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Differential anti-insect activity of natural products isolated from *Dodonaea viscosa* Jacq. (Sapindaceae)

Martina Díaz[1,2,3], *Carmen Elisa Díaz*[2], *Ricardo Guillermo Álvarez*[2], *Andrés González*[1], *Lucía Castillo*[1], *Azucena González-Coloma*[3], *Gustavo Seoane*[4], *Carmen Rossini*[1]*

[1] Laboratorio de Ecología Química, Facultad de Química, Universidad de la República, Montevideo, 11800, Uruguay
[2] Instituto de Productos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas, La Laguna, Tenerife, 38206, Spain
[3] Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, Madrid, 28006, Spain
[4] Departamento de Química Orgánica, Facultad de Química, Universidad de la República, Montevideo, 11800, Uruguay

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Abstract: Botanical biopesticides constitute an important tool for Integrated Pest Management practices. Uruguay has a great potential for developing botanical biopesticides from its abundant native flora. *Dodonaea viscosa* Jacq. is a native Sapindaceae that in preliminary studies was shown to possess a potential deterrent activity against insect models. In this work, ethanolic extracts of the leaves of this species were studied. Bioguided fractionation and supercritical fluid extraction led to the isolation of active compounds. For that purpose four insect models were used: *Epilachna paenulata, Spodoptera littoralis, Myzus persicae*, and *Rhopalosiphum padi*, which are pests of crops of economic importance. Lupeol, stigmasterol, stigmast-7-en-3-ol, and a labdane diterpene were isolated and showed differential activity against the models.

Key words: *Epilachna paenulata*, labdane, *Myzus persicae, Rhopalosiphum padi*, triterpenoid, *Spodoptera littoralis*

Introduction

Dodonaea viscosa Jacq. is an evergreen shrub widely distributed in tropical and subtropical areas of both hemispheres. It belongs to the Sapindaceae family, which includes several genera of plants from which many species have been studied for their anti-insect capacity. From these studied species, many active compounds have been isolated (Diaz and Rossini 2012). The popular medicinal plant, *D. viscosa*, is used in folk medicine as a remedy for fever, rheumatism, inflammation, and pain (Wagner *et al*. 1987; Mata *et al*. 1991). Recent studies on the hepatoprotective activity of the constituents of *D. viscosa* and studies on the capacity of these constituents to treat hyperpigmentation-associated diseases have been published (Yang *et al*. 2013; Ali *et al*. 2014). The anti-insect activity of *Dodonaea* spp. has been described mainly against Lepidoptera (Malarvannan and Subashini 2007; Malarvannan *et al*. 2008; Malarvannan *et al*. 2009; Sharaby *et al*. 2009). In recent studies, many compounds have been isolated from *D. viscosa*, including various flavonoids, diterpenic acids, and saponins. These compounds have mainly been isolated from the Asian specimens (Niu *et al*. 2010). In this study, the phytochemical characterization of the foliar ethanolic extracts from Uruguayan specimens of *D. viscosa* is reported. The work was mainly based on a bioguided fractionation resulting in the isolation of various active compounds against different insect models.

*Corresponding address:
crossini@fq.edu.uy

Materials and Methods

General

The Nuclear Magnetic Resonance (NMR) spectra were recorded on a AMX-500 Spectrometer (400 and 500 MHz for ^{1}H and 100, 125 and 150 MHz for ^{13}C) in $CDCl_3$ (deuterated chloroform) in the case of compound **1**. In the case of compounds **2** to **4**, spectra were recorded on a Bruker DPX Advance Spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) in $CDCl_3$ as well.

In the case of compound **1**, mass spectrometric analyses were performed using a Bruker micrOTOF-Q II Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA), equipped with Electro Spray Ionization (ESI). The instrument was operated at a capillary voltage of 4.5 kV with an end plate offset of −500 V, a dry temperature of 180°C using N_2 as dry gas at 4.0 l · min^{-1} and a nebulizer pressure of 0.4 bar. For compounds **2** to **4**, low resolution mass spectra were obtained in a Shimadzu 2010 Plus Gas Chromatograph coupled to a Shimadzu QP2010 Mass Spectrometer, with direct sample introduction and electronic impact ionization.

