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Insect growth regulatory effects of some extracts and sterols from *Myrtillocactus geometrizans* (Cactaceae) against *Spodoptera frugiperda* and *Tenebrio molitor* ☆

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Abstract

A methanol extract from the roots and aerial parts of *Myrtillocactus geometrizans* (Cactaceae) yielded peniocerol **1**, macdougallin **2**, and chichipegenin **3**. The natural products **1**, **2** their mixtures, MeOH and CH_2Cl_2 extracts showed insecticidal and insect growth regulatory activity against fall armyworm [*Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae)], an important insect pest of corn, and [*Tenebrio molitor* (Coleoptera)], a pest of stored grains in Mexico. The most active compounds were **1**, **2**, and a mixture ($\mathbf{M_2}$) of **1** and **2** (6:4). All these extracts, compounds and the mixture had insect growth regulating (IGR) activity between 5.0 and 50.0 ppm and insecticidal effects between 50 and 300 ppm in diets. The extracts were insecticidal to larvae, with lethal doses between 100 and 200 ppm. These compounds appear to have selective effects on the pre-emergence metabolism of Coleoptera, because in all treatments of the larvae of *T. molitor*, pupation were shortened and this process show precociousness in relation to controls. In contrast to *S. frugiperda* larvae, onset of pupation was noticeably delayed. Emergence in both cases was drastically diminished. In both pupae and in the few adults that were able to emerge, many deformations were observed. The results of these assays indicated that the compounds were more active than other known natural insect growth inhibitors such as gedunin and methanol extracts of *Cedrela salvadorensis* and *Yucca periculosa*. Peniocerol, macdougallin and chichipegenin, as well as mixtures of these substances, may be useful as natural insecticidal agents.

Keywords: Phytoecdysteroids; Ecdysone mimics; *Myrtillocactus geometrizans*; Cactaceae; Sterols; Insect growth regulation; IPM; *Spodoptera frugiperda*; Fall armyworm; *Tenebrio molitor*

1. Introduction

A large number of natural products is produced by arid land plants, including species of the Cactaceae. These compounds, which are stored in roots and aerial parts, include terpenoids, alkaloids, phenolics and amino acids. Some of the compounds that occur in the leaves and stems prevent water loss and protect the plant from sunlight. Another ecological role of secondary metabolites is related to defense against phytophagous insects and pathogens.

Interest in the application of plant secondary metabolites for insect pest management has led us to search for new environmentally friendly and biodegradable, but biologically active, natural products with low mammalian toxicity in order to avoid some of the deleterious

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effects on the environment produced by synthetic pesticides (Kubo, 1997; González and Estevez-Braun, 1998; Céspedes et al., 2000, 2001a,b, 2004; Torres et al., 2003). Some previous studies have focused on nortriterpenoids (limonoids) from the family Meliaceae because of their potent effects on insect pests linked to low toxicity (Kubo and Klocke, 1982b; Champagne et al., 1989; Klocke et al., 1989; Carpinella et al., 2002, 2003). One such compound, gedunin (Arnason et al., 1987), has proven to have excellent properties (Céspedes et al., 2000, 2004). Other triterpenoids with insect growth regulator activities are phytoecdysteroids such as β-ecdysone, ajugasterone C, and cyasterone (Kubo et al., 1981, 1983, 1987; Kubo and Klocke, 1983; Zhang et al., 1992). Sterols and triterpenes have an important biological function as key compounds in the acquirement of cholesterol by insects. Mammals obtain cholesterol either by dietary absorption or by biosynthesis from mevalonate. Because insects have no capacity for de novo sterol synthesis, they rely exclusively on exogenous sources for their normal growth, development, and reproduction (Ikekawa et al., 1993).

Research on the site and mechanism of action of allelochemicals responsible for insect control indicates that many terpenoid compounds are involved in insecticidal and insect growth regulation (IGR) activities. These substances are important enzymatic and metabolic inhibitors (Kubo and Klocke, 1983; Klocke et al., 1989; Hammond and Kubo, 1999; Kubo and Kinst-Hori, 1999; Céspedes et al., 2000, 2001; Kubo et al., 2000, 2003a,b; Calderón et al., 2001; Panzuto et al., 2002). In addition, many metabolites of angiosperms have antifeedant effects on phytophagous insects (Feeny, 1968, 1976; Rhoades and Cates, 1976; Swain, 1979). Some of them bind to proteins, acting as precipitating agents for nutritional protein, thereby inhibiting insect digestive enzymes (Feng et al., 1995; Duffey and Stout, 1996; Korth and Dixon, 1997; Tamayo et al., 2000), and reducing digestibility (Feeny, 1976; Rhoades, 1979).

We have previously demonstrated that a diverse array of secondary metabolites has different sites of action and different molecular targets when the compounds interact with enzymes and processes of metamorphosis (Céspedes et al., 2000, 2001a,b, 2004; Calderón et al., 2001; Kubo et al., 2003a,b; Torres et al., 2003).

Our field observations in the Mexican states Guanajuato, Jalisco, México, Michoacán, Puebla, Oaxaca, San Luis Potosí and Tlaxcala and in the Sierra Gorda of Querétaro and Hidalgo indicated that the common cactus "garambullo" (*Myrtillocactus geometrizans*) is very resistant to insect attack and especially to the lepidopteran *Spodoptera frugiperda*, an insect pest that is widely distributed and is an important insect pest in corn crops in Mexico. We chose to investigate the chemical bases for the insecticidal activity of *Myrtillocactus* species. Species of this genus have not previously been considered important as either medicinal or agronomic plants. These species are endemic to Mexico, where they grow in arid areas and in "barrancas" on the slopes of disturbed mountain ranges. They survive under environmentally stressed conditions and are not attacked by insects (Arias et al., 1997). The genus consists of tree-type cacti with short trunks, sometimes bluish in color with white or cream flowers. Many of these small flowers can develop from one areole at the same time. The name of the genus is from the Greek and signifies "berry-cactus". These plants bear a small oblong blue to purple edible berrylike fruit.

Only four species of the genus *Myrtillocactus* appear to have been studied chemically (Djerassi et al., 1957; Sandoval et al., 1957; Knight et al., 1966). In the present study, the terpenoids peniocerol **1** and macdougallin **2**, and chichipegenin **3**, isolated from the roots and aerial parts, respectively, of *M. geometrizans* were evaluated as insect growth inhibitors. The triterpenes and sterols found in this species have previously been isolated from other cactus species such as *Lamaireocereus chichipe*, (Sandoval et al., 1957) and *Myrtillocactus cochal*, *M. schenckii* and *M. eichlamii* (Djerassi et al., 1957), but the biological activity of these compounds has not yet been studied. *Myrtillocactus geometrizans* proved to be rich in sterol and triterpenoid composition.

