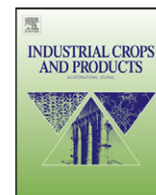
Industrial Crops and Products 42 (2013) 78–86

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

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



The insecticidal, molting disruption and insect growth inhibitory activity of extracts from *Condalia microphylla* Cav. (Rhamnaceae)

Carlos L. Cespedes[a,*], Sofia C. Molina[a], Evelyn Muñoz[a], Claudio Lamilla[a], Julio Alarcon[b], Sara M. Palacios[c], Maria C. Carpinella[c], Jose G. Avila[d]

[a] Laboratorio de Fitoquimica-Ecologica, Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad del Bio-Bio, Casilla 447. Avenida Andrés Bello s/n, Chillan, Chile
[b] Laboratorio de Síntesis y Biotransformacion, Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad del Bio-Bio, Casilla 447. Avenida Andrés Bello s/n, Chillan, Chile
[c] Laboratorio de Fitoquímica Fina, Facultad de Ciencias Químicas, Universidad Católica de Córdoba, Córdoba, Argentina
[d] Laboratorio de Fitoquímica, UBIPRO, FES-Iztacala, UNAM, México D.F., Mexico

ARTICLE INFO

Article history:
Received 10 January 2012
Received in revised form 30 April 2012
Accepted 5 May 2012

Keywords:
Condalia microphylla
Extracts
Rhamnaceae
Insect growth inhibitory activity
Insecticides
Molting disruption
Biopesticides
Defense mechanism

ABSTRACT

Extracts obtained from a common shrub that occurs as part of vegetative species growing on arid lands of North-Central Chile and adjacent central Argentina known as “piquilin” *Condalia microphylla* (Rhamnaceae) showed insect growth inhibitory activity against the fall armyworm *Spodoptera frugiperda*, yellow meal worm *Tenebrio molitor* and fruit fly *Drosophila melanogaster* larvae in artificial diet feeding assays. The effects of these extracts on mortality, antifeedancy and growth inhibition were examined. The phytochemical profile of the most active extract was examined with conventional chromatographic and spectroscopic procedures. This *n*-hexane extract showed a high percentage of hentriacontane and triacontane. The observed mortality strongly correlates with the contents of these long-chain *n*-alkanes compounds, the LD_{50} for *n*-hexane, ethyl acetate and methanol extracts against *S. frugiperda*, were 3.89, 9.4, and 9.7 ppm; against *T. molitor* 5.2, 14.2, and 20.4 ppm, and against *D. melanogaster* 3.23, 7.65 and 17.9 ppm, respectively.

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1. Introduction

Plants in general produce a great variety of secondary metabolites that do not have apparent function in fundamental physiological or biochemical processes; these compounds (or allelochemicals) are often important for mediating interactions between plants and their biotic environment (Berenbaum, 2002; Kessler and Baldwin, 2002). Additional information indicates that many natural products can be models of active defense against insect predators (Crombie, 1999) and inhibitors of enzymes such as tyrosinase or acetylcholinesterase (Keane and Ryan, 1999; Ortego et al., 1999; Kubo et al., 2003a,b). The increasing interest in the possible application of secondary metabolites for pest management has directed the investigation towards search for new sources of biologically active natural products, with new mode, sites, and mechanisms of action, selectivity, and specific action (Conner et al., 2000; Eisner et al., 2000; Meinwald, 2001); these characteristics may enhance their value as commercial pesticides (Akhtar et al., 2008; Isman, 2006).

The literature has reported that different *Condalia* species contain cyclopeptide alkaloids as in *Condalia buxifolia* showing several biological activities (Morel et al., 2002) and that the bark and root of *Condalia microphylla* have tannins (Gimenez et al., 2008).

Condalia microphylla Cav. (Rhamnaceae) is known in Chile and Argentina as “piquilin”. This densely branched shrub that occurs on arid lands in central Argentina (montane grasslands and shrublands) and in foothills of north-central Chile (Mediterranean forests, woodlands, and shrublands) grows to approximately 2 m tall. Although this shrub has not been studied in Argentina or Chile, the presence of hydrocarbons and fatty acids has been reported in the related *Condalia montana* species complex (Zygadlo and Guzman, 1991; Zygadlo et al., 1992).

This shrub is the cause of “mal del piquilin” in cattle; it is unclear whether it is a neurotoxic or hepatotoxic disease (Bedotti et al., 2006). The toxicological activity of *Condalia* species has also been reported by Delgado et al. (2011). Additionally, it has been reported that the use of aerial parts (bark, leaves and stems) of *C. microphylla* induces the accumulation of long-chain n-alkanes, producing ataxia and “paraffin-liver” in cattle (Delgado et al., 2011; Halse et al., 1993). Based on this information and the strong resistance of the wood and leaves to insect and pathogen attack, further studies were carried out to determine insecticidal and insect growth

* Corresponding author. Tel.: +56 42 253277; fax: +56 42 253046.
E-mail address: cespedes.leonardo@gmail.com (C.L. Cespedes).

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http://dx.doi.org/10.1016/j.indcrop.2012.05.002

regulatory effects of the methanol, *n*-hexane, ethyl acetate and aqueous extracts of aerial parts of this shrub.

The present paper deals with the effects of methanol, *n*-hexane, ethyl acetate and aqueous extracts from aerial parts of *C. microphylla* on growth and development of *Spodoptera frugiperda* (fall armyworm), *Drosophila melanogaster* (fruit fly) and *Tenebrio molitor* (yellow meal worm). These data are important for studies of insect control (Cespedes et al., 2006).