High Performance Liquid Chromatography (HPLC) was developed in a Shimadzu LC-20AT Prominence, equipped with a SPD-M20A Prominence diode array detector and Silica Phase columns. Flash and Liquid Chromatographies were developed in silica gel MN Kieselgel

60 M (0.04–0.063 mm/230–400 mesh). For flash columns a FMI Lab Pump, RP-SY and an IWAKI Air Pump, AP-115S were used. Fractions were monitored by thin layer chromatography developed in aluminium silica gel 60 G/UV254 plates (Macherey-Nagel). Spots were visualized by heating after spraying with Oleum reagent (16 ml of water, 80 ml of acetic acid and 4 ml of sulfuric acid) or anisaldehyde/H_2SO_4 (95 ml of ethanol, 4 ml of sulfuric acid and 1 ml of anisaldehyde) (Wagner and Bladt 1996).

Extraction and isolation

Vegetal material was collected in Punta Gorda, Montevideo (Latitude: –34.4893783°, Longitude: –59.090125°) and Los Pinos, Colonia (Latitude: –34.435536°, Longitude: –57.249008°), Uruguay. Then it was dried (48 h, 40°C) and ground.

The foliar extract was obtained by maceration in ethanol of 145 g of dried leaves, extracting the vegetal material three times in 24 h, yielding 25%. The extract (36 g) was fractionated by Vacuum Liquid Chromatography (VLC) in silica gel (using a 13 cm height and 6 cm diameter column; silica Kiesegel 60 M). Fractions were eluted with 2–3 dead volumes (200 ml) of mixtures of hex/AcOEt and AcOEt/MeOH, increasing the polarity over fractions. Sixteen fractions were obtained. Subsequent flash chromatography and preparative HPLC of fractions eluted with hex/AcOEt (8 : 2) yielded three isolated compounds (**1**–**3**). In HPLC, a Beckman Ultrasphere Si-100 (5 mm, 250 × 10 mm) column, with hex/AcOEt as the mobile phase, and a flow of 8 ml · min^{-1}, was used in the case of compound **1**. A Macherey Nagel Nucleosil-100 (7 mm, 250 × 16 mm) column, with DCM/MeOH as the mobile phase, and a flow of 8 ml · min^{-1}, was used in the case of compounds **2** and **3**.

Supercritical extractions were carried out on pilot-scale, automated equipment according to the procedures previously described (Cassel *et al*. 2011). The powdered plant material (30 g) was extracted at a constant temperature (40, 50 or 60°C) while the pressure was successively increased from 90 to 120, 120 to 150, and 150 to 200 bar. The extractions were performed following the experimental procedure defined by Cargnin *et al*. (Cargnin *et al*. 2010), i.e. 100 min at 90 bar, 30 min at 120 bar, 30 min at 150 bar, and 30 min at 200 bar, using the same plant material. The supercritical carbon dioxide flow rate was 6.7×10^{-4} kg · s^{-1} (through the extraction vessel) using a flowmeter assay (Sitrans F C Massflo 2100 Siemens) with an accuracy of < 0.1%. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with a precision of 71.0 bar. The temperature controller was connected to thermocouples (PT-100) with an accuracy of < 0.5. A later HPLC of the fraction obtained at 120 bar yielded compound **4**.

Insects

Epilachna paenulata Germar (Coleoptera: Coccinellidae) is an olyphagous insect with a Cucurbitaceae dietary specialization. Adults were maintained on squash (*Cucurbita pepo* L.) under controlled conditions of temperature (20±2°C) and photophase (14L : 10D).

Spodoptera littoralis Boisduval (Lepidoptera: Noctuidae), a generalist species, was kept on an artificial diet (Pitout and Bues 1970) at 25±1°C, > 70% relative humidity, and with a photoperiod of 16L : 8D.

Rhopalosiphum padi L. (Hemiptera: Aphididae) is a grass specialist species. These aphids were reared on *Hordeum vulgare* L. foliage and maintained at 20±1°C, > 70% relative humidity, and with a photoperiod of 16L : 8D.

Myzus persicae Sulzer (Hemiptera: Aphididae), is a generalist aphid. These aphids were reared on bell pepper foliage (*Capsicum annuum* L.) at 25±1°C, > 70% relative humidity, and with a photoperiod of 16L : 8D.

