Khellin and Visnagin, Furanochromones from *Ammi visnaga* (L.) Lam., as Potential Bioherbicides

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Supporting Information

ABSTRACT: Plants constitute a source of novel phytotoxic compounds to be explored in searching for effective and environmentally safe herbicides. From a previous screening of plant extracts for their phytotoxicity, a dichloromethane extract of *Ammi visnaga* (L.) Lam. was selected for further study. Phytotoxicity-guided fractionation of this extract yielded two furanochromones, khellin and visnagin, for which herbicidal activity had not been described before. Khellin and visnagin were phytotoxic to model species lettuce (*Lactuca sativa*) and duckweed (*Lemna paucicostata*), with IC₅₀ values ranging from 110 to 175 μM. These compounds also inhibited the growth and germination of a diverse group of weeds at 0.5 and 1 mM. These weeds included five grasses [ryegrass (*Lolium multiflorum*), barnyardgrass (*Echinochloa crus-galli*), crabgrass (*Digitaria sanguinalis*), foxtail (*Setaria italica*), and millet (*Panicum* sp.)] and two broadleaf species [morningglory (*Ipomea* sp.) and velvetleaf (*Abutilon theophrasti*)]. During greenhouse studies visnagin was the most active and showed significant contact postemergence herbicidal activity on velvetleaf and crabgrass at 2 kg active ingredient (ai) ha⁻¹. Moreover, its effect at 4 kg ai ha⁻¹ was comparable to the herbicide pelargonic acid at the same rate. The mode of action of khellin and visnagin was not a light-dependent process. Both compounds caused membrane destabilization, photosynthetic efficiency reduction, inhibition of cell division, and cell death. These results support the potential of visnagin and, possibly, khellin as bioherbicides or lead molecules for the development of new herbicides.

KEYWORDS: *Ammi visnaga*, bioherbicide, furanochromone, herbicide, khellin, phytotoxic, visnagin

INTRODUCTION

Billions of tons of agricultural production are lost annually due to weeds. Herbicides are the most important method of weed management, as this technology has been much more effective than previous weed management approaches. After more than 70 years of the dominance of synthetic herbicides for weed control, evolved resistance to herbicides has become a major problem.¹⁻³ This problem is exacerbated by the fact that there have been no herbicides with new modes of action introduced in more than 30 years to help manage evolved herbicide resistance.⁴ Natural phytotoxins are a source of compounds with new modes of action, which has fueled interest in their development.⁵⁻⁷ Furthermore, the biggest pest management need of organic farmers is economical and efficient natural herbicides approved for use in the organic marketplace.⁸⁻¹⁰

Plant natural products provide an attractive alternative in finding effective and environmentally safe phytotoxic compounds, with high structural diversity and novel modes of action.¹¹ Such compounds may be formulated and directly used as bioherbicides or used as lead structures for the development of new products by chemical modifications.

Our group has developed a systematic process to search for, evaluate, and select plant extracts with promising phytotoxic activity. Active extracts could then be used to discover new herbicidal molecules. As a result of this screening process of nearly 2400 plant extracts for their herbicidal activity, an extract from *Ammi visnaga* (L.) Lam. was selected for further studies.

*A. visnaga*, also known as toothpick weed, visnaga, or khella, is an annual or biennial herb belonging to the Apiaceae (Umbelliferae) family, growing to about 1 m height. The stem is erect and highly branched, and leaves are dissected into many small linear to lance-shaped segments up to 20 cm long. The inflorescence of *A. visnaga* is a compound umbel of white flowers, and fruits are compressed oval-shaped structures around 3 mm in length.¹⁰⁻¹¹ This herb is native to the Mediterranean region of Europe, Asia, and North Africa, and it can be found as an introduced species in Argentina, Brazil,
Chile, Uruguay, North America, Southwest Asia, and some Atlantic islands. It grows preferentially under high sun exposure in clay soils, which are well drained and quickly descimated on the surface, in the semiarid superior and subhumid bioclimatic zones. In some regions, this plant has become an invasive weed of cultivated fields.

Fruits of A. visnaga have been described in pharmacopoeias as an antispasmodic, muscle relaxant, and vasodilator. Other uses in traditional medicine include treatment of mild angina symptoms, supportive treatment of mild obstruction of the respiratory tract in asthma or spastic bronchitis, and post-operative treatment of conditions associated with the presence of urinary stones. This herb has also been used as treatment for gastrointestinal cramps, as a diuretic, and for treatment of vitiligo, diabetes, and kidney stones.

Aqueous and ethanolic extracts as well as the essential oil of A. visnaga have antibacterial activity. Also, different extracts of this species and their constituents have antioxidant activity and prevented renal crystal deposition and cell damage caused by oxalate.

With regard to its pesticide uses, alcoholic and aqueous extracts and essential oil of A. visnaga have insecticidal properties on different insect species. Previous work on the allelopathic potential of A. visnaga crude extracts reported some phytotoxicity toward legumes and maize and toward weeds associated with wheat cultivation. However, the compounds responsible for these activities of the crude extracts were not isolated and identified.

The objectives of the present work were to isolate and identify the phytotoxic compounds from a crude extract of A. visnaga and to evaluate their herbicidal effects on different model and weed species in laboratory and greenhouse tests. The possible modes of action of the isolated compounds were also explored.

MATERIALS AND METHODS

**Chemicals.** Acetochlor [2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide] (90%) and glyphosate [N-(phosphonomethyl)glycine] (48%) were provided by Dr. Ricardo Pavon from BASF, San Jeronimo, Argentina. 4,5-Dimethoxy-7-methyl-5H-furo[3,2-g]chromene-3-one (MDL MFCD00410875), 4-methoxy-7-methyl-5H-furo[3,2-g]chromene-3-one (visnagin), 4,9-dimethoxy-5-oxo-5H-chromen-3-one (MDL MFCD18432237), and 4,9-dimethoxy-7-methyl-5H-furo[3,2-g]chromene-3-one (MDL MFCD18432237), were purchased from Sigma-Aldrich (St. Louis, MO, USA). The nonionic surfactant AGRI-DEX (Helena Chemical Co., Collierville, TN, USA), consisting of heavy range paraffinic oil, polyol fatty acid esters, and polyethoxylated derivatives (99%), was purchased from Oxford Farm and Ranch (Oxford, MS, USA). Solvents were of HPLC grade, and all other chemical supplies were purchased from Sigma-Aldrich.

**Plant Material and Extraction Procedures.** A. visnaga (L.) Lam. foliage (Figure S1) was collected near Los Telares, Salavina Department, near Los Telares, Salavina Department, polyol fatty acid esters, and polyethoxylated derivatives (99%), was collected near Los Telares, Salavina Department, polyol fatty acid esters, and polyethoxylated derivatives (99%), was collected near Los Telares, Salavina Department.

