

Research Paper

Phytotoxic compounds from roots of *Centaurea diffusa* Lam.

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An extract of roots of *Centaurea diffusa* (diffuse knapweed) yielded caryophyllene oxide and linoleic acid which were shown to be phytotoxic. Also isolated were germacrene B, a previously-known phytotoxin as well as the inactive polyene aplotaxene. A combination of these compounds, if transferred to the soil, could be one factor in the invasive behavior of this weed. Contrary to a literature report, 8-hydroxyquinoline was not detected in root exudates of in vitro grown *C. diffusa* nor could it be identified in the root extract. However, a recent report from a different group maintains that 8-hydroxyquinoline can be released from roots of *C. diffusa* following a diurnal rhythm.

Introduction

Diffuse knapweed, *Centaurea diffusa* Lam., is an introduced weed which has spread throughout the northwest United States with deleterious effects on range and agricultural areas.¹ It has been suggested that plant-plant allelopathy, perhaps caused by phytotoxins exuded or leached from the knapweed, might account for the invasive behavior of this weed. Thus, Muir and Majak² reported seed germination and seedling growth inhibition by shoot extract fractions and that a sesquiterpene-containing fraction of shoot extracts as well as a polar extract were phytotoxic.³ On the other hand, these researchers could not demonstrate any effects of knapweed litter on plants in range sites and saw no effect on seedlings of other plants grown in the presence of knapweed. Soil extracts from knapweed-infested sites yielded *p*-coumaric acid, but it only inhibited seed germination and seedling biomass reduction at the highest levels tested. No active compounds were identified from these studies and root exudate extracts yielded only "a series of long-chain saturated and unsaturated carboxylic acids", which were not tested for phytotoxicity.³ A more recent report⁴ described the isolation of 8-hydroxyquinoline as a sole phytotoxin from root exudates of in vitro grown *C. diffusa*, and stated that it was abundant in soil extracts from *C. diffusa*-invaded North American fields. In contrast, Norton et al.,⁵ reported that they were unable to detect 8-hydroxyquinoline in experimental or field collected soils from knapweed-infested sites. Roots of *C. diffusa* have not previously been studied chemically and the novelty of

8-hydroxyquinoline as a plant natural product suggested that analysis of the roots as the likely storage site of this compound might provide useful basic information for a later exploration of possible transport mechanisms for the quinoline into exudates. In conjunction with this, we elected to repeat the exudate analysis and to determine if elicitors could affect the process.

Results and Discussion

Compound isolation and identification. The crude ethanolic root extract of *C. diffusa* inhibited the growth of *A. thaliana* seedlings significantly (Fig. 1). Of the partitioned extract residues, the hexane and CH₂Cl₂ residues showed some visual phytotoxic effects on *A. thaliana* seedlings, whereas the *n*-BuOH fraction did not (data not shown). The hexane fraction exhibited a significantly higher toxicity level than did the CH₂Cl₂ fraction (Fig. 2), so it was investigated further. Bioassay-driven chromatographic separation of the hexane residue yielded 12 fractions. Fractions 1, 5 and 7 were found to be phytotoxic (data not shown). A GC-MS study on fraction 1 (513 mg) yielded germacrene B (1) (3 mg), (-)-caryophyllene oxide (2) (1 mg), and aplotaxene (3) (15 mg). Analysis of the fraction 5 (305 mg) by GC-MS yielded ethyl linoleate (2 mg), ethyl oleate (3 mg), linoleic acid (4) (20 mg), and (-)-caryophyllene oxide (2) (5 mg) (see Fig. 3). Germacrene B (1) (C₁₅H₂₄) was identified by a GC-MS peak at 11.25 min with mass spectrum (*m/z* 204, 189, 161, 147, 135 and 12), identical to that of the literature (*m/z* 204, 189, 161, 147, 136, 121). The structure of caryophyllene oxide (2) (C₁₅H₂₄O) was deduced by a GC-MS peak at 12.7 min (*m/z* 220, 87, 177, 161, 149) which was identical to that of the standard commercial sample (*m/z* 220, 187, 177, 161, 149). In addition, the structures of compounds 3 and 4 were also confirmed by comparison of the NMR spectral data with those reported in the literature.^{6,7} The ¹H NMR spectrum of 3 showed the presence of a terminal vinyl group attached to a methylene group [4.88 (1H, ddd), 4.98 (1H, ddd), 5.78 (1H, ddt)] six olefinic protons [5.30–5.40 (6H, m)], two methylenes α to two double bonds [2.77 (4H, m)], three allylic methylenes [1.98–2.08 (6H, m)] and three adjacent methylenes [1.20–1.40 (6H, m)]. The signal at δ 0.94 (3H, t) was assigned to methyl protons in an ethyl group. These data were identical with those previously reported for aplotaxene.⁶