2. Material and methods

2.1. Plant material

Samples of aerial parts (leaves, heartwood and bark) of *Condalia microphylla* were collected during spring 2010 (November) on the slope of Andes Mountains at roadside to Los Andes–Portillo highway near Los Andes City in Chile (collection 1) and at Ecological Reserve on Campus of Catholic University of Cordoba, Cordoba, Argentina (collection 2). Voucher specimens were deposited on the Herbarium of Basic Science Department, University of Bio-Bio, Chillan, Chile (Voucher CLC/0035) and Herbarium "Dr. Marcelino Sagayo" (UCCOR). Specimens were identified by Prof. Gustavo Ruiz, Ph. D. (Agronomic Engineering), Director Herbarium "Dr. Marcelino Sagayo" (UCCOR–acronym not registered), Faculty of Agricultural Sciences, Catholic University of Cordoba, Argentina. The samples of *C. microphylla* were dried and milled, then were macerated with methanol and concentrated under vacuum rotatory evaporator until viscose consistency.

2.2. Extracts and fractions

Plant material was dried at room temperature. The sample was crushed and extracted with methanol for one day, filtered, and the process repeated five times. The resulting MeOH extract was filtered and concentrated under vacuum at 40 °C and 200 mb to obtain a crude residue (475 g). A portion of the total methanolic extract of *C. microphylla* (234 g), was solvent partitioned by dissolving in a mixture of MeOH/H_2O (1:1), transferred to a separatory funnel and extracted 20 times with *n*-hexane (150 ml per extraction), the *n*-hexane phases combined and concentrated under reduced pressure. An identical process was repeated with ethyl acetate and finally obtained a residual water/methanol.

2.3. Phytochemical analysis

A sample of *n*-hexane extract was analyzed by gas chromatography–mass spectrometry in an Agilent Technologies GC–MS System Model G2630A/G3170A gas chromatograph equipped with a DB WAX column (30 m × 0.32 mm). The temperature of the column was programed from 80 to 220 °C at 8 °C min. The injector and detector temperatures were 225 °C. The gas carrier was He, at a flow rate of 1 ml min. Peak areas were measured by electronic integration. The relative amount of the individual components was based on the peak areas and data processor provided information for qualitative and quantitative analysis of the alkane mixture. The temperature of the column and the injector were the same as those from GC. Mass spectra were recorded at 70 eV. Authentic samples and commercial standards were used as patterns for identification. Equivalent chain lengths (ECL) were calculated and compared with results previously reported (Zygadlo et al., 1992). For alkane determination the *n*-hexane extract was evaporated to dryness and redissolved in CH_2Cl_2, the mixture was chromatographed. Alkanes were identified by retention time with respect to pattern reference compounds run under identical conditions and compared with authentic samples.

2.4. Insect rearing techniques bioassays

2.4.1. Antifeedant test against S. frugiperda

S. frugiperda J.E. Smith (Lepidoptera: Noctuidae) larvae for this assay were obtained from Fine Chemicals and Natural Products Laboratory at Catholic University of Cordoba, Argentina. The antifeedant activity was evaluated through choice tests according to Valladares et al. (1997). Briefly, one larva per Petri dish was deposited together with two lettuce circles of 1 cm^2, one of this was sprayed with 10 μl of an ethanolic solution (100 ppm) of the solution to be tested and the other disk with the same quantity of ethanol. Under the lettuce, a round piece of filter paper moistened with distilled water was placed to reduce the dehydration of the plant material. Ten repetitions were made for each extract. The measurements were made for 12 and 24 h, establishing the percentage of consumed area (estimated visually through the use of a grid) and the antifeedant inhibition rate was calculated $AI\% = [(1 - T/C) \times 100]$, where *T* is the average area treated leaf extract consumed and *C* is the average area of leaf consumed without treatment. Once selected the samples with a higher AI%, there was a gradient with different concentrations of these extracts and their most significant effects were measured at 24 h and are showed in Table 2.

2.4.2. Insecticidal activity against larvae of fruit fly (D. melanogaster)

The bioassay for insecticidal activity against larvae of *D. melanogaster* was carried out as follows (Miyazawa et al., 2000): five concentrations (2.0, 5.0, 10.0, 20.0, and 50.0 ppm of sample) were used for determining LD_{50} values. Test compounds were dissolved in 50 μl of EtOH and mixed in 1 ml of artificial diet [brewers' yeast (60 g), glucose (80 g), agar (12 g), and propionic acid (8 ml) in water (1000 ml)]. A control diet was treated with 50 μl of EtOH only. About 100 adults from colonies of *D. melanogaster* were introduced into a new flask with fresh diet and then allowed to oviposit at 25 °C and relative humidity > 60% for 3 h, after 10 eggs were collected and transplanted onto each diet-test (1 ml) in glass tubes and reared at 25 °C and relative humidity > 90% for 8 days. The larvae were hatched and fed each test samples (*n*-hexane, ethyl acetate, methanol, and aqueous) mixed with the artificial diet at different concentrations (2.0, 5.0, 10.0, 20.0, and 50.0 ppm). At 25 °C, larvae generally change to pupate after 7 days. In each instance, the developmental stage was observed, and the numbers of pupae were recorded and compared with those of a control. Ten new eggs were used in each of the five replicates. The LD_{50}, the concentration that produces 50% mortality, was determined by log-probit analysis.