(Figure S1) was collected near Los Telares, Salavina Department, Argentina. Ground dried leaves (27 g) of A. visnaga were consecutively extracted by maceration at 25 °C with two solvents: dichloromethane (DCM) first and then ethanol (EtOH). Plant material was extracted twice (24 h each) with each solvent, followed by Buchner funnel filtration and concentration under reduced pressure in a rotary evaporator. For each extraction step, 10 mL of solvent was added per gram of plant material. This procedure provided the following extracts: flowervDCM (1.74 g), flowervEtOH (3.57 g), and leavesvDCM (1.2 g), and leavesvEtOH (2.5 g).

**Germination and Plant Growth Bioassays with Lettuce (Lactuca sativa), Creeping Bentgrass (Agrostis stolonifera), and Ryegrass (Lolium multiflorum).** Extracts of A. visnaga and column chromatography fractions were evaluated for their phytotoxic activity on lettuce and creeping bentgrass bioassay as described by Dayan et al. Briefly, a filter paper (Whatman no. 1) and five lettuce seeds (L. sativa L. cv. iceberg A from Burpee Seeds) or 10 mg of creeping bentgrass (A. stolonifera var. Penncross from Turf-Seed Inc.) seeds were placed in each well of a 24-well plate. Stock solutions (10 mg mL⁻¹) of test extracts or fractions were prepared in acetone. Distilled water (180 mL) was added to each well together with 20 μL of the stock solution or acetone in solvent control. The final concentration per well was 1 mg mL⁻¹ for extracts or fractions and 10% v/v for acetone. This percentage of acetone was used only for these miniaturized assays, and two controls were done, with and without solvent, to verify that germination and growth of seedlings were comparable between control in solvent and control in water. Plates were sealed with Parafilm and incubated at 26 °C in a Conviron (Winnipeg, Canada) growth chamber set at 173 μmol photons m⁻² s⁻¹ continuous photosynthetically active radiation. Phytotoxic activity was qualitatively evaluated by visually comparing germination and growth in each well with solvent control at 7 days. A qualitative estimation of phytotoxicity was obtained by using a visual rating scale from 0 to 5, where 0 was no effect (control), 1 was ≤50% seedlings shorter than control, 2 was >50% seedlings shorter than control, 3 was ≤50% seeds germinated (only radicles observed), 4 was ≤50% seeds germinated (only radicle tips observed), and 5 was no germination of seeds. This procedure was also used for testing the phytotoxicity of pure compounds and for the dose–response assay on lettuce (L. sativa var. Waldmann’s green, Giacchi SRL Argentina) and ryegrass (L. multiflorum provided by Dr. Ricardo Pavon, BASF Argentina), instead of creeping bentgrass because ryegrass is a weed species with a higher agronomical impact. The experiment with this grass was carried out in 12-well plates. Each well contained 10 seeds and 300 μL of test solution. Eight concentrations of the pure compounds (from 0 to 1 mM) were tested. Germination percentage and plant growth (length of plants) were measured at 7 days to determine the concentration required for 50% germination and growth inhibition (IC₅₀) with respect to control. All experiments were performed in triplicate.

**Bioassay-Guided Fractionation.** The flowervDCM extract of A. visnaga (1.6 g) was divided into two 800 mg portions, and each was subjected to column chromatography using an Isola One system (Biotage, Uppsala, Sweden), equipped with a UV detector (302 and 365 nm) and an automatic fraction collector. Separation was performed by a normal-phase chromatography column using a SNAP Cartridge KP-Sil (37 × 157 mm, 50 μm irregular silica, 100 g, Biotage) and a prepackaged SNAP Sampllet Cartridge KP-Sil (37 × 17 mm, Biotage). A full gradient of hexane/ethyl acetate was used for elution, from 100:0 to 1:100 over 3000 mL. The flow rate was 40 mL min⁻¹, and 25 mL fractions were collected. Equal fractions were recombined on the basis of similarities in their TLC and chromatogram profiles, providing 19 fractions named 1–19. All fractions were evaluated for their phytotoxicity on the germination bioassay of lettuce and creeping bentgrass.

**Chemical Analysis and Compound Identification.** The crude flowervDCM extract of A. visnaga and fractions XIV and XVII were analyzed by GC-MS on an Agilent Technologies 7890A GC system coupled to a 5975C Inert XL MS. The GC was equipped with a DB-5 fused silica capillary column (30 μm × 0.25 mm, film thickness of 0.25 μm) operated under the following conditions: injector temperature, 240 °C; column temperature, 60–240 °C at 3 °C/min then held at 240 °C for 5 min; carrier gas, He; injection volume, 1 μL (splitless). MS mass range was from m/z 40 to 650; filament delay, 3 min; target TIC, 20,000; prescan ionization, 100 μs; ion trap temperature, 150 °C; manifold temperature, 60 °C; and transfer line temperature, 170 °C. 1H and 13C NMR spectra were recorded in CDCl₃ on a Bruker 500 MHz spectrometer (Billerica, MA, USA). High-resolution mass (ESI-
MS) spectra of isolated compounds in MeOH were acquired by direct injection of 20 μL of sample (approximately 0.1 mg mL\(^{-1}\)) in a JEOL USA, Inc. (Peabody, MA, USA) AccuTOF (JMS-T100LC).

**Khellin (fraction XIV)**: high-resolution ESI-MS \(m/z\) 521.1448 [\(2M + H\)]\(^+\) calculated for \(C_{28}H_{32}O_{10}\) 521.14477, mass difference (mmu) \(-0.19\); \(m/z\) 543.12622 [\(2M + Na^+\)] calculated for \(C_{28}H_{32}Na_{2}O_{10}\) 543.12672, mass difference (mmu) \(-0.5\); \(^{13}H\) NMR (in CDCl\(_3\)) \(\delta\) 7.55, 6.92, 5.96, 4.09, 3.96, 2.20, in agreement with published values.\(^{35}\)\(^{13}\)C NMR (in CDCl\(_3\)) \(\delta\) 178.02, 163.84, 148.6, 147.09, 146.86, 145.35, 129.66, 119.15, 113.44, 110.38, 104.95, 62.09, 61.27, 19.8, in agreement with published values.\(^{36,37}\)

**Visnagin (fraction XVII)**: high-resolution ESI-MS \(m/z\) 231.06653 [\(2M + H\)]\(^+\) calculated for \(C_{14}H_{12}O_{5}\) 231.06653, mass difference (mmu) 0.82; \(m/z\) 461.12647 [\(2M + H\)]\(^+\) calculated for \(C_{28}H_{32}O_{10}\) 461.12634, mass difference (mmu) 0.23; \(m/z\) 483.11048 [\(2M + Na^+\)] calculated for \(C_{28}H_{32}Na_{2}O_{10}\) 483.10559, mass difference (mmu) 4.89; \(^{13}H\) NMR (in CDCl\(_3\)) \(\delta\) 7.09, 7.09, 6.92, 5.95, 4.07, 2.21, in agreement with published values.\(^{38,39}\)\(^{13}\)C NMR (in CDCl\(_3\)) \(\delta\) 177.98, 163.68, 157.51, 155.67, 153.25, 144.94, 116.67, 112.09, 110.53, 105.07, 94.77, 61.49, 19.72, in agreement with published values.\(^{38,39}\)