The aim of this work was to correlate the phytochemical composition of *Myrtillocactus geometrizans* with inhibitory behavior on growth and development using *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) as model systems for the study of pest insects. Our data indicate that it is possible to correlate some IGR parameters, i.e., developmental physiological activities (such as delay of pupation and moulting, emergence, and deformities) with the chemical structure of compounds from *M. geometrizans*; these data are important for insect control studies (Klocke and Kubo, 1982; Kubo and Klocke, 1982a; Berenbaum, 1988; Hedin et al., 1991; Dhadialla et al., 1998; Agarwal et al., 2000; Kessler and Baldwin, 2002). On the other hand, these parameters are accepted as indirect measures of yet other physiological processes (Camps, 1988; Céspedes et al., 2000, 2004; Kubo et al., 2003a,b; Torres et al., 2003) that are affected by the chemicals assayed.

The present paper specifically deals with the effects of isolates from MeOH and CH_2Cl_2 extracts of the root and aerial parts of *Myrtillocactus geometrizans* [sterols **1**, **2**, triterpene **3**, derivative **1b**, and mixtures $\mathbf{M_1}(\mathbf{1}+\mathbf{2})$ (1:1) and $\mathbf{M_2}(\mathbf{1}+\mathbf{2})$ (6:4)], against growth parameters of fall armyworm (FAW, *S. frugiperda*) and yellow mealworm (*T. molitor*). Aspects examined included insecticidal and growth regulatory activity, rate of development, time of pupation, adult emergence, and deformities in insects at each of the various stages. The effects

of these substances from *M. geometrizans* were evaluated and compared to those of gedunin and to *Yucca periculosa* (Me-Yuc) (Agavaceae) and *Cedrela salvadorensis* (Meliaceae) MeOH (Me-Ced) extracts, all known growth inhibitors of *S. frugiperda* (Céspedes et al., 2000; Calderón et al., 2001; Torres et al., 2003).

2. Results and discussion

In our screening program which is designed to discover interesting biological activities of subtropical Mexican plants from arid lands, it was found that *Myrtillocactus geometrizans* showed insecticidal activity in a preliminary trial. Based on this information, we have carried out several studies of the root and aerial parts of this plant.

From a mixture **M** of sterols (**1** + **2**), obtained from the methanolic extract of the roots of *M. geometrizans*, the sterols peniocerol **1** and macdougallin **2**, and the triterpene chichipegenin **3** (3β,16β,28β,22α-tetrahydroxyoleanan) were obtained from MeOH and CH_2Cl_2 extracts of aerial parts. These compounds were identified by comparison of their spectral features with those reported in the literature and by comparison with authentic samples (Djerassi et al., 1957, 1958, 1965; Sandoval et al., 1957; Knight et al., 1966; Knight and Pettit, 1969) (Fig. 1).

Peniocerol **1**, macdougallin **2** and chichipegenin **3** were converted into their acetyl (**1a**, **2a**, **3a**) and tosyl derivatives (**1b**, **2b**), which were evaluated in bioassays as the quantity of material permitted. Only **1b** was used in insecticidal bioassays, as the yield of **1a**, **2a**, **2b** and **3a** was very small. These substances were used for structural determination only. In order to obtain more satisfactory data for insecticidal activity, the bioassay was carried out at lower concentrations with compounds **1**, **2**, **3**, and **1b**, and the MeOH and CH_2Cl_2 extracts, and mixtures $\mathbf{M_1}$ (**1** + **2**, 1:1) and $\mathbf{M_2}$ (**1** + **2**, 6:4). Gedunin, Me-Yuc, and Me-Ced extracts were used as positive controls.

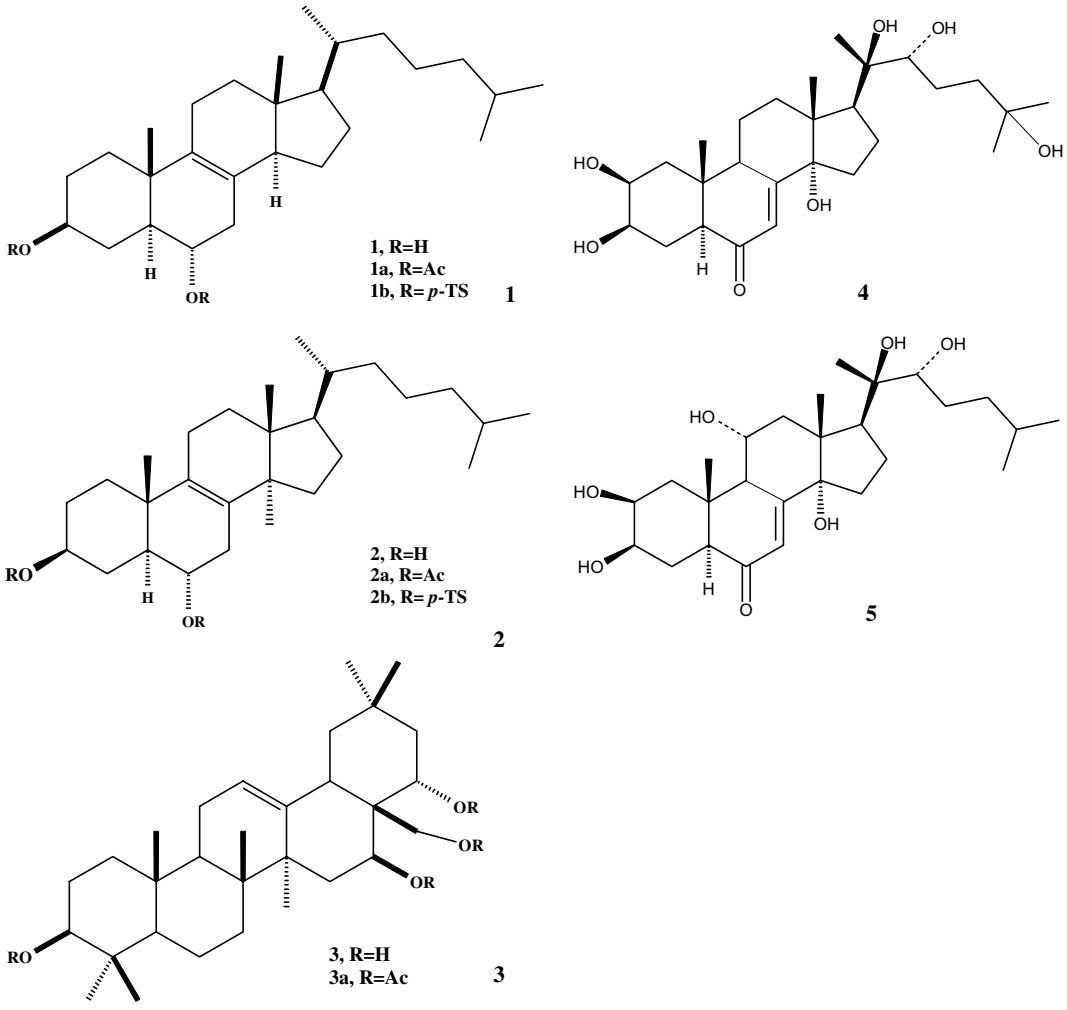

Fig. 1. Chemical structures of peniocerol **1**, macdougallin **2,** chichipegenin **3**, their derivatives **1a**, **1b**, **2a**, **2b**, **3a**, β-ecdysone **4** and ajugasterone C **5**.