Aphid anti-settling bioassay

The activity against aphid settling was tested in choice experiments as described elsewhere (Gutierrez *et al*. 1997). The extracts were tested in plastic boxes (3 × 3 × 1.5 cm) lined at the bottom with 2% agar (20 replicates per extract). Two pieces of leaves (ca. 1 cm^2) cut from the appropriate host plant (*C. annuum* and *H. vulgare* for *M. persicae* and *R. padi*, respectively) were placed on the agar and treated either with the extract at 100 μg · cm^{-2} (in the case of pure compounds a 50 μg · cm^{-2} dose was used) or the same amount of solvent (MeOH). Ten aphids were placed in the box and the percentage of aphids settled on each surface was recorded after 24 h of exposure. A preference index (PI) (Díaz *et al*. 2011) was calculated for each replicate as:

$$PI = [(\%C - \%T)/(\%C + \%T)],$$

where %T and %C are the percentages of aphids settled on the treated and control leaf pieces, respectively.

Feeding deterrence bioassay

The antifeedant (phagodeterrent) activity was evaluated in choice-bioassays in Petri dishes (9 × 1 cm) lined at the bottom with a layer of agar (2%). Insects were offered four leaf discs (1 cm^2) of the appropriate host plant (*C. pepo* for *E. paenulata* and *C. annuum* for *S. littoralis*). Two of the discs (T) were coated with 100 μg of the extract or 50 μg of pure compounds (10 μl of a 10% or 5% MeOH solution, respectively), and the other two (C) were treated with 10 μl of MeOH. The insects were tested individually as adults for *E. paenulata* (10–15 replicates per extract) and as larvae for *S. littoralis* (5–6 replicates per extract). To measure deterrent activity, a visual score of area consumed (0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5 or 100%) was assigned for all discs within the plate. A preference index (PI) (Díaz *et al*. 2011) was determined for each replicate using the formula: PI = (C – T)/(C + T), where C and T are the amounts consumed for the control and the treatment of the leaves, respectively (Bellomo *et al*. 2009).

Statistics

Bioassay data were analyzed by Wilcoxon rank tests (Lowry 1998–2009). Anti-settling activity determination was based on the number of aphids settled on the leaf

treated with solvent (the control) compared to the number settled on the leaf treated with the substance tested (the treatment). Replicates with fewer than five aphids settled in total (on both leaves) were not considered. Antifeedant (deterrent) activity determination was based on the proportions of the consumption of the leaf treated with solvent (the control) compared to the proportions of the consumption of the leaf treated with the substance tested (the treatment). Replicates where no consumption was detected were not considered.

All significance levels were set at $p < 0.05$.

Results and Discussion

The ethanolic foliar extract of *D. viscosa* showed deterrent activity against *R. padi* as well as against *E. paenulata*. However, after VLC fractionation many of the fractions obtained were also shown to be active against *M. persicae* and in some cases against *S. littoralis* (Table 1). When active, all of the fractions were deterrent with the exception of the first fraction eluted with hex/AcOEt (8 : 2) (fraction number 5 in table 1), which showed a settling stimulation towards *R. padi*. Medium polarity fractions (the ones eluted with hexane: AcOEt 9 : 1 to AcOEt 100%) seemed to concentrate the deterrent activity (most of them were active against more than one of the insect models). From the second fraction eluted with hex/AcOEt (8 : 2) (fraction number 6 in table 1; 0.45 g) which showed differential activity when tested against the insect models, the pentacyclic triterpene lupeol (**1**, 15 mg) (Burns *et al*. 2000), and the sterols stigmasterol (**2**, 6 mg) (Kojima *et al*. 1990; Kolak *et al*. 2005), and stigmast-7-en-3-ol (**3**, 3.5 mg) (Kojima *et al*. 1990; Lee *et al*. 2005) were isolated (Fig. 1). In those cases where the mass was enough, these compounds were tested against the insect models.

A supercritical (120 bar) extract from twigs of *D. viscosa* was fractionated by preparative HPLC [mobile phase: hex/AcOEt (6 : 4)], obtaining compound **4** (Fig. 1). Compound **4** was shown by Thin Layer Chromatography (TLC) to be present in high quantities in that extract and showed evidence of being present also in the foliar ethanolic crude extract.

