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Plant Growth Inhibitory Activity of *p*-Hydroxyacetophenones and Tremetones from Chilean Endemic *Baccharis* Species and Some Analogous: A Comparative Study

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Plant growth inhibitory effects of acetophenones **1**−**6**, tremetones **7**−**12**, and MeOH and CH_2Cl_2 extracts from the aerial parts of *Baccharis linnearis*, *Baccharis magellanica*, and *Baccharis umbelliformis* collected in Chile were assayed as growth inhibitory activity in ranges of 10−500 μM and 0.1−150 ppm, respectively. The effects on seedling growth, germination, and respiration of ryegrass, lettuce, green tomato, and red clover weedy target species were measured. In addition to the inhibitory activity on bleaching of crocin induced by alkoxyl radicals, these compounds also demonstrated scavenging properties toward 2,2-diphenyl-1-picrylhydrazyl in thin-layer chromatography autographic and spectrophotometric assays. In addition, acetophenones and tremetones also showed inhibition of H^+ uptake and oxygen uptake respiration in isolated chloroplasts and mitochondria, respectively. Our results indicate that **1**, **4**, **7**−**12**, and CH_2Cl_2 extracts interfere with the dicot preemergence properties, mainly energy metabolism of the seeds at the level of respiration. These compounds appear to have selective effects on the radicle more than shoot growth of dicot seeds. Also, the levels of radicle inhibition obtained with some compounds on *Physalis ixocarpa* and *Trifolium pratense* are totally comparable to those of ovatifolin, a known natural growth inhibitor. This behavior might be responsible for its plant growth inhibitory properties and its possible role as an allelopathic agent.

KEYWORDS: Acetophenones; plant growth inhibitor; *Baccharis* species

INTRODUCTION

The large genus *Baccharis* (Compositae-Asteraceae) is a strictly American genus with approximately 350 species. Only a few are important medicinal plants, and their uses seem to be rather diverse; more than 50 species have been investigated chemically (*1*−*3*). The most widespread compounds found are diterpene glycosides, clerodane derivatives, and labdane and kaurane derivatives (*4*). Furthermore, typical acetylenic compounds, baccharis oxide (a unique triterpene), and derivatives of *p*-hydroxyacetophenone are present. In continuation of our investigations on South American *Asteraceae* representatives, we have studied *Baccharis linnearis*, *Baccharis magellanica*, and *Baccharis umbelliformis* endemic species of Austral Southern Chile.

In the semiarid zones from Atacama to the Patagonia region of Austral Southerm Chile grow *B. linnearis, B. magellanica*, and *B. umbelliformis* (tribe, Eupatorieae; family, Asteraceae). *B. linnearis* is a dominant shrub that can be found growing either in communities as with associated flora or in solitary form throughout central to south-central regions (*3*); *B. magellanica* and *B. umbelliformis* grow in semiarid regions of Austral Southern Chile (*5*−*7*). These plants exhibit strong effects of survival under different stress environmental conditions (*8*). Chromenes, *p*-hydroxyacetophenones, and benzofurans (tremetones) have been isolated from Chilean *Baccharis* (*9*). These compounds have been shown to have some biological (*2*, *10*), antifungal (*18*), and insecticidal (*13*, *14*) activities. In addition, they possess repellency toward honey bees, rodent, and bird (*11*, *12*), are pheromonal constituents (*15*), occur as phytoalexin in tropical plants (*16*, *17*), and are DNA synthesis inhibitors (*19*).

Some investigations on mechanisms of allelopathic action report that different phenolic (isoprenoid and nonisoprenoids) compounds are seedling growth and metabolic inhibitors in chloroplasts and mitochondria (*20*−*23*), and in some cases, acetophenones enhance and promote the growth root of myrtle (*24*).

We have previously demonstrated that diverse secondary metabolites have a different mechanism of action and different molecular targets when they interact with the germination, seedling growth, and photosynthetic electron transport chain (*25*−*33*). Uncoupled properties of some acetophenones and

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benzofurans on electron transport rate in chloroplasts have been reported (*21*, *34*, *35*).

The aim of this work was focused to correlate structure−activity relationships with the inhibitory behavior on germination respiration, the main macroscopic parameter of the development and growth of weeds. Our data indicate that it is possible to correlate some antioxidant activities (i.e., crocin, DPPH) against germination and respiration; these data are important for allelopathic studies (*31*). On the other hand, these parameters are accepted as indirect measures of other different physiological processes (*36*) affected by the assayed chemicals.

Here, we report the plant growth inhibitory effects of *p*-hydroxyacetophenone (**1**), *p*-*O*-glycoside acetophenone (**2**), *p*-bromo-acetophenone (**3**), *p*-methoxy-acetophenone (**4**), 4′-acetyl-acetophenone (**5**), acetophenone (**6**), tremetone (**7**), 10, 11-epoxy-tremetone (**8**), 10,11-dihydrotremetone (**9**), 4′-hydroxy-tremetone (**10**), 4′-acetyl-tremetone (**11**), 10,11-epoxy-4′-hydroxy-tremetone (**12**), and MeOH and CH_2Cl_2 extracts on germination and other physiological activities (shoot−root elongation, seed respiration, O_2 uptake, H^+ uptake, and redox inhibition in chloroplasts and mitochondria). Dicots assayed were lettuce (*Lactuca sativa* cv. Roman), green tomato (*Physalis ixocarpa*), and red clover (*Trifolium pratense* cv. Kenland). The only monocot assayed was ryegrass (*Lolium multiflorum* cv. Gulf). 2,4-Dichlorophenoxyacetic acid (2,4-D) was introduced into the bioassay as internal standard in the plant growth bioassay, and ovatifolin was used as a pattern substance (*31*). In addition, tetrahydroxyquinone (THQ), quercetin, butylated hydroxy anisole (BHA), and gallic acid were used as internal standards in assays concerned with the reduction of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and antioxidant measurements, respectively.

As far as we know, the effects of **1**−**12** on germination and respiration of weed seeds, antioxidants, radical scavengers, oxygen uptake, and photosynthesis have not been investigated. This is the first report on the allelopathic effects of the main compounds and some of their synthetic derivatives and extracts from the South American *Baccharis* species.