The ¹H NMR spectrum of 4 presented four olefinic protons [5.23–5.40 (4H, m)], a methylene α to two double bonds [2.75 (2H, m)], a methylene adjacent to an ester group [2.25 (2H, m)], two allylic methylenes [1.98 (4H, m)], a methylene [1.66 (2H, m)], seven adjacent methylenes between [(14H, m)] and a terminal

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methyl group [0.90 (3H, t)]. The spectral properties of this compound were comparable with those reported for linoleic acid.⁷

The ¹H NMR spectrum of fraction 7 (20 mg) showed a complex mixture which was not further investigated.

Fatty acids appear to be chemotaxonomic and biosynthetically less evolved than their counterpart polyacetylenes and are widespread among higher plants. Polyacetylenes are present in a more limited number of family plants, mainly Asteraceae and Apiaceae and commonly occur in a number of *Centaurea* species,⁸ but they were not detected in any of the *C. diffusa* root extracts. Aplotaxene and its derivatives are characteristic compounds of the closely related genera *Cirsium* and *Centaurea*.^{9,10} To what further extent compounds like aplotaxene are of chemotaxonomic importance in these genera is unclear⁶ and more data are needed.

Germacrene B and related compounds are widespread in *Centaurea* species.^{11,12} However, neither germacrene B nor other compounds identified in this study were previously described from *C. diffusa*.

8-hydroxyquinoline. The hexane, CH₂Cl₂, and *n*-BuOH root extracts were analyzed by LC-MS as described above along with a standard commercial sample of 8-hydroxyquinoline (14.8 min retention time) but none of the quinoline was detected.

Root exudates. Root exudates elicited with MeJA (12 mg), SA (7 mg), and a non-elicited control (10 mg) were analyzed by LC-MS and NMR. Basically, all showed strong NMR resonances for the presence of a complex mixture of fatty acids with only a trace due to flavonoid-type peaks. The resonances in the crude ¹H NMR spectrum in comparison with those data reported in the literature,⁷ suggested the presence of oleic acid (5) as a major component. Muir et al.³ also reported only long chain fatty acids from their study of *C. diffusa* root exudates, but these were not further studied.

Definite structures of the other compounds present in the root exudates could not be confirmed due to the small amounts present in comparison to the fatty acid components. Based on EIMS (*m/z* 300), UV (273.2 nm, 368.3 nm), and the crude ¹H NMR spectrum the flavonoid hispidulin (6; 5, 7, 4'-trihydroxy-6-methoxyflavone)¹³ was identified as a possible component. Hispidulin is a common compound of aerial and root parts of *Centaurea* species.¹⁴⁻¹⁶ Phytotoxic activity of this flavonoid on the germination and growth of crop species is known.¹⁷

