be noted that the food rejection effect for both compounds appeared at similar dosages against *E. paenulata*, while a strong difference was observed with *X. luteola* which had an ED_{50} of 6.13 and 178.51 μg/cm^2 for **1** and **4**, respectively. The difference in potency of **1** and **4** depending on the insect species suggests that **1** could have different mode of action than **4** (Table 2). Compound **1** was about 13 times less active than azadirachtin **5** ($ED_{50} = 0.59$ μg/cm^2) (Table 2).

Reported ED_{50} values of different flavonoids on *S. litura* showed that quercetin was inactive, although rotenone and flavone had ED_{50} values of 59.1 and 24.6 μg/cm^2, respectively (Morimoto et al., 2003). Chrysin, a flavone analogue of **1**, had an ED_{50} of 635 μg/cm^2 against the spodopteran *S. litura* (Morimoto et al., 2003). Hydroxylated flavones, such as 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone and 5-hydroxy-3,6,7,8-tetramethoxyflavone, with values at 42.8 and 7.16 μg/cm^2, respectively (Morimoto et al., 2000) were similar. These results indicate that pinocembrin has greater antifeedant activity than most other flavonoids.

In comparisons of the ED_{50} of **1** with the reported activity for other antifeedant secondary metabolites, selected because of their known high potency for controlling pest insects, such as meliartenin, isolated from *Melia azedarach* (Carpinella et al., 2002) or toosendanin and artemisinin, isolated from *Melia toosendan* (Chen et al., 1995) and *Artemisia annua* (Klayman et al., 1984), respectively, **1** was seen to be about 10 times less active than meliartenin ($ED_{50} = 0.80$ μg/cm^2) and 2 times less active than toosendanin ($ED_{50} = 3.69$ μg/cm^2) against *E. paenulata* (Carpinella et al., 2003). However, pinocembrin was almost twice as active as the sesquiterpene artemisinin ($ED_{50} = 14.7$ μg/cm^2) (Maggi et al., 2005) against this insect species.

It is known that flavonoids play an important role in the selection of food by insects, but it is still unclear whether it is the overall profile of flavonoids and other compounds, or the presence or absence of specific compounds that determine either host acceptance or food rejection. It is still difficult to predict from flavonoid structures whether they might or might not influence insect behavior (Simmonds, 2001). The antifeedant profile of many flavonoids is known to change with a change in dosage (Simmonds, 2003). At low doses many flavonoids become phagostimulant but at higher doses they may be antifeedants (Simmonds, 2003). The feeding behavioral responses of *E. paenulata* to **1**, **2**, **3** and **4** are shown in Fig. 2. Pinocembrin was almost inactive at 0.01 and 0.1 μg/cm^2 although **3** and **4** were clearly strongly phagostimulant. At a dosage of 1–10 μg/cm^2, both **1** and **4** were antifeedant with comparable potency, whereas either **2** or **3** were stimulants. Strong feeding inhibition was observed for **1** and **4** at 50–100 μg/cm^2. At this same concentration, compounds **2** and **3** showed medium inhibitor potency. These results suggest that **1** is consistently an antifeedant substance, in contrast to **2**, **3**, and **4** which exert different

Table 1
Effects of **1** on feeding by *Epilachna paenulata, Xanthogaleruca luteola* and *Spodoptera frugiperda* in leaf-disk choice assay.

Dosage of **1** (μg/cm^2)	AI%[a]
E. paenulata	
1	12
5	21
10	62**
50	90**
100	99.1**
X. luteola	
1	0
5	62
10	62*
50	94*
100	98**
S. frugiperda	
5	21
10	70**
50	91**
100	92**

[a] Antifeedant index calculated as (AI%) = [1 − (T/C)] × 100, values are means of 10 replicates. Significant differences between consumption on treated and control leaves (Wilcoxon signed rank test).
* $p < 0.05$.
** $p < 0.01$.

Table 2
Effective dosage (ED_{50}) of pinocembrin (**1**) against *Epilachna paenulata*, *Xanthogaleruca luteola* and *Spodoptera frugiperda* in leaf-disk choice assay.

	Insect	ED_{50} (μg/cm^2) (values and 95% confidence interval)
Pinocembrin (**1**)	*E. paenulata*	7.98 (5.84–10.89)
	X. luteola	6.13 (4.58–8.21)
	S. frugiperda	8.86 (5.51–14.25)
Naringenin (**2**)	*E. paenulata*	160.15 (70.50–363.79)
Catechin (**3**)	*E. paenulata*	47.6 (47.64–47.65)
	X. luteola	50.22 (25.06–112.61)
Quercetin (**4**)	*E. paenulata*	9.26 (4.84–17.73)
	X. luteola	178.52 (89.75–355.07)
Azadirachtin (**5**)	*E. paenulata*	0.59 (0.07–4.37)

behavioral responses (phagostimulant and antifeedant) with *E. paenulata*, depending on the dosage.

In light of these results, pinocembrin appears to constitute the major protection strategy of *F. oolepis* against insect herbivory. Further studies with the aim of finding insecticides of plant origin are desirable as a strategy for the discovery of new environmentally safe plant protection compounds. In Argentina, as well as Europe, organic production must use only natural insecticides and not synthetic ones for pest control (Cabizza et al., 2004). Although some natural insecticides are found on the market, new compounds with activity against a variety of insects are always necessary either to prevent the emergence of resistance in insects or to guarantee the ready availability of natural insecticides through more widely distributed sources. For these reasons we propose use of **1** as a natural insect antifeedant. This study suggests that pinocembrin and plants containing it might be used as new tools for protecting plants from harmful insects, especially in organic agriculture.

Acknowledgements

Financial support for this work was provided by Agencia Nacional de Promoción Científica y Técnica, FONCYT, PICT 33593. GDN gratefully acknowledges receipt of a fellowship from CONICET.

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