2.4.3. Insecticidal bioassay with S. frugiperda larvae

Larvae of *S. frugiperda* J.E. Smith (Lepidoptera: Noctuidae) used for this experiment were obtained from culture at the Fine Chemicals and Natural Products Laboratory at Catholic University of Cordoba, Argentina, and maintained under previously described conditions (Cespedes et al., 2000). An artificial diet (1 kg) containing 800 ml of sterile water, 10.0 g of agar, 50.0 g of soya 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, was used and prepared by the procedure described earlier (Mihm, 1987). Polystyrene multidishes (24-well) were filled with the liquid diet, and allowed to solidify for 20 min at room temperature under sterile conditions. The 3.4 ml wells measured 17 mm in depth, 15 mm in diameter with a 1.9 cm^2 culture area. All samples were dissolved in 95% ethanol and were sprayed in layers on top of each well with artificial diet

Table 1
Alkane composition (% total) of two populations of *Condalia microphylla* (collections 1 and 2, for collections see Section 2.1) compared with two populations of Cordoba Area, Argentina.[a]

Compounds[d]	Collection 1	Collection 2	Population 6[b]	Population 14[c]
Nonadecane (C-19)	4.0	4.8	4.9	3.2
Heinecosane (C-21)	2.2	2.0	2.1	2.0
Tricosane (C-23)	3.0	2.9	3.1	4.0
Pentacosane (C-25)	8.1	7.9	8.1	6.2
Heptacosane (C-27)	4.7	4.5	4.5	9.9
Octacosane (C-28)	3.8	3.8	5.5	6.3
Nonacosane (C-29)	31.2	29.0	26.0	30.0
Triacontane (C-30)	3.9	3.9	4.8	5.0
Hentriacontane (C-31)	33.7	32.7	34.3	12.4
Tritriacontane (C-33)	5.0	6.1	6.0	15.5
Unknown	0.4	2.4	0.7	5.5

[a] For populations 6 and 14, please see ref. Zygadlo et al. (1992).
[b] *C. microphylla* f. xanthocarpa (Cordoba area, Argentina).
[c] *C. microphylla* f. melanocarpa (Cordoba area, Argentina).
[d] For structural determination, please see Section 2.3.

at 5 concentrations (Tables 2–4) and a control (1 ml 95% ethanol). Ethyl acetate, *n*-hexane and methanol extracts were used, as these extracts showed the highest inhibitory activity in preliminary trials (data not shown). For each concentration used and for the controls, a single *S. frugiperda* neonate larva was placed on the diet mixture in each well for 7 days. Thus each treatment included 72 larvae in total (i.e., three plates of 24 wells). 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 day. Other life cycle data such as time to pupation, mortality of larvae and adult emergence and deformities were recorded. All experiments were carried out in a controlled environmental chamber with an 18L:6D photoperiod, 19 and 25 °C night and day temperature, respectively, and a relative humidity of 80 ± 5%. There were five replications for each treatment. Controls contained the same numbers of larvae, volume of diet, and ethanol as the test solutions (Torres et al., 2003; Cespedes et al., 2000, 2005).

2.4.4. Bioassays with yellow meal worm (T. molitor)

Larvae of *T. molitor* L. (*Coleoptera*: *Tenebrionidae*) were fed with wheat bran in plastic boxes at 24.0 ± 1° C, with a 16:8/L:D photoperiod, these larvae maintained into a chamber under these environmental conditions were used in the test. 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 topically applied to ventral abdominal segments with a microsyringe 2 μl/larva; equivalent to 0.2 μg/larva of the assayed compounds for each one of 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 three replicates. After treatment insects were placed in Petri dishes (5 cm diameter), with 3 g of sterilized wheat bran, a plug of 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) (Cespedes et al., 2005).

2.4.5. 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 2.0, 10.0, 25.0 and 50.0 ppm, for each of the insect species. The solvent used was acetone (10.5 μl) which was administered with a microsyringe Hamilton of 25 μl. The control was only treated with 10.5 μl of acetone. After 24 h, survivals were recorded. Ten larvae were used for each concentration, respectively (Torres et al., 2003).

2.4.6. Relative growth index and growth index

The relative growth index (RGI) and growth index (GI) were calculated according to Zhang et al. (1993). Different experiments were used for to evaluate these indexes.

2.5. Statistical analyses

Data are average results obtained by means of three or five 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 6.1 statistical and graphs PC program.

3. Results and discussion

In our screening program designed to discover interesting biological activities of plants from temperate regions it was found in a preliminary trial that *C. microphylla* showed insecticidal activity. Based on this information and the observed resistance to insect and pathogen attack on the plant, we carried out an insect grow regulatory study of the aqueous, ethyl acetate, methanol, and *n*-hexane extracts of aerial parts of this shrub.

Milled sample of aerial parts from *C. microphylla* was macerated with MeOH and further partitioned with *n*-hexane, ethyl acetate and water, respectively. These extracts were used in a preliminary bioassay trial. Subsequently, in order to obtain more satisfactory data for insecticidal activity, some bioassays were carried out at lower concentrations. Gedunin and methanol extract from *Cedrela salvadorensis* (Me–Ced) were used as patterns and positive controls (Cespedes et al., 2000, 2004, 2005; Torres et al., 2003).