2.1. Insecticidal activity against larvae of Spodoptera frugiperda

The effects of MeOH and CH_2Cl_2 extracts on growth and development of larvae of first instar of *S. frugiperda* and larvae of last instar of *T. molitor* were evaluated initially at a concentration of 300 ppm. The results of studies with *S. frugiperda* larvae outlined in Tables 1–3 showed that the MeOH extract induced a significant decrease in larval survival at 7 days. In a similar manner, the CH_2Cl_2 extract produced a decrease in the number of live larvae. At 21 days, the number of larvae and pupae decreased drastically in all treatments. At a concentration greater than 50.0 ppm for **1**, **2**, and MeOH extract and higher than 35.0 and 20.0 ppm for $\mathbf{M_1}$ and $\mathbf{M_2}$, respectively, pupation was low. When pupation did occur, serious abnormalities were observed. Ecdysis and sclerotization were incomplete. Ultimately, all pupae died at those concentrations.

At concentrations higher than 300 ppm, compounds **1**, **2**, mixtures $\mathbf{M_1}$, $\mathbf{M_2}$, MeOH and CH_2 Cl_2 extracts showed acute toxicity to the insect larvae of both species. In experiments carried out against larvae of the first instar of *S. frugiperda* during the first 7 days, the effects of these extracts were 100% lethal (outlined in Table 1). The 95% lethal dose (LD_{95}) of these compounds and mixtures are: peniocerol **1** (125.1 ppm), macdougallin **2** (285.0 ppm), $\mathbf{M_1}$ (384.8 ppm), $\mathbf{M_2}$ (135.0 ppm), MeOH extract (484.8 ppm) and CH_2 Cl_2 extract (477.0). At 100.0 ppm, peniocerol **1**, macdougallin **2**, and the mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$ produced significant larval mortalities (>80%), whereas the triterpene chichipegenin **3** only produced 95% larval mortality at concentrations greater than 1000 ppm. Thus, **1**, **2**, $\mathbf{M_2}$, MeOH and CH_2Cl_2 extracts all exhibited 100% larval mortality and gave the highest insecticidal activity. It is important to point out that peniocerol **1**, possessing an LD_{95} of 125.0 ppm, was more active as an insecticide than gedunin or either of the two extracts (Me-Ced and Me-Yuc) used as positive controls.

2.2. Insect growth inhibitory activity for Spodoptera frugiperda larvae

In additional experiments, peniocerol **1** (10 ppm), macdougallin **2** (100 ppm), MeOH (100 ppm) and CH_2-Cl_2 (200 ppm) extracts, and mixtures $\mathbf{M_1}$ (100 ppm) and $\mathbf{M_2}$ (10 ppm) specifically inhibited each larval growth stage, e.g., growth (up to 75% of length), when incorporated into diets (Table 1). Moreover, **1**, **2**, MeOH and CH_2Cl_2 extracts, and mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$ produced total inhibition (100%) of growth and weight increase at 21 days (data not show). The tosylated derivative **1b** did not show inhibition of larval weight and growth. Values were very similar to those of the control. At 21 days, larval growth reduction by $\mathbf{M_1}$ and $\mathbf{M_2}$ was significant at concentrations between 10.0 and 35.0 ppm ($p < 0.05$). On the other hand, compounds **1** and **2**, at 35.0 ppm, showed a high larval growth inhibition with 72.2% mortality (Table 2).

The percentage of larvae that reached pupation decreased drastically with almost all compounds from *Myrtillocactus geometrizans* including mixtures and extracts (except **3** and the tosylated derivative **1b**). Thus, **1** (35.0 ppm, 5.0%), **2** (35.0 ppm, 5.6%), $\mathbf{M_1}$ (20 ppm, 13.9%), $\mathbf{M_2}$ (10 ppm, 37.5%), **MeOH** (100 ppm, 5%), $\mathbf{CH_2Cl_2}$ (100 ppm, 5%), gedunin (50 ppm 25.0%), and Me-Yuc (25 ppm, 37.5%) and Me-Ced (50 ppm, 25.0%) extracts all showed significant delay of pupation. At 50 ppm, no larvae survived to pupation with **1**, **2**, $\mathbf{M_1}$, $\mathbf{M_2}$, or extracts (Table 2). Delays in time to pupation (>24 days) for **1** (1.0 ppm), **2** (10 ppm), $\mathbf{M_1}$ (50 ppm) and $\mathbf{M_2}$ (35 ppm) were observed (data not show). Furthermore, concentrations of **1**, **2**, $\mathbf{M_1}$ and $\mathbf{M_2}$ between 10.0 and 50 ppm significantly reduced pupal weights. **MeOH** and $\mathbf{CH_2Cl_2}$ extracts produced the greatest effect on pupal weights at 50.0 ppm (data not show), whereas Me-Ced and Me-Yuc extracts produced the greatest effect on pupal weights at 10.0 ppm, as previously reported (Céspedes et al., 2000; Torres et al., 2003).

The percentage of emergence of adults from the pupae was also drastically affected by these substances. The greatest reductions were shown with **1** (20 ppm, 8.3%), **2** (20 ppm, 5.6%), $\mathbf{M_1}$ (20 ppm, 20.0%), $\mathbf{M_2}$ (10 ppm, 18%), **MeOH** (35 ppm, 9.7%) and $\mathbf{CH_2Cl_2}$ (35 ppm, 9.7%) (Table 2). Thus, at higher concentrations, these substances from *Myrtillocactus geometrizans* (compounds, mixtures and extracts) completely blocked adult emergence, as no viable adults emerged from the pupae at this step.

During insect development the shedding of the cuticle, known as molting, or ecdysis, occurs. Molting affects the entire body wall and all internal parts that are formed as invaginations of the wall. Collectively, all changes that involve growth, molting, and maturation are known as morphogenesis. The molting process begins when epidermal cells respond to hormonal changes by increasing their rate of protein synthesis. The first step of molting is apolysis: the separation of epidermal cells from the inner surface of the old endocuticle and the formation of the subcuticular space. A molting gel (including enzymes) is secreted into this space. An insect larva that is actively constructing new exoskeleton is said to be in a pharate condition (Marks, 1980). In this study, analysis of the test insects fed with *M. geometrizans* extracts revealed a developmental disruption in which the insects died (at 50–300 ppm) during pharate conditions after initiation of molting (the apolysis step), without completion of morphogenesis.