Contrary to a previous report,⁴ 8-hydroxyquinoline was not detected in the root exudates and these results are complementary to the Norton et al.,⁵ studies in which it was not found in extracts of *C. diffusa* soils, either greenhouse-grown or field-collected. However, it should be noted that in a recent study 8-hydroxyquinoline was reported to be secreted by the roots of hydroponically-grown *C. diffusa* plants following a diurnal pattern.¹⁸ In this study the authors reported that 8-hydroxyquinoline was secreted at its maximum by the roots after 6 continuous hours of sun light following a dark period, after 8 hours of sun light the compound started to degrade and after 10 h the compound could not be detected. These studies

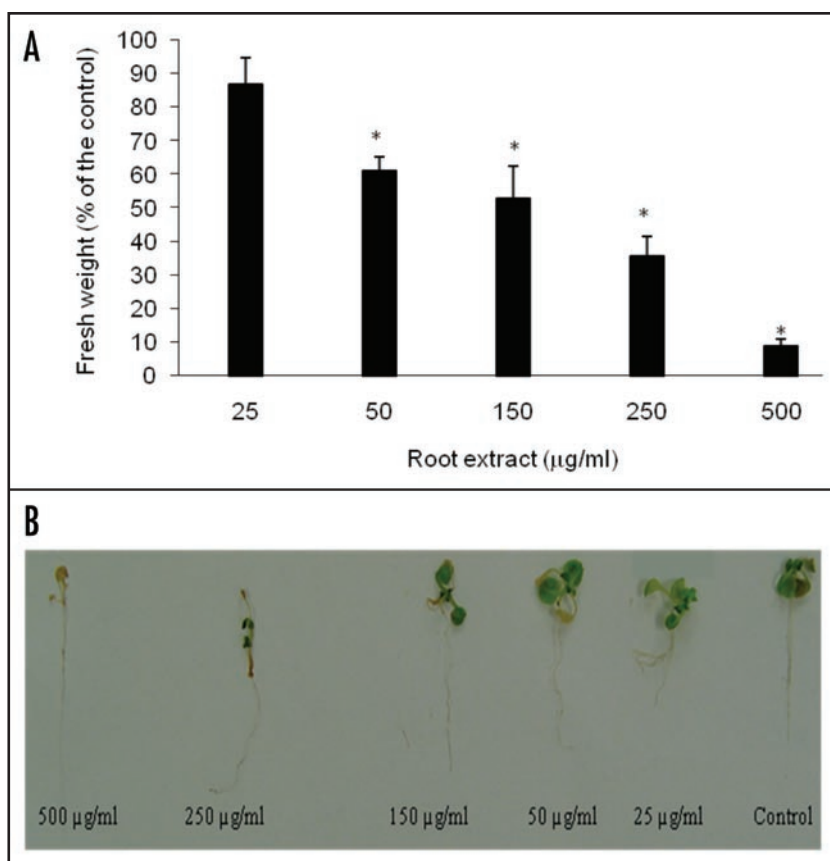


Figure 1. Growth inhibition of *C. diffusa* root extract on *A. thaliana* seedlings. (A) Addition of 50 µg/ml reduced 40% the growth of *A. thaliana*. Values are presented as percentage differences from the control. Errors bars are the s.e. of the mean, *n* = 3. **p* < 0.05. (B) *A. thaliana* seedlings treated with 500, 250, 150, 50 and 25 µg/ml *C. diffusa* root extracts. 50 µl of MeOH was added to control wells.

highlight the transient nature of some root secreted compounds and stress the fact that further studies of 8-hydroxyquinoline are needed to corroborate its presence (or absence) under field conditions. Interestingly, a root secreted compound from *Centaurea maculosa*, catechin, has been found under field conditions in high concentrations but only at certain times of the year.¹⁹