Table 1. Anti-insect activity of fractions eluted from the Vacuum Liquid Chromatography (VLC) of the ethanolic crude extract from leaves of *Dodonaea viscosa*

Fraction number	Mobile phase	Mass [g]	Preference index (PI ±SE)			
			insect species			
			Myzus persicae	*Rhopalosiphum padi*	*Spodoptera littoralis*	*Epilachna paenulata*
Crude foliar extract			0.19±0.04	0.19±0.08*	0.3±0.2	0.6±0.2*
1	hex 100%	0.013	–	0.2±0.1**	0.4±0.2	–
2	hex/AcOEt (95 : 5)	0.107	–	0.2±0.1	0.3±0.2	0.4±0.2
3	hex/AcOEt (9 : 1)	0.053	–	0.1±0.1	0.1±0.3	0.4±0.3
4		0.119	0.3±0.1*	–0.2±0.1	0.3±0.2	0.5±0.2*
5	hex/AcOEt (8 : 2)	0.252	0.5±0.1*	–0.3±0.1*	–0.1±0.3	0.4±0.3
6		0.450	0.5±0.1*	0.1±0.2	0.35±0.07*	0.4±0.3*
7	hex/AcOEt (1 : 1)	3.180	–	0.0±0.1	0.4±0.2	0.0±0.3
8		2.800	0.4±0.1*	0.0±0.1	0.4±0.2	0.90±0.09*
9		1.160	0.5±0.1*	0.31±0.09*	0.7±0.1*	0.2±0.3
10	AcOEt 100%	2.060	0.1±0.1	0.01±0.09	0.1±0.3	0.5±0.1*
11		1.660	0.4±0.1*	0.3±0.1*	0.2±0.3*	0.6±0.3*
12	AcOEt/MeOH (98 : 2)	0.530	0.2±0.1	0.0±0.1	0.3±0.1*	0.2±0.4
13	AcOEt/MeOH (8 : 2)	3.550	0.7±0.2	0.1±0.1	0.4±0.3	–0.4±0.4
14		4.530	0.71±0.07*	0.2±0.1	0.17±0.09	0.0±0.4
15	MeOH 100%	–	0.5±0.1*	0.04±0.09	0.3±0.2	0.1±0.3
16		–	–	–	–	0.7±0.3

*denotes significant differences between control and treatment (deterrent, $p < 0.05$, Wilcoxon 2 tail rank test)

**denotes significant differences between control and treatment (deterrent, $p < 0.05$, Wilcoxon 1 tail rank test); SE – standard error

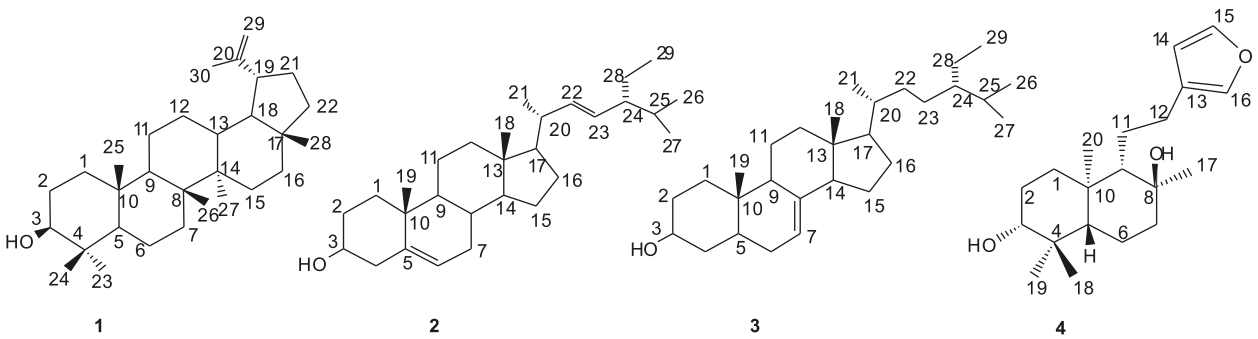

Fig. 1. The chemical structure of compounds **1–4**

Table 2. Chemical properties of lupeol, stigmasterol and stigmast-7-en-3-ol [calculated using Advanced Chemistry Development (ACD/Labs) software V11.02]*