During a molt, ecdysteroid levels first rise to stimulate onset of apolysis and cuticle synthesis, but then must fall

Table 1
Growth inhibitory effects of compounds **1**, **2**, **3**, **5**, mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$, MeOH-Yuc, MeOH-Ced extracts and gedunin on fall armyworm growth bioassay[a]

Treatment	μg/ml (ppm)	Mean weight gained (mg)[b]	% of weight[c]	Mean length gained (cm)[d]	% of length[c]	Mortality%	LD_{95}[e]
Control		110.9 ± 8.1a	100	1.20 ± 0.055	100	4.2	
1	5.0	89.1 ± 3.5	81	0.95 ± 0.019	79.2	6.9	125.1
Peniocerol	10.0	27.5 ± 5.6	25	0.22 ± 0.015	18.0	13.9	
	20.0	16.6 ± 2.7	15	0.20 ± 0.014	16.7	20.8	
	35.0	10.9 ± 2.2	9.8	0.20 ± 0.011	16.7	30.6	
	50.0	7.7 ± 0.9	7.0	0.40 ± 0.095	16.7	72.2	
	100.0	3.2 ± 0.4	2.9	0.15 ± 0.079	12.5	93.0	
	200.0	3.2 ± 0.4	2.9	0.15 ± 0.079	12.5	96.0	
	300.0	**0**	**0**	**0**	**0**	**100.0**	
2	5.0	112.1 ± 4.8a	101	1.21 ± 0.031	100	4.2	285.0
macdougallin	10.0	109.5 ± 2.5b	98.7	1.10 ± 0.034	91.7	5.6	
	20.0	97.1 ± 2.1b	87.5	0.95 ± 0.049	79.2	6.9	
	35.0	74.3 ± 1.9c	67.0	0.91 ± 0.037	75.8	8.3	
	50.0	44.1 ± 1.3c	41.3	0.80 ± 0.035	66.7	72.2	
	100.0	40.2 ± 0.5	36.2	0.29 ± 0.035	24.2	83.3	
	300.0	**39.5 ± 0.8c**	**38.8**	**0.21 ± 0.022**	**17.5**	**98.0**	
3	100.0	110.8 ± 3.1b	100	1.20 ± 2.2b	100	4.2	N.D.
chichipegenin	200.0	109.9 ± 4.0b	99.1	1.17 ± 2.1b	97.5	16.7	
	300.0	99.1 ± 2.6b	89.4	1.14 ± 1.9b	95.0	29.2	
5	100.0	96.6 ± 2.0b	87.1	1.17 ± 1.8b	97.5	10.0	N.D.
tosylate	200.0	72.5 ± 2.7b	65.4	1.08 ± 1.7b	90.0	12.5	
	300.0	60.0 ± 2.1b	54.1	0.95 ± 1.5b	79.2	31.6	
M_1(1 + 2)	5.0	88.3 ± 4.8a	79.6	1.11 ± 0.031	92.5	4.2	384.8
(1:1)	10.0	69.1 ± 2.5b	62.3	1.10 ± 0.034	91.7	5.6	
	20.0	57.1 ± 2.1b	51.5	0.75 ± 0.049	62.5	6.9	
	35.0	34.3 ± 1.9c	30.9	0.55 ± 0.037	45.8	8.3	
	50.0	24.1 ± 1.3c	21.7	0.38 ± 0.035	31.7	72.2	
	100.0	**16.0 ± 0.3c**	**14.4**	**0.19 ± 0.038**	**15.8**	**83.3**	
	300.0	**10.6 ± 0.3c**	**9.5**	**0.19 ± 0.3c**	**15.8**	**88.0**	
M_2	5.0	89.1 ± 3.5	81	0.95 ± 0.019	79.2	6.9	135.0
(1 + 2/6:4)	10.0	27.5 ± 5.6	25	0.22 ± 0.015	18.0	13.9	
	20.0	16.6 ± 2.7	15	0.20 ± 0.014	16.7	20.8	
	35.0	10.9 ± 2.2	9.8	0.20 ± 0.011	16.7	30.6	
	50.0	7.7 ± 0.9	7.0	0.40 ± 0.095	16.7	72.2	
	100	3.2 ± 0.4	2.9	0.15 ± 0.079	12.5	93.0	
	200	3.2 ± 0.4	2.9	0.15 ± 0.079	12.5	96.0	
	300	**0.0**	**0.0**	**0.0**	**0.0**	**100**	
MeOH extract	100	76.0 ± 5.1b	68.5	0.22 ± 0.051c	18.3	16.7	484.8
	200	55.6 ± 4.8b	50.1	0.20 ± 0.045c	16.7	30.6	
	300.0	45.6 ± 3.7c	41.1	0.16 ± 0.031c	13.3	65.0	
CH_2Cl_2 extract	100	16.0 ± 0.3b	14.4	0.39 ± 0.059c	32.5	16.7	477.0
	200	10.6 ± 0.3b	9.5	0.29 ± 0.044c	24.2	31.6	
	300.0	7.5 ± 0.3c	6.7	0.19 ± 0.031c	15.8	58.0	
MeOH-Yuc	10.0	67.2 ± 1.9a	66	0.73 ± 0.051	61	63	
	25.0	28.5 ± 1.1c	28	0.26 ± 0.036	22	81	
	50.0	11.2 ± 0.9c	11	0.11 ± 0.005	9	98	
Gedunin	10.0	7.2 ± 0.4c	7.1	0.39 ± 0.019	32.5	33	10.8
	25.0	3.40 ± 0.2c	3.3	0.24 ± 0.012	20.0	38	
	50.0	1.90 ± 0.1c	1.9	0.20 ± 0.010	16.7	70.8	
MeOH-Ced.	10.0	24.3 ± 1.5b	23.8	0.65 ± 0.032	54.2	51.8	
	25.0	8.9 ± 0.6c	8.7	0.46 ± 0.023	38.3	78.9	
	50.0	6.5 ± 0.4c	6.4	0.40 ± 0.020	33.3	98.6	

[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 ±SE. 95% Confidence limits.
[c] Percentage with respect to control.
[d] Mean length total increase from eclosion.
[e] Lethal doses for 95% of death.

to facilitate release of eclosion hormone (EH) (Truman et al., 1983) and the ecdysis-triggering hormone (ETH) (Zitnan et al., 1996, 1999). These last substances act in concert to trigger insect ecdysis during the final stages of the molt. Sterols may disrupt ecdysteroid metabolism resulting in inhibition of emergence behavior, or may, alternatively, act directly to inhibit the release of ETH (Hesterlee and Morton, 1996).