3.1. Phytochemical analysis

Table 1 shows the *n*-alkanes composition (%) of *C. microphylla*. In the present work, the yield of *Condalia* neo-triacontanes and

Table 2
Results obtained in antifeedant election tests, using different concentrations of extracts of *C. microphylla* on *S. frugiperda* larvae. Antifeedant inhibition (AI) percentage.[a]

Concentration (ppm)	Control	Aqueous	Ethyl acetate	*n*-Hexane	MeOH	Me–Ced	Gedunin
1	0	−5	5	5	13	5	21
5	0	−10	20	25	33	25	33
10	0	−15	40	75	66	37	45
25	0	−40	80	80	79	62	51
50	0	−51	85	98	90	89	89
100	0	−79	90	100	100	99	95

Control contain only solvent (ethanol 15%), Me–Ced and gedunin were used as positive control.
[a] AI (%) = Antifeedant inhibition = $[(1 - T/C) \times 100]$.

triacontanes (C-27, C-29, C-30, C-31, and C-33, respectively), using conventional extraction procedures (>35%), was comparable with literature data (Zygadlo et al., 1992; Frontera et al., 2000). On the other hand, *n*-hexane direct extraction was a little more efficient in its isolation from the plant (data not show) than that conventional method with methanol. Interestingly, the yield of *n*-alkanes of the Chilean collection (collection 1) was slightly higher than yield obtained for Argentinian collection (collection 2), this phenomena could be explained by normal variation in the plant and by the ecological and environmental conditions of the places of collections, "Montane grasslands and shrublands" in Argentina and "Mediterranean forests woodlands and shrubs" in Chile, respectively, being more arid in Chile than Argentina. The *n*-alkanes composition determined in this work is similar to the composition of *Condalia* complex in the *n*-hexane extracts published by Zygadlo et al. (1992).

3.2. Antifeedant activity against *Spodoptera frugiperda*

The methanol, *n*-hexane and ethyl acetate extracts of *C. microphylla* showed a high feeding dissuasive activity against *S. frugiperda* larvae in the election assays (Table 2). The consumption of leaves treated with these extracts was significantly lower in contrast to the controls and in the case of the *n*-hexane extract, this showed high levels of feeding inhibition, according to Hassanali and Lwande (1989) who consider a AI (antifeedant inhibition percentage) as high when it is greater than 75%, and moderate when it is between 50 and 75%, while in the ethyl acetate extract just a significant reduction of the consumption was observed, therefore the effect of this extract on the larvae can be considered as moderate. In the case of the aqueous extract, it showed the opposite effect, where the larvae preferred the treated leave instead of the control, this, presumably because of the high sugar content in this fraction of the plant.

In this bioassay, methanol extract at 10 ppm showed a reduced to moderate effect (66%), while ethyl acetate extract showed no significant effect at this concentration and the most active extract always was *n*-hexane with 75% showing high deterrence power.

3.3. Insecticidal activity

In the results obtained in assays of not choice for each one of the extracts shows that three of four extracts possesses a lethal effect on the *T. molitor*, *S. frugiperda* and *D. melanogaster* larvae (Table 3).

Against *S. frugiperda* the extracts of *n*-hexane, ethyl acetate and methanol at 10 ppm showed 80, 70 and 70% of mortality effect,

Table 3
Results obtained in tests with not choice measured in mortality percentage of *S. frugiperda*, *T. molitor* and *D. melanogaster* larvae, after application of the extracts at different concentrations in larvae's diet.

Sample	CONC. [ppm]	*S. frugiperda*	*T. molitor*	*D. melanogaster*	LD_{50} *S. frugiperda*	LD_{50} *T. molitor*	LD_{50} *D. melanogaster*
	Control	0	0	0.0			
Aqueous	10	0	0	0.0	n.d.	n.d.	n.d.
	25	0	0	0.0			
	50	0	0	0.0			
	100	0	0	0.0			
Ethyl acetate	10	70 ± 0.6b	45 ± 0.6b	61.0 ± 0.6a	9.4	14.2	7.65
	25	55 ± 0.7b	70 ± 0.7b	90.0 ± 0.6b			
	50	100 ± 0.8c	83.4 ± 0.8c	100.0 ± 1.0c			
	100	100 ± 1.0c	100 ± 1.0c	100.0 ± 1.0c			
Hexane	10	80 ± 0.2b	73.5 ± 0.3d	94.5 ± 0.6a	3.89	5.2	3.23
	25	90 ± 0.9c	84.7 ± 0.5b	100 ± 0.6a			
	50	100 ± 1.0c	95 ± 0.6b	100 ± 0.6b			
	100	100 ± 1.0c	100 ± 1.0c	100.0 ± 1.0c			
Methanol	10	70 ± 0.5b	40 ± 0.7b	30.0 ± 1.52	9.7	20.4	17.9
	25	50 ± 0.7b	55 ± 0.7b	65.0 ± 3.4b			
	50	100 ± 1.0c	80 ± 0.5b	100.0 ± 4.47c			
	100	100 ± 1.0c	100 ± 1.0c	100.0 ± 4.47c			
Me–Ced	10	54.0 ± 0.4d	25 ± 0.9a	70 ± 0.6a	48.0[a]		
	25	79.0 ± 0.3b	49 ± 0.8d	100 ± 1.0c			
	50	99.0 ± 0.9c	88 ± 0.7c	100 ± 1.0c			
Gedunin	10	37.0 ± 0.2a	22 ± 0.8a	69 ± 0.6a	10.8[a]		
	25	45.5 ± 0.3d	59 ± 0.9d	100 ± 1.0c			
	50	73.5 ± 0.6b	89 ± 0.4c	100 ± 1.0c			

Each value corresponds to the average of the five different experiments ± SE. The values followed by the same letter are not significantly different. The significance level $p < 95\%$.
The time for *S. frugiperda* was after 21 days, for *T. molitor* was 25 days, and for *D. melanogaster* 72 h.
[a] This value correspond to LD_{95} (Cespedes et al., 2005).