MATERIAL AND METHODS

Plant Material. Aerial parts (steam, leaf, and flowers) from *B. linnearis* were collected in Lampa near Santiago, Chile (Metropolitan Region); *B. magellanica* and *B. umbelliformis* were collected in Antillanca, X Region, Chile, January, 1997. Voucher specimens (R. Rodríguez and C. Marticorena) can be found at the ethnobotanical collection of the Herbarium (CONC), Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile.

Chemicals and Solvents. All reagents used were either A. R. grade or chromatographic grade. *m*-Chloroperbenzoic acid (MCPBA), $NaBH_4$, pyridine, 2,4-D, 1,1′-dimethyl-4,4′-bipyridinium dichloride (methyl viologen), DPPH, THQ, BHA (2[3]-*tert*-butyl-4-hydroxyanisole; 2[3]-*tert*-butylhydroquinone monomethyl ether), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin, Percoll, 4'-methoxyacetophenone, 4'-bromoacetophenone, acetophenone, β-carotene, gallic acid, quercetin, saffron, sorbitol, tricine, and trizma-hydrochloride were purchased from Sigma-Aldrich Quimica, S. A. de C. V., Toluca, Mexico. Methanol, CH_2Cl_2, $CHCl_3$, NaCl, KCl, NaOH, KOH, *tert*-butyl alcohol, *tert*-butyl hydroperoxide, $CuSO_4$, NH_4Cl, $MgCl_2$, acetic anhydride, silica gel GF_{254} analytical chromatoplates, silica gel grade 60 (70-230, 60A°) 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. ^1H nuclear magnetic resonance (NMR) spectra were recorded at 300, and ^{13}C NMR spectra were recorded at 75 MHz, respectively, on Varian VXR-300S and VXR-500S spectrometers; chemical shifts (ppm) are related to $(CH_3)_4Si$ as the internal reference. $CDCl_3$ and acetone-d_6 from Aldrich Chemical Co. were used as solvents. Coupling constants are quoted in Hertz. IR spectra were obtained in KBr or $CHCl_3$ on a Perkin-Elmer 283-B and a FT-IR Nicolet Magna 750 spectrophotometers. A Spectronic model Genesys 5 spectrophotometer was used for biological activities. Optical rotation was measured on a JASCO DIP-360 spectropolarimeter. Melting points were obtained on a Fisher-Johns hot-plate apparatus and remain uncorrected. Oxygen evolution (uptake) was determined with a Clark type electrode connected to YSI (Yellow Spring Instruments) oxygraph model 5300. Fluorimetric measurements were determined with TURNER Barnstead-Thermolyne, model Quantech S5 Fluorometer, with 420, 440, 470, 550, and 650 Turner filters.

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

		I_{50} (ppm)	
plant species	sample tested	*L. multiflorum*	*P. ixocarpa*
B. linnearis	AcOEt extract	ND	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
B. magellanica	AcOEt extract	ND	ND
	CH_2Cl_2 extract	28.5	23.0
	MeOH extract	135.2	125.9
	F_0-1	92.0	80.0
	F_0-2	35.0	33.0
	F_0-3	19.9	15.1
	F_0-4	15.5	14.5
B. umbelliformis	AcOEt extract	ND	ND
	CH_2Cl_2 extract	27.2	20.0
	MeOH extract	121.0	120.0
	F_0-1	87.0	84.0
	F_0-2	41.5	43.0
	F_0-3	10.5	7.8
	F_0-4	13.5	20.0

[a] I_{50} = concentration that inhibits 50% germination.

General Experimental Procedures. High-performance liquid chromatography (HPLC) was performed on a WATERS model 600E, equipped with μBondapack RP-18 column, 250 mm × 8 mm, flow rate 1.5 mL/min; UV detector 280 nm, mobile phase MeOH/H_2O 7:3 v/v. Analytical thin-layer chromatography (TLC) was performed on Silica gel 60 F_{254} Merck plates, and the spots were visualized by spraying with a 10% solution of H_2SO_4, followed by heating at 110 °C for 3 min.

Bioactivity-Guided Isolation and Purification of Acetophenones, Tremetones, and Their Derivatives. The plant materials were *B. magellanica* (5.9 kg), *B. linnearis* (6.5 kg), and *B. umbelliformis* (5.5 kg). The dried and milled aerial part of the three species was extracted with MeOH, and furthermore, this extract was partitioned between CH_2Cl_2 and MeOH/H_2O (1:1 v/v). Evaporation of the solvent in a rotatory evaporator afforded the crude extracts, which were weighed to calculate the yield.

The MeOH, CH_2Cl_2, and ethyl acetate extracts of the milled aerial part of the three *Baccharis* species were assayed under the initial phytotoxic activity of the resulting extract; it was evaluated for its growth effects (roots and hypocotyl development) on *L. multiflorum* and *P. ixocarpa* seeds and seedlings, using the Petri dish bioassay (**Table 1**) (*27*).

The most active extract of each one of the species was CH_2Cl_2, which was tested for phytogrowth activity (**Table 1**) and then submitted to column chromatography using SiO_2 (G 60, Merck) as solid phase. Elution of each one of the CH_2Cl_2 extracts with *n*-hexane:ethyl acetate mixtures and ethyl acetate followed by methanol afforded 20 fractions that after evaporation of the solvent to dryness were analyzed by TLC and bioautographic assay (*28*) using different solvent systems (*n*-hexane: ethyl acetate and DCM:MeOH mixtures). It was found that four fractions (F-14−F-17) contained the active compounds.

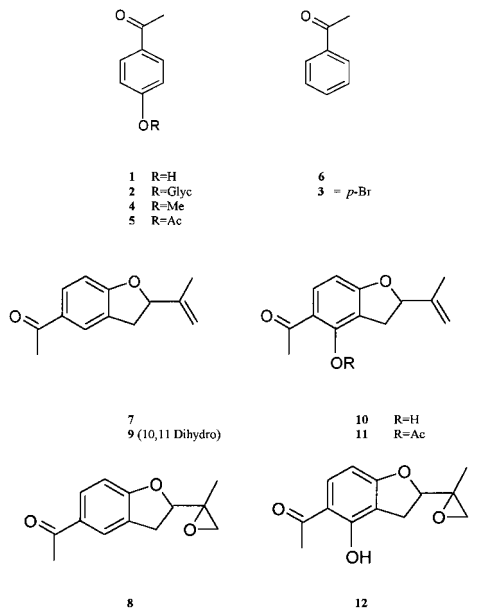

Figure 1. Structures of 4-hydroxyacetophenone (**1**), 4-glucosyl acetophenone (**2**), 4-methyl acetophenone (**3**), 4-bromo acetophenone (**4**), 4-acetyl acetophenone (**5**), acetophenone (**6**), tremetone (**7**), 10,11-epoxy tremetone (**8**), 10,11-dehydro tremetone (**9**), 4-hydroxy tremetone (**10**), 4-acetyl tremetone (**11**), and 10,11-epoxy-4-hydroxy tremetone (**12**).