Property**	Lupeol	Stigmasterol	Stigmast-7-en-3-ol
KoC	1E7	7.18E6	1E7
LogD	10.46	10.07	10.61
LogP	10.46	10.072	10.607

**SciFinder*, version 2006; Chemical Abstracts Service: Columbus, OH, 2006; KoC value for RN 50-52-2 (Accessed: November 12, 2006); calculated using ACD/Labs software, version 8.14; ACD/Labs 1994–2006

**KoC – organic carbon adsorption coefficient; LogD – distribution coefficient; LogP – partition coefficient

Table 2 shows the anti-insect activities of the isolated compounds. Lupeol (**1**) was active against *M. persicae* and *E. paenulata*. Stigmasterol (**2**) showed activity only against the aphids. Stigmast-7-en-3-ol (**3**) was active only against *M. persicae.* It could be speculated that the differential activity of compound **2** against *R. padi* may be related to its higher polarity as shown by its lower KoC, LogP, LogD (Table 3). Furthermore, the activity of compound **2** against *R. padi* compared to the inactivity of compound **3** against that aphid, may be related to the differences on their chemical structure: the different location of the double bound in the cycles, and the existence of a double bound in the side chain in compound **2**. Since none of the compounds were active against the lepidopteran, it can be suspected that these compounds were not responsible for the activity shown by the original fraction against that insect (Table 3) although the loss of a synergic effect could not be ruled out. The labdane (**4**) isolated from the superctirical extract was only active against *E. paenulata*.

In the case of lupeol (**1**), which is reported here for the first time in this vegetal species, our results show deterrent activity against *M. persicae* and *E. paenulata*. Such activity was also reported in an independent work when lupeol (**1**) was isolated from *Allophylus edulis* (A St.Hil.) Radlk., another Sapindaceae (Díaz *et al*. 2014). There is a previous study in which the inactivity of lupeol (**1**) against *M. persicae* is reported (Gonzalez-Coloma *et al*. 2011). The divergence between these results may be related to the different aphid instars used in the assays. Within the frame of phytochemical and biological activity studies of plant extracts, compound **1** has been isolated from many plant species (Brimson *et al*. 2012; Dong *et al*. 2012; dos Santos *et al*. 2012; Kumari and Kakkar 2012). Specifically, compound **1** has been reported to be a component of a *Nothofagus dombeyi* Mirb. (Oerst.) (Nothofagaceae) plant fraction active against the leaf miners *Ctenopseustis obliquana* (Walker) (Lepidoptera: Tortricidae) in feeding deterrence bioassays (Thoison *et al*. 2004). Likewise, as it happened in the present study with the lepidopteran, when lupeol was tested as a pure compound, it did not show activity against those larvae.

Stigmasterol (**2**) has been reported on its activity against various insects. For example, the feeding deterrence against *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) and *Spilosoma obliqua* Walker (Lepidoptera: Arctiidae) (Tandon *et al*. 1998), and cytotoxic activity against *S. litura* (Huang *et al*. 2009). Stigmasterol, compound **2,** was also reported as a component of an active extract against the bug *Collaria oleosa* Distant (Hemiptera: Miridae) (Saez *et al*. 1998) as well as of an active extract against *M. persicae* (Santana *et al*. 2012). There are no previous reports in the literature concerning the activity of stigmasterol against aphids, as far as we are aware of.

The activity of stigmast-7-en-3-ol (**3**) against other insects has been slightly studied. Its antifeedant activity against the cotton boll weevil *Anthonomus grandis* Boh (Coleoptera: Curculionidae) has been previously reported (Miles *et al*. 1990). However, to the best of our knowledge, we are the first to report the deterrent activity of Stigmast-7-en-3-ol (**3**) against *M. persicae*.