Table 4
Activity of extracts from *C. microphylla* on pupation and emergences parameters of fall armyworm (after 21 days of incubation).[a]

Treatment	Conc. (ppm)	Mean time pupation (days)[b]	Pupation *SP* (%)[e]	Mean weight pupae (mg)[c]	Mean emergence (days)[d]	Emergence (%)[f]	Male (%)	Female (%)
Control		22.0	88.2	309.5 ± 15.47a	33	77.50	35	42.5
n-Hexane	2.0	22.0	60.6	190.5 ± 11.43a	31	8.3	8.3*	–
	10.0	22.5	22.8	180.9 ± 9.78b	33	8.3	8.3*	–
	25.0	24.0	16.8	122.7 ± 8.79b	–	0.0	–	–
	50.0	n.d.	0	n.d.	–	0.0	–	–
Ethyl acetate	2.0	21.5	68.3	227.6 ± 11.38a	33	16.7	8.3	8.3
	10.0	24.0	25.7	150.8 ± 7.54b	36	8.3	8.3*	–
	25.0	25.0	12.5	148.8 ± 7.44b	–	0.0	–	–
	50.0	25.0	6.3	n.d.	–	0.0	–	–
MeOH	2.0	22.0	52.3	205.3 ± 10.26a	32	16.6	8.3*	8.3
	10.0	25.0	26.3	119.9 ± 5.49b	35	8.3	8.3*	–
	25.0	25.0	6.3	109.0 ± 4.44b	–	–	–	–
	50.0	25.0	0	n.d.	–	–	–	–
Gedunin	10.0	22.5	49.8	111.5 ± 5.57b	34	16.6	8.3	8.3
	25.0	23.0	24.2	67.1 ± 3.35c	35	15.6	5.2	10.4
	50.0	24.0	4.17[d]	55.1 ± 2.75c	36	4.17	4.17*	–
MeOH–Ced	2.0	21.5	78.4	235.9 ± 1179a	33	33.3	11.1	22.2
	10.0	22.0	56.2	148.2 ± 7.41b	33	30.5	10.2	20.3
	25.0	23.5	29.5	124.3 ± 6.21b	34	20.8	10.4	10.4
	50.0	24.5	18.2	119.2 ± 5.54b	34	16.7	–	16.7

[a] The values for growth bioassay were from weight, values taken at 22 ± 1 day 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 aqueous extract were omitted because are irrelevant and this extract 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.

respectively. Against *T. molitor* at 10 ppm only *n*-hexane showed a significant effect with a high percentage of mortality 73.5%. Against *D. melanogaster*, the *n*-hexane extract showed the highest mortality percentage with 94.5% at 10 ppm after 72 h and above 25 ppm it showed a mortality of 100% (Table 3).

Additionally, the effects of ethyl acetate, methanol and *n*-hexane extracts on growth and development of larvae of first instar of *S. frugiperda*, were evaluated initially at concentration of 2.0 ppm, the results are outlined in Table 4. Interestingly, methanol and ethyl acetate extracts induced a significant decrease in larval survival at 7 days. After 21 days, the number of larvae and pupae decreased drastically in all treatments. For example, at a concentration greater than 10.0 ppm for *n*-hexane, 25.0 ppm for ethyl acetate and to concentrations higher than 30.0 ppm for methanol extracts the pupation was very low (data not show). When pupation did occur, serious abnormalities were observed, and this phenomenon was observed in similar form to *D. melanogaster*. Ecdysis and sclerotization were incomplete. All pupae under *n*-hexane, ethyl acetate and methanol treatments died at concentrations above 35.0 ppm and at concentrations greater than 50 ppm, all extracts produced acute toxicity in the insect larvae of the three species (data not show).

In experiments of not choice carried out against larvae of first instar of *S. frugiperda* and four instar of *D. melanogaster* during the first 6 days, the effects of the ethyl acetate and methanol extracts were 100% lethal at concentration greater than 50 ppm (data not show). The concentrations that show 95% lethal doses (LD_{95}) of these extracts against *S. frugiperda* are: methanol (31.0 ppm), *n*-hexane (27.0 ppm), ethyl acetate (42.0 ppm) (data not show). At 30.0 ppm all extracts produced significant larval mortalities (> 80%), whereas the aqueous extract did not produced larval mortality at concentrations greater than 300 ppm (data not show).

3.4. Insect growth inhibitory activity against *Spodoptera frugiperda* larvae

At intermediate concentrations (between 10.0 and 25.0 ppm) of extracts specifically inhibited each larval growth stage, e.g., growth and weight gained (up to 75% of length) when incorporated into diets (Table 4). Moreover, *n*-hexane extract produced the strongest inhibition (58.5 and 39.6%, at 10.0 and 25.0 ppm, respectively) of growth and weight increase at 21 days (Table 4). On the other hand, the three extracts (*n*-hexane, ethyl acetate and methanol), above 25.0 ppm, showed a high growth inhibition, and after 21 days these extracts showed 100% of mortality, respectively (Table 4).