**Duckweed (Lemna paucicostata) (L.) Hegelm.** Bioassay. Phytotoxic activities of khellin and visnagin were evaluated on duckweed by using a previously described method.\(^{40}\) Briefly, duckweed stocks were grown from a single colony consisting of a mother and two daughter fronds in a beaker on modified Hoagland medium. The medium was adjusted to pH 5.5 with 1 M NaOH and filtered through a 0.2 μm filter (no. 431118, Corning Inc.) Each well of nonpyrogenic polystyrene sterile 6-well plates (CoStar 3506, Corning Inc.) was filled with 4950 μL of Hoagland medium and 50 μL of double-distilled water (ddH\(_2\)O) or 50 μL of acetone as solvent control. The assay described by Sampietro et al.\(^{41}\) was performed in triplicates and the plant growth (length of plants) was measured for each condition at 6 days (4 days of treatment). The assay was repeated with Aphelantus hypochondriacus (A. hypochondriacus) as a positive control or water with acetone (1% v/v) as solvent control. For one of the experiments, seedlings of velvetleaf were 6 days old and those of crabgrass 8 days old at treatment. Khellin and visnagin were tested at 2 μg ai ha\(^{-1}\). Teevet 20 (1% v/v) was added to the solution as a surfactant, and the control consisted of water with acetone and surfactant. For another experiment was conducted with plants at the two to three true-leaf stage. Velvetleaf plants was sprayed on 13-day-old velvetleaf and 16-day-old crabgrass and barnyardgrass at 4 kg ai ha\(^{-1}\). The postemergence activity of visnagin was compared with the bioherbicide pelargonic acid at the same rate. AGRI-DEX (1% v/v) was used as a surfactant, and the control consisted of water with acetone and surfactant.
Electrolyte Leakage Assay. The effects of pure furanochromones (Sigma-Aldrich) on membrane stability were studied as described by Dayan and Watson. Cucumber cotyledon disks were exposed to each furanochromone at 100 and 300 μM. Control tissues were exposed to the same solvent as treated tissues but without the compounds. Conductivity measurements were carried out at the beginning of the dark incubation period, a second measurement was made after 16 h, at which time the samples were placed under high light intensity, and final measurements were made after 8 and 24 h of light exposure. Each experiment consisted of three replicates. Maximum conductivity was measured by boiling three samples of each treatment for 20 min. To study if electrolyte leakage caused by khellin and visnagin was light-dependent, two sets of Petri plates were prepared with cucumber cotyledon disks exposed to test compounds and control in both. One set was treated as described before (dark 16 h/light 26 h), whereas the other one was kept in darkness for 42 h. Conductivity measurements were done at the indicated times.

Effect of Compounds on Photosynthetic Efficiency. The effect of pure khellin and visnagin was evaluated by chlorophyll fluorescence measurements according to the procedure of Dayan and Zaccaro. Cucumber cotyledon disks were exposed to different dilutions of each furanochromone (10, 30, 100, and 300 μM). Control tissues were exposed to the same solvent as treated tissues but without the test compounds. The cotyledon disks were incubated in darkness for 18 h before exposure to light for 24 h. Photosynthetic quantum yield and electron transport rate (ETR) were measured. ETR values were expressed as percent of the ETR average values observed in control treatments. A time course experiment was performed by measuring induced fluorescence of cotyledon disks after treatment at 3 h in darkness (start) and after 18 h in darkness, after which the samples were placed in the light, and further measurements were made after 6 and 24 h of light exposure. Three replicates were performed for each experiment.

Detection and Measurement of Reactive Oxygen Species (ROS). ROS cellular localization was determined by confocal microscopy using the fluorescent probe 2′,7′-dichloroﬂuorescein diacetate (DCFDA). Cucumber cotyledon disks (1 cm) were treated as described for the electrolyte leakage assay. Five disks were placed in 5 cm Petri plates and exposed to different dilutions of khellin and visnagin (0, 100, and 300 μM). Disks were incubated in darkness for 16 h before exposure to high light intensity for 5 h or in darkness for 30 h. As positive controls, disks were exposed to the same solvent as treated tissues but with 10 mM hydrogen peroxide for 30 min in the light. After each treatment, they were vacuum-infiltrated in the dark with 50 μM DCFDA in 10 mM Tris-HCl pH 7.5, and ROS were visualized in an Eclipse TE-2000-E2 Nikon confocal microscope with excitation at 488 nm and emission at 515/530 nm. Green fluorescence intensities were quantified using the image processing package Fiji of ImageJ software.

Effect on Cell Division. Onion seed (Allium cepa L. Evergreen Longwhite Bunching, Burpee & Co., 2012, EE.UU) germination was carried out for 7 days with a 14 h photoperiod in 9 cm diameter Petri dishes on a filter paper disk that was moistened with a dilution (2.5 mL) of test compound or control. Stock solutions of test compounds (100x) were prepared in acetone, and aliquots were diluted in ddH2O to get the final concentration. The control consisted of water with the same proportion of acetone (1% v/v) applied in the treatments.

At 7 days of incubation, samples were processed for mitotic index analysis according to the method of Armbruster et al. Twenty root tips (1 cm sections) were fixed in glacial acetic acid/absolute ethanol (1:3 v/v) for 30 min. The segments were incubated with 5 N HCl at 25 °C for 1 h and washed several times with distilled water. After that, segments were stained with Schiff’s reagent for 45 min in the dark at 25 °C. Stained meristematic regions were identified as purple tips. The root segments were transferred to tweezers to a drop of 45% acetic acid in water on a microscope slide. The tips were cut with a razor blade, and a coverslip was carefully placed over the tips and gently squashed by applying slight and constant pressure directly over the tissues. The edge of the coverslip was sealed with nail polish to delay the evaporation of acetic acid. The mitotic index was calculated by tallying the cells in various stages of mitosis. At least 1000 cells slide−1 and in triplicate (3000 cells per treatment) were counted for suitable statistical analysis of data. An Olympus BX60 microscope (Olympus, Center Valley, PA, USA) was used, and cells with abnormal mitotic configurations were counted as a separate class. This procedure was slightly modified to evaluate if cell division inhibition caused by furanochromones was reversible. For this experiment onion seeds were incubated with solutions containing khellin or visnagin for 3 days. After this period, all seeds were washed three times with distilled water and placed in new Petri dishes on moistened filter paper disks with ddH2O (wash + treatment). Seeds were then incubated for an additional 4 days, after which a mitotic index analysis was performed. As a reference to compare results, another set of onion seeds was kept with test compounds or control for 7 days (wash – treatment) before analysis.