Repeated TLC of these fractions led to the isolation of the secondary metabolites, which were purified by prep-TLC. Identical compounds were collected and identified by TLC with authentic samples. *p*-Hydroxyacetophenone (**1**, 700 mg), the minor known compounds *p*-*O*-glycoside acetophenone (**2**, 80 mg), acetophenone (**6**, 250 mg), and the dihydrobenzofurans tremetone and 4-hydroxytremetone (**7** and **10**, 1.5 g and 935 mg, respectively) (**Figure 1**) were obtained as pure natural products, which were analyzed and characterized by their R_f, IR, UV, ^{1}H NMR, and ^{13}C NMR data.

The identification of acetophenones and tremetones was made by these spectroscopic methods and direct comparison with authentic samples (*37*−*41*); as **1**, **2**, **7**, and **10** were purified in sufficient amount, these were used in the bioassays and derivatization. Ovatifolin was available from previous work (*31*). Compound **9** was characterized as the 10,11-dihydro derivative of **7**. Compounds **5**, **11**, **8**, and **12** were characterized as the acetate and epoxy derivatives, respectively.

Acetylation of Compounds 1 and 10. Derivatization of the C_4-hydroxyl group by acetylation with acetic anhydride/pyridine of 4-hydroxyacetophenone (**1**) gave 4-(acetate)acetophenone (**5**). An amount of 55 mg of **1** 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 49 mg of **5** and then purified by TLC using silica gel GF_{254} preparative chromatoplates, 2 mm, Macherey-Nagel, eluted with a *n*-hexane/ethyl acetate (80:20) solvent system; using UV_{254} detection, its structure was established by high-resolution spectroscopic methods, and data are in agreement as previously reported (*41*). A similar procedure was used by acetylation of **10** to give **11**.

Epoxidation of Tremetone. Fifty milligrams of **7** was dissolved in 10 mL of dried methylene chloride (DCM), and then, 50 mg of sodium acetate was added. Afterward, 50 mg of MCPBA dissolved in dried DCM was added dropwise with stirring. After 1 h, the reaction was stopped, and the workup was carried out as follows: the reaction mixture was extracted with NaOH(aq) 5% (2×) to separate the remainder of the MCPBA and *m*-chlorobenzoic acid; then, the pH of the organic phase was adjusted to nearly 7.0 by washing with distilled water (2×). All of the aqueous phases were extracted with DCM, and the combined organic phases were dried on anhydrous sodium sulfate. After separation by prep-TLC, compound **8** was obtained in a crystalline form (81%), and its structure was established by high-resolution spectroscopic methods. A similar procedure was used by epoxidation of **10** to give **12**.

Reduction of Tremetone (7). A 2 mL portion of methanolic solution with 15 mg of **7** was kept in a Dewar glass at 0 °C. While the solution was stirring, $NaBH_4$ (1:1.4 molar relationship) was added during the first 5 min of reaction. After 1 h, the reaction was stopped by addition of 2 mL of distilled water. Extraction with AcOEt and HPLC purification (hexane/ethyl acetate mix) yielded the 10,11-dihydroderivative (**9**, 85%).

Post- and Preemergence Activities. *Seeds Germination Bioassays. L. sativa, L. multiflorum, T. pratense,* and *P. ixocarpa* were purchased from "SEMILLAS-COBO" S. A. de C. V. Mexico D. F., México. 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 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 foil 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 volume of water and methanol as the test solutions. Seeds were selected for uniformity of size; the damaged ones were discarded (*27*).

Growth Bioassays. Coleoptile or hypocotyl 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 (*29*).

Seeds Respiration. Seed respiration was measured polarographically at 25 °C with a Clark type electrode as oxygen uptake during the germination process using an YSI oxygraph model 5300. 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% (*26*).

Preparation of Thylakoid Membranes. Isolated chloroplasts were prepared from market spinach leaves (*Spinacia oleracea*) as reported (*25*), and the pellet was resuspended. These were homogenized in ice-cold extraction buffer (330 mM sorbitol, 50 mM tricine-NaOH, 10 mM $MgCl_2$, 5 mM Na-ascorbate, 2 mM EDTA, and 0.05% bovine serum albumin, pH 7.8) and filtered through a layer of miracloth and four layers of cheesecloth. The filtrate was centrifuged at 1100*g* for 5 min at 4 °C. The chloroplast pellet was resuspended in ice-cold lysing buffer (25 mM tricine-NaOH, 10 mM $MgCl_2$, pH 7.8) and centrifuged at 3000*g* for 5 min. The pellet was resuspended, unless indicated, in a buffer (100 mM sorbitol, 25 mM tricine-NaOH, 10 mM $MgCl_2$, and 10 mM NaCl, pH 7.8). For the oxygen evolution experiments, the thylakoid membranes were diluted to 4 mg of chlorophyll/mL according to Arnon as modified by Hiscox and Israelstam (*42*).

Measurement of Proton Uptake and Adenosine Triphosphate (ATP) Synthesis. Proton uptake was measured as the pH rose from 8.0 to 8.1 (*29*) with a combination microelectrode connected to a potentiometer (model 225 Research pH/ion-meter, Denver Instrument Company, Arvada Colorado) with expanded scale. The reaction medium was 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, and 1 mM Na+-tricine, pH 8. ATP synthesis was measured titrimetrically according to the procedure of Dilley (*43*); 50 μM MV was added as an electron acceptor for the Hill reaction.

Measurement of Electron Transport. Photosynthetic noncyclic electron transport rates from water to MV were monitored with a YSI model 5300 oxygen monitor connected to a Clark type electrode. The reaction medium was the same as that used in the H^+ uptake assay except for the tricine concentration (15 mM), and in the case of the uncoupled electron transport measurement, 6 mM NH_4Cl was added. All reaction mixtures were illuminated with light from a projector lamp (GAF 2660) filtered through 5 cm of 1% $CuSO_4$ solution at 20 °C (*29*, *44*).