Table 2
Activities of compounds **1**, **2**, mixtures $\mathbf{M_1}$,$\mathbf{M_2}$, MeOH, CH_2Cl_2, MeOH-Ced extracts and gedunin on growth bioassay[a], pupation[b] and emergence[b] parameters of fall armyworm (mean of three replicates)

Treatment	Doses (ppm)	Mean weight gained (mg)[c]	%[d]	Mortality%[d]	Pupation[c] SP [%][e]	Emergence (%)[f]
Control	0.0	520.5 ± 15.6a	100	4.2	97.2a	100
1	5.0	498.9 ± 22.0b	95.8	12.5	20.8	48.5
	10.0	255.2 ± 10.2b	49.0	27.8	16.7	27.8*
	20.0	110.9 ± 4.4c	21.3	30.6	12.5	8.3*
	35.0	99.4 ± 2.4c	19.1	72.2	5	4.2*
	50.0	89.5 ± 2.1c	17.2	93.0	0	0
	100.0	0	0	100	0	0
2	5.0	515.8 ± 18.7a	99.1	4.2	91.7a	72.8
	10.0	499.3 ± 13.5a	95.9	6.9	70.8a	42.2
	20.0	266.9 ± 11.7a	51.3	27.8	9.7c*	5.6*
	35.0	188.9 ± 8.4b	36.3	72.2	5.6c*	3.0*
	50.0	104.5 ± 6.2b	20.1	97.2	0	0
	100.0	0	0	100	0	0
$\mathbf{M_1}$	5.0	405.0 ± 22.1a	77.8	44.0	45.8b	33.3
	10.0	393.1 ± 21.8a	75.5	55.0	19.4c	21.4
	20.0	143.3 ± 15.1b	27.5	96.0	13.9c	20.0*
	35.0	44.2 ± 6.2c	8.5	97.2	0	0
	50.0	0.0	0.0	100	0	0
$\mathbf{M_2}$	5.0	201.5 ± 24.0a	38.7	30.6	62.5b	20*
	10.0	190.0 ± 18.8a	36.5	50.6	37.5c	18*
	20.0	91.2 ± 10.9c	17.5	93.0	0	0
	35.0	22.1 ± 1.8c	4.2	97.2	0	0
	50.0	0.0	0.0	100	0	0
MeOH extract	100	240.6 ± 25.1a	46.2	63.0	5	0
	200	95.6 ± 6.8b	18.4	72.2	0	0
	300	65.5 ± 3.7c	12.6	97.2	0	0
CH_2Cl_2 extract	100	355.0 ± 20.3a	68.2	58.0	5	0
	200	110.6 ± 10.5b	21.2	83.3	0	0
	300	77.5 ± 5.3c	14.9	97.2	0	0
Gedunin	10.0	9.86 ± 0.55c	2.05	11.6	54.2b	15
	25.0	6.50 ± 0.19c	1.35	8.4	41.7b	13
	50.0	3.81 ± 0.11c	0.79	6.6	25.0c	0
MeOH-Ced.	2.0	421.1 ± 22.50a	87.45	71.0	54.2b	15
	10.0	289.1 ± 14.90a	60.04	52.3	41.7b	13
	25.0	166.6 ± 7.83a	34.60	36.3	33.3c	8
	50.0	101.2 ± 4.51b	21.01	27.6	25.0c	0

[a] The values for growth bioassay were from weight, values taken at 22 ± 1 days before pupation, the criteria followed was to account larvae that formed pupae, the larvae that not formed pupae were counted as died larvae.
[b] Values taken after pupation. The values for **3** and **5** were omitted because are irrelevant and these compounds not showed any effect at all assayed concentrations.
[c] 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), 95% Confidence limits.
[d] Percentage with respect to control.
[e] SP: survival pupation = number of surviving pupae × 100/total larvae for pupation.
[f] % = Number of adults emerged × 100/total number of pupae. The asterisks indicates deformities.

2.3. Insect growth inhibitory activity for *Tenebrio molitor*

The MeOH extract caused a decrease in the number of larvae of *T. molitor* that reach pupation (50%); mixture $\mathbf{M_2}$ has this same action, but is less active (20%) (Table 4). With this extract, as well as $\mathbf{M_1}$, $\mathbf{M_2}$, peniocerol **1**, macdougallin **2** and **MeOH**, the larvae had a more brief time of pupation and emergence; however, many of the pupae were not viable and died (Table 4). At higher levels (100 ppm), peniocerol **1** (5.0% survival), macdougallin **2** (3%), $\mathbf{M_2}$ (0%) and **MeOH** extract exhibited potent acute toxicity on larvae and pupae of *T. molitor*. In addition to a shorter pupal stage for those pupae that emerged, many pupae did not emerge. That effect was observed at 300, 200, and 100 ppm for **1**, **2**, $\mathbf{M_2}$ and **MeOH** extract, respectively (Table 4).

These results suggest that compounds and mixtures from *Myrtillocactus geometrizans* have effects on ecdysone

Table 3
GI and RGI of *Spodoptera frugiperda* as a function of increased concentrations of compounds **1**, **2**, **3**, **5**, mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$, MeOH, and CH_2Cl_2 extracts from *Myrtillocactus geometrizans*[a]

Compounds	Concentration (ppm)	GI[b]	RGI[c]
Control		0.95	1.0
1	5.0	0.93	0.97
	10.0	0.86	0.90
	20.0	0.79	0.83
	35.0	0.69	0.73
	50.0	0.28	0.29
	100.0	0.07	0.07
	200.0	0.04	0.04
2	5.0	0.95	1.0
	10.0	0.94	0.99
	20.0	0.93	0.98
	35.0	0.916	0.96
	50.0	0.28	0.29
	100.0	0.174	0.175
	300.0	0.027	0.029
3	100.0	0.95	1.0
	200.0	0.83	0.87
	300.0	0.55	0.58
5	100.0	0.88	0.93
	200.0	0.87	0.92
	300.0	0.69	0.73
$\mathbf{M_1}$	5.0	0.95	1.0
	10.0	0.94	0.99
	20.0	0.93	0.98
	35.0	0.916	0.96
	50.0	0.28	0.29
	100.0	0.174	0.175
	300.0	0.125	0.136
$\mathbf{M_2}$	5.0	0.93	0.98
	10.0	0.86	0.90
	20.0	0.79	0.83
	35.0	0.69	0.73
	50.0	0.28	0.29
	100.0	0.071	0.073
	200.0	0.040	0.040
MeOH extract	100.0	0.83	0.87
	200.0	0.69	0.73
	300.0	0.33	0.35
CH_2Cl_2 extract	100.0	0.83	0.87
	200.0	0.69	0.73
	300.0	0.41	0.44

[a] Mean of three replicates.
[b] Means followed by the same letter within a column after ± SE error values are not significantly different in a Student–Newman–Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% confidence limits.
[c] $RGI_{treatment} = GI_{treated}/ GI_{control}$. (GI = growth index = number of surviving larvae/total larvae used. RGI = GI treated/GI control).

receptors (Dinan, 2001). From Table 4, it is possible to infer that MeOH extract accelerates the time of pupation for larvae of *T. molitor*. This extract contains a high percentage of sterols (>30%) and does not show similar activity to $\mathbf{M_1}$ or $\mathbf{M_2}$ and exhibited acute toxicity to larvae of this insect with regard to the number of larvae that reached the pupal stage. Notably, our compounds, mixtures and extracts proved be more potent than stigmasterol, sitosterol, cholesterol and their epoxy, hydroxy and chloride derivatives (Meyer et al., 1998), and other sterols (Miles et al., 1994).

