Comparative Study of Ovatifolin Antioxidant and Growth Inhibition Activities

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A comparative study on the effect of arturin (**1**), ovatifolin (**3**), deacetylovatifolin (**5**), and their 1-acetoxyarturin (**2**), 8-acetoxyovatifolin (**4**), 1,10-epoxyovatifolin (**6**), and 11,13-dihydroovatifolin (**7**) derivatives, isolated from *Podanthus ovatifolius* and *Podanthus mitiqui*, on the seedling growth, germination, and respiration of several monocot and dicot weedy target species was carried out. In addition to the inhibitory activity on the bleaching of crocin induced by alkoxyl radicals, these compounds also displayed scavenging properties toward DPPH in TLC autographic and spectrophotometric assays. The results indicate that ovatifolin (**3**), deacetylovatifolin (**5**), epoxyovatifolin (**6**), dihydroovatifolin (**7**), and the CH_2Cl_2 extract interfere with pre-emergence of seedlings at the level of respiration. These compounds appear to have selective effects on the radicle and shoot growth of *Physalis ixocarpa* and *Trifolium pratense*. Their allelopathic effects are comparable to those of parthenolide, a know natural growth inhibitor.

Keywords: *Podanthus ovatifolius; P. mitiqui; ovatifolin; germacrane; sesquiterpene lactone; Asteraceae; phytogrowth inhibition; seed respiration; radical scavengers; DPPH*

INTRODUCTION

In the continuation with our search for natural products with biological activities, we have studied ovatifolin, a germacrane-type sesquiterpene lactone and the main natural product of *Podanthus mitiqui* and *Podanthus ovatifolius* (*1*). Extracts of *P. mitiqui* and *P. ovatifolius* display antitumoral and antibacterial activities (*2*−*4*). The germacrane-type sesquiterpene lactone, ovatifolin acetate, showed antineoplasic activity and cytotoxic effects by modifying cell proliferation kinetics of mouse marrow erythrocytes and also increased micronucleated cells (*5*). In addition, genotoxic effects of this compound have also been shown (*5*, *6*). We have recently reported the anti-inflammatory activity of ovatifolin from *P. ovatifolius* (*7*).

Over 5000 sesquiterpene lactones have been described (*8*−*10*), and some are noted for their biological activities (*11*−*14*). They may show potent pharmacological (*5*, *15*), allelopathic (*16*−*19*), and plant growth regulatory activities (*20*), and some are powerful inhibitors of weed seed germination (*17*, *18*, *21*−*25*). Insecticidal (*26*−*28*) and herbicidal (*29*) activities have also been described. These compounds have potential for the development of herbicides possessing novel structures, and they significantly inhibit several photosynthetic reactions in spinach chloroplasts (*30*−*32*). However, very little is known about their structure−activity relationships or their possible modes of action in plants.

Several sesquiterpenoid lactones have been isolated from Chilean members of the Asteraceae (*1*, *33*). A phytochemical study of *P. ovatifolius* led to the isolation of ovatifolin (*34*, *35*), the stereochemistry of which was previously determined by Gopalakrishna's group (*36*). *P. mitiqui* and *P. ovatifolius* are small shrubs that grow in the rain forest of the Pacific slopes, especially in the Araucanian region of southern Chile (*37*).

Studies of allelopathy report that different sesquiterpene lactones are seedling growth and photosynthetic inhibitors (*29*, *31*). Our recent data indicate that it is possible to correlate some antioxidant activities (i.e., crocin and DPPH) with germination and respiration. These data are important for allelopathic interpretation activities of secondary metabolites.

We have previously demonstrated that diverse secondary metabolites have different mechanisms of action and different molecular targets when interacting with the energetic systems (*38*−*44*). This is the first report on the allelopathic effects of arturin, deacetylovatifolin, ovatifolin, and some of their synthetic derivatives as well as extracts from *Podanthus* species.

Here we report the plant growth inhibitory effects of arturin (**1**), arturin acetate (**2**), ovatifolin (**3**), ovatifolin acetate (**4**), deacetylovatifolin (**5**), 1,10-epoxyovatifolin (**6**), 11,13-dihydroovatifolin (**7**), and MeOH and CH_2Cl_2 extracts on germination. The seedling growth activity and the behavior of these compounds toward metabolic energetic reactions on dicotyledonous and a monocotyledon standard target species are also reported. Dicots assayed were lettuce (*Lactuca sativa* L. cv. Roman), green tomato (*Physalis ixocarpa*), and red clover (*Trifolium pratense* cv. Kenland). The monocot assayed was ryegrass (*Lolium multiflorum* cv. Gulf). Our focus was on the development and growth of weedy plants (germination, root and shoot elongation, and respiration of seeds), and we attempted to correlate that with antioxidant activity. These parameters are accepted as indirect measures of other physiological processes (*17*) affected by the assayed chemicals. A commercial herbicide, 2,4-D, was introduced into the bioassay as an

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internal standard in the plant growth bioassay. In addition, quercetin and gallic acid were used as internal standards in assays concerned with the reduction of DPPH and antioxidant measurements, respectively.

MATERIALS AND METHODS

Plant Material. Aerial parts (stems, leaves, and flowers) of *P. ovatifolius* (5.4 kg) were collected in Rocoto Concepción, Chile, and aerial parts of *P. mitiqui* (6.1 kg) near Pichidangui, Chile, in December 1997. Voucher specimens were deposited at the ethnobotanical collection of the Herbarium of the Universidad of Concepción, Chile (CONC), Facultad de Ciencias Naturales y Oceanográficas, Departamento de Botánica, Universidad de Concepción (voucher: C. Marticorena and R. Rodríguez).

Chemicals and Solvents. All reagents used were of either AR grade or chromatographic grade. 2,4-Dichlorophenoxyacetic acid (2,4-D), 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, gallic acid, quercetin, saffron, sorbitol, and tricine were purchased from Sigma-Aldrich Quimica, S.A. de C.V., Toluca, Mexico. Methanol, CH_2Cl_2, $CHCl_3$, KCl, $CuSO_4$, NH_4Cl, $MgCl_2$, *m*-chloroperbenzoic acid (*m*-cpba), $NaBH_4$, pyridine, acetic anhydride, silica gel GF_{254} analytical chromatoplates, silica gel grade 60 (70−230, 60 Å) 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.

