

Insect Growth Regulator and Insecticidal Activity of β -Dihydroagarofurans from *Maytenus* spp. (Celastraceae)

Carlos L. Céspedes^{a*}, Julio Alarcón^c, Eduardo Aranda^b, José Becerra^d and Mario Silva^d

^a Instituto de Química, Universidad Nacional Autónoma de México, Coyoacán 04510, Mexico DF, Mexico. Fax: +52-5-616-2203. E-mail: ccespede@servidor.unam.mx

^b Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México

^c Dpto. Ciencias Básicas, Facultad de Ciencias, Universidad del Bío-Bío. Chillán, Chile

^d Dpto. Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Chile

* Autor for correspondence and reprints requests

Z. Naturforsch. **56c**, 603–613 (2001); received February 14/March 27, 2001

β -Dihydroagarofurans, Acute Toxicity, Acetylcholinesterase

From the aerial parts of *Maytenus disticha*, we have isolated 9 β -benzoyloxy-1 α ,2 α ,6 β ,8 α ,15-penta-acetoxy-dihydro- β -agarofuran (**1**) and from seeds of *Maytenus boaria* 9 β -furoyloxy-1 α ,6 β ,8 α -tri-acetoxy-dihydro- β -agarofuran (**2**). These compounds and their MeOH and hexane/ethyl acetate (1:1 v/v) extracts were evaluated for their effects on the fall armyworm (*Spodoptera frugiperda*). Toosendanin, a commercial insecticide derived from *Melia azedarach* was used as a positive control. When tested for activity using neonate larvae in a no-choice artificial diet bioassays, the agarofurans **1**, **2** and toosendanin as well as the MeOH and hexane/EtOAc extracts caused significant growth inhibitory effects with GC₅₀ of 7.55; 3.84; 1.75; 14.0 and 7.3 ppm at 7 days, respectively. Compounds **1** and **2** caused 100% larval mortality at 25 and 15 ppm, respectively. MeOH and hexane/EtOAc extracts caused 100% larval mortality at 25.0 ppm, respectively, they also increased the development time of surviving larvae and a significant delay for the time of pupation and adult emergence. These compounds showed comparable potency of activity with toosendanin. Acute toxicity against adults of *S. frugiperda* was also found, for hexane/EtOAc extract and **2** had the most potent activity with LD₅₀ value of 4.7 and 1.9 ppm, respectively. MeOH extract, hexane/EtOAc extract, **1** and **2** caused acetylcholinesterase inhibition with 78.0, 89.2, 79.3 and 100% inhibition at 15.0 ppm, respectively. Therefore, the furoyloxy agarofuran may be responsible for the insecticidal activity of these plants.

Introduction

The search to find new pesticides of botanical origin has been currently focused on limonoids from the Meliaceae family due to their potent effects on insect pest and low toxicity. Our interest is centered on the study of possible insecticidal activities of shrubs and trees of moderate height belonging to the Celastraceae family occurring in the south of Chile, because they are strongly resistance to attack by different families of insects pest.

Maytenus disticha (Hook) Urban Celastraceae commonly known as “maitencito” or “romasillo”, is a small tree, which grows on rainfall forest in the south Pacific, slope ranging from Araucanian Region to Tierra del Fuego in the Patagonian Region of Chile. We have isolated six agarofurans

from *M. disticha* (Alarcón *et al.*, 1991, 1998), similar to those isolated from Peruvian and Mexican Celastraceae (González *et al.*, 1983). Some of these compounds have been tested in insecticidal bioassays against the Fall Armyworm (*Spodoptera frugiperda*, Lepidoptera: Noctuidae) (Céspedes *et al.*, 1999).

There are reports on diverse effects of *Maytenus* extracts on human health and on animals (Bhakuni *et al.*, 1973; González *et al.*, 1992). Several species of Chilean *Maytenus* have been investigated (Muñoz and San Martín, 1991). The biologically active compounds of these *Maytenus* genera are β -agarofuran type sesquiterpenoids. We have previously reported the photosynthetic inhibitory activity of dihydro- β -agarofurans sesquiterpenes from *M. disticha* and *M. boaria* (Céspedes *et al.*, 2000b). There are reports of the insecticidal

activity of these compounds (González *et al.*, 1992, 1997). β -Agarofurans has been reported from *M. magellanica* and *M. boaria* (Alarcón *et al.*, 1995). Minor sesquiterpenes and β -agarofurans have been reported in *M. canariensis*, *M. magellanica* and *M. chubutensis* and their possible anti-feedant activity (Alarcón *et al.*, 1998). In addition, there are reports of the presence of triterpenoids in *M. scutioides* (González *et al.*, 1999), *M. macrocarpa* (Chávez *et al.*, 1998), *M. undata* (Muhammad *et al.*, 2000), *M. blepharodes* (González *et al.*, 2000), brassinosteroids in *M. boaria* (Franke *et al.*, 1999) and phenolic compounds in *M. chubutensis* and *M. disticha* (Muñoz *et al.*, 1999); also insecticidal activity against *Cydia pomonella* of nortriterpene quinone methides from Paraguayan *Maytenus* species (Avilla *et al.*, 2000).

A phytochemical re-examination of the aerial parts of *M. disticha* and seeds of *M. boaria* collected in Chillán City, Chile, were undertaken because these plants are highly resistant to attack by insects and no insecticidal work has been carried out on this plant. The present study deals with the mechanism by which this plant exhibits its insecticidal effects on the feeding behaviour, growth and development of the Fall Armyworm (FAW) *S. frugiperda* (J. E. Smith).

The resistance to insecticides results from three main mechanisms: 1) short penetration of insecticide. 2) efficiency of esterases, mixed function oxidases or glutathione transferases and 3) the target of the insecticide is modified (Fournier *et al.*, 1992). The resistance to insecticides also may be due to acetylcholinesterase activity. It is a key enzyme in the insect nervous system in which the cholinergic system is essential (Fournier *et al.*, 1992). This property can be used for insect pests control. Both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) occur in vertebrates while mainly AChE activity is demonstrated in insects, BuChE may occur in aphids (Brestkin *et al.*, 1992). We investigated the inhibition of AChE activity by extracts from *Maytenus* spp. These extracts contained mainly sesquiterpene of β -agarofuran type. There are no reports about inhibition of AChE by these compounds. The field observations showed that these plants are not attacked by any insects, therefore it seems important to investigate the capacity of inhibition on the growing of insect and to correlate with ace-

tylcholinesterase inhibition by the main compounds of these plants.