2.4. Growth inhibition and relative growth index for *Spodoptera frugiperda*

Larvae that reached pupal stage belonged to the lowest concentration groups; nonetheless, the pupae that emerged showed many deformities. In all treatments, the average time to reach the mean weight of the adult stage relative to the time needed for control larvae to reach the adult stage was significantly delayed. The growth index (GI or number of surviving larvae/total larvae used) and relative growth index (RGI or $GI_{treated}/GI_{control}$) showed (Table 3) that the strongest effects were shown at 50 ppm by **1** (RGI 0.29), **2** (RGI 0.29), $\mathbf{M_1}$ (RGI 0.29), $\mathbf{M_2}$ (RGI 0.29), and at 300 ppm by the MeOH (RGI 0.35) and CH_2Cl_2 extracts (RGI 0.44). These parameters together with the LD_{95} (the lethal dose producing 95% of death) values (Table 1), established that the greatest effect was shown at 50 ppm by **1** (72.2% mortality), **2** (72.2%), $\mathbf{M_1}$ (72.2%) and $\mathbf{M_2}$ (72.2%). These substances produced the greatest insecticidal effects (Table 1).

The compounds of this study are not phytoecdysteroids. Although they may have similar activity, they do not have the same type of structures as ecdysteroids. Their action also is similar to juvenile hormone mimics that occur in higher plants. The compounds from *Myrtillocactus geometrizans* have similar activity to known juvenile hormone mimics, but do not have the same structures. Insect growth regulatory activity on *S. frugiperda* was shown by phytoecdysteroids from *Ajuga remota* (Labiatae) (Kubo et al., 1981) on two polyphagous (*Spodoptera littoralis* and *Ostrinia nubilalis*) and a monophagous species (*Bombyx mori*) (Marion-Poll and Descoins, 2002).

Although there is an ample body of literature about biological activities of phytoecdysteroids (Simon and Koolman, 1989; Sláma and Lafont, 1995; Schmelz et al., 1999; Sáez et al., 2000; Savchenko et al., 2000; Dinan et al., 2001), there are no reports for insecticidal activity of these sterols.

2.5. Acute toxicity on last stage larvae of *Spodoptera frugiperda* and *Tenebrio molitor*

In order to determine a possible correlation between insect growth regulatory (IGR), acute toxicity, and ecdysis caused by these steroidal compounds, oral injection of 25 ppm of sterol **1** and **2** into 10 larvae of 21 days of *S. frugiperda* was carried out. This concentration promoted apolysis to the fifth instar, but inhibited ecdysis,

Table 4
Growth inhibitory activities on *Tenebrio molitor* as a function of increased concentrations of compounds **1**,**2**, **M_2**, and MeOH extract from *Myrtillocactus geometrizans*[a]

Samples	Doses	Number of pupae formed				Duration of pupal stage[b]	Successfully pupation%[c]	Emergence%[d]
		5 Days	12 Days	20 Days	25 Days			
Control	(ppm)	3	9	27	57	15.5 ± 0.4a	95	95
1	300	20	29	44	45	n.d.	75.0	0
	200	24	33	48	48	n.d.	80.0	0
	100	20	29	43	45	14.5 ± 0.7a	75.0	5*
	50	18	20	40	44	13.5 ± 0.6b	73.3	5*
2	300	15	30	45	44	n.d.	73.3	0
	200	17	34	48	47	n.d.	78.3	0
	100	15	29	50	45	12.5 ± 0.8b	75.0	3*
	50	5	25	39	43	14.5 ± 0.6a	71.7	5*
M_2	300	22	31	52	48	n.d.	80.0	0
	200	19	35	50	49	n.d.	81.7	0
	100	11	36	48	47	n.d.	78.3	0
	50	5	30	43	45	10.9 ± 0.6c	75.0	5*
MeOH extract	300	5	15	25	30	n.d.	50.0	0
	200	3	12	24	36	n.d.	60.0	0
	100	3	10	23	38	n.d.	63.3	0
	50	2	11	22	31	11.5 ± 0.7c	51.7	1*

[a] Twenty larvae by assay and by triplicate, larvae of last stage, topical application.
[b] Average duration, the criteria used were to measure until emergence of survival pupae, n.d. meaning correspond to pupae that not produce any adult. Means followed by the same letter within a column after ± SE values are not significantly different in a Student–Newman–Keuls (SNK) test at $p < 0.05$ (treatments are compared by concentration to control), 95% Confidence limits.
[c] Percentage with respect to control.
[d] The asterisk indicate adults with deformities.

whereas oral injection of 25 ppm of chichipegenin **3** resulted only in a delay of the normal molt to the fifth instar. Doubling the oral dose of both sterols to 50 ppm induced prothetely as evidenced by the appearance of precocious pupal structures in the larvae, in some (30%) of the treated fourth instar larvae after 48 and 72 h. These larvae molted directly to pupae. Prothetely can sometimes be elicited experimentally in larvae by application of juvenile hormone or juvenile hormone mimics (Truman and Riddiford, 2002). Peniocerol-induced prothetely resulted in precociousness and browning of pupae in roughly half of the controls (data not show).

The same bioassay was carried out on last instar larvae of *Tenebrio molitor*. At 50 ppm, peniocerol **1**, macdougallin **2**, and mixtures **M_1** and **M_2** and the **MeOH** and **CH_2Cl_2** extracts produced strong acute toxicity with only 12.5%, 25.0%, 35.0% and 10% survival, respectively (data not show).

On the other hand, **1**, **2**, **M_2** and **MeOH** extract, and at higher levels (100 ppm), peniocerol **1** (5.0% survival), macdougallin **2** (3%), **M_2** (0%) and **MeOH** extract (0%) exhibited potent acute toxicity to larvae of *T. molitor* (Table 4).

3. Concluding remarks

Based on these results, we suggest that the insect growth inhibition caused by a mixture of peniocerol and macdougallin could be due to synergistic effects as in **M_2** (**1** + **2**, 1:1). These plant sterols may be considered to be efficient insect growth regulators (IGR), as well as having activity similar to phytoecdysteroids, as was evidenced by their significant inhibition of molting processes.

The sterols peniocerol **1**, macdougallin **2** and the mixture **M_2** all had potent insecticidal and growth inhibitory activities. Conversion of the hydroxyl group to the tosyl derivative **1b** results in significant loss of activity. This finding suggests that hydroxyl groups play an important role in both the insecticidal and IGR activity of compounds **1** and **2**.