Determination of Mitochondrial Oxygen Consumption. The procedure for the isolation of bean root mitochondria is based on previously described protocols (*45*) with minor modifications, as follows. Approximately 700 g of *Phaseolus vulgaris* seeds was sterilized

for 30 min in a 10% (v/v) solution of sodium hypochlorite and then washed with sterile water successively. Seeds were growing in a sterile sphagnum peat moss mixed with vermiculite (1:1) (purchased from Hummert de Mexico, S. A. de C. V., Cuernavaca, Mexico) and grown in complete darkness for 72 h at 28 °C. Roots were obtained by cut with scissors from the coleoptyle; all subsequent procedures were carried out at 4 °C as rapidly as possible. The chilled tissue was ground in a mortar and pestle with silica gel 60 (0.063−0.200 mm) in 2 volumes of grinding buffer (0.4 M sorbitol, $MgCl_2$ 5mM, KCl 10 mM, tricine 30 mM, pH 8.0, with KOH) and filtered through Miracloth into 250 mL centrifuge bottles. The filtrate was then centrifuged at 3000*g* for 5 min in a Sigma-B.Braun model 2-15 rotor, the supernatant was removed, and the mitochondria were pelleted by centrifugation at 10 000*g* for 15 min. One to five milliliters of wash buffer (0.4 M sorbitol, $MgCl_2$ 5mM, KCl 10 mM, tricine 30 mM, pH 7.8, with KOH) was added per tube, and the pellet was resuspended using a soft paintbrush. The suspension was placed in 50 mL tubes and centrifuged at 3000*g* for 5 min in a Sigma-B.Braun model 2-15 rotor. The supernatant was then carefully transferred to another 50 mL tube and centrifuged at 10 000*g* for 15 min to repellet the mitochondria, which were then resuspended in 1−2 mL of resuspension buffer (0.4 M sorbitol, $MgCl_2$ 5mM, KCl 10 mM, tricine 30 mM, pH 7.2, with 5 M KOH). To purify the mitochondria from residual contaminating plastids, the crude mitochondrial suspension was loaded onto 26% (v/v) Percoll (SIGMA) in resuspension buffer (ca. 40 mL) in a 50 mL polycarbonate tube, and a density gradient was generated by centrifugation at 40 000*g* for 90 min in a Sigma-B.Braun model 2-15 rotor. A buff-colored band of mitochondria was visible below a band containing plastids. The upper layer was removed by aspiration, and the mitochondrial band was recovered (ca. 1−2 mL) and diluted with 20 mL of resuspension buffer. Mitochondria were then recovered by centrifugation at 12 000*g* for 15 min in a Sigma-B.Braun model 2-15 rotor. The resulting pellet was very loose, and the supernatant had to be removed by aspiration with care.

This wash procedure was repeated, and the final pellet was resuspended in 0.4−1 mL of resuspension buffer and stored on ice. Total mitochondrial protein concentration was determined using the Bradford procedure (*46*), adjusted to 0.3−0.5 mg for each experiment, and the freshly prepared mitochondria were used directly in reaction of inhibition. The O_2 uptake of mitochondria was monitored with an YSI model 5300 oxygen monitor connected to a Clark type electrode.

The integrity of mitochondria was verified using HCN as respiratory chain inhibitor. All compounds were dissolved in 0.5% methanol. Respiration control and ADP:O ratios were calculated for each mitochondrial isolation (*47*). A constant state 3 respiratory rate (*48*), with 10 μM succinate was obtained before measuring with our compounds. The ADP:O ratio for succinate was 1.5.

Reduction of DPPH [2,2′-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl] Radical. TLC autographic assay: after they were developed and dried, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. The plates were examined 30 min after spraying. Active compounds appeared 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 appeared as pale yellow spots against a white background. Spectrophotometric assay (*49*, *50*): 50 μL 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. 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 ^1H and ^{13}C NMR data. The test was carried out according to Bors (*49*). An aqueous solution containing 10 μM crocin, 1 mM *t*-BuOOH, 0.5 M *t*-BuOH, and various dilutions of the compounds to be tested were prepared. The solutions were placed under UV_{254} light. Following the decrease of absorbance, bleaching of crocin and fluorescence emission at 440 and 470 nm were monitored with time each 5 min.

Statistical Analysis. Data shown in figures and tables are the mean results obtained with means of three replicates and independent seeds, crocin, and DPPH preparations and are presented as mean ± standard errors of the mean (SEM). 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. Completely statistical analysis was performed by means of the MicroCal Origin 4.1 statistical and graphs PC program.

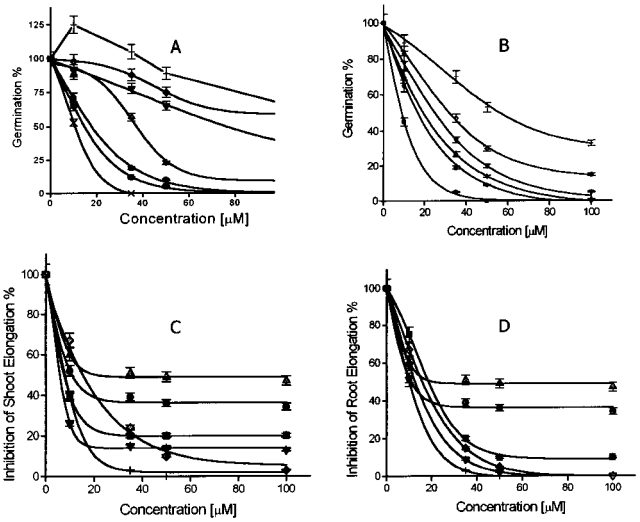

Figure 2. Effects of compounds **1** (■), **2** (+), **3** (♦), **4** (●), **5** (▼), **6** (▲), and **12** (×) on seed germination of *L. multiflorum* (**A**); **7** (♦), **8** (▲), **9** (▼), **10** (●), **11** (+), and **12** (■) on seed germination of *P. ixocarpa* (**B**); inhibition of **1** (●), **4** (▲), **7** (■), **8** (♦), **10** (▼), and **12** (■) on shoot development of seedlings of *P. ixocarpa* seeds (**C**); and **1** (●), **4** (▲), **7** (■), **8** (♦), **10** (▼), and **12** (+) on root development of seedlings of *P. ixocarpa* seeds (**D**), respectively. Compounds **1** (●), **4** (▲), **7** (■), **8** (♦), **10** (▼), and **12** (+) are expressed as percent of control.