Materials and Methods

General experimental procedures

HPLC was performed on a WATERS Model 600E, equipped with Bondapack RP 18 column, 250 $\times$ 8 mm, speed flux 1.5 ml/min, speed paper 0.5 cm/min. UV detector 280 nm, movil phase MeOH/H₂O 7:3 v/v. Analytical and preparative TLC were performed on Si gel 60 F254 E Merck plates, and the spots were visualised by spraying with a 10% solution of H₂SO₄, followed by heating at 110 °C.

Plant material

Aerial parts (stem, leaf and flowers) from *M. disticha* and seeds of *M. boaria* were collected in Chillán, VIII Region of Chile. Voucher specimens (R. Rodríguez and C. Marticorena) can be found at the ethnobotanical collection of the Herbarium (CONC), Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile.

Isolation and purification of the β -agarofurans 1 and 2

Compound 1. The aerial part of *M. disticha* was percolated with methanol and partitioned between CHCl₃ and *n*-hexane/EtOAc (1:1 v/v) extracts (the hex/EtOAc extract was used in bioassays). Agarofuran **1** was isolated from hexane: EtOAc extract as reported (Céspedes *et al.*, 2000b) by conventional chromatographic methods (80 mg). Its molecular formula is C₃₂H₄₀O₁₃, M⁺ *m/z* 632 and ¹³C-NMR spectra exhibited 32 carbon signals. All spectral data were identical as in Alarcón *et al.* (1991) (Fig. 1).

Compound 2. Seeds of *M. boaria* were milled and extracted with MeOH, and this extract was used in insect bioassays. The extract was solvent partitioned using CHCl₃, EtOAc and H₂O. The CHCl₃ extract was chromatographed on a silica gel column using petrol-EtOAc mixtures. Polar fractions were combined and separated by TLC (petrol-EtOAc, 1:1 v/v) to give impure agarofuran **2**, which was further purified by HPLC (RP 18, 250 $\times$ 8 mm, MeOH-H₂O, 7:3) 20 mg (R_t 8.9 min), their chromatographic and spectral data

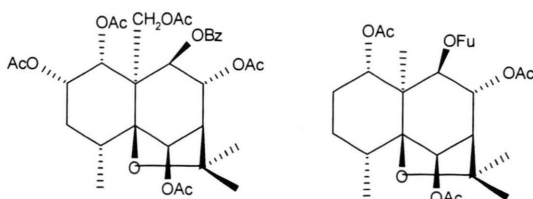


Fig. 1. The chemical structures of 9-benzoyloxy-1,2,6,8,15-pentaacetoxy-dihydro- β -agarofuran (**1**) and 9-furanoxy-1,6,8-triacetoxy-dihydro- β -agarofuran (**2**), where Bz = benzoyloxy and Fu = furanoxo.

were identical with reported by Alarcón *et al.* (1995) (Fig. 1) (Céspedes *et al.*, 2000b).

Apparatus

^1H -NMR spectra were recorded at 300 and 500 MHz, ^{13}C -NMR at 75 and 125 MHz respectively, on Varian VXR-300S and VXR-500S spectrometers, chemical shifts (ppm) are related to $(\text{CH}_3)_4\text{Si}$ as internal reference. CDCl_3 and acetone- d_6 from Sigma-Aldrich Quimica, S. A. de C. V., Toluca, Mexico were used as solvents, and coupling constants are quoted in Hz. IR spectra were obtained as KBr pellets on Perkin Elmer 283-B and FT-IR Nicolet Magna 750 spectrophotometers. Electron impact mass spectra were taken on a JEOL JMS-SX102A instrument (70 eV). UV spectra were determined on a Shimadzu UV-160 spectrophotometer; CHCl_3 was used as solvent. Optical rotations were measured on a JASCO DIP-360 spectropolarimeter; CHCl_3 was used as solvent. Melting points were obtained on a Fisher-Johns hot-plate apparatus and remain uncorrected. Nunc 24-well polystyrene multidishes were purchased from Nalge Nunc International. LAB-LINE Chamber model CX14601A, with adjustable Hi-Lo protection thermostats safeguard samples. LAB-LINE refrigerator/freezer, model 3559-10.

A Spectronic model GENESYS 5 spectrophotometer was used to carry out the spectrophotometric measurements in the acetylcholinesterase activity. The centrifuge used in this study was B. BRAUN, model SIGMA 2-15.

Chemicals and solvents

Thiamine, sorbate, methyl-paraben, ascorbate, acetic acid, acetaldehyde, acetylcholinesterase (AChE), acetylthiocholine (ATC), 5,5'-dithio-

bis(2-nitrobenzoic acid) (DTNB), choline-chloride, calcium pantothenate, niacinamide, riboflavin, folic acid, biotin and vitamin B-12 were purchased from Sigma-Aldrich Quimica, S. A. de C. V., Toluca, Mexico. Methanol, ethyl-acetate, CuSO_4 , KCl, NaHCO_3 , MgCl_2 , NH_4Cl , pyridine, acetic anhydride, silica-gel GF₂₅₄ analytical chromatoplates, silica gel grade 60, 70-230, 60 Å, for column chromatography were purchased from Merck Darmstadt, Germany. Pre-coated TLC plates SIL G-100 UV₂₅₄, 1.0 mm, preparative were purchased from Macherey-Nagel, Düren, Germany.

Bioassays with fall armyworm

Larvae used for the experiments were obtained from a culture at the Institute of Chemistry, UNAM. The larval culture was maintained under previously described conditions (Céspedes *et al.*, 2000a). An artificial diet containing 800 ml of sterile water, 10.0 g of agar, 50.0 g of soy meal, 96.0 g of corn meal, 40.0 g of yeast extract, 4.0 g of wheat germ, 2.0 g of sorbic acid, 2.0 g of choline chloride, 4.0 g of ascorbic acid, 2.5 g of *p*-hydroxybenzoic acid methyl ester, 7.0 ml of Wesson salt mixture, 15.0 ml of Vanderzant vitamin mixture for insects, 2.5 ml of formaldehyde, 0.1 unit of streptomycin, 5.0 g of aureomycin, and 20.0 g of milled ear of corn grain (for 1 kg of diet) were used for the bioassay, which was prepared by the procedure described earlier (Céspedes *et al.*, 2001). 24-well polystyrene multidishes were filled with the liquid diet, then left for twenty minutes at room temperature in sterile conditions. The 3.4 ml wells measure 17 mm in depth $\times$ 15 mm diameter with a 1.9 cm² culture area. All test compounds were dissolved in 95% ethanol and layered on top of each well with the artificial diet using four concentrations (Table I) and a control (1 ml 95% ethanol) allowing evaporation of solvent. For each concentration used and control, a single *S. frugiperda* neonate larvae were placed on the diet mixture in each well and left for 7 days. After 7 days, length of surviving larvae were measured and weighed, then transferred to separate vials containing fresh stock diet. Larval weight gains and mortality were recorded after 21 days of incubation, since pupation occurred after 23 ± 1 days. Other life-cycle measurements were recorded, such as time to pu-