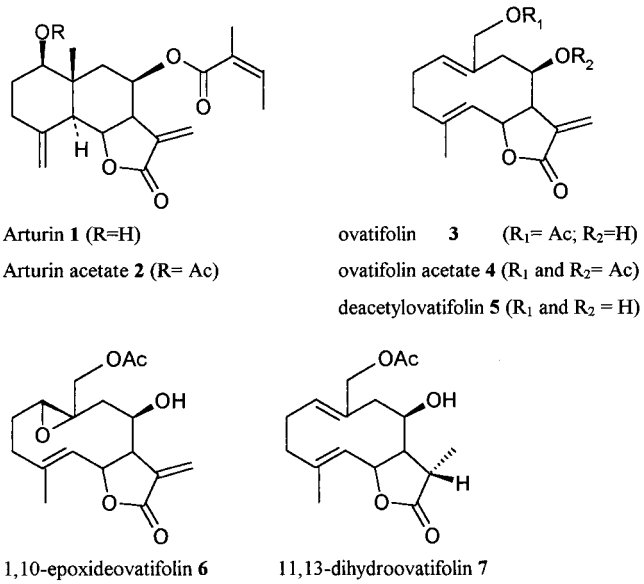
Apparatus. ^{1}H NMR spectra were recorded at 300 and 500 MHz and ^{13}C NMR spectra at 75 and 125 MHz, respectively, on Varian VXR-300S and VXR-500S spectrometers; chemical shifts (parts per million) are related to $(CH_3)_4Si$ as an internal reference. $CDCl_3$ and acetone-d_6 (Aldrich Chemical Co.) were used as solvents. Coupling constants are quoted in hertz. IR spectra were obtained in $CHCl_3$ with Perkin-Elmer 283-B and FT-IR Nicolet Magna 750 spectrophotometers. UV spectra for pure compounds were determined with a Shimadzu UV-160; biological activities were determined with a Spectronic model Genesys 5 spectrophotometer. Optical rotation was measured with a JASCO DIP-360 spectropolarimeter. Melting points were recorded and are uncorrected. Oxygen evolution (uptake) was determined with a Clark-type electrode connected to a YSI oxygraph (model 5300). Fluorometric measurements were determined with a Turner Barnstead-Thermolyne Quantech S5 fluorometer using 420, 440, 470, 550, and 650 Turner filters.

General Experimental Procedures. HPLC was performed with a Waters model 600E, equipped with a Bondapack RP 18 column (250 × 8 mm), flow = 1.5 mL/min, UV detection at 280 nm. The mobile phase was MeOH/H_2O (7:3 v/v). Analytical TLC was performed on silica gel 60 F_{254} E. Merck plates, and components were visualized by spraying with a 10% solution of H_2SO_4 followed by heating at 110 °C.

Isolation and Purification of Ovatifolin and Their Derivatives. Ovatifolin (400 mg), deacetylovatifolin (80 mg), and arturin (50 mg) (Figure 1), were extracted from the aerial parts of *P. mitiqui* (6.1 kg) with MeOH and CH_2Cl_2 extracts, followed by chromatography on silica gel as previously reported (*6*, *34*, *35*) to yield a mixture of these and other minority compounds (2.91 g, 0.26%). The yield was 5 times higher than that previously obtained from *P. ovatifolius* plants (*45*). In addition, an ethyl acetate extract was obtained.

*Arturin (**1**, 1β-Hydroxy-8β-angeloyloxyeudesmane-4(15),11-(13)-diene-6,12-olide).* Further elution of the column with *n*-hexane/ethyl acetate 1:1 (v/v) afforded arturin (**1**) as an oil: UV λ_{max}^{MeOH} nm 210 (ϵ 5200); IR ν_{max} $CHCl_3$ cm^{-1} 3460, 2960, 2860, 1760, 1720, 1650, 1230, 1150, 1040, 1020, 990, 960, and 750; MS, *m*/*z* M^+ 346 (calcd for $C_{20}H_{26}O_5$ 346.178), 247, 99 ($C_5H_7O_2^+$).

*Ovatifolin (**3**)* was isolated as brown orthorhombic crystals: mp 137 °C; UV λ_{max}^{MeOH} nm 213 (ϵ 24500); IR ν_{max} $CHCl_3$ cm^{-1} 3450, 2980, 1750, 1660, 1235, and 725; MS, *m*/*z* M^+ 306 (calcd for $C_{17}H_{22}O_5$ 306.35), 264, 246, 228, 217, 213, 166, 121, 107, 91, 43; ^{1}H MNR (300 MHz, TMS internal standard, $CDCl_3$, δ values) 1.64 (s, H-15), 2.07 (s, Me of acetate), 2.18 (d, br, H-9β), 2.32−2.5 (m, H-1, H-3), 2.76 (ddd, H-7α), 2.96 (dd, br, H-9α), 4.57 (d, br, H-14′), 4.69 (d, H-8α), 4.87 (d, br, H-5), 4.81 (d, br, H-14), 5.12 (dd, H-1), 5.23 (dd, H-6β), 5.59 (d, H-13′), 6.35 (d, H-13), 7.3 (s, OH-8). This compound was identical to an authentic sample of ovatifolin (*6*, *34*, *35*).



Arturin **1** (R=H)
Arturin acetate **2** (R= Ac)
ovatifolin **3** (R_1= Ac; R_2=H)
ovatifolin acetate **4** (R_1 and R_2= Ac)
deacetylovatifolin **5** (R_1 and R_2 = H)
1,10-epoxideovatifolin **6**
11,13-dihydroovatifolin **7**

Figure 1. Structures of artutrin (**1**), arturin acetate (**2**), ovatifolin (**3**), ovatifolin acetate (**4**), deacetylovatifolin (**5**), 1,10-epoxideovatifolin (**6**), and 11,13-dihydroovatifolin (**7**).