The percentage of larvae that reached pupation decreased drastically with almost all extracts assayed. Thus, *n*-hexane (10.0 ppm, 22.8%), ethyl acetate (10.0 ppm, 25.7%), and methanol (10 ppm, 26.3%) extracts showed significant delay of pupation (Table 4). Above 50 ppm, no larvae survived to pupation with *n*-hexane, ethyl acetate and methanol extracts (Table 4). Delays in time to pupation (>24 days) for *n*-hexane (>10.0 ppm), ethyl acetate (>15 ppm), and methanol (>35.0 ppm), were observed (data not show). Furthermore, at low concentrations of *n*-hexane, ethyl acetate and methanol between 2.0 and 10 ppm significantly reduced pupal weights. Being *n*-hexane the extract that produced the greatest effect on pupal weights between 1.0 and 5.0 ppm (data not shown).

The percentage of adults emergence from the pupae was also drastically affected by these substances. The greatest reductions were showed by *n*-hexane (2.0 and 10 ppm, 91.7%), ethyl acetate (2.0 and 10 ppm, 83.4 and 91.7%, respectively) and methanol (2.0 and 10 ppm, 83.4 and 91.7%, respectively), and at these concentrations deformities could be also observed. Moreover, at concentrations between 2.0 ppm and 0.5 ppm these extracts from *C. microphylla* significantly blocked the percentage of adult

Table 5
Insect growth regulatory activity of the ethyl acetate, MeOH and *n*-hexane extracts from *C. microphylla*, Me–Ced and gedunin against *S. frugiperda* larvae in a no-choice bioassay.[a]

	7	Days		21	Days	Pupation
Treatment	GWI_{50} [b]	GLI_{50} [c]	MC_{50} [d]	EI_{50} [b]	pI_{50} [e]	PI_{50} [f]
n-Hexane	8.6	5.3	3.9	0.55	0.26	3.46
Ethyl acetate	3.1	3.1	9.7	0.77	0.11	2.11
MeOH	4.0	8.4	3.5	3.10	0.49	4.62
Gedunin	2.7	5.9	27.9	0.66	0.18	9.96
MeOH–Ced	5.5	14.5	7.8	13.4	1.13	12.4

[a] The parameters in ppm values.
[b] The GWI_{50} and EI_{50} correspond to the growth inhibition in weight at 7 and 21 days, respectively, and was calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by the computer program ANOVA ($p < 0.05$) under Microcal Origin 6.1.
[c] GLI_{50} correspond to the growth inhibition in length at 7 days, and was calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by the computer program ANOVA ($p < 0.05$) under Microcal Origin 6.1.
[d] MC_{50} is the concentration producing 50% mortality.
[e] pI_{50} correspond to $-\log EI_{50}$.
[f] PI_{50} correspond to concentration producing 50% of pupation, and was calculated as the dose corresponding to midpoint between complete inhibition (100% of control) and no effect by the computer program ANOVA ($p < 0.05$) under Microcal Origin 6.1.

emergence, because no viable adults can emerge from pupae in 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 insect fed with *C. microphylla* extracts, revealed a developmental disruption in which the insects died (between 10 and 25 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 to facilitate the release of eclosion hormone (EH) (Truman et al., 1983; 2002) 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. Thus, the *n*-hexane extract may disrupt several steps of ecdysteroid metabolism to result in an inhibition of emergence behavior, or alternatively may act directly by inhibiting the release of ETH (Hesterlee and Morton, 1996). In our case and in base to toxicological information the accumulation of long-chain alkanes as hentriacontane for instance, by means of an unknown mechanism of action could be affecting the complete morphogenesis of the insects used in our study. Since the hydrophobic environment prevents the action of enzymes, which require an aqueous medium for its action (Jung and Deetz, 1993), all these considerations are supported under the evidence showed in Table 5, where the pI_{50} values are very low for *n*-hexane (0.26) and ethyl acetate (0.11), for instance.

3.5. Growth inhibition and relative growth index for S. frugiperda

Larvae reaching the pupal stage in the groups of lowest concentration completely not pupate and pupae that emerged showed deformities. Thus, 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 $GI_{treated}/GI_{control}$) (Table 6) shown that the strongest effects are between 2.0 and 10.0 ppm by *n*-hexane extract (RGI 0.25), and at 25 ppm by methanol (RGI 0.25). These parameters together with the LD_{95} (the lethal dose producing 95% of death) and LD_{50} values, established that the greatest effect were showed at 25.0 ppm by *n*-hexane (100% mortality), and by ethyl acetate and methanol at 35.0 ppm, respectively (100% mortality).

Interestingly, the phytochemical composition of the *n*-hexane extract of this study is conformed mainly by long-chain *n*-alkanes (Table 1) (Delgado et al., 2011; Halse et al., 1993). As they have activity on morphological (molting) processes, is possible that they act in similar manner to structures as ecdysteroid, but with a new mode of action. Its action is similar to juvenile hormone mimics that occur in higher plants. However, this extract from *C. microphylla* with similar activity to known juvenile hormone mimics does not have exactly the same chemical structure of phytoecdysteroids. Insect growth regulatory activity on *S. frugiperda* was shown by phytoecdysteroids from *Ajuga remota* (Labiatae) on two polyphagous (*Spodoptera littoralis* and *Ostrinia nubilalis*) and a monophagous species (*Bombyx mori*) (Marion-Poll and Descoins, 2002). Similar