Cell Death Determination. To evaluate cell death in roots, onion seeds were germinated and treated as described for the postemergence assay with weed species. Onion seedlings were exposed to different doses of each furanochromone (0, 100, and 300 μM) during 4 days. For the experiment with leaf disks 3-week cucumber plants were used. One disk (1 cm) was placed in each well of a 12-well plate together with 1 mL of a 2% w/v sucrose/1 mM MES, pH 6.5, solution containing each of the compounds tested at the appropriate concentration (0, 100, or 300 μM) and acetone (1% v/v). Each assay was performed with leaf disks from different plants. Plates were sealed with Parafilm and incubated in a growth chamber at 21–27 °C with a 16/8 h light/dark cycle for 7 days.

Cell death was determined by Evans blue staining. Root tips (5 mm sections) and leaf disks were incubated for 30 min in 0.25% w/v Evans blue aqueous solution at 25 °C on a rotary shaker. After staining, unbound dye was removed by extensive washing with deionized water. Three root tips (three replicates) or one leaf disk (four replicates) were ground in a tissue grind tube with 500 μL of 1% w/v sodium dodecyl sulfate (SDS). The resulting suspension was centrifuged for 20 min at 20000 g, and the supernatant was used for dye quantification by monitoring the absorbance at 600 and 680 nm. Relative cell death was expressed as A600 for root tips and A600/A500 ratios for leaf disks.

Statistical Analysis. Data from dose-response experiments were analyzed by a log–logistic model using the dose–response curve module of R software version 2.2.1. Concentrations required for 50% germination or growth inhibition relative to control (IC50 values) were obtained from estimated parameters in the regression curves. The standard error of each estimation is provided. Data from phytotoxicity bioassays in the laboratory and greenhouse, ROS, and cell death quantifications were analyzed by ANOVA using InfoStat statistical software version 2015, and Scheffe’s test was employed to compare the means at α = 0.05.

Table 1. Phytotoxic Activity of Extracts Prepared with Flowers and Leaves of A. visnaga Using Two Different Solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>lettuce</th>
<th>creeping bentgrass</th>
</tr>
</thead>
<tbody>
<tr>
<td>flowersDCM 3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>leavesDCM 2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>flowersEtOH 2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>leavesEtOH 1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*Bioassay rating based on scale of 0–5: 0 = no effect and 5 = no growth or germination.
extract of flowers (flowers\textsubscript{DCM}) exhibited the highest inhibitory effect on seed germination and growth. Therefore, its bioassay-guided fractionation was carried out to isolate the phytotoxic compounds.

The flowers\textsubscript{DCM} crude extract was subjected to normal-phase flash column chromatography, providing 19 fractions, I–XIX. All fractions were evaluated for their phytotoxicity on lettuce and creeping bentgrass at 1 mg mL\textsuperscript{−1}. Four fractions, XIV–XVII, were the most active, causing complete germination inhibition of both species at 7 days (Table 2). The \textsuperscript{1}H NMR spectra of these four active fractions revealed that fractions XIV and XVII were identical to those of \textit{A. visnaga} \textit{furanochromones} isolated from \textit{A. visnaga} (Figure 1). Five structural analogues commercially available (Figure 1) were also tested (Table ST1). Interestingly, benzo-\(\gamma\)-pyrone (chromone), the structure of which is smaller and simpler than those of khellin and visnagin, showed a similar level of activity to them. This may indicate that the rest of the structure contributes to activity, but it would not be essential for the phytotoxicity. Also, the replacement of the methyl group in C-7 of khellin by a carboxyl group (as in 4,9-dimethoxy-5-oxo-3,2\textsuperscript{-}furo[3,2-\textit{g}]chromene-7-carboxylic acid) did not increase the phytotoxicity, although it did improve the aqueous solubility of the molecule. Except for these two compounds, the effects of which were close to those of khellin and visnagin, none of the other analogues was as phytotoxic as these furanochromones. Accordingly, only the herbicidal activities of khellin and visnagin were studied in more detail.

### Table 2. Phytotoxic Activity of Fractions Obtained by Flash Column Chromatography from the Flowers\textsubscript{DCM} Extract of \textit{A. visnaga}

<table>
<thead>
<tr>
<th>fraction\textsuperscript{a}</th>
<th>mass (mg)</th>
<th>lettuce</th>
<th>creeping bentgrass</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>46.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>62.5</td>
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<td>0</td>
</tr>
<tr>
<td>III</td>
<td>57.6</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>10.7</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>V</td>
<td>28.1</td>
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<td>2</td>
</tr>
<tr>
<td>VI</td>
<td>15.8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>VII</td>
<td>58.5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>VIII</td>
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<td>3</td>
</tr>
<tr>
<td>IX</td>
<td>38</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>XI</td>
<td>45.6</td>
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<td>4</td>
</tr>
<tr>
<td>XII</td>
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<td>4</td>
</tr>
<tr>
<td>XIV</td>
<td>28.5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>XV</td>
<td>296.4</td>
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</tr>
<tr>
<td>XVI</td>
<td>88.7</td>
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<td>5</td>
</tr>
<tr>
<td>XVII</td>
<td>76.6</td>
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<td>5</td>
</tr>
<tr>
<td>XVIII</td>
<td>144.2</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>XIX (MeOH wash)</td>
<td>412.2</td>
<td>0</td>
<td>4</td>
</tr>
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</table>

\textsuperscript{a}Bioassay rating based on scale of 0 to 5: 0 = no effect and 5 = no growth or germination. \textsuperscript{b}Each fraction was tested at 1 mg mL\textsuperscript{−1}.

Figure 1. Structures of phytotoxic furanochromones khellin and visnagin isolated from \textit{A. visnaga} and the structural analogues tested for phytotoxicity.
activity when they were applied to ryegrass seeds (Figure S11). Additionally, the postemergence application of khellin and visnagin at 500 μM on 2-day-old seedlings of ryegrass in Petri dishes significantly reduced the growth of this weed (Figure 2).

The plant growth at 6 days was much lower after treatment with visnagin than with khellin. The growth inhibitory effect caused by visnagin at 500 μM on ryegrass was similar to the one caused by the postemergence herbicide glyphosate at 750 μM (Figure 2A).

Moreover, the phytotoxic activity of khellin and visnagin was observed on other problematic weeds. To evaluate if this effect was selective, these compounds were tested on a diverse group of target weeds, which included grasses such as barnyardgrass (E. crus-galli), crabgrass (D. sanguinalis), foxtail (S. italica), and millet (Panicum sp.), and broadleaf species such as morning-glory (Ipomea sp.) and velvetleaf (A. theophrasti). As it was observed that these molecules caused the highest reduction in plant growth at 500 μM or more (Figures S8, S10, and S11), they were used at 0.5 and 1 mM to determine their herbicidal potential, and the effect was compared with the pre-emergent herbicide acetochlor.