RESULTS AND DISCUSSION

Phytotoxicity. Table 1 summarizes the phytotoxic activity of the extracts and fractions from the CH_2Cl_2 extract. The IC_{50} values of phytogrowth inhibitory activity, from fraction F-10 to F-14 are shown. F-13 from *B. linnearis* showed the highest inhibitory activity with 9.1 and 5.8 ppm for *L. multiflorum* and *P. ixocarpa*, respectively. From this active fraction, acetophenones and tremetones were isolated with highest yields. In addition to compounds **1**, **2**, **7**, and **10** (**Figure 1**) the derivatives **3**−**6**, **8**, **9**, **11**, and **12** and CH_2Cl_2 extract were evaluated against seed germination inhibition of one monocot and three dicot species (**Tables 2**−**4**).

Seed Germination. Figure 2A shows the phytotoxic effect of the compounds **1**−**6** and **12** assayed on seed germination of *L. multiflorum*. The hydroxyl **1** and methoxyl derivative **4** showed higher inhibition profiles than **2**, **3**, **5**, and **6**. Here, it is possible to observe that the glucosyl compound at low concentration enhances the germination activity, and at very high concentration, it inhibits the plant growth of monocot seeds. Moreover, no significant effect was shown by acetophenones **2**, **3**, **5**, and **6** against dicot seeds. In addition to **3**, **5**, and **6**, compound **2** did not interfere with the seedling growth of dicot seeds. However, this effect is very clear with tremetones (**Figure 2B**−**D**), where the inhibitory effects of **7**−**12** on seed germination of *P. ixocarpa* are shown. In general, dicot seeds (*L. sativa*, *P. ixocarpa*, and *T. pratense*) were more sensitive to these

Table 2. Effect of Acetophenones, Tremetones, and Their Derivatives on Seed Germination[a]

	GI_{50} values (μM)[b]			
	monocot plants		dicot plants	
compd	L. multiflorum	L. sativa	P. ixocarpa	T. pratense
1	14.4	45.3	15.0	28.5
2	*c*	*c*	*c*	*c*
3	*c*	*c*	*c*	*c*
4	18.1	51.2	23.5	125.9
5	75.3	41.0	*c*	233.2
6	127.5	47.0	*c*	137.3
7	37.5	45.0	31.8	35.5
8	65.6	36.1	19.2	22.3
9	31.5	25.9	24.7	27.9
10	25.5	21.0	16.6	17.8
11	79.9	85.5	55.2	40.0
12	10.4	11.5	8.7	7.1

[a] Means of three experiments. [b] Concentration that inhibits 50% of seed germination. [c] GI_{50} was nondetermined due to lack of response.

compounds than monocots and ~100% inhibition was observed. The low values of GI_{50} indicate that **1**, **8**, **10**, and **12** are the most powerful inhibitors with values of 15.0 and 28.5, 19.2 and 22.3, 16.6 and 17.8, and 8.7 and 7.1 μM for *P. ixocarpa* and *T. pratense*, respectively (**Table 2**). According to Hatfield and Karlen (*51*) and Mohr and Schopfer (*52*), preemergent selective inhibitors are applied after planting but before emergence of weeds or crops, as was done in this work.

Compounds **2** and **3** and acetates **5** and **11** (data not shown) have the lowest potency against monocot seed germination, and >500 μM was required for 100% inhibition and no activity against dicots, whereas the other chemicals were 10 times more active (<50 μM) as the tremetones assayed in this study. These results suggest that in the plant species the oxygenated moiety of these natural compounds allows, as in the mixture of acetophenones and tremetones (into the plant) and the pure compounds **1**, **2**, **7**, and **10**, to play an important role in the inhibition of these activities, which may be either due to their lipophilicity or to the fact that the hydrophilicity of the C-4, C-10 of tremetone (at physiological pH) makes it easy for these compounds to reach the target. On the other hand, the glucoside **2** showed a root-promoting activity in similar form to the findings in myrtle (*Myrtus communis*) (*24*), which is indicative of the regulating effect of glycoside substituents.

The inhibitory potential of these compounds against seed germination, root, and coleoptile development of *P. ixocarpa*, *T. pratense*, *L. multiflorum*, and *L. sativa* shows that the acetophenone partially inhibited seedling growth in monocot species and compound *O*-glycoside was regulatory. These facts indicate that *O*-glycosidation of the hydroxyl group increases the regulatory activity of acetophenones.

Monocot and Dicot Growth. Growth inhibition followed a dose-dependent pattern, which stimulated or inhibited germination. **Figure 2C**,**D** shows the inhibitory effects of the pure compounds **1**, **4**, **7**, **8**, **10**, and **12** on the root and coleoptyle development of *P. ixocarpa*, respectively. The compounds **9** and **11** do not show any effect.

The ID_{50} of the pure compounds and CH_2Cl_2 extract was obtained by determining the concentration that induced 50% of growth inhibition of development of roots and shoots (**Table 3**). Root development is affected to a larger extent as indicated by the lowest ID_{50} values, as compared with coleoptyle or hypocotyle development. The growth of the monocot was less sensitive to inhibition by compounds **2**, **3**, **5**, **6**, **8**, **9**, and **11** as shown by higher ID_{50} values (**Table 3**, **Figure 2C**,**D**).