Phytotoxic activity of compounds. Compounds 2 and 4 were phytotoxic against *A. thaliana* seedlings (Fig. 4), while aplotaxene (3) was not at the doses tested. Germacrene B (1) has previously been shown to be phytotoxic,²⁰ but we had insufficient material to retest it in this study. The sesquiterpene 2 was the most active compound against *A. thaliana* seedlings growth. A previous study had shown that fractions containing 2 completely suppressed *Schizachyrium scoparium* germination but had no effect on lettuce.²¹ The pure compound itself was not tested. Our results showed that 2 decreased the fresh weight of *A. thaliana* seedlings by 70% even at 50 µg/ml and caused significant bleaching of the seedlings at phytotoxic concentrations. A bleaching effect has been reported for monoterpene derivatives isolated from another Asteraceae species plant family²² and they were shown to be inhibitors of phytoene desaturase, a key enzyme in carotenoid pigment biosynthesis. Since carotenoids protect chlorophyll from photooxidation, their lack would result in loss of chlorophyll. Some carotenoid biosynthesis inhibitors (Norflurazon and fluridone, for example) are commercial

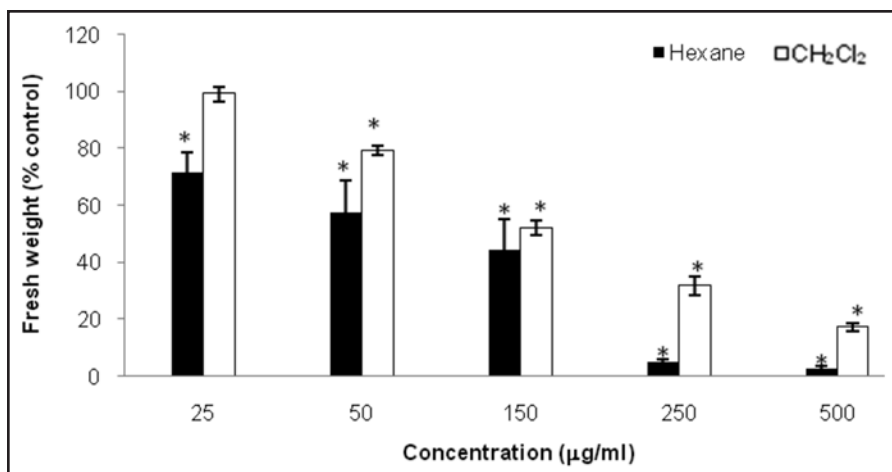


Figure 2. Phytotoxic activity of hexane and CH₂Cl₂ root extracts from *C. diffusa* on *A. thaliana* seedlings. Values are presented as percentage differences from the control. Errors bars are the s.e. of the mean, $n = 4$. * $p < 0.05$.

herbicides for control of grasses and aquatic weeds.²³ The bleaching of *A. thaliana* seedlings caused by caryophyllene oxide (2) suggests that it too could be a carotenoid biosynthesis inhibitor.

Compound 4 also produced a strong inhibitory effect on *A. thaliana*, reducing the growth of seedlings by 60% and 80% when the compound was applied in doses between 125 and 500 µg/ml,

respectively. Phytotoxicity of this fatty acid on zooplankton²⁴ and germination of some species²⁵ has been previously reported, but its growth inhibitory activity at later stages of plant development, has never been evaluated.

The root-exudate compound 5 decreased growth seedlings by 70% at the two highest concentrations but was inactive at the rest of concentrations tested. The phytotoxicity of 5 has been evaluated in algae²⁴ but this is the first report about its inhibitory growth activity in higher plants.

The higher activity of linoleic acid compared to oleic acid presented in this study is in agreement with the findings of Kakisawa,²⁶ who showed that toxicity of fatty acids increases with an increase in double bonds. Phytotoxic properties of fatty acids are well known and understood. Allelopathic properties of long-chain fatty acids have reported

previously on algae by Proctor²⁷ and Kroes.²⁸ Song et al.,²⁹ identified fatty acids among other compounds as putative allelochemicals in rice root exudates.

Further investigations in natural ecosystems where *C. diffusa* occurs are necessary to confirm the phytotoxic activity observed in vitro against *A. thaliana*.