Khellin and visnagin showed a pre-emergence, nonselective effect because they significantly reduced the growth of the various weed species tested by application on the seeds (Figure 3A). Germination of the grasses but not the broadleaf species was affected by these treatments (Figure 3B). Interestingly, at 0.5 mM, visnagin caused a reduction in the growth and germination of weeds comparable to acetochlor at the same dose. Foxtail and crabgrass were shorter after treatment with visnagin than with acetochlor. By increasing the concentration of both furanochromones to 1 mM, germination of grasses and growth of all species were significantly more affected than at 0.5 mM (Figure 3), and the natural compounds were more phytotoxic to weeds than 0.5 mM acetochlor.

To evaluate if khellin and visnagin were also effective on weeds in soil, their herbicidal activity was tested in greenhouse conditions.

Table 3. Mean Inhibitory Concentrations (IC₅₀) of Khellin and Visnagin Required To Reduce Growth and Germination of Three Different Species after 7 Days of Exposure

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀ growth ± SE (μM)</th>
<th>IC₅₀ germination ± SE (μM)</th>
<th>IC₅₀ growth ± SE (μM)</th>
<th>IC₅₀ germination ± SE (μM)</th>
<th>IC₅₀ growth ± SE (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>duckweed</td>
<td>lettuce</td>
<td>ryegrass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>khellin</td>
<td>162 ± 29</td>
<td>701 ± 93</td>
<td>110 ± 11</td>
<td>637 ± 112</td>
<td>244 ± 37</td>
</tr>
<tr>
<td>visnagin</td>
<td>122 ± 28</td>
<td>740 ± 98</td>
<td>175 ± 16</td>
<td>502 ± 115</td>
<td>214 ± 23</td>
</tr>
</tbody>
</table>

Figure 2. Phytotoxicity of khellin and visnagin on ryegrass compared to that of glyphosate after postemergence treatment: (A) ryegrass plant growth at 6 days (+ days of treatment); (B) photographs of ryegrass plants exposed to different treatments during 4 days. Each data point represents the mean of three experiments ± SD. Different letters above the bars indicate significant differences between treatments (p < 0.05).

Figure 3. Phytotoxicity of khellin and visnagin on six different weed species compared to acetochlor after pre-emergence treatment: (A) plant growth as percent of control at 7 days; (B) germination as percent of control at 7 days; (C) weeds after different treatments. Bars represent, from left to right in each grouping, morningglory, velvetleaf, barnyardgrass, foxtail, millet, and crabgrass. Each data point represents the mean of three experiments ± SD. Different letters above the bars indicate significant differences between treatments (p < 0.05).
studies. As these compounds showed a generally nonselective effect, two weed species were selected for these assays: crabgrass (*D. sanguinalis*) as representative of grasses and velvetleaf (*A. theophrasti*) as representative of broadleaf species. No significant pre-emergence activity 11 DAT was observed when these compounds were sprayed at 2 kg ai ha\(^{-1}\) (data not shown). This result is in contrast to what was detected in laboratory assays in Petri dishes. Many natural phytotoxins may decrease their activities in soil due to several factors.\(^{65,66}\) There are physical, chemical, and microbiological factors that can reduce herbicide activity by making them biologically unavailable or by degrading them by chemical or biological processes.\(^{67}\)

On the other hand, during this greenhouse assay, both furanochromones produced a significant postemergence effect 11 DAT at 2 kg ai ha\(^{-1}\) (Figure 4). Visnagin was the most active in these conditions, causing a biomass reduction of >50% in crabgrass and velvetleaf with respect to control (Figure 4A). It also reduced significantly the height of both weeds, affecting crabgrass more severely (Figure 4B). Plant growth reduction was observed with both compounds but was greater with visnagin. Necrosis was observed on the leaf edges of both weeds sprayed with these compounds, were significantly smaller than the control (Figure 5A). A similar reduction was observed on weed plant height, except for barnyardgrass, the height of which was not affected by visnagin or pelargonic acid (Figure 5B). As in the previous greenhouse study, necrotic lesions of leaves after treatment with visnagin at 4 kg ai ha\(^{-1}\) indicated it can act as a contact herbicide. This was observed with both visnagin and pelargonic acid (Figure 5C). The oldest leaves, which received the spray with these compounds, were significantly more injured than newer leaves.

Pelargonic acid is a natural fatty acid, which is sold as a broad-spectrum bioherbicide for nonselective vegetative burn-down in many situations. It disrupts plant cell membranes, causing rapid loss of cellular functions.\(^{68}\) This fatty acid is more effective than other bioherbicides such as acetic acid or corn gluten meal that must be applied at rates of tons per hectare. Pelargonic acid is recommended at rates of 10−15 kg ai ha\(^{-1}\).\(^{69}\) The potential of visnagin as a bioherbicide seems comparable to that of pelargonic acid according to the results of the greenhouse studies. Both compounds caused a significant and comparable reduction in biomass and height of weeds at equivalent doses.

The postemergence effect of visnagin at 11 DAT was comparable to the one caused by pelargonic acid at the same dose (Figure 5). The dry weight of the three weeds sprayed with these two compounds was significantly smaller than the control (Figure 5A). A similar reduction was observed on weed plant height, except for barnyardgrass, the height of which was not affected by visnagin or pelargonic acid (Figure 5B). As in the previous greenhouse study, necrotic lesions of leaves after treatment with visnagin at 4 kg ai ha\(^{-1}\) indicated it can act as a contact herbicide. This was observed with both visnagin and pelargonic acid (Figure 5C). The oldest leaves, which received the spray with these compounds, were significantly more injured than newer leaves.

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Figure 4. Postemergence effects of khellin and visnagin on velvetleaf and crabgrass during greenhouse assays: (A) plant dry weight 11 DAT; (B) plant height 11 DAT; (C) photographs of plants 11 DAT. Bars represent, from left to right in each grouping, crabgrass and velvetleaf. Furanochromones were tested at 2 kg ai ha\(^{-1}\) on 6−8-day-old plants. Data represent means of three replicates ± SD. Different letters above the bars indicate significant differences between treatments (p < 0.05).

The postemergence effect of visnagin at 11 DAT was comparable to the one caused by pelargonic acid at the same dose (Figure 5). The dry weight of the three weeds sprayed with these two compounds was significantly smaller than the control (Figure 5A). A similar reduction was observed on weed plant height, except for barnyardgrass, the height of which was not affected by visnagin or pelargonic acid (Figure 5B). As in the previous greenhouse study, necrotic lesions of leaves after treatment with visnagin at 4 kg ai ha\(^{-1}\) indicated it can act as a contact herbicide. This was observed with both visnagin and pelargonic acid (Figure 5C). The oldest leaves, which received the spray with these compounds, were significantly more injured than newer leaves.