Table 6
GI and RGI of *S. frugiperda* as a function of increased concentrations of Ethyl acetate, MeOH and *n*-hexane extracts from *C. microphylla*, Me–Ced and gedunin.[a]

Compounds	Concentration (ppm)	GI[b]	RGI[c]
Control	–	0.99 ± 0.045a	–
Me–Ced	2.0	0.99 ± 0.050b	1.00
	10.0	0.84 ± 0.085b	0.85
	25.0	0.75 ± 0.031b	0.75
	50.0	0.69 ± 0.055b	0.70
Ethyl acetate	2.0	0.75 ± 0.031b	0.75
	10.0	0.59 ± 0.040b	0.60
	25.0	0.25 ± 0.035c	0.25
	50.0	0.00	0.00
MeOH extract	2.0	0.99 ± 0.050b	1.00
	10.0	0.69 ± 0.055b	0.70
	25.0	0.25 ± 0.040b	0.25
	50.0	0.00	0.00
Hexane extract	2.0	0.25 ± 0.015c	0.25
	10.0	0.03 ± 0.015c	0.03
	25.0	0.00	0.00
	50.0	0.00	0.00
Gedunin	10.0	0.77 ± 0.060b	0.77
	25.0	0.51 ± 0.040b	0.51
	50.0	0.10 ± 0.010c	0.10

[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] $RGI_{treatment} = GI_{treated}/GI_{control}$.

Table 7
Acute toxicity of ethyl acetate, MeOH and *n*-hexane extracts against larval of last stage of *S. frugiperda*.[a]

Compounds	Concentration (ppm)	% Survival [b] *S. frugiperda*	% Survival[b] *T. molitor*	LD_{50} [c] *S. frugiperda*	LD_{50}[d] *T. molitor*
Control	0.0	100.0	100		
Ethyl acetate	2.0	79.5 ± 3.80b	91.5 ± 5.1a	7.99	13.74
	10.0	41.2 ± 2.00b	65.2 ± 3.9b		
	25.0	15.9 ± 0.85b	15.9 ± 0.77c		
	50.0	0	0		
n-Hexane	2.0	67.0 ± 3.70b	77.4 ± 2.9b	4.56	5.43
	10.0	30.0 ± 2.25b	27.3 ± 2.21c		
	25.0	7.0 ± 0.55a	10.1 ± 0.67d		
	50.0	0	0		
MeOH	2.0	90.9 ± 4.69b	90.0 ± 4.9a	24.0	24.2
	10.0	78.9 ± 3.95b	78.0 ± 3.8b		
	25.0	50.2 ± 3.51c	50.0 ± 3.3c		
	50.0	0	0		
Me–Ced	2.0	95.1 ± 4.75		36.65	
	10.0	78.1 ± 3.90			
	25.0	59.3 ± 2.96			
	50.0	45.0 ± 2.25			
Gedunin	10.0	54.7 ± 2.73b		10.78	
	25.0	14.1 ± 0.71c			
	50.0	0			

[a] After 24 h, survival of adults was recorded (percent relative to controls).
[b] Mean of three replicates. 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] The LD_{50} is the lethal dose producing 50% survival.

activities have been also showed by ponasterone A, B and C and 20E, inokosterone and other terpenes that have been isolated from *Podocarpus nakaii* (Nakanishi et al., 1966), from *Podocarpus elatus* (Galbraith and Horn, 1966), and from *Podocarpus nagi* and *Podocarpus gracilior* (Ying and Kubo, 1993; Zhang et al., 1992).

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; Saez et al., 2000; Savchenko et al., 2000; Dinan, 2001), there are no reports about insecticidal activity of extracts from *C. microphylla* species with this type of

Table 8
Growth inhibitory activities on *T. molitor* as a function of increased concentrations of extracts from *C. microphylla*.[a]

Samples	Doses	Number of pupae formed				Duration of pupal stages[b]	Successfully pupation %[c]	Emergency %[d]
		5 days	12 days	20 days	25 days			
Control	[ppm]	3	9	27	57	15.5 ± 0.4a	95	95
n-Hexane	2	5	25	39	43	14.5 ± 0.6a	73	5*
	5	11	23	28	30	10.9 ± 0.6b	45	5*
	10	10	25	26	27	n.d.	15	0
	25	5	12	17	20	n.d.	10	0
	50	3	10	11	12	n.d.	1	0
MeOH	2	5	25	40	45	16.1 ± 0.4b	76	5*
	5	11	30	40	46	14.5 ± 0.5a	79	5*
	10	12	25	27	30	n.d.	15	3*
	25	10	20	23	24	n.d.	13	0
	50	5	15	17	18	n.d.	5	0
Ethyl acetate	2	11	11	17	18	10.7 ± 0.6c	39	3*
	5	5	9	12	16	n.d.	20	1*
	10	3	9	13	17	n.d.	20	0
	25	3	10	15	18	n.d.	10	0
	50	3	5	7	10	n.d.	10	0
Me–Ced	10	10	15	20	32	15.9 ± 0.8a	25	5
	25	11	16	22	31	10.7 ± 0.6b	15	5
	50	7	12	19	22	9.0 ± 0.6b	10	3
Gedunin	10	7	15	22	29	9.2 ± 0.7b	3	0
	25	5	13	21	25	n.d	1	0
	50	3	11	20	24	n.d.	1	0

[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.

activity and this is the first report about insecticidal activity in this species.