*Deacetylovatifolin (**5**).* Needle crystals were obtained after recrystallization: mp 166 °C; UV λ_{max}^{MeOH} nm 210 (ϵ 8500); IR ν_{max} $CHCl_3$ cm^{-1} 3400, 1740, 1660, 1300, 1240, 1155, 1040, 950, 840, 740, and 690; MS, *m*/*z* M^+ 264 (calcd for $C_{15}H_{20}O_4$ 264.31), 257, 246, 228, 210, 165, 136, 120, 105, 91, 77; ^{1}H MNR (300 MHz, TMS as internal standard, $CDCl_3$, δ values) 1.64 (s, H-15), 2.40 (d, br, H-9β), 2.32−2.5 (m, H-1 and H-3), 2.77 (ddd, H-7α), 2.95 (dd, br, H-9α), 4.55 (d, br, H-14′), 4.69 (d, H-8α), 4.87 (d, br, H-5), 4.81 (d, br, H-14), 5.12 (dd, H-1), 5.23 (dd, H-6β), 5.59 (d, H-13′), 6.35 (d, H-13), 6.9 (s, OH-14), 7.3 (s, OH-8. This compound was identical to an authentic sample of deacetylovatifolin (*6*, *34*, *35*).

Acetylation of Ovatifolin. Derivatization of the C_8-hydroxyl group by acetylation with acetic anhydride/pyridine of ovatifolin (**3**) gave ovatifolin acetate (**4**) (*6*, *34*, *35*). Ovatifolin (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 usual to yield 41 mg of (**4**) and then purified by preparative TLC; a sample was developed with *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 (*1*, *6*, *34*−*36*).

Epoxidation of Ovatifolin. Fifty milligrams of **3** was dissolved in 10 mL of dried dichloromethane (DCM), and then 50 mg of sodium acetate was added. Afterward, *m*-cpba (50 mg), dissolved in dried DCM, was added dropwise with stirring. After 1 h, the reaction was stopped. The reaction mixture was extracted with NaOH (aq) 5% (two times) to separate the remaining *m*-cpba and *m*-chlorobenzoic acid; then, the pH of the organic phase was adjusted to nearly 7.0 by washing with distilled water (two times). All of the aqueous phases were extracted with DCM, and the combined organic phases were dried over anhydrous sodium sulfate. After separation by preparative TLC (*n*-hexane/ethyl acetate 40:60, v/v), compound **6** was obtained in crystalline form (81%) and its structure established by high-resolution spectroscopic methods.

Reduction of Ovatifolin. To a stirred solution of 15 mg of **3** in 2 mL of methanol in a Dewar glass at 0 °C was added $NaBH_4$ (1:1.4 molar relationship) during the first 5 min of

Table 1. Growth Inhibitor Activity of AcOEt, CH_2Cl_2, and MeOH Extracts and Primary Fractions on Germination of *L. multiflorum* and *P. ixocarpa*

sample	I_{50}[a] (ppm)	
	L. multiflorum	*P. ixocarpa*
AcOEt extract	ND[b]	ND
CH_2Cl_2 extract	25.2	21.0
MeOH extract	119.0	125.0
F_0-1	88.0	80.0
F_0-2	39.0	35.0
F_0-3	9.1	5.8
F_0-4	12.5	19.5

[a] I_{50} = concentration that inhibits 50% germination. [b] ND, not determined.

reaction. After 1 h, the reaction was stopped by the addition of 2 mL of distilled water. Extraction with AcOEt and HPLC purification (hexane/ethyl acetate mixture 2:8, v/v) yielded the dihydro derivative **7** (85%).

Seed Germination Bioassays. *Lactuca sativa* L. var. Roman, *Lolium multiflorum* var. Gulf, *Trifolium pratense* var. Kenland, and *Physalis ixocarpa* were purchased from semillas COBO S.A. de C.V., Mexico D.F., Mexico. For these experiments 25 seeds of *L. multiflorum*, *T. pratense*, and *P. ixocarpa* were placed on a Petri dish; however, 50 seeds of *L. sativa* were required for the assays. The number of seeds used for each experiment was selected so that an appreciable change in O_2 uptake could be detected by the oxygraph. Seeds were placed on filter paper (Whatman No. 1) in Petri dishes (85-mm diameter). In three replicate experiments, the paper was wet with 8 or 10 mL of deionized water or test solution (MeOH <1%). The dishes were wrapped with Parafilm (laboratory film) and incubated at 28 °C in the dark at intervals of 48 h. The number of germinated seeds was determined according to the criteria of 1-mm extrusion of the radical. The replication was three for each germination assay. Control seed dishes contained the same amount of seeds and volumes of water and methanol as the test solutions. Seeds were selected for uniformity of size; the damaged ones were discarded (*40*).

Growth Bioassays. Coleoptyle or hypocotyle and root lengths for all germinated seeds were measured after 120 h, in three replicates following this design, after which the germinated plants were dried to constant weight at 40 °C (*43*). I_{50} values for plant development of the pure compounds and CH_2Cl_2 extract were obtained by determining the concentration that induced 50% of growth inhibition of development of roots and shoots (Figure 3 and Table 3).

Seeds Respiration. Seed respiration was measured polarographically as oxygen uptake during the germination process. The oxygen uptake, in the presence of different concentrations of tested compounds, was evaluated over 5 and 10 min, in a nonilluminated cell. The requirement for oxygen was plotted as percentage, taking the control as 100% (*39*).

Bioactivity-Guided Isolation of Ovatifolin. Milled aerial parts of *P. mitiqui* were extracted with MeOH, CH_2Cl_2, and ethyl acetate, and the initial phytotoxic activity of the resulting extract was evaluated for its growth effects (roots and hypocotyle development) on *L. multiflorum* and *T. vulgare* seeds and seedlings, using the Petri dish bioassay (Table 1) described above.

The more active CH_2Cl_2 extract was fractionated by silica gel column chromatography to yield four primary fractions (F-1−F-4), which were tested for activity. It was found that fraction F-3 contained the active compounds, which were analyzed and characterized by their R_f, IR, UV, ^{1}H NMR, and ^{13}C NMR data. Part of this mixture (50 g) was acetylated and yielded arturin and ovatifolin acetates (**2** and **4**) (*6*).