Table 3. Effect of Acetophenones, Tremetones, and Their Derivatives on Seedling Growth during Seed Germination[a]

	I_{50} values (μM)[b]							
	monocot		dicots					
	L. multiflorum		*L. sativa*		*P. ixocarpa*		*T. pratense*	
compd	shoot	root	shoot	root	shoot	root	shoot	root
1	17.1	19.0	48.5	45.5	10.8	10.8	34.4	29.5
2	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
3	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
4	17.9	18.9	49.5	44.3	20.1	20.5	120.0	126.2
5	77.3	75.5	39.1	38.9	*c*	*c*	234.0	221.3
6	125.9	127.9	49.5	45.7	*c*	*c*	137.0	133.5
7	35.0	36.1	45.0	44.2	7.7	18.6	35.5	34.9
8	65.9	72.6	33.0	33.8	5.8	15.7	14.3	16.2
9	29.3	31.9	17.3	17.4	20.1	19.1	23.2	28.6
10	26.2	22.3	19.0	16.7	15.2	12.7	47.6	46.3
11	87.5	82.1	89.5	87.5	65.5	70.1	45.0	45.0
12	11.2	11.9	10.5	10.9	7.7	9.9	7.2	7.1

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

The acetate derivatives promote root and hypocotyl development for the monocots **2** and **3**, and acetates **5** and **11** 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 hypocotyl growth was slightly stimulated at lower concentrations up to 50 μM for *P. ixocarpa* and to 200 μM for *T. pratense* and, thereafter, partially inhibited by these compounds.

Similar to the effects shown on seed germination, the compounds **2**, **3**, **5**, **6**, and **11** have the lowest inhibitory effects on growth (30% inhibition or lower) at 500 μM for monocots (data not shown), while for compounds **7**, **8**, **10**, and **12** 100% inhibition was achieved above 100 μM for *P. ixocarpa* (**Figure 2C**,**D**) and above 175 μM for *T. pratense* and *L. sativa* (data not shown).

The results showed in this work regarding the differences in behavior of the acetate derivatives and the pure monohydroxys **1** and **10**, methoxy **4**, epoxys **8** and **12**, and dihydro **9** derivatives indicate that the mechanisms of action of the phytotoxins may be different for growth and germination (*53*) and confirm the findings obtained by Marles's group (*54*); the mechanism of action established by those authors claims that compounds that involve two functional groups (O=C−C=CH_2), which is most commonly part of the α-methylene-γ-lactone, are necessary for activity but may be present in a β-position, e.g., compounds such as a β-unsubstituted cyclopentenone with a hydroxyl group in a very near carbon or other ester or ketone, reacting by a Michel type addition to a biological nucleophile, particularly the sulfhydryl group of reduced glutathione and L-cysteine, or undergoing a Wagner−Meerwein rearrangement; these features may occurs with our compounds.

On the other hand, higher phytotoxic effects are observed from cyclic alcohols to cyclic ketones (<0.1 mM) (*55*). The greater toxicity of terpene ketones with respect to alcohols has been demonstrated in several cell systems (*55*, *56*). Compounds capable of Michel addition but lacking a hydroxyl group or with hydroxyl groups in other positions are inactive. Thus, these compounds inhibit cellular enzyme activities and metabolism; this may relate to hydrogen-bonding requirements for appropriate fits into a receptor molecule, as in our molecules where a

Table 4. Mean Concentrations of Compounds **1**, **4**, **8**, **10**, **12**, MeOH, and CH_2Cl_2 Extracts Expressed as Concentrations that Inhibit 50% of O_2 Uptake as a Function of Control Seed Respiration[a]

	I_{50} values (μM)[b]			
	monocot	dicots		
compd	*L. multiflorum*	*L. sativa*	*P. ixocarpa*	*T. pratense*
1	41.0	43.0	52.9	53.0
2	*c*	*c*	*c*	*c*
3	*c*	*c*	*c*	*c*
4	40.0	55.9	54.6	57.6
5	*c*	*c*	*c*	*c*
6	*c*	*c*	*c*	*c*
7	*c*	*c*	*c*	*c*
8	67.9	58.0	57.5	49.1
9	*c*	*c*	*c*	*c*
10	39.0	27.0	28.7	22.0
11	*c*	*c*	*c*	*c*
12	18.0	11.0	15.8	10.1
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 to the 24 h. [d] Values in parts per million.

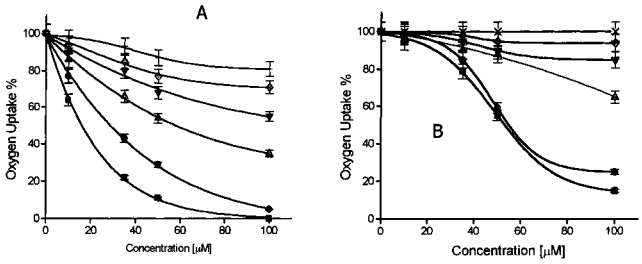

Figure 3. Oxygen evolution of tremetones **7** (♦), **8** (▲), **9** (+), **10** (●), **11**(▼), and **12** (♦) (**A**) and **1** (■), **2** (×), **3** (▼), **4** (●), **5** (▲), and **6** (♦) (**B**) on seed respiration of *P. ixocarpa* (dicot). Means of three experiments. Values correspond to the concentration that inhibits seed respiration during germination. Values at 72 h.

carbonyl group is very near to a hydroxyl group, throughout a bridge of two carbon atoms.

2,4-D shows a pronounced effect on root and shoot length and on the germination of seeds; 100% of inhibition was obtained at 70 μM with ID_{50} of 0.4 and 0.5 μM for root and shoot, respectively (data not shown). Anaya (*57*) has also reported a similar type of finding.

Seed Respiration during Seed Germination. The respiratory rate of all seeds decreases with concentration of the phytochemicals **1**, **4**, **10**, **12**, MeOH and CH_2Cl_2 extracts in a concentration-dependent manner (**Table 4**). The only exception is for the compounds **2**, **5**, and **11** on *L. multiflorum* seed respiration where enhancement at 30 μM is observed, as the time of inhibition increases (data not show). However, at higher concentrations, 300−500 μM, respiration was also inhibited in a similar form as shown by compounds **1**, **4**, **8**, **10**, and **12** at lowest concentrations (**Figure 3A**,**B**). These results suggest that **1**, **4**, **8**, **10**, and **12** may act as uncouplers to phosphorylation at a lower concentration, but at higher concentrations, it inhibits either the energy transduction or the respiration redox enzymes. **Table 4** shows the RI_{50} values (the concentration of phytochemicals that induce 50% seed respiration inhibition) for **1**, **4**, **8**, **10**, **12**, and CH_2Cl_2 extract tested. According to their RI_{50} values, dicot seeds (*P. ixocarpa* and *T. pratense*) are more sensitive to inhibition. On the other hand, *L. multiflorum* seeds showed the highest resistance to respiration inhibition.