3.6. Acute toxicity on last stage larvae of S. frugiperda and T. molitor

In order to determine a possible correlation between insect growths regulatory (IGR), acute toxicity, and molting disruption caused by these extracts, oral injections of 2, 10, 25 and 50 ppm of all extracts samples into ten larvae of 21 days of *S. frugiperda* and of 25 days of *T. molitor* was carried out (Table 7). At 2.0 ppm of *n*-hexane extract was promoted the apolysis to the fifth instar, but inhibited molting, whereas oral injection at 10.0 ppm resulted only in a delay of the normal molt to the fifth instar. Increasing the oral dose of three extracts (*n*-hexane, ethyl acetate and methanol) to 10, 25 and 50 ppm, was induced the appearance of precocious pupal structures in the larvae (prothetely) (Marks, 1980), in some cases (>30%) of the treated fourth instar larvae. 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). Thus *n*-hexane extract induced prothetely expressed in precociousness, browning and dead of pupae and in a high adult mortality (data not show). Consequently, *n*-hexane extract exhibited 100% larval mortality and gave the highest insecticidal activity.

3.7. Insect growth inhibitory activity against Tenebrio molitor

The *n*-hexane extract caused a strong decrease in the number of larvae of *T. molitor* that reach pupation (45% at 5.0 ppm) and ethyl acetate extract has this same effect without significant differences (39% at 2.0 ppm) (Table 8). With these extracts, as well as methanol, the larvae had a more brief time of pupation and emergence; however, many of the pupae were not viable and died (Table 8). At higher levels (>10 ppm), these extracts 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 10, 25, and 10 ppm for *n*-hexane, methanol and ethyl acetate extracts, respectively (Table 8).

These results suggest that compounds *in n*-hexane and ethyl acetate extracts from *C. microphylla* have effects on ecdysone receptors (Dinan, 2001). From Table 8, it is possible to infer that *n*-hexane and ethyl acetate extracts accelerates the time of pupation for larvae of *T. molitor*. The *n*-hexane extract contains a high percentage of long-chain *n*-alkanes (Table 1) and exhibited acute toxicity to larvae of this insect with regard to the number of larvae that reached the pupal stage.

4. Concluding remarks

Based on these results, we suggest that the insect growth inhibition caused by *n*-hexane and ethyl acetate extracts could be due to synergistic effect. These plant extracts 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. These extracts had potent insecticidal and growth inhibitory activities. Probably the presence of long-chains *n*-alkanes compounds in *n*-hexane extract results in an increasing of IGR activity. This finding suggests that these components could play an important role in both the insecticidal and IGR activity of *C. microphylla* extracts.

The most active *n*-hexane extract, contain a number of a relatively non-polar compounds (long-chain *n*-alkanes) that are under a complete metabolomic analysis. *n*-Hexane and ethyl acetate extracts have very good potency that was comparable to gedunin, methanol extract from *Yucca periculosa* (MeOH-Yuc), methanol extract from *Myrtillocactus geometrizans* (MeOH-Myrt), and methanol extract from *Cedrela salvadorensis* (MeOH-Ced) previously reported (Cespedes et al., 2000, 2004, 2005, 2006).

Preceding experimental observations suggest that acute toxicity and growth inhibition of our extracts may be due to inhibition of a proteinase, ETH and other polyphenol oxidases (PPO) that could be bind to these type of compounds (*n*-alkanes) or move throughout cellular membranes and could produce white substance accumulation (deposits) in vacuoles in similar form to reported by Delgado et al. (2011) and Halse et al. (1993). This target has been demonstrated for other compounds of natural origin (Cespedes et al., 2005; Karban and Baxter, 2001; Kessler and Baldwin, 2002).

The sites and mode of action of these extracts and their isolated components are being investigated and probably correspond to a combination of antifeedant action, as well as, neurodegenerative effects, midgut phenol oxidase, proteinase, ETH, tyrosinase or other PPOs and cuticle synthesis inhibition, as well as molting disruption and/or sclerotization toxicity, as has been found for other natural compounds (Kubo et al., 2003a,b; Cespedes et al., 2000, 2004, 2005, 2006; Torres et al., 2003) and extracts (Feng et al., 1995).

Thus, the effect of *n*-hexane, ethyl acetate and methanol extracts on reducing insect growth, increasing or shortening development time, modifying the apolysis during molting and producing a high mortality on *T. molitor, S. frugiperda* and *D. melanogaster* were more powerful than gedunin, and MeOH-Ced extract from *Cedrela salvadorensis*, (Cespedes et al., 2000, 2004, 2005, 2006; Torres *et al.*, 2003). Although chemically distinct, the level of insecticidal activity of metabolites and mixtures derived from this *Condalia* species is comparable to that of the known insect growth regulator, gedunin and may be due to a synergistic effect shown by the ecdysone-like activity of the *n*-hexane extract in the test system used. Based on the present investigations, materials from Chilean Rhamnaceae should prove to be valuable sources of interesting biologically active compounds, including insecticides (Alarcon et al., 2011; Cespedes and Alarcon, 2011). New biological activity studies of the isolated from these extracts are in progress.

Acknowledgements

We thank Ana Ma. Garcia-Bores UBIPRO-FES Iztacala, UNAM for her technical assistance during Gas/Mass experiments. This research was supported byCONICYT-Chile (Comisión Nacional de Investigación Científica y Tecnológica) through FONDECYT program grant #1101003, and by Dirección de Investigación, University of Bio Bio, grant # 091909-1/R. EM and CL thanks to FONDECYT #1101003 for fellows.

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