Reduction of DPPH Radical and β-Carotene. *TLC Autographic Assay.* After developing and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. The plates were examined 30 min after spraying. Active compounds appear as yellow spots against a purple background. In similar form, TLC plates were sprayed with 0.05% β-carotene solution in $CHCl_3$. The plates were examined under UV_{254} light until the background became discolored (bleached). Active compounds appear as pale yellow spots against a white background.

Spectrophotometric Assay (46, 47). Fifty microliters of a solution containing the compound to be tested was added to 5 mL of a 0.004% MeOH solution of DPPH; quercetin was used as internal standard reference. Absorbance at 517 nm was measured after 30 min, and the percent of activity was calculated.

Bleaching of Crocin. Crocin was isolated from commercial saffron (Sigma) by extraction with MeOH followed by HPLC (RP-18, MeOH/H_2O 1:1). It was identified by its ^{1}H and ^{13}C NMR data. Bleaching of crocin was performed according to the method of Bors (*46*). Aqueous solutions containing 10 μM crocin, 1 mM *t*-BuOOH, 0.5 M *t*-BuOH, and various dilutions of the compounds to be tested were prepared and irradiated with UV_{254} light. Bleaching was monitored by following the decrease of absorbance of crocin and fluorescence at 440 and 470 nm with time each 5 min.

Statistical Analysis. Data shown in the figures and tables are the means of three replicates of seedling growth, crocin, and DPPH and are presented as means ± standard errors. Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by GLM procedures. The 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 on the basis of the percentage of inhibition obtained at each concentration of the samples. I_{50} is the concentration producing 50% inhibition. Complete statistical analyses were via the MicroCal Origin 4.1 statistical and graphs PC program.

RESULTS AND DISCUSSION

Structural Determination of Compounds 1, 3, and 5. The structure of compound **3**, ovatifolin, was assigned and based on the following evidence: IR absorption bands at 3400 cm^{-1} showed the presence of a hydroxyl group, and absorption bands at 1750 and 1660 cm^{-1} indicate an α-methylene-γ-lactone. This chromophore in the UV spectrum was confirmed by the loss of *m/z* 95 ($C_5H_3O_2^+$) units in the mass spectrum and signals for two protons at 6.35 (d) and 5.59 (d) ppm for H-13 and H-13′, respectively, in the ^{1}H NMR spectrum. The four doublets of four protons appearing at 4.57 (d, br), 4.69 (d), 4.87 (d, br), and 4.81 (d, br) ppm confirm the presence of a germacranolide skeleton with an 8β-hydroxyl group. These assignments were identical with those previously reported (*34*).

The structure of arturin (**1**) followed immediately from comparison of the spectral data with those of compound **3**. Accordingly, one hydroxyl signal was evident (6.85 s). The following signals indicated the presence of a eudesmanolide skeleton with 1β-hydroxyl and 8β-angeloyloxy groups; a characteristic pair of doublets for H-13a,b was also observed. All signals in the ^{1}H NMR and ^{13}C NMR spectra were assigned and are identical to those reported by Hoeneisen (*34*).

The structure of deacetylovatifolin (**5**) was determined by comparing the above spectral signals that were typical for a germacranolide skeleton with 8β,14-dihydroxyl groups. IR absorption at 3400 cm^{-1} showed the presence of hydroxyl groups, and the absorptions at 1740 and 1660 cm^{-1} showed the presence of an α-methylene γ-lactone. In the ^{1}H NMR spectra, the signal for the methyl group of the acetate substituent has disappeared and two signals to lower field appear for two

Table 2. Effect of Ovatifolin and Its Derivatives on Seed Germination[a]

	GI_{50}[b] (μM)			
	monocots		dicots	
compd	*L. multiflorum*	*L. sativa*	*P. ixocarpa*	*T. pratense*
1	*c*	*c*	*c*	*c*
2	*c*	*c*	*c*	*c*
3	66.9	*c*	41.5	*c*
4	*c*	*c*	*c*	*c*
5	72.3	41.0	22.5	33.2
6	93.0	47.0	30.2	37.3
7	127.5	45.0	32.5	35.5

[a] Means of three experiments. [b] Concentration that inhibits 50% of seed germination. [c] GI_{50} not determinated due to lack of response.

Table 3. Effect of Ovatifolin and Its Derivatives on Growth of Seedlings during Seed Germination[a]

	I_{50}[b] (μM)							
	monocots				dicots			
	L. multiflorum		*L. sativa*		*P. ixocarpa*		*T. pratense*	
compd	shoot	root	shoot	root	shoot	root	shoot	root
3	15.5	34.4	20.8	19.0	11.4	11.2	34.4	29.5
5	33.9	82.6	13.0	13.8	10.7	11.5	14.3	16.2
6	ND[c]	119.9	17.3	17.4	12.5	10.2	23.2	28.6
7	36.2	52.3	79.0	96.7	23.8	14.2	67.6	46.3
CH_2Cl_2 extract[d]	27.5	32.1	9.5	7.5	23.0	12.3	15.0	15.0

[a] Means of three experiments. [b] Each value corresponds to the concentration that inhibits 50% of either root or coleoptyle/hypocotyle development during seedling stage. [c] Concentration of inhibition not determinated. [d] Values in ppm.

hydroxyl groups. The spectroscopic data were very similar to those reported by Romo de Vivar (*48*) for budlein B.

Growth Inhibition/Seed Germination. Table 1 summarizes the seed germination activity of the extracts and fractions from the CH_2Cl_2 extract. The I_{50} values of growth inhibitor activities (fractions F_0-1−F_0-4) are shown. F_0-3 showed the highest inhibition activities at 9.1 and 5.8 ppm for *L. multiflorum* and *P. ixocarpa*, respectively. Germacrane-type sesquiterpene lactones were isolated from this active fraction by chromatographic methods. In addition to the extracts and fractions, the compounds ovatifolin (**3**), epoxyovatifolin (**6**), dihydroovatifolin (**7**), and deacetylovatifolin (**5**) (Figure 1) were evaluated for their germination inhibition of one monocot and three dicot species (Tables 2−4).