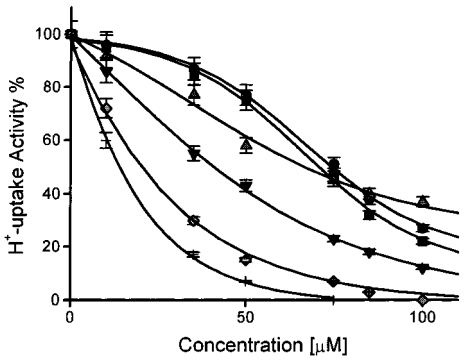

Figure 4. Oxygen uptake of mitochondrial respiration against the presence of **1** (●), **4** (▲), **8** (▼), **10** (♦), and **12** (+) at 50 μM; control (■) (conditions in Materials and Methods).

Table 5. Respiratory I_{50} in Purified Mitochondria Preparations[a]

compds	I_{50} (μM)[d]	compds	I_{50} (μM)[d]
1	121.2	**10**	45.0
4	151.8	**12**	17.1
8	109.1	CH_2Cl_2 extract[c]	36.5

[a] Mean concentrations of compounds **1**, **2**, **8**, **10**, **12**, and CH_2Cl_2 able to inhibit mitochondria respiration by 50% of O_2 uptake as a function of control seed respiration. [b] Means of three experiments. [c] Values in parts per million. [d] The control was mitochondria in buffer suspension with MeOH 0.5%.

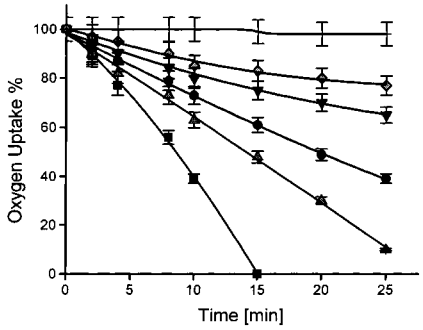

Figure 5. Effects of **1** (●), **4** (▲), **7** (■), **8** (▼), **10** (♦), and **12** (+) on H^+ uptake activity in isolated chloroplasts.

Mitochondrial Respiration. The effect of acetophenones and tremetones compounds on mitochondrial respiration is shown in **Figure 4**. The I_{50} for **1**, **4**, **8**, **10**, and **12** was 121.2, 151.8, 109.1, 45.0, and 17.1 μM, respectively (**Table 5**). These data corroborate the results obtained with seed respiration of *P. ixocarpa*, *T. pratense*, and *L. sativa*. We still registered an increase in oxygen uptake for concentrations of compounds **2** and **5** as high as 70 and 90 μM, respectively, and for compound **11** applications up to 50 μM. As in the seed respiration, compound **12** (I_{50} = 17.1 μM) was found to be the lowest. The cleaning of mitochondria with buffer suspensions, with the recovery of full respiratory activity, annulled the effect of compounds. Our results showed that tremetones **10** and **12** are powerful inhibitors of mitochondria respiration, with similar concentrations to those reported by Moreland and Novitzky (*58*). The site of action is being studied.

Effect of Acetophenone and Tremetones on ATP Formation, Proton Uptake, and Electron Transport Rate. To assess the effects of acetophenones and tremetones on photosynthesis, their effects on different photosynthetic reactions were tested. These compounds only partially inhibited ATP synthesis (data not shown) and H^+ uptake (**Figure 5**) in a concentration-dependent manner. The assay concentration ranged from 1 to

Table 6. I_{50} of Compounds **1**, **4**, **7**, **8**, **10**, and **12** on H^+ Uptake Activity in Isolated Chloroplasts[a]

compds	I_{50}	compds	I_{50}	compds	I_{50}
1	74.5	**6**	nt	**10**	20.4
2	nt[b]	**7**	70.7	**11**	nt
3	nt	**8**	40.7	**12**	13.8
4	65.7	**9**	nt	CH_2Cl_2 extract	15.1
5	nt				

[a] The procedures are under the Materials and Methods section. [b] Not tested.

Table 7. Effect of Natural Compounds and Their Derivatives and MeOH and CH_2Cl_2 Extracts on DPPH Reduction[a] and I_{50} Values (μM)[b]

compd	I_{50}	compd	I_{50}
THQ	9.37	**7**	nd
BHA	26.4	**8**	62.6
1	nd[c]	**9**	nt
2	nt[d]	**10**	53.2
3	nt	**11**	nt
4	nd	**12**	30.6
5	nt	MeOH extract[e]	39.3
6	nt	CH_2Cl_2 extract	9.5

[a] Means of three experiments. [b] Concentration that produced 50% of scavenging radicals from DPPH. [c] Not determined. [d] Not tested. [e] Values in parts per million.

500 μM in 25 μM increments. The only activity inhibited significantly was H^+ uptake. **Figure 5** shows that compound **12** inhibits proton uptake completely to 75 μM and compound **10** up to 100 μM, while the compounds **1**, **4**, **7**, and **8** inhibit partially at 100 μM (**Table 6**). The compounds **8**, **10**, and **12** show this effect at concentrations between 10 and 50 μM, while the compounds **1**, **4**, and **7** show similar effects between 100 and 500 μM. These results suggest that the presence of an oxygenated function at C-4 and C-10 positions (hydroxyl, methoxy, and epoxy substituent) significantly enhances the inhibitory potency of these compounds as energetic inhibitors. On the other hand, the *O*-glycoside and acetate groups at the OH− position are an important structural requirement for the observed regulatory effect, and this fact suggests that a glycoside group at this position significantly reduced their inhibitory potency on photophosphorylation.

Radical Scavenging Properties Antioxidant Activity. Radical scavenging properties of the compounds **1**, **7**, **8**, **10**, and **12** were evaluated against the DPPH radical, using DPPH as a TLC spray reagent (**Table 7**). These compounds (10 μM) appeared as yellow spots against a purple background, while the same amount of **2**−**6**, **9**, and **11** did not react with the radical. Compounds **1**, **7**, **8**, **10**, and **12** were also tested against DPPH in a spectrophotometric assay; this method confirms the observation on TLC that the compounds **8**, **10**, and **12** exhibited the strongest radical scavenging activity in this assay. Compounds **2**−**6**, **9**, and **11** were less sensitive than **1**, **7**, **8**, **10**, and **12**. It is worth mentioning that in addition to quercetin, flavonols with five hydroxyl groups, THQ and BHA, were used as reference compounds. Those compounds possess strong antioxidant properties and are useful as antioxidants in foods, as well (**Figure 6**).