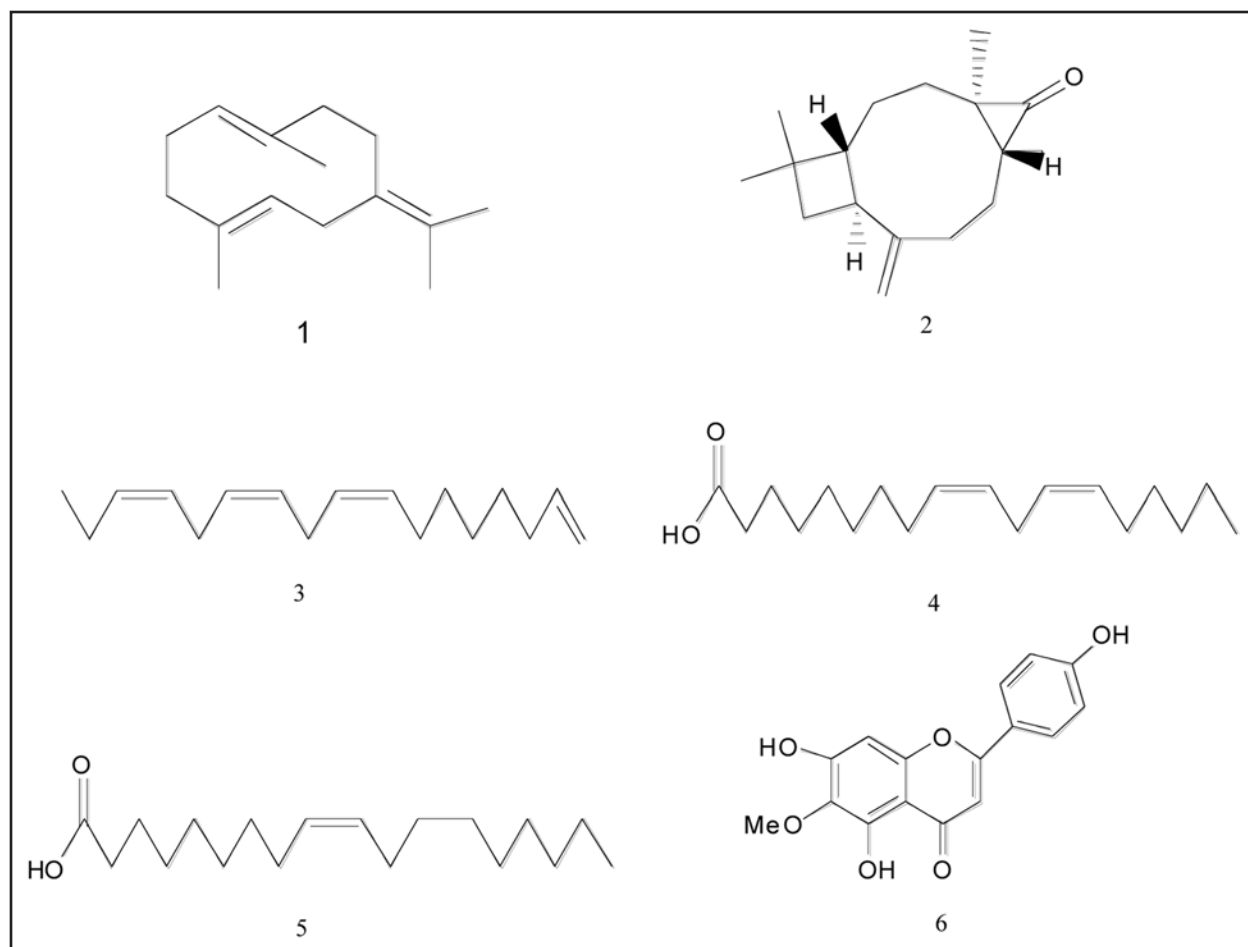


Figure 3. Compounds present in *C. diffusa* roots and root exudates.