pation, weight of pupae, mortality of larvae, adult emergence and deformities. All treatments were carried out in a controlled environment chamber with an 18L: 6D photoperiod, at 25 °C day and 19 °C night temperature regime, and a relative humidity of $80 \pm 5\%$. There were three replications for each concentration. Control assay (24-wells) contained the same amount of larvae (24), volume of diet and ethanol as the test solutions (Céspedes *et al.*, 2000a).

Acute toxicity on S. frugiperda

Acute toxicity was determined by topical application to larvae of last stage of *S. frugiperda*. The larvae were cooled on ice to stop their movement and treated on their abdomens with each of the test compounds and extracts, at concentrations of 15.0; 12.0; 3.0 and 0.5 ppm, additional concentration (25.0 ppm) was used for toosendanin (Table V). The solvent used was 10.5 µl of acetone injected with a 50 µl microsyringe. Control larvae were only treated with 10.5 µl of acetone. After 24 hrs the survival of the larvae was recorded. Five larvae were used for each concentration, respectively, including control. LD₅₀ (the lethal dose for 50% mortality) was determined from extrapolation of a curve from values in Table V.

Inhibition of acetylcholinesterase

An enzyme extract containing acetylcholinesterase (AChE) was obtained according to the method of Grundy and Still (1985). About 100 adults were frozen at -20 °C for 7 days. The heads of frozen adults were detached, then milled and homogenised in 20 ml of 0.1 M phosphate buffer at pH 8.0. The crude homogenate was centrifuged at $15,000 \times g$ for 15 min at 5 °C, and the supernatant was used for the enzyme activity. ATC (cholinesterase substrate) was dissolved in 0.1 M phosphate buffer (pH 8.0). DTNB (3-carboxy-4-nitrophenyl-disulfide), Ellman's reagent a sensitive sulfhydryl reagent 39.6 mg of this compound was dissolved in 10 ml of 0.1 M phosphate buffer at pH 7.0, and 15.0 mg of NaHCO₃ was added.

Inhibition of AChE was determined according to Ellman's procedure (colorimetric method) (Ell-

man, *et al.*, 1961) using both the control (EtOH) and test solutions. The reaction mixture contained 0.2 ml of the enzyme solution and 0.1 ml of DTNB added to 2.4 ml of 0.1 M phosphate buffer (pH 8.0). The reaction mixtures were added to each of the test compounds dissolved in 50 ml of EtOH. The control solution was similarly prepared by the addition of 50 ml of EtOH. Both control and each of the test solutions were preincubated at 25 °C for 10 min. After preincubation, the enzyme reaction was started by the addition of 40 ml of ATC followed by incubation at 25 °C for 20 min. After 20 min, the absorbance at 420 nm was measured spectrophotometrically and compared with that of the control immediately after adding an enzyme to the above reaction mixtures. Reading was repeated for 5 min at 30 sec intervals to verify that the reaction occurs linearly. Blank reaction was measured by substituting saline for enzyme. AChE activity was calculated with the absorption coefficient 1.49 mmol/min. All experiments were repeated three times and the results were analyzed by SAS ANOVA and GLM procedures and graph by Microcal Origin version 4.1.

Relative growth index and growth index

The relative growth index (RGI) and growth index (GI) were calculated according to Zhang *et al.* (1993), (Fig. 2).

Data analysis

Data for all the insect bioassays were analysed by ANOVA and GLM procedures (SAS Institute, 1982) and Microcal Origin version 4.1 ($p < 0.05$). GC₅₀, MC₅₀, LD₅₀ and AC₅₀ values for each activity were calculated by Probit analysis (Finney, 1971) on the basis of the percentage of mortality or inhibition obtained from the concentration of the compounds compared with control. Differences between treatment means were established with a Student-Newman-Keuls (SNK) test. MC₅₀ is the concentration producing 50% mortality. GC₅₀ is the concentration inhibiting 50% of first instar larval growth (weight). LD₅₀ is the concentration producing 50% of acute toxicity. AC₅₀ is the concentration producing 50% of acetylcholinesterase inhibition.

Results and Discussion

Insecticidal and insect growth inhibitory activities

The insecticidal effects of **1**, **2**, toosendanin, methanol and hexane/ethylacetate extracts against first instar larvae of *Spodoptera frugiperda* are outlined in Table I. **1**, **2**, and MeOH extract at concentration of 12.0 ppm show significant mortalities in this larval stage (>65%). However, hex/EtOAc extract produced higher mortality (79.1%) at the same concentration. On the other hand **2** and hex/EtOAc extract show highest insecticidal activity producing 100% and 93.4% of larval mortality at 15.0 ppm, respectively. When larvae were fed with a diet containing 25.0 ppm or higher doses of all extracts and tested compounds all of the larvae died (Table I). It is important to note that the 50%

lethal concentration (MC₅₀) of larvae at 7 days for hex/EtOAc extract was found at 6.0 ppm of diet concentration.

β-Agarofurans **1** and **2** inhibit larval growth after 7 days when incorporated into diets with GC₅₀ of 7.55 and 3.84 ppm, respectively (Table I). After 23 days, this growth reduction is clearly significant in the 6.5 ppm for **1** and **2**, respectively (Table II). When expressed as the percentage of respective controls, **2** is more active than toosendanin on larval growth inhibition. In relation to the total length of the larvae the effect was more pronounced at the 23 days (Table II) for all compounds and extracts tested. Although in our results **2** (furoyloxy substituent) showed greater inhibitory effect than **1** and toosendanin on *S. frugiperda* larval growth as compared to all com-

Table I. Growth inhibitory effects of MeOH, *n*-hexane/EtOAc extracts, compounds **1**, **2** and toosendanin on Fall Armyworm growth bioassay^a.