Seed Germination. Figure 2 shows the seed germination inhibition effects of compounds **3** and **5**−**7**. Greater inhibition was observed with dihydroxy compound **5** than with **3**, **6**, or **7**. In general, dicot seeds (*L. sativa*, *P. ixocarpa*, and *T. pratense*) were more sensitive to these compounds and deacetylovatifolin given ~100% inhibition. The low germination I_{50} values indicate that deacetylovatifolin (**5**) and epoxyovatifolin (**6**) are the most powerful inhibitors for seed germination of *P. ixocarpa* and *T. pratense* (I_{50} values are 22.5 and 33.2, and 30.2 and 37.3 μM, respectively; Table 2). According to Hatfield and Karlen (*49*) and Mohr and Schopfer (*50*), pre-emergence selective inhibitors are applied after planting but before emergence of weeds or crops, as was done in this work.

Compounds **1**, **2**, and ovatifolin acetate (**4**) (data not shown) have the lowest potency against seed germination, >300 μM being required for 100% inhibition, whereas the other chemicals were 10 times more active

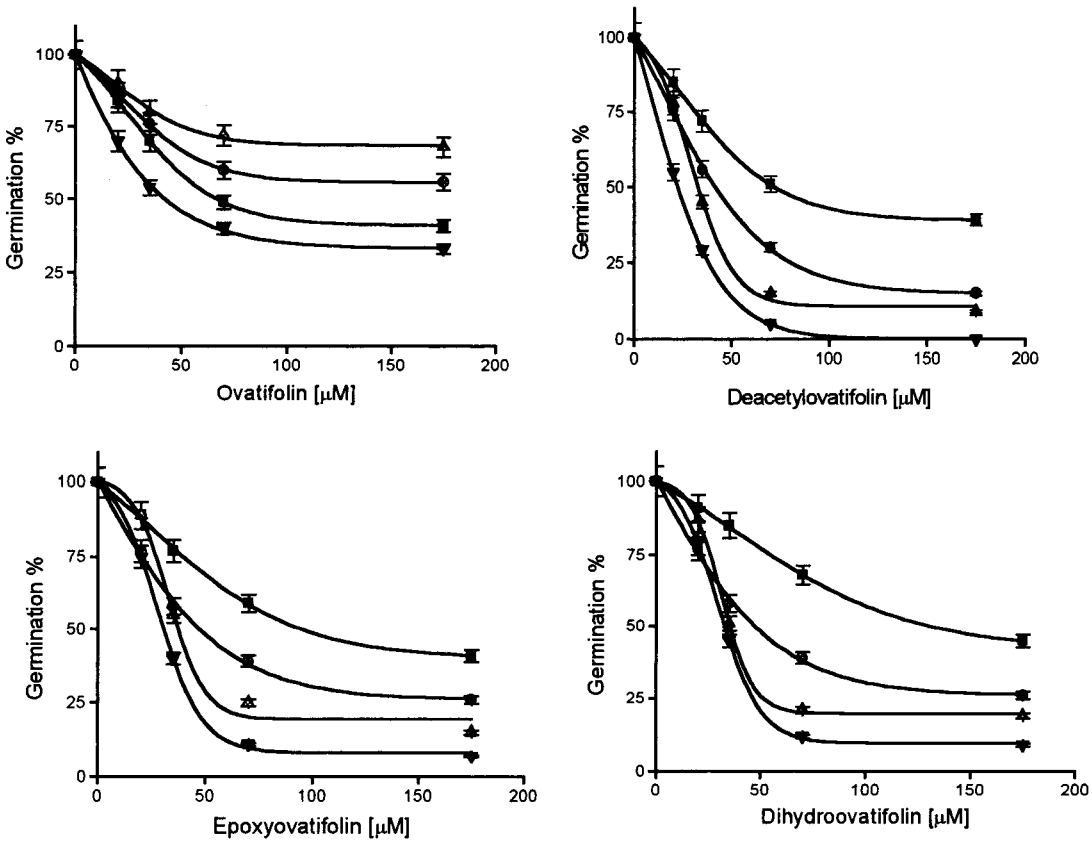


Figure 2. Effects of ovatifolin (**3**), deacetylovatifolin (**5**), epoxyovatifolin (**6**), and dihydroovatifolin (**7**) on germination of *L. multiflorum* (■), *L. sativa* (●), *P. ixocarpa* (▼), and *T. pratense* (▲) seeds, expressed as percent of control germination.

Table 4. Mean Concentrations of Compounds 3 and 5−7 and MeOH and CH_2Cl_2 Extracts Expressed as Concentration That Inhibits 50% of O_2 Uptake as a Function of Control Seed Respiration[a]

	I_{50}[b] (μM)			
	monocot	dicots		
compd	*L. multiflorum*	*L. sativa*	*P. ixocarpa*	*T. pratense*
3	32	*c*	45	*c*
5	51	42	25	33
6	93	41	30	34
7	120	39	35	39
MeOH extract[d]	119	*c*	125	*c*
CH_2Cl_2 extract[d]	25	15	21	19

[a] Means of three experiments. [b] Each value corresponds to the concentration that inhibits 50% of seed respiration during germination. Values at 72 h. [c] I_{50} was undetermined due to lack of seed respiration response within 24 h. [d] Values in ppm.

(~30 μM). These results suggest that the oxygenated moiety of these natural compounds allows the mixture of sesquiterpene lactones. The pure compounds **3** and **5**−**7** play an important role in the inhibition activities, which may be due either to their lipophilicity or to the fact that the hydrophilicity of the C-8, C-10, and lactonic moiety of ovatifolin (at physiological pH) makes it easy for these compounds to reach the target.

Monocot and Dicot Growth. Figures 3 and 4 show the inhibitory effects of the pure compounds on the root and coleoptyle development of the dicot and monocot seeds.

Root development is more sensitive, on the basis of I_{50} values, as compared with coleoptyle or hypocotyle development. Growth of the monocot apparently was less sensitive to inhibition by compounds **3** and **5**−**7** as determined by I_{50} values (Table 3; Figures 3 and 4).