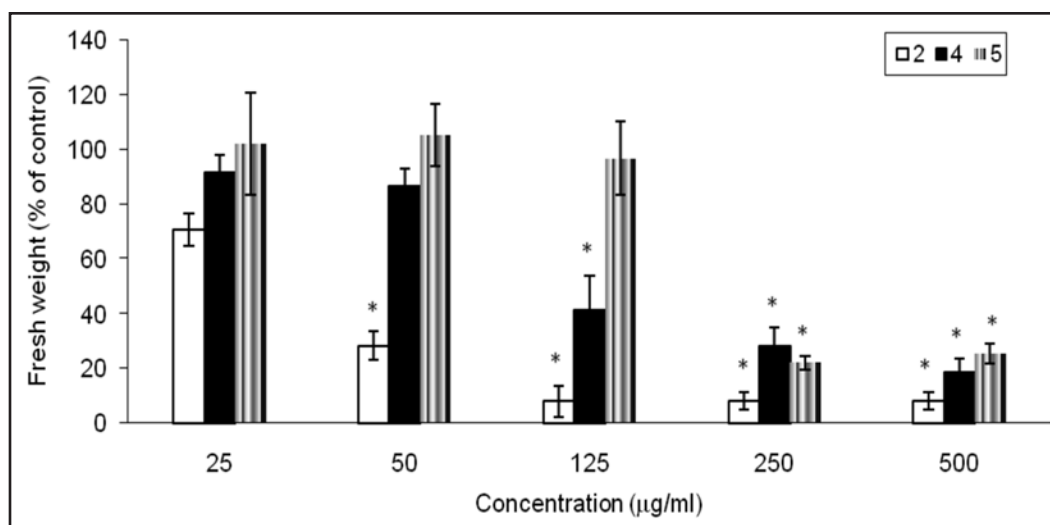


Figure 4. Comparison of effect of compounds 2, 4 and 5 found in *C. diffusa* roots and root exudates on *A. thaliana* seedling growth. Values are presented as percentage differences from the control. Errors bars are the s.e. of the mean, $n = 4$. * $p < 0.05$.

The growth stimulating effect of an exudate lipid fraction on ectomycorrhizal fungi in the rhizosphere has been reported before.³⁰ *C. diffusa* is known to have associations with vesicular arbuscular mycorrhizal fungi (VAM),³¹ but nothing is known about its ectomycorrhizal fungi associations.

Muir and Majak² reported that germinating seeds of *C. diffusa* did not inhibit the seed germination of other species, but suggested that seed chemical production could affect plant growth at later stages. In our study, the *C. diffusa* seed leachate was evaluated for growth inhibition on *A. thaliana* (Fig. 5). Significant growth inhibition ($p < 0.05$) was only observed at the highest dose applied (500 µg/ml). This weak activity is unlikely to have any ecological relevance.

The manner in which *C. diffusa* has become an invasive species in the U.S. is probably complex and not due to any one factor. It possesses some morphological and physiological characteristics that could promote its aggressiveness in the field.¹ In addition to its prolific production of viable seeds which germinate under a broad range of environmental conditions, its high tolerance to drought and its monocarpic perennial character should give this species a significant advantage in the ecosystem. As we have discussed, the allelopathic potential of *C. diffusa* has been a subject of contradictory and controversial studies.^{2-5,18} Phytotoxic compounds identified in this report could be one of the factors involved in the success of this noxious weed in the colonization of new ecosystems. In addition, lipids present in the root exudates could promote the growth of mycorrhizal fungi in the *C. diffusa* rhizosphere, which would make it more resistant to soil-borne pathogen attacks.

Materials and Methods

General. NMR spectra were performed on a Varian INOVA 400 instrument, operating at 400 MHz. The spectra were run in CDCl₃; chemical shifts (δ) are given in ppm and the coupling constant (J) in hertz (Hz), using TMS as an internal standard.