Treatment	µg/ml [ppm]	Mean weight gained [mg] ^b	% of weight ^c	Mean long gained [cm] ^d	% of length ^c	GC ₅₀ [ppm] ^e	Death [%]	MC ₅₀ [ppm] ^f
Control		79 ± 8.0a	100	1.1 ± 0.2	100		7.50	
1						7.55		8.2
	6.5	7 ± 0.1b	9.4	0.62 ± 0.1	57.1		41.4	
	12.0	6 ± 0.5b	8.0	0.39 ± 0.1	36.1		67.4	
	15.0	6 ± 0.3b	7.6	0.28 ± 0.4	26.0		89.2	
	25.0						100	
2						3.84		7.5
	6.5	6 ± 0.2c	7.5	0.42 ± 0.1	39.0		43.9	
	12.0	3 ± 0.3c	3.6	0.12 ± 0.1	11.0		70.5	
	15.0						100	
	25.0						100	
MeOH extract						14.0		9.4
	6.5	67 ± 0.5a	85.0	0.79 ± 0.19	71.8		31.1	
	12.0	20 ± 0.1c	26.0	0.60 ± 0.10	54.5		65.9	
	15.0	18 ± 0.3c	23.3	0.55 ± 0.25	49.5		89.1	
	25.0						100	
<i>n</i> -hex/EtOAc						7.3		6.0
	6.5	23 ± 0.1c	29.8	0.61 ± 0.15	55.5		50.9	
	12.0	13 ± 0.1c	15.9	0.45 ± 0.10	40.9		79.1	
	15.0	7 ± 0.1c	9.0	0.30 ± 0.10	27.3		93.4	
	25.0						100	
Toosendanin						1.75		8.5
	5.0	5 ± 2.5c	6.27	0.90 ± 0.23	94.7		40.1	
	15.0	2 ± 1.7c	2.82	0.77 ± 0.18	81.0		70.0	
	25.0	1 ± 1.6c	1.23	0.57 ± 0.20	60.0		89.9	

^a After 7 days of incubation, mean of three replicates.

^b Means followed by the same letter within a column are not significantly different in a Student-Newman-Keuls (SNK) test at *P* < 0.05 (treatments are compared by concentration to control). Means are ± standard error.

^c Percentage with respect to control.

^d Mean length total increase from eclosion.

^e The GC₅₀ is the inhibitory concentration for reduction of first instar larval growth (weight) rates by 50% in “no choice” test calculated by ANOVA program (*P* < 0.05).

^f The MC₅₀ is the concentration producing 50% mortality in “no choice” test calculated by ANOVA program (*P* < 0.05).

Table II. Activity of compounds **1**, **2**, toosendanin, MeOH and *n*-hexane/EtOAc extracts of *M. disticha* on larval growth parameters of *S. frugiperda*^a.

Treatment	Concentration [ppm]	Mean weight gained [mg] ^b	% ^c	Mean length gained [mm]	% ^c	GC ₅₀ [ppm] ^d
Control		464.5 ± 4.99a	100.0	41.1 ± 5.1	100.0	0.43
1	6.5	29.2 ± 1.3c	6.3	11.9 ± 0.1	28.90	
	12.0	11.3 ± 1.2c	2.4	6.5 ± 0.8	15.8	
	15.0	6.1 ± 0.7c	1.3	0.6 ± 0.4	1.46	
2	6.5	22.0 ± 0.4c	4.7	7.7 ± 0.3	18.7	0.37
	12.0	—	0	—	0	
MeOH	6.5	180.3 ± 2.3b	38.8	3.4 ± 0.4	8.27	4.8
	12.0	98.9 ± 1.7b	21.3	1.1 ± 0.8	2.67	
	15.0	—	0	—	0	
<i>n</i> -hexane/EtOAc extract	6.5	134.5 ± 0.83b	28.9	9.1 ± 0.16	22.1	0.37
	12.0	72.80 ± 0.74b	15.7	5.2 ± 0.71	12.6	
	15.0	42.20 ± 0.36c	9.1	0.89 ± 0.20	2.16	
Toosendanin	5.0	21.5 ± 2.5c	4.58	7.0 ± 0.5	25.0	2.68
	15.0	17.0 ± 3.1c	3.62	6.1 ± 0.6	21.7	
	25	11.2 ± 1.6c	2.38	2.8 ± 0.4	10.0	

^a Before pupation, at 23 days.^b Means followed by the same letter within a column are not significantly different in a Student-Newman-Keuls (SNK) test at $P < 0.05$ (treatments are compared by concentration to control). Means are ± standard error.^c Percentage with respect to control.^d The GC₅₀ is the inhibitory concentration for reduction of five instar larval growth (weight) rates by 50% in “no choice” test calculated by ANOVA program ($P < 0.05$).

pounds and extracts tested, we also found that **1** (benzoyloxy substituent) significantly reduced larval length growth as compared to control (Table II). In addition, MeOH and hexane/EtOAc extracts also showed a significant length inhibition at 12.0 ppm (2.67 and 12.6% respectively) and in this aspect toosendanin showed a significant effect with 10% of inhibition at 25 ppm.

The percentage of larvae that reached pupation decreased in both tested compounds in comparison to control. The most important effect was observed with **2** at 6.5 ppm, which almost fully reduced the pupation and adult emergence to 92% and 83% respectively (Table III). Moreover, these compounds and extracts show dose-response dependent activity.

Significant delays in time to pupation were observed between 6.5 to 12.0 ppm for **1**-treated, between 3.0 to 6.5 ppm for **2**-treated and between 15 to 25 ppm toosendanin-treated. Development of pupae was significant delayed at 12.0 ppm of **1**-treatments and between 1.5 to 6.5 ppm with **2**-treatments (Table III). Of the compounds tested

only **2** significantly reduced pupal weights of males and females at 6.5 ppm.

Survival to the adult stage, as compared to the pupal stage, showed no further reductions with the test compounds. However, **2** produced additional mortality and lower survivorship to the adult stage for this compound in low concentrations. Adult weights were significantly delayed in mean time to the adult stage were seen in many of the treatments (Table III). In all cases the adult weight is clearly reduced, but the main effect was observed for **2** and toosendanin inhibiting the adult weight in 19.2 and 25.7% at 6.5 and 5.0 ppm, respectively. However, toosendanin showed the greatest effect inhibiting the emergence times in 4.17% at 15 and 25 ppm.

The effect of the agarofuran **2** on reducing insect growth, increasing development time and mortality of *S. frugiperda* is similar to that of other compounds such as limonoids (Kubo, 1993). The mode of action of this compound is being investigated and it may be due to a combination of antifeedant action and postdigestive toxicity, as found for

Table III. Activity of compounds **1–2** MeOH and *n*-hexane/EtOAc extracts on pupation and emergency parameters of *S. frugiperda*^a.