The acetate derivatives promote root and hypocotyle development for the monocot. Arturin (**1**), arturin acetate (**2**), and ovatifolin acetate (**4**) alone enhance, rather than inhibit, the growth of both *T. pratense* and *L. sativa* roots as concentration increases to 50 and 300 μM (data not shown). In the case of *P. ixocarpa*, only root development was partially inhibited at 200 μM by the acetate derivatives. The hypocotyle growth was slightly stimulated at lower concentrations up to 50 μM for *P. ixocarpa* and up to 200 μM for *T. pratense* and, thereafter, partially inhibited by these compounds.

Compounds **1**, **2**, and **4** have the lowest inhibitory effects on growth (30% inhibition or lower) at 500 μM for monocots (data not shown), similar to the effects shown on seed germination, whereas for compounds **5**−**7**, 97, 95, and 100% inhibition, respectively, was achieved above 175 μM for dicots (Figures 3 and 4).

The results shown here regarding the differences in behavior of the acetate derivatives and the pure dihydroxy (**5**), epoxy (**6**), and dihydro (**7**) derivatives indicate that the mechanisms of action of these compounds may be different for growth and germination (*51*). 2,4-D shows a pronounced effect on root and shoot length, as well as on the germination of seeds, as 100% inhibition was obtained at 70 μM with I_{50} values of 0.4 and 0.5

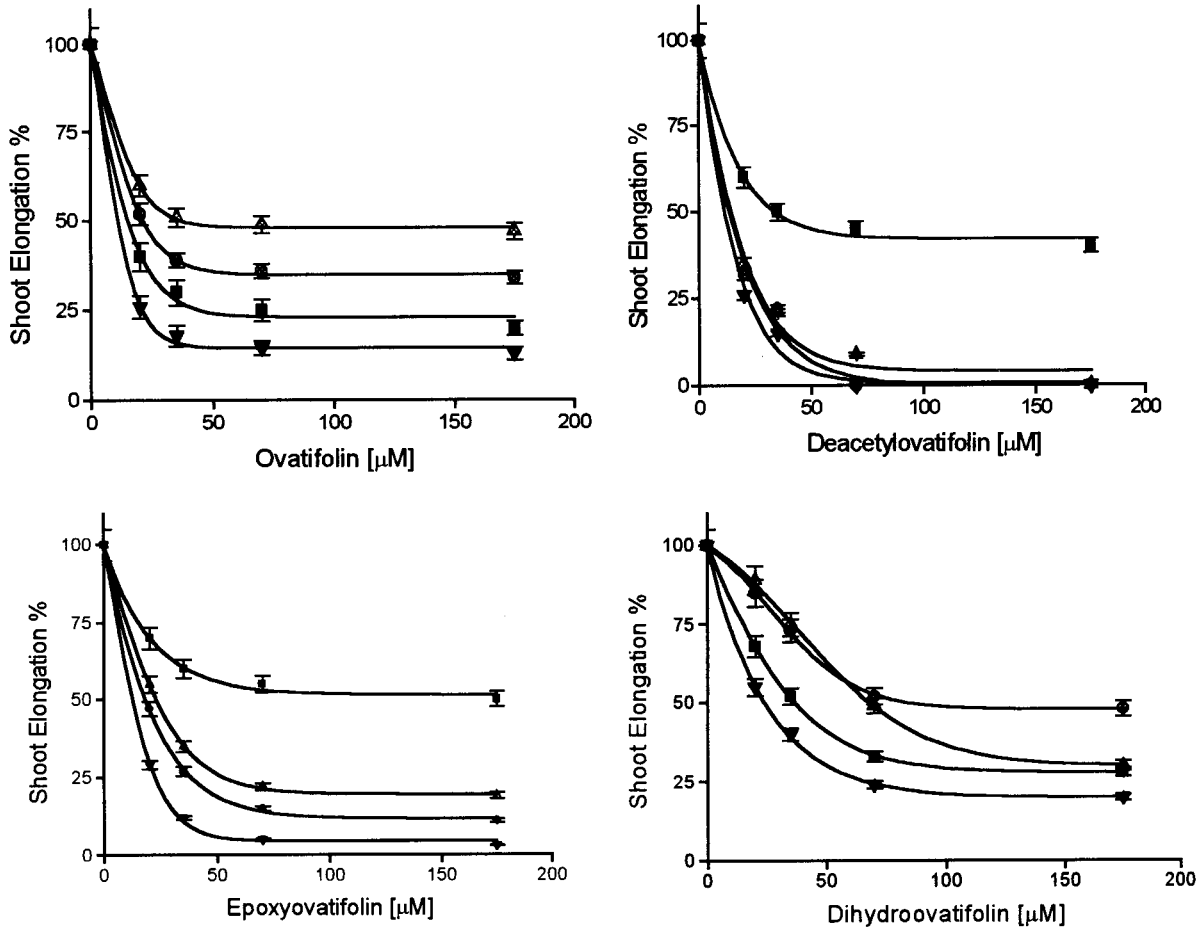


Figure 3. Effects of **3** and **5**−**7** on shoot development of *L. multiflorum* (■), *L. sativa* (●), *P. ixocarpa* (▼), and *T. pratense* (▲) seeds, expressed as percent of control.