Table 3. Anti-insect activity of the fraction eluted from the Vacuum Liquid Chromatography (VLC) of the ethanolic crude extract from *Dodonaea viscosa* leaves which produced pure compounds that were isolated. The pure isolated compounds are listed

Fraction number/ Compound	Preference index (PI ±SE)			
	insect species			
	Myzus persicae	*Rhopalosiphum padi*	*Spodoptera littoralis*	*Epilachna paenulata*
Fraction 6 [hex/AcOEt (8 : 2)]	0.50±0.10*	0.10±0.20	0.35±0.07*	0.4±0.3*
Lupeol (**1**)	0.60±0.10*	–0.03±0.07	0.20±0.20	0.6±0.2*
Stigmasterol (**2**)	0.30±0.10*	0.29±0.05*	0.09±0.06	–0.2±0.2
Stigmast-7-en-3-ol (**3**)	0.61±0.09*	0.00±0.10	–	–
Labdane (**4**)	0.20±0.10	–0.01±0.09	0.20±0.20	0.5±0.3**

*denotes significant differences between control and treatment (deterrent, $p < 0.05$, Wilcoxon 2 tail rank test)

**denotes significant differences between control and treatment (deterrent, $p < 0.05$, Wilcoxon 1 tail rank test)

Sterols play a critical role in all the organisms of the animal kingdom, owing to their basic role as components of cell membranes and in some cases as hormone precursors (including molting hormones). However, herbivorous insects do not have the capacity to synthesise cholesterol. That is why they depend on the ingestion of phytosterols which could be metabolised to cholesterol (Behmer and Elias 1999). Despite this fact, in many cases anti-insect activity has been found on some plant sterols. For phytosterols that have their function in the basic metabolism of plants and also present defensive activity against insects, it would be interesting to find out if the plants that produce them, do so in different concentrations, deposit them differentially in their organs, and/or are able to increase the biosynthesis in response to damage (assuming a dose-dependent effect).

Compound **4**, isolated from the supercritical extract of the twigs of this species, was noted in its infrared spectra bands at 3,395 cm^{-1} indicating the presence of at least an hydroxyl group; at 871 and 1,462 cm^{-1}, typical of *β* substituted furans (Ortega *et al*. 2001). In its mass spectra, the molecular ion m/z = 320, suggested the molecular formula $C_{20}H_{32}O_3$. It was revealed through NMR analysis, that it was a structure containing three quaternary carbons plus one carbon without H and with an heteroatom, six methynic carbons, six methylenic carbons and four methylic substituents. This finding indicated the same molecular formula that was confirmed afterwards by HRESIMS. With mono- and bidimensional NMR studies, it could be confirmed that compound **4** corresponded to ent-15, 16-epoxy-9aH-labda-13(16)14-diene-3b,8a-diol (Mata *et al*. 1991).

In our study, compound **4** was found to only be active against *E. paenulata*. Even though compound **4** has been isolated before from *D. viscosa* (Mata *et al*. 1991), and reported to have antispasmodic properties (Rojas *et al*. 1996), the anti-insect activity of compound **4** had not been tested before. Nevertheless, there are various research reports describing the biological activities of labdane diterpenes, isolated from this and other vegetal species. That is the case of labdanes with anti-inflammatory activity isolated from *D. viscosa* (Wabo *et al*. 2012) and from *Callicarpa longissima* Hemsl. (Merr.) (Verbenaceae) (Liu *et al*. 2012). Regarding activity against insects, diterpens of this kind have been reported to be active against dipterans (Geris *et al*. 2008), lepidopterans (Gonzalez-Coloma *et al*. 2005), and also against aphids (Rose *et al*. 1981).

Although in this study, compound **4** revealed activity only against the tested coleopteran, seeing the activity reported about other labdane diterpenes and the potential anti-insect activity showed by this vegetal species, it is possible to assume that other active diterpenes are present in other fractions. The evidence of the presence of compound **4** in the foliar extract from which it was not isolated by conventional extractions, shows the need for developing different methods of extraction in the same vegetal species in order to isolate all the potential anti-insect compounds.

The ethanolic foliar extract of *D. viscosa* showed a low toxic effect on honeybees in a previous study (Castillo *et al*. 2009). Moreover, it has been widely used in folk medicine (Wagner *et al*. 1987; Mata *et al*. 1991). It may be assumed that the ethanolic foliar extract of *D. viscosa* does not exhibit significant toxicity on mammals. These facts, together with the wide distribution of *D. viscosa* in Uruguay (Garay *et al*. 2008), point to this shrub's great potential as a source of botanical pesticides. Therefore, further studies on the domestication capacity of *D. viscosa* must be pursued.

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