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Figure 5. Postemergence herbicidal effect of visnagin on three weeds compared to the bioherbicide pelargonic acid during greenhouse assays: (A) plant dry weight 11 DAT; (B) plant height 11 DAT; (C) representative photos of velvetleaf 11 DAT. Bars represent, from left to right in each grouping, barnyardgrass, crabgrass, and velvetleaf. Visnagin and pelargonic acid were tested at 4 kg ai ha\(^{-1}\) on 13−16-day-old (2–3 true leaf stage) plants. Data represent means of three replicates ± SD. Different letters above the bars indicate significant differences between treatments (p < 0.05).
Formulation and chemical modification of lead molecules can greatly improve herbi
cide efficacy.70–72 The analogues tested in the course of this study were less active than visnagin or khellin, but different analogues could have improved activity. However, their identification and study go beyond the objectives of the present work.

Studies on the Possible Mode of Action of Khellin and Visnagin. The herbi
cidal activity or phytotoxicity of these two furanochromones has not been described before. Therefore, we conducted several assays that can provide information about their mode(s) of action. Because the IC50 values of these compounds to inhibit growth of duckweed, lettuce, and ryegrass were in the range from 110 ± 11 to 244 ± 37 μM, doses in the range of the IC50 values were used for these assays.

The integrity of the plant plasma membrane is a good biomarker to help identify modes of action of herbicides and their dependence on light.43 Stress conditions are often accompanied by the accumulation of high levels of ROS exceeding the detoxification mechanisms of plant cells. This can lead to membrane lipid peroxidation resulting in the uncontrolled release of cellular electrolytes.75 Khellin and visnagin produced a destabilization of the cell membranes at 100 and 300 μM, leading to significant electrolyte leakage (Figure 6). The stress caused by these furanochromones may trigger this phenomenon through direct or indirect effects.

![Figure 6](image)

Figure 6. Electrolyte leakage induced by khellin (A, B) and visnagin (C, D) under different conditions of illumination: (A, C) 16 h dark + 26 h light (arrows indicate the start of light exposure). Bars represent, from left to right in each grouping, start, 16 h dark, 8 h light, and 26 h light. (B, D) 42 h dark. Bars represent, from left to right in each grouping, start, 16 h, 24 h, and 42 h. Data represent means of three replications ± SD. The dotted line represents maximum leakage obtained by boiling the cotyledon disks.

It has been recently suggested that electrolyte leakage, which stimulates proteases and endonucleases, and programmed cell death are often linked to each other when plant cells are severely stressed.74 In cucumber leaf disks exposed to 100 μM khellin or visnagin, an increase (~35%) in relative cell death was detected with respect to the control, as estimated by the Evans blue staining procedure74 (Figure 7A, B). This rose to a 3.5-fold increase when the concentration was 300 μM, indicating severe damage to leaf tissue at the higher dose. Such processes of plasma membrane destabilization and cell death induced by these furanochromones would explain the necrosis observed in plants sprayed with these compounds in greenhouse assays.

At 42 h of incubation, either in the dark or after 26 h of high light intensity exposure, both furanochromones triggered significant electrolyte leakage on cucumber cotyledon disks (Figure 6). Ion leakage caused by 100 and 300 μM khellin and visnagin in the dark (Figure 6B,D) indicates that the mode of action of these compounds is not light-dependent and involves cellular leakage. This was also shown when the phytotoxicity of khellin and visnagin was compared under light and dark conditions. There were no significant differences in their phytotoxic effects on lettuce in light and darkness (Figure 8).

However, the most intense electrolyte leakage was observed after incubation of cucumber cotyledon disks with khellin and visnagin plus 26 h of high light intensity (Figure 6A,C). Under these conditions, the effect of these compounds at 100 and 300 μM was comparable to the positive control obtained by boiling the cotyledon disks and bleaching was also observed (Figure S13). This may be a consequence of the higher level of ROS produced in light, combined with the stress caused by the furanochromones.

Most biocides cause ROS generation as a side effect before cell death occurs. To evaluate if there was an increase in ROS levels after treatment with khellin and visnagin, cucumber cotyledon disks were studied under presymptomatic conditions (before the detection of high levels of electrolyte leakage). As for cell death and electrolyte leakage assays, each compound was tested at 100 and 300 μM. The tissues were incubated in darkness (16 h) before exposure to high light intensity for 5 h. Cucumber cotyledon disks were subsequently treated with the ROS-dependent fluorescent probe DCFDA for visualization by confocal microscopy.

In control disks and disks treated with the compounds at 100 μM, most of the label was recovered in chloroplasts as expected in light conditions, co-localizing with chlorophyll autofluorescence (Figure 9A). Image analysis indicates that ROS levels in cotyledon disks exposed to 300 μM khellin or visnagin were significantly higher than in the control (Figure 9B) and comparable to the treatment with 10 mM H2O2. Under these conditions, additional green fluorescence was detected in other cellular compartments and membranes (Figure 9A), indicating increased peroxidation.

According to these results, the treatment with these furanochromones at 300 μM, together with high light intensity, caused an increase in cellular ROS levels prior to the plasma membrane destabilization. However, no significantly higher generation of ROS was detected after treatment with compounds at 100 μM plus 5 h of high light intensity (Figure 9), even though visnagin caused electrolyte leakage at this dose after 8 h in the light. A longer exposure to light is probably required to detect ROS generation. Thus, the increase in ROS may not be directly associated with the molecular target site of these compounds, but is more likely to be a secondary or tertiary effect of what is causing cellular leakage. Moreover, after incubation in complete darkness for 30 h, ROS generation was not detected in cucumber cotyledon disks with either khellin or visnagin at 300 μM (Figure S14). This observation suggests that the main source of ROS in furanochromones-treated plants is associated with photosynthetic activities. Moreover, whereas ROS propagation may contribute to damage in the light, the basic mechanism of visnagin and khellin toxicity would be ROS-independent.
Chlorophyll fluorescence measurements showed that khellin and visnagin significantly reduce the photosynthetic efficiency of cucumber cotyledon disks. The ETR was reduced ∼35 and ∼50% after 24 h of incubation in the light (42 h total) with these compounds at 100 and 300 μM, respectively (Figure 10). These results suggest that photosynthesis is not a primary target of khellin or visnagin. Considering the long time period of incubation and irradiation required for a significant photosynthetic efficiency decline, khellin and visnagin probably affect photosynthesis indirectly, most likely altering chlorophyll fluorescence as a consequence of the membrane peroxidation and destabilization previously detected under these conditions (Figures 6 and 9).