Treatment	[ppm]	Pupation [days]	Weight pupae [mg] ^c	%	SP ^d [%]	Adult weight [mg] ^c	% ^f	Emergence [days] ^c	Emergence [%] ^g
Control		22.0 ± 1.0	263.4 ± 8.9a	100	89.9	51.0 ± 2.1a	100	31.5 ± 1.0a	83.3
1	6.5	26.0 ± 2.0 ^b	77.0 ± 1.0 ^c	29.2	22.0	20.0 ± 0.5 ^c	39.2	32.5 ± 1.5 ^b	25.7
	12.0	27.5 ± 1.5 ^b	71.4 ± 0.3 ^c	26.9	12.0	15.0 ± 0.1 ^c	29.4	33.0 ± 0.5 ^c	10.5
2	6.5	24.0 ± 0.5 ^b	20.4 ± 3.1 ^c	7.6	6.0	9.8 ± 0.2 ^c	19.2	35.0 ± 0.5 ^c	16.7
	12.0	–	–	0	–	–	0	–	–
MeOH extract	6.5	26.5 ± 1.0 ^b	108.5 ± 2.1 ^b	41.2	10.5	34.0 ± 0.4 ^b	66.6	32.5 ± 1.0 ^b	30.0
<i>n</i> -hexane/ EtOAc extract	12.0	27.0 ± 1.0 ^b	78.5 ± 1.8 ^c	29.8	9.1	22.4 ± 0.5 ^b	43.9	33.0 ± 1.0 ^c	27.0
	6.5	27.0 ± 1.0 ^b	79.3 ± 0.5 ^c	30.1	7.3	24.0 ± 0.4 ^b	47.1	33.5 ± 1.5 ^c	26.1
	12.0	27.0 ± 1.0 ^b	52.4 ± 0.4 ^c	19.9	5.2	12.4 ± 0.3 ^c	24.3	35.0 ± 1.0 ^c	19.5
Toosendanin	5	23.5 ± 0.5	95.0 ± 2.1 ^c	36.1	17.9	13.1 ± 0.33 ^c	25.7	34.0 ± 0.5 ^c	16.7
	15	24.0 ± 1.0 ^b	45.1 ± 1.9 ^c	17.1	4.17 ^e	10.1 ± 0.19 ^c	19.8	35.0 ± 1.0 ^c	4.17
	25	24.0 ± 1.0 ^b	38.0 ± 1.3 ^c	14.4	4.17 ^e	3.0 ± 0.25 ^c	5.8	36.0 ± 1.0 ^c	4.17

^a Mean of three experiments.^b Means within a column are significantly different from control in a Kruskal-Wallis chi-squared approximation test at $P < 0.05$.^c Means followed by the same letter within a column are not significantly different in a Student-Newman-Keuls (SNK) test at $P < 0.05$ (treatments are compared by concentration to control). Means are ± standard error.^d SP: Survival Pupation = number of pupae × 100/total larvae for pupation.^e These values correspond to one survival larva.^f % of adult weight compared to control.^g % = number of adults emerged × 100/total number of pupae.

other limonoids (Isman *et al.*, 1995). The activity of these compounds is comparable to the commercial insecticide toosendanin (Chen *et al.*, 1995), which suggests potential for further development of these natural products.

This study differs than the reported by González *et al.* (1997) on the Egyptian cotton leafworm *Spodoptera littoralis* in respect to that some of the reported compounds here are more potent for *S. frugiperda* and at lower concentration act as insect growth regulator. However, they used a different methodology for feeding the active compounds to the insect and it implies the need of more studies in this field with compounds of this type.

Growth inhibition and regulatory growth index

In many of the treatments, mean adult weight was significantly delayed in the average time to reach the adult stage relative to control larvae. GI and RGI clearly indicate (Fig. 2) the stronger effect showed by compound **2**, with RGI value of 0.20 at 6.5 ppm, (Table IV). In addition to toosendanin also showed a pronounced effect with RGI of 0.34 and 0.10 at 15.0 and 25.0 ppm, respectively

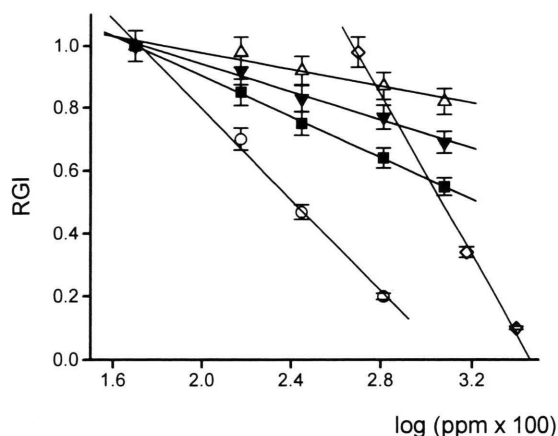


Fig. 2. Effect on growth development expressed as RGI vs log (ppm × 100) of agarofuran **1** (■), agarofuran **2** (○), *n*-hexane/ethylacetate (▼), MeOH extract (△) and toosendanin (◇) on *S. frugiperda*. Each value represents means ± SE (N = 5) from three determinations in error bars.

(Table IV). Noteworthy, the slope of curves in Fig. 2 shows the potency of **2** and toosendanin, respectively. These parameters together with the LD₅₀ values (Table V), corroborate the highest effect of compound **2**, since it caused the greatest

Table IV. GI and RGI of *S. frugiperda* as a function of increased concentrations of **1**, **2**, MeOH and *n*-hexane/EtOAc extract from *P. argentatum* and toosendanin (3)^a.

Compounds	Concentration [ppm]	GI ^b	RGI
Control		0.99 ± 0.049a	
1	3.0	0.91 ± 0.043b	0.75
	6.5	0.52 ± 0.081b	0.64
	12.0	0.24 ± 0.067c	0.55
2	3.0	0.35 ± 0.049b	0.47
	6.5	0.22 ± 0.038c	0.20
	12.0	0.89 ± 0.048b	0.92
MeOH extract	3.0	0.74 ± 0.037b	0.87
	6.5	0.69 ± 0.057b	0.82
	12.0	0.74 ± 0.037b	0.83
<i>n</i> -hexane/EtOAc extract	3.0	0.69 ± 0.055b	0.77
	6.5	0.51 ± 0.039b	0.69
	12.0	0.99 ± 0.050b	0.98
Toosendanin	5.0	0.50 ± 0.031b	0.34
	15.0	0.10 ± 0.015c	0.10
	25.0		

^a Mean of three replicates.