The most active compounds, **1** and **2**, contain a small and relatively lipophilic group at C-17, hydroxyl groups at C-3 and C-6, and a double bond between C-8 and C-9 (Δ^8) (endo position), whereas β-ecdysone (20-hydroxyecdysone) (20*E*) **4** and ajugasterone C **5** have this same functionality between C-7 and C-8 (Δ^7) and a carbonyl group (α,β-unsatured) at C-6. Compounds **1** and **2** had potency that was comparable to **4** and **5** (Kubo and Klocke, 1983). In contrast, compound **3**, with four hydrophilic hydroxyl groups, but without a double bond and lacking a lipophilic group (as in β-ecdysone, **4**), exhibited the lowest activity level of activity.

These results confirm previous findings on structure activity relationships (SAR) for phytoecdysteroids and derivatives, namely that the growth inhibitory activity of the respective natural product depends on the number of hydroxyl groups and the presence of a moderate by bulky group at C-17 (Dinan, 2001). Based on previous empirically derived SAR studies by Dinan, compounds

with *cis*-A/B-ring junctions, a Δ^7-double bond, a 6-keto group, a full 8-carbon side-chain and a 7-en-6-one or a 8(9)-en-6-hydroxy moiety (as in our case) should not have activity (Dinan, 2001). Nonetheless, our compounds are quite active.

The preceding experimental observations suggest that acute toxicity and growth inhibition of our compounds may be due to inhibition of a proteinase, ETH and other polyphenol oxidases (PPO) that bind to these steroids. This target has been demonstrated for other compounds of natural origin (Kubo et al., 1986; Carrizo et al., 1998; Tamayo et al., 2000; Karban and Baxter, 2001).

In summary, the insecticidal activity of **1**, **2** and mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$ from roots and aerial parts of *Myrtillocactus geometrizans* may be due to a synergistic effect shown by the ecdysonelike activity of the extracts in the test system used in this investigation. These compounds show structural similarities to ecdysones from other natural sources (Baltaev, 2000; Dinan et al., 2001).

The sites and mode of action of these compounds and extracts are being investigated and probably correspond to a combination of antifeedant action, midgut phenol oxidase, proteinase, ETH, tyrosinase or other PPOs, and cuticle synthesis inhibition, as well as moulting sclerotization toxicity, as has been found for other natural compounds (Kubo et al., 2000, 2003a,b; Kubo, 2000; Kubo and Kinst-Hori, 1999; Taýbi et al., 2003; Berghiche et al., 2003) and extracts (Feng et al., 1995).

Thus, the effect of compounds **1**, **2**, and mixtures $\mathbf{M_1}$ and $\mathbf{M_2}$ on reducing insect growth, increasing or shortening developmental time, modifying apolysis during molting and producing high mortality in *S. frugiperda* and *T. molitor* larvae were more powerful than gedunin, MeOHYuc and MeOH extract from *Cedrela salvadorensis* (Calderón et al., 2001; Céspedes et al., 2000). Although chemically distinct, the level of insecticidal activity of metabolites and mixtures derived from *M. geometrizans* is comparable to that of the known insect growth regulator, gedunin. Based on the present investigations, materials from *M. geometrizans* and related species should prove to be valuable sources of interesting biologically active compounds, including insecticides. Studies of the biological activity of the isolated compounds are in progress.

4. Materials and methods

4.1. Plant materials

Plant material of *Myrtillocactus geometrizans* was collected on the mountain slopes between the States of Hidalgo and Querétaro, Mexico, in October 2002. Voucher samples (MEXU-1075998 and 1075999) were deposited at the National Herbarium (MEXU), Institute of Biology, UNAM.

4.2. Spectral data

IR spectra were recorded on a Nicolet Magna-IR 750 spectrometer. ^{1}H NMR spectra were recorded at 300 and 500 MHz, and ^{13}C NMR spectra at 75 and 125 MHz, respectively, on Varian VXR-300S and VXR-500S spectrometers. Chemical shifts (ppm) are relative to $(CH_3)_4Si$. $CDCl_3$, MeOD-d_4, and acetone-d_6 from Aldrich Chemical Co. were used as solvents. Coupling constants are quoted in Hz. EIMS data were determined on a JEOL JMS-AX505HA mass spectrometer at 70 eV FABMS were obtained on a JEOL JMS-SX102A mass spectrometer operated with an acceleration voltage of 10 kV. Samples were desorbed from a nitrobenzyl alcohol matrix using 6 keV Xenon atoms. UV spectra of pure compounds were determined on a Shimadzu UV-160 instrument. Optical rotation was measured on a JASCO DIP-360 spectropolarimeter. Melting points were obtained on a Fisher-Johns hot-plate apparatus and remain uncorrected.

4.3. Chemicals and solvents

All reagents were either analytical reagent or chromatographic grade. Methanol, CH_2Cl_2, $CHCl_3$, KCl, $CuSO_4$, NH_4Cl, $MgCl_2$, *p*-toluenesulfonyl chloride, pyridine, acetic anhydride, silica gel GF_{254} analytical chromatoplates, silica gel grade 60, (70–230, 60Å) for column chromatography, *n*-hexane, and ethyl acetate were purchased from Merck-Mexico, S.A., Mexico. Pyridine and acetic anhydride were distilled prior to use. Column chromatography was carried out on Silica Gel G (Merck, Darmstadt, Germany).

4.4. Extraction, isolation and derivatization of triterpene and sterols

Dried and ground roots and aerial part (8.5 and 2.7 kg, respectively) of *M. geometrizans* were processed, extracted, and purified. These samples were first extracted with a mixture of MeOH/CH_2Cl_2 (1:1), the two phases separated by water/methanol partition, to yield two extracts MeOH (EM) and CH_2Cl_2 (ED). These extracts were used for preliminary bioassay evaluation.

The most active extract of each of the samples was the MeOH extract, which was tested for insecticidal activity and then submitted to open column chromatography on SiO_2 Merck (G 60) as solid phase. In addition to the MeOH extract (EM), column chromatography was carried out with the CH_2 Cl_2 extract (ED). Elution of both columns was carried out with solvent systems (hexane:ethyl acetate:methanol mixtures) that afforded different active fractions. These fractions were subsequently analyzed by TLC and insecticidal assay. Repeated TLC of these fractions led to isolation of individual secondary metabolites that were purified by prep-TLC.

Identical compounds were collected and identified by TLC and comparison with authentic samples.

Peniocerol **1** (2.5 g, molecular weight 402.64 g/mol) was isolated from EM. This compound was isolated previously from *Peniocereus fosterianus* (Djerassi et al., 1965). Macdougallin **2** (1.9 g, molecular weight 416.6 g/mol) was isolated by repeated crystallization from MeOH and later from aerial plant parts following the procedures reported by Djerassi (Djerassi et al., 1957). The MeOH extract was also hydrolyzed with acid the resulting mixture extracted with ethyl acetate, dried with sodium sulfate, and subsequently purified by column chromatography to give a large yield of chichipegenin **3** (>5.5 g, molecular weight 474.6 g/mol) (Fig. 1). This compound was isolated previously from *M. geometrizans* and from *Lemaireocereus chichipe* (Sandoval et al., 1957; Khong and Lewis, 1975).