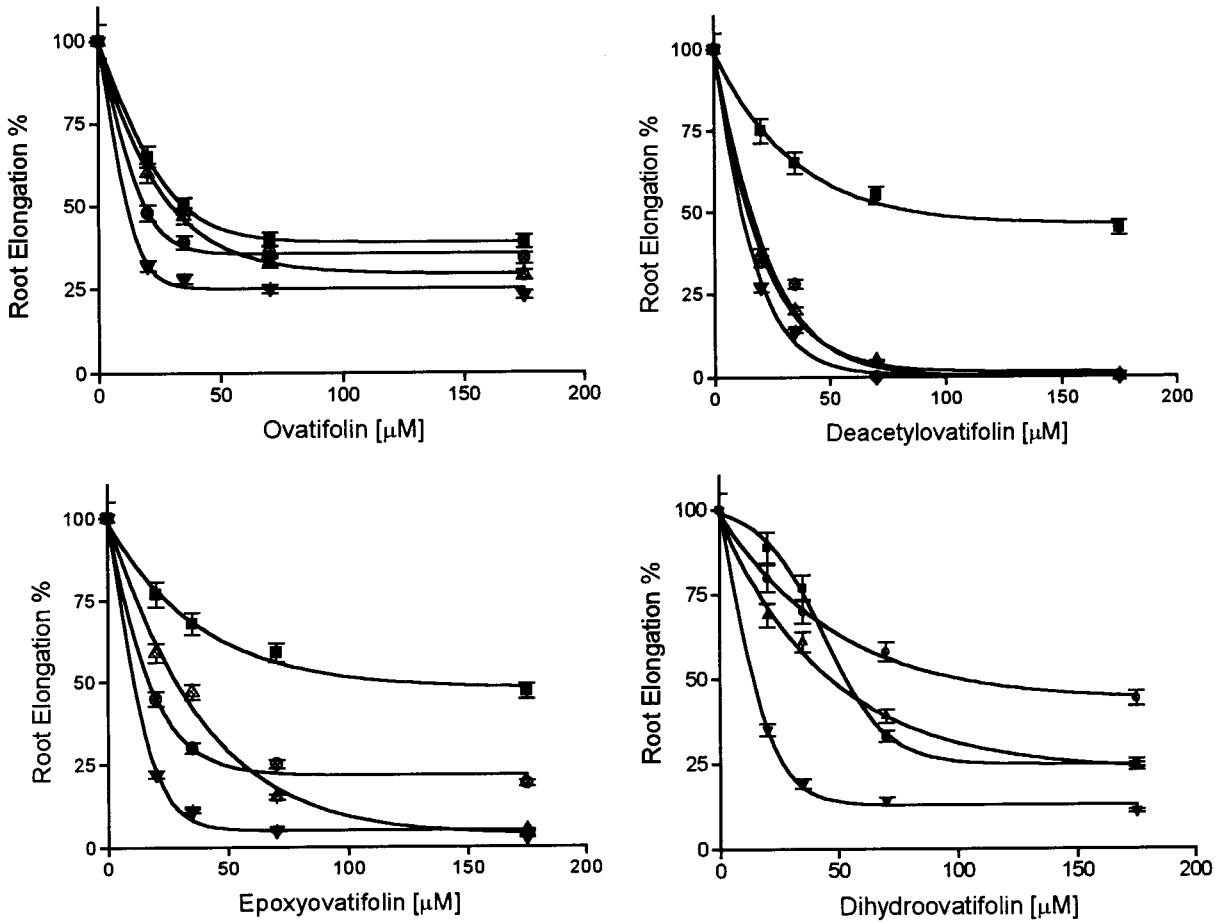


Figure 4. Inhibition of **3** and **5**−**7** on root development of seedlings of *L. multiflorum* (■), *L. sativa* (●), *P. ixocarpa* (▼), and *T. pratense* (▲) seeds, expressed as percent of control.

μM for root and shoot, respectively. Anaya (*52*) has also reported similar results.

Seeds Respiration during Seed Germination. The respiratory rates of all seeds decrease with exposure to compounds **3** and **5**−**7** and CH_2Cl_2 extract in a concentration-dependent manner (Table 4). The only exceptions are for compounds **1**, **2**, and **4** on *L. multiflorum* seed respiration, for which enhancement at 70 μM is observed, as the time of imbibition increases. However, at higher concentrations of 70−300 μM, respiration was also inhibited in a manner similar to that shown by compounds **3** and **5**−**7** at lowest concentrations (Table 4). These results suggest that ovatifolin, deacetylovatifolin, epoxyovatifolin, and dihydroovatifolin may act as uncouplers of phosphorylation at lower concentrations, but at higher concentrations they inhibit either energy transduction or respiration redox enzymes. Table 4 shows the RI_{50} values (the concentration of phytochemicals that induces 50% seed respiration inhibition) for **3**, **5**−**7**, and MeOH and CH_2Cl_2 extracts tested. According to their RI_{50} values, dicot seeds (*P. ixocarpa* and *T. pratense*) are the more sensitive to inhibition. On the other hand, *L. multiflorum* seeds showed the highest resistance to respiration inhibition.

Radical Scavenging Properties. Radical scavenging properties of compounds **1**−**7** were evaluated against the DPPH radical, using DPPH as a TLC spray reagent. Compounds **3** and **5**−**7** (10 μM) appeared as yellow spots against a purple background, whereas the same amounts of compounds **1**, **2**, and **4** did not react with the radical. Compounds **3** and **5**−**7** were also tested against DPPH in a spectrophotometric assay, and this method confirmed that compounds **3** and **5**−**7** exhibited the strongest radical-scavenging activity in this assay. Compounds **1**, **2**, and **4** were less sensitive than compounds **3** and **5**−**7**. It is worth mentioning that quercetin, a flavonol with three hydroxyl groups, was used as a reference compound that possesses strong antioxidant properties (Figure 5; Table 5).

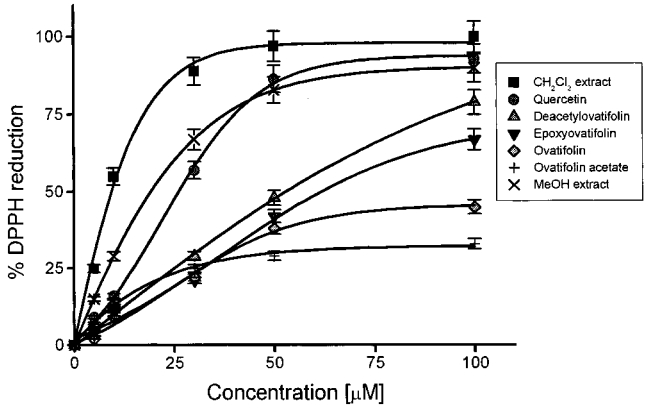


Figure 5. Scavenging activity of compounds **3**−**6** and MeOH and CH_2Cl_2 extracts on DPPH radical: quercetin (●); CH_2Cl_2 extract (■); deacetylovatifolin (▲); epoxyovatifolin (▼); ovatifolin (◆); ovatifolin acetate (+); MeOH extract (×). Measurements were made at 517 nm with determination after 30 min. Values of MeOH and CH_2Cl_2 extracts are in parts per million.