^b Means followed by the same letter within a column after ± standard error values are not significantly different in a Student-Newman-Keuls (SNK) test at *P* < 0.05 (treatments are compared by concentration to control).

^c $RGI_{treatment} = GI_{treated}/GI_{control}$.

(GI = Growth index = number survival larvae/total larvae used).

inhibitory effect of growth inhibition in weight at 7 and 23 days, respectively (Tables I and II) and growth inhibition in length at the 7 days and 23 days, respectively (Tables I and II).

It is important to note that similar insect growth regulatory activity on *Ostrinia nubilalis* (European Corn Borer) was studied by Jiménez *et al.* (1997). These authors reported that the nortriterpenoids humilinolide A, B, C, and D, cedrelanolide and toosendanin induced larval mortality between 5.0 to 50.0 ppm ranges with percentages between 36 to 60%, respectively. However, our compounds showed lower range between 15.0 to 25.0 ppm.

Acute toxicity on adults of *S. frugiperda*

MeOH extract from *M. boaria* showed moderate acute toxicity with 63 and 44% of survival at 12.0 and 15.0 ppm, respectively. However, compounds **1**, **2**, *n*-hexane/EtOAc extract of *M. disticha* and toosendanin showed a potent activity of acute toxicity on adults of *S. frugiperda* at concentrations greater than 12.0, 6.5, 15.0 and 15.0 ppm, respectively. The LD₅₀ values of **1**, **2**, *n*-hexane/

EtOAc extract and toosendanin were 6.4, 1.9, 4.7 and 6.7 ppm, respectively (Table V). Moreover, these values show the potency of *Maytenus* compounds and their extracts. In order to determine the site of action on the insect growth regulatory activity (IGR) and the acute toxicity, the effect of **1**, **2**, MeOH and Hex/EtOAc extracts, and toosendanin on acetylcholinesterase activity was studied.

Inhibition of acetylcholinesterase

The inhibitory activity of **1**, **2**, MeOH, hexane/EtOAc extracts and toosendanin on AChE is outlined in Table V. Compound **2**, hexane/EtOAc extract and toosendanin showed the greatest effect with 98.5, 89.2 and 98.0% inhibition at 12.0, 15.0 and 15.0 ppm, respectively. However, **2** and toosendanin showed a strong inhibitory effect by 100%, at 15.0 and 25.0 ppm, respectively. While MeOH and hexane/EtOAc extracts showed inhibitory activity in similar form to **1**, both extracts, **1** and **2** inhibited AChE activity in a dose-dependent manner confirming that these compounds are active inhibitors of AChE in *Maytenus* spp. In addition, the presence of a furanic ring increases the strength of these compounds on inhibition of AChE. We assume that inhibitory activities of hexane/EtOAc extract were caused not by one strong inhibitor but by synergistic activity. This confirms the results obtained by Ortego *et al.* (1999), with furanic limonoids as azadirone and a mixture of 1,7-di-O-acetylhananensin and 3,7-di-O-acetylhananensin from *Trichilia havanensis* (Meliaceae). These compounds, significantly reduce digestive protease and esterase activities in the midgut of Colorado potato beetle (*Leptinostarsa decemlineata*) (Ortego, *et al.*, 1999). Inhibition of AChE activities by terpenoids have been reported on related insecticidal effects (Céspedes *et al.*, 2001; Gracza, 1985; Grundy *et al.*, 1985).

Compound **2** had a potent inhibitory activity. Obviously, the nature of the ester substituents at C-1, C-6, and C-8 plays an important role in the inhibitory activity of the agarofurans, confirming the observation made by Gonzalez *et al.* (1997). The most active compound (**2**) contained small and relatively hydrophilic three-acetyl groups, whereas compound **1** with bulky and more lipophilic ester groups exhibited a little minor activity level.

Table V. Acute toxicity and inhibitory activity of compounds **1**, **2** MeOH and *n*-hexane/EtOAc extracts against adults^a and acetylcholinesterase from adults heads^b of *S. frugiperda*, respectively.

Compounds	Concentration [ppm]	% Survival ^{a,c}	LD ₅₀ ^d	% Inhibition ^{b,e}	AC ₅₀ ^f
Control		100		— ^g	
1			6.4		5.7
	3.0	66 ± 3.3b		39.8 ± 2.0b	
	6.5	56 ± 2.8b		53.5 ± 2.7b	
	12.0	25 ± 1.3c		65.3 ± 3.3c	
	15.0	20 ± 1.0c		79.3 ± 3.9c	
2			1.9		2.9
	3.0	35 ± 1.8c		55 ± 2.8b	
	6.5	20 ± 1.0c		71 ± 3.5c	
	12.0	0.0		98.5 ± 1.5c	
	15.0	0.0		100	
MeOH Extract			15.0		9.7
	3.0	77 ± 3.9b		—	
	6.5	70 ± 3.5b		34.2 ± 1.7b	
	12.0	63 ± 3.2b		63.1 ± 3.1b	
	15.0	44 ± 2.2c		78.0 ± 3.5c	
<i>n</i> -hexane/EtOAc extract			4.7		6.0
	3.0	61 ± 3.1b		33.7 ± 1.7b	
	6.5	43 ± 2.2c		58.9 ± 2.9b	
	12.0	29 ± 1.5c		67.1 ± 3.6c	
	15.0	11 ± 0.6c		89.2 ± 4.1c	
Toosendanin			6.7		8.8
	5.0	58 ± 2.9c		21 ± 1.1b	
	15.0	21 ± 1.1c		98 ± 2.0c	
	25.0	0.0		100	

^a After 24 h, survival of adults was recorded (percent relative to controls).^b After incubation for 20 min, changes in absorbance at 412 nm were recorded and compared with control.^c Means followed by the same letter within a column are not significantly different in a Student-Newman-Keuls (SNK) test at $P < 0.05$ (treatments are compared by concentration to control). Means are ± standard error.^d LD₅₀, lethal dose for 50% mortality was determined by test calculated by ANOVA program ($P < 0.05$).^e Means followed by the same letter within a column are not significantly different in a Student-Newman-Keuls (SNK) test at $P < 0.05$ (treatments are compared by concentration to control). Means are ± standard error.^f AC₅₀ is the concentration producing 50% of acetylcholinesterase inhibition test calculated by ANOVA program ($P < 0.05$).^g No effect was observed.