GC-MS analyses were carried out in an Agilent 5973N GC/MS instrument operated in the electron ionization mode (70 eV) and equipped with a methyl silicone column (30 x 0.25 mm, film thickness 0.25 µm), using He as carrier gas (1 ml min⁻¹), and 2 µl injection

size. The injector and MS transfer line temperature were set at 275 and 290°C, respectively. The oven temperature was programmed from 80°C (2 min) to 250°C (5 min) at 10°C min⁻¹. Identification of the different root constituents was performed by a comparison of their retention time and mass spectra with those of standard sample or by mass spectra with those stored in a MS database (Wiley 6N).

For HPLC, a Dionex Co., (Sunnyvale, CA, USA) system was equipped as follows: a P680 pump connected to an ASI-100 automated sampler injector; a PDA-100 photodiode array detector; C₁₈ column (4.6 x 150 mm) with particle size 5 µm; flow rate 0.7 ml min⁻¹; mobile phase 0.1% (v/v) acetic acid in H₂O-MeOH; gradient 0 to 10% MeOH 3 min, 10 to 90% MeOH 40 min, 90% MeOH for 15 min.; injection volume 20 µl. The HPLC was coupled to a MSQ-MS detector system (Thermo Electron Co., Waltham, MA, USA), operating in positive mode; electrospray ionization with a N₂ flow at 80 psi, a cone voltage of 70 V, needle voltage of 3 kV, and cone temperature 600°C.

Linoleic acid, ethyl linoleate, ethyl oleate, (-)-caryophyllene oxide, 8-hydroxyquinoline, oleic acid, methyl jasmonate and salicylic acid were purchased from Sigma (St. Louis, MO, USA).

Plant material and extract preparation. Roots of *C. diffusa* Lam., (2 kg fresh weight) were collected in May 2007 from near Boulder, Colorado, (40° 0' 46" N, 105° 17' 48" W) USA. The plant was identified by Timothy Seastedt, Prof. of Ecology and Evolutionary Biology, University of Colorado (Boulder, CO, USA).

Freeze-dried roots (526.6 g) were ground into a powder and soaked in 95% EtOH (2 L x 3) at room temp for 3 days. The extract was filtered and evaporated to dryness in vacuo. The concentrated crude extract (16.5 g) was dissolved in MeOH-H₂O and partitioned into hexane (5 g), CH₂Cl₂ (2 g) and *n*-BuOH (2.5 g) extract residues, successively.

Part of the hexane extract (2 g) was purified on a CombiFlashTM RETRIEVE[®] (ISCO, Lincoln, NE, USA) system, using a normal phase flash column (RediSepTM, ISCO), eluting with hexane and increasing amounts of EtOAc (flow 50 ml/min), to give 12 fractions, which were tested for phytotoxic activity. Active fractions were further analyzed by GC-MS and ¹H NMR.

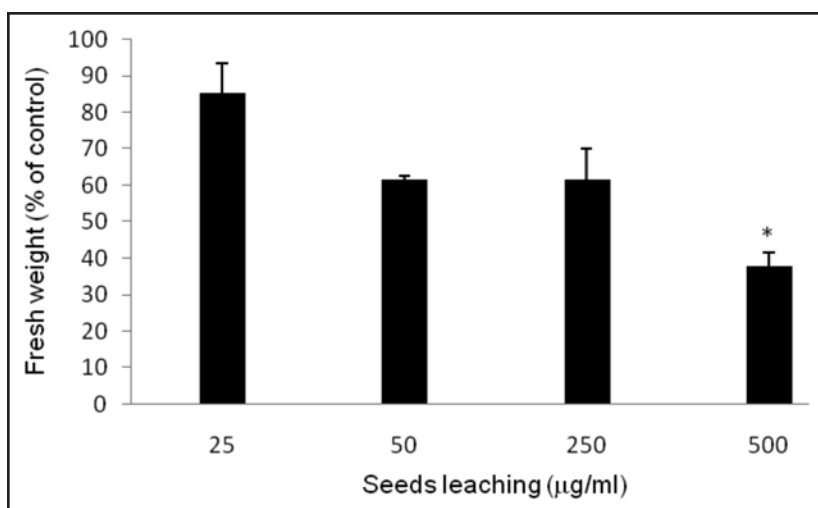


Figure 5. Effect of seed exudates on *A. thaliana* growth. Values are presented as percentage differences from the control. Errors bars are the s.e. of the mean, $n = 4$. * $p < 0.05$.