The effect of khellin and visnagin on cell division was evaluated by mitotic index analysis of root meristem cells of onion (A. cepa). Because the sensitivity of this plant species to these furanochromones was unknown, a broader range of concentrations was tested (0–1000 μM). Both compounds inhibited cell division in a dose-dependent manner (Figure 11A). Visnagin was more active than khellin and completely inhibited onion cell division at 300 μM. Inhibition was not associated with the arrest of a particular phase of mitosis. The percentage of cells observed in each mitotic phase after exposure to these furanochromones was smaller than in the control. However, when there was not a total inhibition, the relative proportion of cells in the mitotic phases after treatment with both compounds was comparable to the control (Figure 12). A different situation was observed regarding cells with abnormal configurations such as chromosome aberrations (chromosome bridges, breaks, and losses), nuclear abnormalities (lobulated nuclei, nuclei carrying nuclear buds, polynuclear cells, etc.), or micronuclei. The proportion of these types of cells with abnormal configurations after treatment with khellin or visnagin was higher than in the control (Figure 12).

Inhibition of cellular division caused by khellin and visnagin at 100 μM was irreversible under the experimental conditions employed (Figure 11B). As in the dose–response assay, we failed to detect alterations in the distribution of dividing cells into the different mitotic phases with respect to the control, except for cells with abnormal configurations, the proportion of which after treatment with furanochromones was higher than in the control (Figure S15). However, root meristem onion cells did not recover their normal division rate after washing the seeds at 3 days with distilled water. This effect might be associated with a cell death process induced by these compounds. In onion roots exposed to 100 μM khellin or

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**Figure 7.** Cell death induced by khellin and visnagin in cucumber leaf disks and onion roots: (A) cucumber leaf disks stained with Evans blue after exposure to different doses of khellin and visnagin at 7 days; (B) relative cell death in cucumber leaf disks estimated by Evans blue staining; (C) onion root tips stained with Evans blue after exposure to different doses of khellin and visnagin at 4 days; (D) relative cell death in onion root tips estimated by Evans blue staining. Bars represent, from left to right in each grouping, khellin and visnagin. Data are means of three replicates ± SD. Different letters above the bars indicate significant differences between treatments (p < 0.05).

**Figure 8.** Comparison of khellin and visnagin phytotoxicity on lettuce under different conditions of illumination. Bars represent, from left to right in each grouping, light and darkness. Acetochlor was included as reference of pre-emergent herbicide, and to get the highest herbicidal effect, all compounds were tested at 1 mM. Data represent means of three replicates ± 1 SD. Different letters above the bars indicate significant differences between treatments (p < 0.05).
visnagin, a 2-fold increase in relative cell death was detected (Figure 7C,D). These furanochromones inhibited cell division, but also caused cell death in onion roots, which was dependent on the dose used. At 300 μM, both compounds induced a 2-fold increase in relative cell death compared to 100 μM and a 3–4-fold increase relative to the control conditions (Figure 7C,D).

Figure 9. ROS production in cucumber cotyledon disks exposed to khellin and visnagin: (A) ROS determined using DCFDA and detected by confocal laser scanning microscopy in cucumber cotyledon disks; (B) quantification of DAF fluorescence. Bars represent, from left to right in each grouping, khellin and visnagin. The dotted line represents the DAF fluorescence obtained by treatment of cotyledon disks with H$_2$O$_2$. Values are means of three replicates ± 1 SD. Different letters above the bars indicate significant differences between treatments (p < 0.05). AU, arbitrary units.

Figure 10. Photosynthetic electron transport rate (ETR) in cucumber cotyledon disks exposed to different concentrations of khellin (A) and visnagin (B). Bars represent, from left to right in each grouping, start, 18 h dark, 6 h light, and 24 h light. Data are means of three replications ± SD. The dotted line represents ETR of untreated solvent control.

Figure 11. Effect of khellin and visnagin on A. cepa root meristem cell division. (A) Amount of dividing cells after exposure to different concentrations of each furanochromone. Bars represent, from left to right in each grouping, khellin and visnagin. (B) Amount of dividing cells with and without washing A. cepa seeds at 3 days with distilled water after an exposure to khellin and visnagin. Bars represent, from left to right in each grouping, without wash and with wash. The amount of dividing cells is expressed as percent of total counted cells at 7 days. Data are means of three replicates.

Figure 12. Dose–response effect of khellin and visnagin on A. cepa root meristem cell division. (A) Amount of dividing cells in each phase of mitosis and with abnormal configurations. Bars represent, from left to right in each grouping, 0 μM (control), 30 μM, 100 μM, 300 μM, and 1000 μM. (B) Photographs of meristem cells of A. cepa at 7 days: (1, 2) control; (3–7) 30 μM visnagin; (8) 100 μM visnagin; (9–11) 300 μM khellin; (12) 1000 μM khellin; P, prophase; M, metaphase; A, anaphase; T, telophase; CA, chromosomal aberrations; MN, micronuclei. The amount of dividing cells is expressed as percent of total counted cells at 7 days.
Our results indicate that the mode of action of khellin and visnagin could be a complex process involving multiple targets. The inhibition of cell division and the increased cell death caused by these furanochromones, together with cell membrane destabilization, would account for the reduction in plant growth. In addition, these effects explain the development of necrosis and abnormal leaves observed after treatment with both compounds.

Because membrane destabilization caused by these furanochromones was intensified after a light irradiation period, and considering their chemical nature, a phototoxic effect might be expected. Some biological activities caused by furanochromones, as well as their possible role in plant defense, have been associated with their photoactivity. However, their phytotoxicity was not light-dependent, because both compounds induced electrolyte leakage in darkness, and the inhibitions of lettuce growth under light and darkness were similar.

Despite the similarities in chemical structures and properties that furanochromones share with furanocoumarins, or psoralens, they differ in their photochemical properties and in their ability to photodamage eu-karyotic cells and form cross-links. Visnagin is much less phototoxic and photomutagenic than bergapten when compared at equimolar concentrations and equal UV-A doses on the green alga Chlamydomonas reinhardtii. According to Martelli et al., visnagin and khellin could react with DNA and generate activated oxygen species upon UV irradiation. However, in later work, the extent of photoaddition was low compared with most furanocoumarins, and oxygen-dependent photo-oxidation of DNA was not observed. The absence of DNA photo-oxidation after treatment with visnagin or khellin plus UV-A suggested that furanochromones do not have any photodynamic effect on DNA. The phototoxicity of these molecules, albeit low when compared to furanocoumarins, might contribute to their herbicidal activity. Under high irradiation conditions, ROS production increased after treatment with khellin and visnagin, resulting in higher oxidative damage to cell membranes and other cellular components. However, the potential phototoxic effect under high light would not explain the phytotoxicity in the dark, indicating that other mechanisms are involved.