Acetylcholinesterase activity of the 100% control correspond to 19.3 mmol ATC split/mg protein × min

In addition, these results confirm previous findings on quantitative structure activity relationship of clerodanes derivatives, namely that the anti-feedant activity of the respective natural product depends on the polarity of B ring and on the size of the ester substituents (Camps and Coll 1993; Rodríguez *et al.*, 1999). On the other hand, this target was demonstrated also for *S. litura* larvae and adult migratory grasshopper's *Melanoplus sanguinipes*. The bark extracts of *Melia toosendan*;

whose major component is toosendanin, inhibited midgut esterase activity (Feng *et al.*, 1995) and also on *Leptinostarsa decemlineata* (Ortego *et al.*, 1999). These data suggest that both toxicity and growth inhibition may be due to the inhibition of acetylcholinesterase. Therefore, the plant terpenes may be available as an AChE antagonist (Miya-zawa *et al.*, 1997; Keane and Ryan, 1999).

In summary, the insecticidal activity of **2** and hexane/EtOAc extract from *M. disticha* may be

due to a synergistic effects shown by the main components of the mixture in the test systems used in this investigation. Comparison of insecticidal activities of EtOH extracts from *Azadirachta indica* and *Melia azedarach* (Mikolajczak *et al.*, 1989) showed an inhibitory effect of 58% and 100% at 16 and 80 ppm, respectively, indicative of the potency of the agarofurans **1**, **2** and MeOH and hexane/EtOAc extracts from *M. disticha* and *M. boaria*, respectively.

Concluding remarks

The effect of the agarofuran **2** and hexane/EtOAc extract on reducing insect growth, increasing development time and mortality of *S. frugiperda* is similar to that of limonoids such as gedunin and cedrelone (Govindachari *et al.*, 1995). The mode of action of these compounds is being investigated and may be due to a combination of anti-feedant action and postdigestive toxicity, as found for other limonoids (Nakatani *et al.*, 1994; Isman *et al.*, 1995). In addition, a furoyloxy group seems to be important for these activities as showed for the most potent compound in this study agarofuran **2** and the hexane/ethylacetate extract.

Furthermore, a great inanition observed may be due to the inhibition of acetylcholinesterase or another esterase as well.

The activity of these sesquiterpenes is comparable to the commercial insecticide toosendanin, which suggests potential for further development of these materials. However, not any terpene has been found with the outstanding activity of azadirachtin (Isman *et al.*, 1995).

Acknowledgements

This work was partially supported by Instituto de Química, UNAM. Dirección de Investigación de la Universidad de Concepción, Concepción, Chile, and DIPRODE, Universidad del Bio-Bio, Chillán, Chile. We thank Isabel Chávez, Rocío Patiño, and Luis Velasco, Instituto de Química, UNAM, for their help in the spectra determinations. Professor Murray B. Isman British Columbia University, Vancouver, Canada, supplied the sample of toosendanin. Dr. Pankaj Sharma, Instituto de Química, UNAM, for helping to correct the manuscript. Zenón Rosas, Natural product chemistry lab., U. de Concepción, Chile, is especially thanked for his technical assistance in the isolation and purification of compounds.

- Alarcón J., Becerra J., Silva M., Jakupovic J. and Tschirzitz F. (1991), β -Agarofurane sesquiterpene esters from *Maytenus disticha*. *Rev. Latinoamer. Quím.* **22**, 65–66.
- Alarcón J., Becerra J., Silva M., Morgenstren T. and Jakupovic J. (1995), β -Agarofurans from seeds of *Maytenus boaria*. *Phytochemistry*, **40**, 1457–1460.
- Alarcón J., Becerra J. and Silva M. (1998), Further information on the chemistry of Chilean Celastraceae. *Bol. Soc. Chil. Quím.* **43**, 65–71.
- Avilla J., Teixido A., Velázquez C., Alvarenga N., Ferro E. and Canela R. (2000), Insecticidal activity of *Maytenus* species (Celastraceae) nortriterpene quinone methides against codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). *J. Agric. Food Chem.* **48**, 88–92.
- Bhakuni D. S., Meyer M., Poyser K. A., Poyser J. P., Sammes P. G. and Silva M. (1973), Anticancer agents from Chilean plants *Maytenus boaria* (Mol.) *Rev. Latinoamer. Quím.* **4**, 166.
- Brestkin A. P., Khovanskikh A. E., Maizel E. B., Moralev S. N., Novozhilov K. V. and Sazonova I. N. (1992), Cholinesterases of aphids. Sensitivity of cholinesterases of spring grain aphid *Schapis gramma* Rond. to some tetraalkylammonium ions and alcohols. *Insect. Biochem. Molec. Biol.* **22**, 769–775.
- Camps F. M. and Coll J. (1993), Insect allelochemicals from *Ajuga* plants. *Phytochemistry* **32**, 1361–1370.
- Céspedes C. L., Alarcon J., Achnine L., Becerra J., and Silva M., (1999), Antifeedant and insecticidal activity of a β -dihydroagarofuran from *Maytenus disticha*. *Rev. Latinoamer. Quím.* **27**, 41–45.
- Céspedes C. L., Calderón J. S., Lina L. and Aranda E. (2000a), Growth inhibitory effects on fall armyworm *Spodoptera frugiperda* of some limonoids isolated from *Cedrela* spp. (Meliaceae). *J. Agric. Food Chem.* **48**, 1805–1809.
- Céspedes C. L., Achnine L., Alarcón J., Becerra J., and Lotina-Hennsen B., (2000b), Photosynthetic inhibitory activity of dihydro- β -agarofurans sesquiterpene from *Maytenus disticha* and *Maytenus boaria* (Celastraceae) *Z. Naturforsch.* **55c**, 631–637.
- Céspedes C. L., Martínez-Vazquez M., Calderón J. S., Salazar J. R. and Aranda E. (2001), Insect growth regulatory activity of some extracts and compounds from *Parthenium argentatum* on fall armyworm *Spodoptera frugiperda*. *Z. Naturforsch.* **56c**, 95–105.
- Chávez H., Estévez-Braun A., Ravelo A. G., and González A. G. (1998), Friedelane triterpenoids from *Maytenus macrocarpa*. *J. Nat. Prod.* **61**, 82–85.
- Chen W., Isman M. B. and Chiu S. F. (1995), Antifeedant and growth inhibitory effects of the limonoid toosendanin.