The antioxidant activity of these compounds was also evaluated spectrophotometrically on the bleaching of the

Table 5. Effect of Quercetin, Ovatifolin, Their Derivatives, and MeOH and CH_2Cl_2 Extracts on DPPH Reduction[a]

compd	I_{50}[b] (μM)
quercetin	26.4
3	ND[c]
4	ND
5	54.5
6	62.7
7	ND
MeOH extract[d]	19.3
CH_2Cl_2 extract	9.5

[a] Means of three experiments. [b] Concentration that inhibits 50% of seed germination. [c] ND, not determined. [d] Values in ppm.

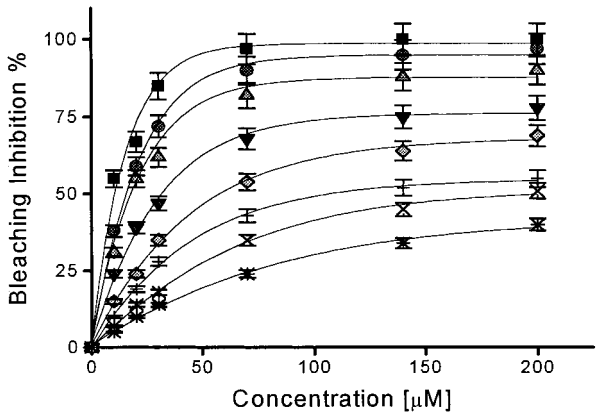


Figure 6. Inhibitory activity of compounds **3**−**6** and MeOH and CH_2Cl_2 extracts on the bleaching of crocin: CH_2Cl_2 extract (■); deacetylovatifolin (●); epoxyovatifolin (▲); gallic acid (▼); ovatifolin (◆); dihydroovatifolin (+); ovatifolin acetate (×); arturin (∗). Measurements were made at 440 nm of fluorometric emission with determination after 20 min. Values of CH_2Cl_2 extract are in parts per million.

H_2O-soluble crocin (*46*). Alkoxyl radicals were generated from *t*-BuOOH by UV photolysis of aqueous solutions containing 10 μM crocin and 1 mM *t*-BuOOH. *t*-BuOH (0.5 M) was added to scavenge the $HO^{\bullet}$ radicals produced. Gallic acid was added as a reference compound. Compounds **3** and **5**−**7** were all active and showed activities comparable to that of gallic acid (Figure 6).

CONCLUDING REMARKS

Interestingly, deacetyl ovatifolin, arturin, and ovatifolin characterized as brown needle crystals could not be detected in the acetate extracts of *P. mitiqui* and *P. ovatifolius*. In contrast, ovatifolin and its deacetylated form are abundant in the CH_2Cl_2 extracts and are found in minor amounts in MeOH extracts. More importantly, these compounds seem to accumulate in the cells and are exuded slowly. As a matter of fact, they seem to be localized in glandular trichomes on the leaves of these plants (*53*). The bitter taste and the yield of sesquiterpene lactones are in direct relationship with the amount of glandular trichomes present on the leaves (*53*−*56*). From this, it may be logical to assume that arturin, deacetylovatifolin, and ovatifolin are synthesized for defensive process. It appears that the releasing mechanism of ovatifolin may be one of the key processes to understanding plant defense.

The seven compounds examined in this study have similar molecular structures, the differences resting in the position of the acetyl and angeloyloxy substituents (Figure 1). Of the seven sesquiterpenes tested for growth inhibition of four plant species, compounds **5** and **6** showed the greatest effect on the dicot weeds (*P. ixocarpa* and *T. pratense*). Ovatifolin and deacetylovatifolin had similar inhibitory effects on the root growth of *L. multiflorum* (Figure 3), whereas deacetylovatifolin had a greater effect on shoots than that shown by ovatifolin (Figure 3). At amounts >30 μg/g of **5** and **6**, the shoots were severely malformed with a corkscrew-like appearance and were significantly smaller ($p < 0.05$, ANOVA) than controls.

Inhibitory effects of some germacrane-type sesquiterpene lactones (GTSL) were investigated by Macias's group (*17*). It was reported that these natural and synthetic products show enhancement and inhibition effects on different pre-emergence properties (i.e., germination and root length) at low and high concentrations, respectively. Similar findings were observed by Fischer and co-workers (*22*, *23*). However, the effects of other GTSL on seed germination and root length were unclear. Ovatifolin and deacetylovatifolin are unique among the GTSL reported to date (*34*, *35*), with the presence of an acetyl group at the C-10 position, a hydroxyl at C-8, and a methylene group in the lactone ring.

The effects of these compounds have certain similarities with those of costunolide and parthenolide and their derivatives (*17*).

In addition, it was possible to correlate the antioxidant activity with the seedling growth inhibitory activity.

In conclusion, our data indicate that deacetylovatifolin and epoxyovatifolin are more selective and potent respiratory inhibitors toward dicots than toward monocots. Respiration processes are involved in the interference action, as these processes were inhibited in a parallel manner by the CH_2Cl_2 extract and the compounds assayed. At the same doses, a higher inhibition was observed on seed germination than on seed respiration, and it was concluded that these extracts and compounds have more than one target of interference.

The treatment concentrations for the mixtures that reduced seedling growth were low (10−40 μM) compared to allelopathic chemicals that have been previously studied under laboratory conditions (*51*, *57*). The reported secondary metabolite potency is in the range from 100 to 1000 μM for growth reduction by many phenolic acids, or ∼10 μM for sorgoleone, or at the micromolar level for juglone, which suppresses the growth of several herbaceous species (*58*, *59*).

Whatever the mechanism(s) of action of the mixture of ovatifolin and their derivatives, they have proved to be good inhibitors of plant growth. They show preemergent phytotoxic properties by inhibiting germination and growth. They also show some degree of selectivity by inhibiting dicotyledonous species more drastically, and these behaviors could be correlated with radical scavenger and antioxidant properties.

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