The antioxidant activity of these compounds was also evaluated spectrophotometrically on the bleaching of the H_2O soluble crocin (*49*). 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 **1**, **7**, **8**, **10**, and **12** were

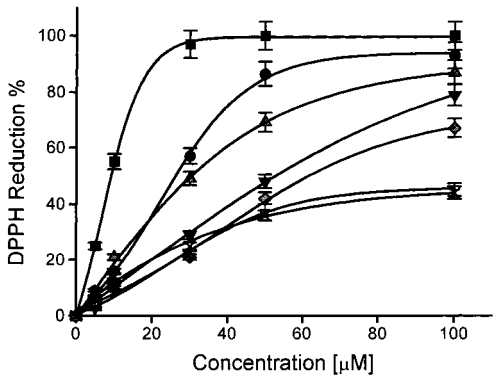

Figure 6. Scavenging activity of compounds THQ (■), BHA (●), **12** (▲), **10** (▼), **8** (♦), **7** (+), and **1** (×) on radical reduction of DPPH. Measurements were taken at 517 nm and determined after 30 min.

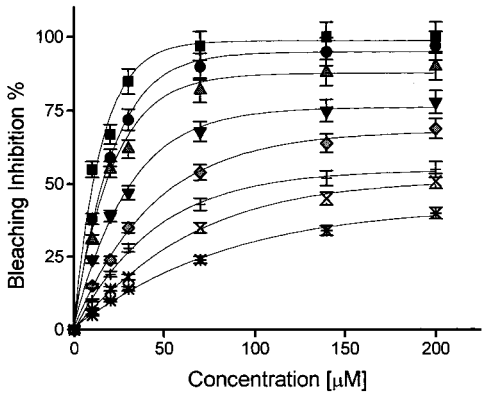

Figure 7. Inhibitory activity of compounds **3**–**6**, MeOH, and CH_2Cl_2 extracts on the bleaching of crocin measurement at 440 nm of fluorimetric emission, determination after 20 min. THQ (■), CH_2Cl_2 extract (●), **12** (▲), gallic acid (▼), **10** (♦), **8** (+), **7** (×), and 1 (×). Values of CH_2Cl_2 extract in parts per million.

all active, with an activity comparable to gallic acid (**Figure 7**).

CONCLUSION

We must emphasize that compounds **1**, **2**, **6**, **7**, **10**, and **12** could not be detected in the acetate extracts of *B. linnearis*, *B. magellanica*, and *B. umbelliformis*. In contrast, lipophilic compounds **6** and **7** are abundant in the CH_2Cl_2 extract and in minor amounts in the MeOH extract. More important, these compounds seem to be accumulated in the cells and are exuded slowly. So, it may be logical to assume that acetophenones and tremetones **1**, **2**, **6**, **7**, and **10** were synthesized for defensive processes. It appears therefore that the releasing mechanism of tremetones and their derivatives may be one of the key processes to understanding the plant defense.

The tremetones examined in this study have similar molecular structures, the sole difference resting in the presence of the hydroxyl, acetyl, and epoxy substituent (**Figure 1**). Of the six tremetones tested for inhibited growth of four plant species, only **10** and **12** showed the greatest effect on the dicot weed (*P. ixocarpa* and *T. pratense*). Compounds **10** and **12** had similar inhibitory effects on the root growth of *P. ixocarpa*, whereas **12** had a greater effect on roots than shown by **10** (**Figure 2B**). At amounts greater than 30 μM of **10** and **12**, the roots were severely malformed with a corkscrew like appearance and significantly smaller ($P < 0.05$ ANOVA) than controls.

Inhibitory effects of some tremetones were investigated by Mata's group (*35*). It was reported that these natural products

show enhancement and inhibition effects on different preemergence properties (i.e., germination and root length) at low and higher concentrations, respectively. Similar findings were observed by Fischer and co-workers (*59*−*61*). However, the effects of other tremetones and acetophenones on seed germination and root length were unclear. The presence of **1** and **10** are unique among the natural products reported till now in *Baccharis* species (*37*), with the presence of the hydroxyl group at the C-4 position a carbonyl at C-13 and methylene group in the furan ring.

The effects of these compounds have certain similarities with costunolide and ovatifolin and their derivatives (*36*, *31*) and may show as plant cells respond to plant and pathogen signals that they accumulate defense molecules. Among these molecules, the phenolic compounds (tremetones and acetophenones, for instance) are probably the most abundant and best-characterized plant metabolites (*20*−*23*). The enrichment of these molecules in cell plant organelles has been shown in a large number of plants (*21*, *62*, *63*). In addition, there are some data on interesting plant growth inhibitory activities: plasma membrane leakage and mitosis disruption produce by sesquiterpene lactones, i.e., artemisinin, zaluzanin C, and dehydrozaluzanin C (*36*, *64*−*67*). Thus, on this point of view, the effects of our molecules are of much help to explain which may be the end of production and the function of secondary metabolites in the plant cell.

On the other hand, it was possible to correlate the antioxidant activity with the seedling growth inhibitory activity. This evaluation is important in view of the growing interest in the search of new sources of nutraceuticals and active oxygen species, whose amounts are also increased upon infection or elicitor treatment in plant cells (*63*, *68*).

In conclusion, our data indicate that the compounds **10** and **12** are more selective and potent toward dicots than 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. When the compounds are acetylated, the potency of inhibition decreased 10 times (data not shown). Thus, it may be suggested that a natural derivative is the active one in vivo.

The treatment concentrations for the compounds that reduced seedling growth were low (10−50 ppm) as compared to allelopathic chemicals that have been previously studied under laboratory conditions (*20*, *54*). The reported secondary metabolites potency is in the range from 100 to 1000 μM for growth reduction by many phenolic acids, around 10 μM for sorgoleone, or at the micromolar level for juglone, which suppresses the growth of several herbaceous species (*69*, *70*).

Whatever the mechanism(s) of action of these compounds and their derivatives, they have proved to be good inhibitors of plant growth. They show preemergent phytotoxic properties by inhibiting germination, respiration (seeds and mitochondria), 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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