- danin and *Melia toosendan* extracts on the variegated cutworm, *Peridroma saucia* (Lep. Noctuidae). J. App. Entomol.-Z. Angew. Entomol. **119**, 367–370.
- Ellman G. L., Courtney K. D., Andres V., Jr. and Featherstone R. M. (1961), A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. **7**, 88–95.
- Feng R. Y., Chen W. K. and Isman, M. B. (1995), Synergism of malathion and inhibition of midgut esterase activities by an extract from *Melia toosendan* (Meliaceae). Pestic. Biochem. Physiol. **53**, 34–41.
- Finney D. J. (1971), Probit Analysis; University Press, Cambridge.
- Fournier D., Bride J.-M., Hoffmann F. and Karch F. (1992), Acetylcholinesterase two types of modifications confer resistance to insecticide. J. Biol. Chem. **267**(20), 14270–14274.
- Franke K., Kuhnt Ch., Schmidt J. and Muñoz O. (1999), 24-epi-castasterone and phytosterols from seeds of *Maytenus boaria* (Celastraceae). Rev. Latinoamer. Quím. **27**, 45–47.
- González A. G., Fraga B. M., González P., González C. M., Ravelo A. G., Ferro E., Domínguez X. A., Martínez M. A., Perales A. and Fayos J. (1983), Crystal structure of orthosphenic acid. J. Org. Chem. **48**, 3759–3761.
- González A. G., Jiménez I. A., Ravelo A. G., Coll J., González J. A. and Lloria J. (1992), Antifeedant activity of sesquiterpenes from Celastraceae. Biochem. Sys. Ecol. **25**, 513–519.
- González A. G., Jiménez I. A., Ravelo A. G., Coll J., González J. A. and Lloria J. (1997), Antifeedant activity of sesquiterpenes from Celastraceae. Biochem. System. Ecol. **25**, 513–519.
- González A. G., Alvarenga N. L., Bazzocchi I. L., Ravelo A. G. and Moujir L. (1999), Triterpene trimers from *Maytenus scutoides*: Cycloaddition compounds? J. Nat. Prod. **62**, 1185–1187.
- González A. G., Rodríguez F. M., Bazzocchi I. L. and Ravelo A. G. (2000), New terpenoids from *Maytenus blepharodes*. J. Nat. Prod. **63**, 48–51.
- Govindachari T. R., Narasimhan N. S., Suresh G., Partho P. D., Gopalakrishnan G. and Krishna-Kumari G. N. (1995), Structure-related insect antifeedant and growth regulating activities of some limonoids. J. Chem. Ecol. **21**, 1585–1600.
- Gracza L. (1985), Molecular pharmacological investigation of medicinal plant substances II. Inhibition of acetylcholinesterase by monoterpene derivatives *in vitro*. Z. Naturforsch. **40c**, 151–153.
- Grundy D. L. and Still C. C. (1985), Inhibition of acetylcholinesterase by pulegone-1, 2-epoxide. Pestic. Biochem. Physiol. **23**, 383–388.
- Isman M. B., Arnason J. T. and Towers G. H. N. (1995), Chemistry and biological activity of ingredients of other species of Meliaceae. In: The Neem Tree *Azadirachta indica* A. Juss and Other Meliaceae Plants. Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and other Purposes (H. Schmutterer, K. R. S. Ascher, M. B. Isman, M. Jacobson, C. M. Ketkar, W. Krauss, H. Rembold, and R. Saxena, eds.), GTZ, Eschborn, Germany, pp. 652–666.
- Jiménez A., Mata R., Pereda-Miranda R., Calderón J., Isman M. B., Nicol R. and Arnason, J. T. (1997), Insecticidal limonoids from *Swietenia humilis* and *Cedrela salvadorensis*. J. Chem. Ecol. **23**, 1225–1234.
- Keane S. and Ryan M. F. (1999), Purification, characterization, and inhibition by monoterpenes of acetylcholinesterase from the waxmoth, *Galleria mellonella* (L.). Insect Biochem. Mol. Biol. **29**, 1097–1104.
- Kubo I. (1993), Insect control agents from tropical plants. In: Phytochemical Potential of Tropical Plants (K. R. Downum, J. T. Romeo and H. A. Stafford, eds.), Plenum Press, New York pp: 133–151.
- Mikolajczak K. L., Zilkowski B. W. and Bartelt R. J. (1989), Effect of meliaceous seed extracts on growth and survival of *Spodoptera frugiperda* (J. E. Smith). J. Chem. Ecol. **15**, 121–128.
- Miyazawa M., Watanabe H. and Kameoka H. (1997), Inhibition of acetylcholinesterase activity by monoterpenoids with a *p*-menthane skeleton. J. Agric. Food Chem. **45**, 677–679.
- Muhammad I., El Sayed K. A., Mossa J. S., Al-Said M. S., El-Ferally F. S., Clark A. M., Hufford Ch. D., Oh S. and Mayer, M. S. (2000), Bioactive 12-oleanene triterpene and secotriterpene acids from *Maytenus undata*. J. Nat. Prod. **63**, 605–610.
- Muñoz O. and San Martín, A. (1991), Los productos naturales en la Facultad de Ciencias Universidad de Chile. Parte I. Fitoquímica de Plantas Terrestres. Rev. Latinoamer. Quím. **22**, 13–25.
- Muñoz O., González A., Ravelo A. and Estevez A. (1999), Triterpenoid and phenolics compounds from two Chilean Celastraceae. Z. Naturforsch. **54c**, 144–145.
- Nakatani M., Huang R. C., Okamura H., Naoki H. and Iwagawa T. (1994), Limonoid antifeedants from Chinese *Melia azedarach*. Phytochemistry **36**, 39–41.
- Ortego F., López-Olgún J., Ruíz M. and Castañera P. (1999), Effects of toxic and deterrent terpenoids on digestive protease and detoxication enzyme activities of Colorado potato beetle larvae. Pestic. Biochem. Physiol. **63**, 76–84.
- Rodríguez B., Rodríguez B., De la Torre M., Simmonds M. S. J. and Blaney W. M. (1999), From a phagostimulant natural product to semisynthetic antifeedants against *Spodoptera littoralis* larvae: chemical transformations of the neo-clerodane diterpenoid scutegalin B. J. Nat. Prod. **62**, 594–600.
- SAS Institute (1982), SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina, USA.
- Zhang M., Chaudhuri S. K. and Kubo I. (1993), Quantification of insect growth and its use in screening of naturally occurring insect control agents. J. Chem. Ecol. **19**, 1109–1118.