Each of these compounds was obtained as a pure natural product, which was analyzed and characterized completely by its Rf, IR, UV, ^{1}H NMR and ^{13}C NMR data. In addition to the spectroscopic methods, the identification of triterpene **3** and sterols **1** and **2** was made by direct comparison with authentic samples (Sandoval et al., 1957). Compounds **1**, **2**, and **3** were purified in sufficient amount for be used for bioassays. Analytical TLC were performed on Silica gel 60 F_{254} (E. Merck) plates and bands visualized by spraying with a 10% solution of H_2SO_4 followed by heating at 110 °C.

4.5. Acetylation and tosylation of peniocerol and macdougallin

Derivatization of the C_3- and C_6-hydroxyl groups by acetylation with acetic anhydride/pyridine of peniocerol (**1**) gave peniocerol diacetate (**1a**) (Knight et al., 1966). Peniocerol (55 mg) was treated with Ac_2O (2 mL) and pyridine (2 mL) at room temperature for 48 h. The reaction mixture was worked up as routine procedures to yield 41 mg of (**1a**), which was then purified by preparative-TLC. A sample of peniocerol acetate was eluted with a *n*-hexane/ethyl acetate (80:20) solvent system, using UV_{254} for detection. Structures were established by high-resolution spectroscopic methods and data are in agreement with previously reported values (Knight et al., 1966).

The tosyl derivative was prepared according to routine procedures. A mixture of peniocerol (10 ml) in chloroform (50 ml), magnetically stirred at 0 °C, was placed in a three necked flask and chlorosulphonic acid (1.0 ml) added over a period of 15 min with cooling and exclusion of moisture. Stirring was continued for 45 min at room temperature and the mixture poured onto crushed ice. The product was extracted with chloroform (100 ml), dried over anhydrous sodium sulphate and freed from solvent in vacuo at 30 °C. The white residue was redissolved in warm pentane, filtered and concentrated (to about 200 ml). The tosyl derivative crystallizes slowly on standing and was washed with pre-chilled pentane (−30 °C); after drying in vacuo over phosphorous pentoxide the yield was 75.0%. Further concentration of the motherliquor gave a second crop (total yield 86%). The product was recrystallised from pentane and dried over phosphorous pentoxide in vacuo. Similar procedure was used for derivatives from macdougallin and acetyl derivative from chichipegenin.

4.6. Bioassays with fall armyworm (*S. frugiperda*)

Larvae used for experiments were obtained from culture at the Centro de Investigación en Biotecnología at the Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México, maintained under previously described conditions (Aranda et al., 1996; Céspedes et al., 2000). 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 (Mihm, 1987). 24-Well polystyrene multidishes were filled with the liquid diet, and then left for 20 min at room temperature under sterile conditions. The 3.4 ml wells measure 17 mm in depth × 15 mm in diameter with a 1.9 cm^2 culture area. Up to six concentrations of each all test compound was dissolved in 95% ethanol and layered on top of each well with the artificial diet (see Table 1) and a control (1 ml 95% ethanol) bollowing evaporation of solvent. Hexane (1.0 ppm) and MeOH extracts (3.5 ppm) were used, as these extracts showed the highest inhibitory activity in preliminary trials (data not shown). For each concentration and for the controls, a single *S. frugiperda* neonate first instar larva was placed on the diet mixture in each well for 7 days. Thus, each experiment contains 72 larvae in total (each plate of 24 wells with three replicates). After 7 days, surviving larvae were measured and weighed and then transferred to separate vials containing fresh stock diet. Larval weight gains and mortality were recorded after 21 days of incubation, as the pupation average is 23 ± 1 days. Other life cycle measurements, such as time to pupation, mortality of larvae and adult emergence and deformities were recorded. All treatments were carried out in a controlled environmental chamber with an 18 L:6D photoperiod, at 25 °C day and 19 °C night temperature regime, and a relative humidity of 80% ± 5%. There were three replications for each assay. Control assays (24-wells) contained the same numbers of larvae, volume

of diet, and ethanol as the test solutions (Torres et al., 2003; Céspedes et al., 2000).

4.7. Bioassays with yellow meal worm (*T. molitor*)

A stock culture of *T. molitor* (Coleoptera: Tenebrionidae) larvae was fed with wheat bran in plastic boxes at 24.0 ± 1 °C, with a 16:8/L:D photoperiod, and these larvae were maintained into a chamber under these environmental conditions. Bioassays were performed with last instar larvae of *T. molitor* based on live weight (103–160 mg). For each compound test solutions Me_2CO/MeOH (9.5:0.5 v/v) were applied topically to ventral abdominal segments with a microsyringe at 2 μL/larva; equivalent to 0.2 μgL/larva of the assayed compounds for each of the concentrations used. Controls were treated with the solvent alone. For each individual compound, there were three replicates of 20 larvae each and the assay was run with three replicates. After treatment, insects were placed in Petri dishes (5 cm diameter), with 3 g of stirilized wheat bran, a moistened cotton for preserve humidity and held at 24.0 ± 1 °C with 16:8 (L:D) photoperiod. The number of larvae that successfully pupated, as well as the duration of the pupal stage (in days) were recorded every 24 h for 30 days (end-point of the experiment).

4.8. Acute toxicity

Acute toxicity was determined by topical application and oral injection of compounds to larvae of the last stage (fifth instar) of *S. frugiperda* and *T. molitor*, respectively. The larvae were iced to stop their movement and treated on their abdomens and mouths with each of the test compounds, at concentrations of 10.0, 25.0 and 50.0 ppm, for both insect species. The solvent used was acetone (10.5 μl) which was administered with a 25 μl microsyringe (Hamilton). The control was treated only with 10.5 μl of acetone. After 24 h, survivals were recorded. Ten larvae were used for each concentration, respectively (Calderón et al., 2001; Torres et al., 2003).

4.9. Relative growth index and growth index

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

4.10. Statistical analyses

Data shown in figures and tables are average results obtained by means of three replicates and independent experiments and are presented as average ± standard errors of the mean (SEM). Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by GLM procedures. Results are given in the text as probability values, with $p < 0.05$ adopted as the criterion of significance. Differences between treatment means were established with a Student–Newman–Keuls (SNK) test. The GI_{50}, RI_{50} and I_{50} values for each activity were calculated by PROBIT analysis based on percentage of inhibition obtained at each concentration of the samples. I_{50} is the concentration producing 50% inhibition of growth. Complete statistical analysis was performed by means of the MicroCal Origin 6.0 statistical and graphs PC program.

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