In conclusion, the mode of action of these furanochromones appears to be a complex process. It is not light-dependent and involves effects on membrane stability, cell division, and cell viability in leaves and roots that may not be related. Both compounds also reduce photosynthetic efficiency through indirect effects and induce oxidative damage under high light intensity. Visnagin had the best contact postemergence herbicidal activity in greenhouse assays. Its effect was comparable to that of the commercial herbicide pelargonic acid at the same rate, indicating visnagin’s potential as a bioherbicide or lead molecule for the discovery of new synthetic herbicides.

**REFERENCES**


**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02462.

Additional data as cited in the manuscript. Phytotoxicity of commercial standards of khellin and visnagin as well as five structural analogues (Table ST1). Pictures of Ammi visnaga (L.) Lam. (Figure S1). 1H NMR data of fractions XIV (khellin) (Figure S2), XV (Figure S4), XVI (Figure S5), and XVII (visnagin) (Figure S6). 13C NMR data of fractions XIV (khellin) (Figure S3) and XVII (visnagin) (Figure S7). Dose–response curves of khellin and visnagin on duckweed (Figure S8), lettuce (Figure S10), and ryegrass (Figure S11). Effect of khellin and visnagin on the tissue of duckweed (Figure S9) and cucumber cotyledon disks under different conditions of illumination (Figure S13). Comparison of germination and growth of lettuce and ryegrass in water and solvent control (Figure S12). Detection and quantification of ROS in cucumber cotyledon disks in darkness (Figure S14). Effect of khellin and visnagin on root meristem cell division of A. cepa (Figure S15) (PDF)

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**Notes**

A provisional patent was filed with the results presented in this work: “Herbicidal composition comprising chromosome derivatives and a method for weed control.” (2015) Provisional patent in the United States 62/272,880. N/ref.: 577 US PROV. The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We are grateful to Robert Johnson, Susan Watson, Amber Reichley, and Solomon Green for their excellent technical assistance.

**ABBREVIATIONS USED**

DCM, dichloromethane; EtOH, ethanol; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; DCFDA, 2′,7′-dichlorofluorescein diacetate


(11) WHO Monographs on Selected Medicinal Plants; Tsai, R., Garden, H., Eds.; WHO Library Cataloguing in Publication Data: Geneva, Switzerland, 2007; Vol. 3.


(38) Chen, X.; Han, H.; Jiang, P.; Nie, L.; Bao, H.; Fan, P.; Lv, S.; Feng, J.; Li, Y. Transformation of β−lycopene cyclase genes from...


Supporting information

Khellin and Visnagin, Furanochromones from *Ammi visnaga* (L.) Lam., as Potential Bioherbicides

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Table ST1. Phytotoxicity of Commercial Standards of Khellin and Visnagin, and Five Structural Analogues.

<table>
<thead>
<tr>
<th>Compounds tested at 1 mM</th>
<th>Phytotoxicity at 7 days*</th>
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<tr>
<td></td>
<td>Lettuce</td>
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<td>Fraction XIV (khellin)</td>
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</tr>
<tr>
<td>Khellin (technical standard from Sigma-Aldrich)</td>
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</tr>
<tr>
<td>Fraction XVII (visnagin)</td>
<td>3</td>
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<td>Visnagin (technical standard from Sigma-Aldrich)</td>
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<tr>
<td>Khelloside</td>
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</tr>
<tr>
<td>Benzo-γ-pyrone</td>
<td>3</td>
</tr>
<tr>
<td>4,9-dimethoxy-5-oxo-5H-furo[3,2-g]chromene-7-carboxylic acid</td>
<td>3</td>
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</tr>
<tr>
<td>4,9-dihydroxy-7-methyl-5H-furo[3,2-g]chromen-5-one</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

*Bioassay rating based on scale of 0 to 5: 0= no effect and 5= no growth or germination.
Figure S1. (A) *Ammi visnaga* (L.) Lam. growing in Santiago del Estero province, Argentina, where plant material was collected; (B) herbarium specimen prepared for identification of the species.
Figure S2. $^1$H NMR (500 MHz) data for fraction XIV (khellin).
Figure S3. $^{13}$C NMR (125 MHz) data for fraction XIV (khellin).
Figure S4. $^1$H NMR (500 MHz) data for fraction XV.
Figure S5. $^1$H NMR (500 MHz) data for fraction XVI.
Figure S6. $^1$H NMR (500 MHz) data for fraction XVII (visnagin).
Figure S7. $^{13}$C NMR (125 MHz) data for fraction XVII (visnagin)
Figure S8. Dose-response curves of khellin and visnagin on duckweed (*Lemna paucicostata*). Percentage of increase between days 1 and 7 was determined relative to baseline area at day zero. Data represents means of three replicates ± SD.
Figure S9. Effect of khellin and visnagin on the tissue of duckweed (L. paucicostata): (A) Tissue condition at 7 d; (B) Comparison between initial (day 0) and final conditions (day 7) of duckweed treated with the highest doses of khellin and visnagin.
Figure S10. Dose-response curves of khellin and visnagin on lettuce (*Lactuca sativa*). The percentage of germination and length of plants were determined at 7 days. Data represents means of three replicates ± SD.
Figure S11. Dose-response curves of khellin and visnagin on ryegrass (*Lolium multiflorum*). The percentage of germination and length of plants were determined at 7 days. Data represents means of three replicates ± SD.
Figure S12. Germination and plant growth at 7 d in water and solvent control with acetone 10% v/v. Black bars correspond to controls of curves with khellin and grey bars correspond to controls of curves with visnagin. Data are the means of three replicates ± SD.
Figure S13. Tissue condition of the cucumber cotyledon discs exposed to khellin and visnagin under different conditions of illumination.
Figure S14. Detection and quantification of ROS in cucumber cotyledon discs in darkness. The dotted line represents the DAF fluorescence obtained by treatment of cotyledon discs with 10 mM H$_2$O$_2$. Black bars correspond to khellin and grey bars to visnagin. Values are means of three replicates ± SD and different letters above the bars indicate significant differences between treatments ($p < 0.05$). AU, arbitrary units.
Figure S15. Evaluation of reversibility of cell division inhibition caused by khellin and visnagin. (A) Amount of dividing cells in each phase of mitosis and with abnormal configurations, without and with washing *A. cepa* seeds at 3 days with distillated water. (B) Photographs of meristem cells of onion at 7 days, after exposure to different treatments: (1-2) control (wash -); (3-4) control (wash +); (5-7) 100 µM khellin; (8-10) 100 µM khellin (wash +); (11) 100 µM Visnagin; (12-16) 100 µM visnagin (wash +). P, prophase; M, metaphase; A, anaphase; T, telophase; CA, chromosomal aberrations; MN, micronuclei; NA, nuclear abnormality. Amount of dividing cells is expressed as percent of total counted cells at 7 days.