Root exudates. *C. diffusa* seeds obtained from the Boulder, Colorado natural populations were soaked overnight at 4°C and surface sterilized in an Eppendorf tube $\frac{3}{4}$ filled with 50% commercial bleach and 0.1% sodium dodecyl sulfate (SDS) (Shelton scientific, Inc., Shelton, CT, USA). The tube was then vortexed for five min and rinsed five times with sterile ddH₂O. Sterilized seeds were germinated on solid Murashige and Skoog (MS) medium (Caisson Laboratories, Inc., Rexburg, ID, USA) supplemented with gibberelic acid (Sigma Chemical Co., St. Louis, MO, USA) in a growth chamber (Percival Scientific, IO, USA) with a 16 h daily light period at 25°C. Ten-day-old seedlings were transferred to previously sterilized 50 ml test tubes (Bellco Biotechnology, Vineland, NJ, USA) with 10 ml of liquid MS medium supplemented with 3% sucrose. Plant cultures were maintained in a rotary shaker at 90 rpm under cool-white fluorescent lights ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16 h daily light period at 25°C. Thirty-day-old plants were elicited with methyl jasmonate (MeJA) and salicylic acid (SA). Solutions of MeJA and SA were prepared in ethanol and added separately to 20 *C. diffusa* seedlings per treatment at a final concentration of 250 μM . The root exudates were collected 3 h after the treatment. A non-elicited control of 20 *C. diffusa* seedlings was also harvested during the same period of time for separate analysis. Elicited and non-elicited root exudates were filtered through Nylon filters of 0.45- μm pore size (Millipore, Durapore® Membrane Filters, Ireland) and extracted with EtOAc (Fisher Co., Fair Lawn, NJ, USA) three times. The resulting EtOAc extracts were concentrated on a rotavapor to yield oily residues which were subjected to NMR and HPLC-MS analyses.

Seed leaching. *C. diffusa* seeds were added to 500 μl of ddH₂O and kept at 4°C overnight. The resultant liquid was filtered through Nylon filters of 0.45- μm pore size (Millipore, Durapore® Membrane Filters, Ireland) prior to lyophilization (Labconco Kansas City, MO, USA).

Phytotoxic activity assay. Root extracts, fractions and pure compounds were evaluated for growth inhibition on *Arabidopsis thaliana* seedlings. *A. thaliana* seeds (Lehle Seeds, Round Rock, TX, USA) were surface-sterilized with sodium hypochlorite (3% v/v) for one min, washed five times in distilled sterile ddH₂O and germinated

on solid MS (Caisson Laboratories, Inc., Rexburg, ID, USA) medium in a growth chamber (Percival Scientific, IO, USA) with a 16 h daily light period at 25°C. The 7-d-old seedlings were transferred into 1 ml of liquid MS medium supplemented with 1% sucrose in 24-well plates (VWR Scientific) and incubated on a rotary shaker at 90 rpm. After 24 h, in four replicate experiments the plants were treated with four concentrations of each test solution (25, 50, 250 and 500 $\mu\text{g ml}^{-1}$) and a control. Samples to be tested were dissolved in MeOH or DMSO, and applied to the 24-well plates containing the seedlings. Pure MeOH or DMSO (50 μl) was added to control wells. After treatment, plates were sealed with Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA), incubated on a rotary shaker and grown for seven days under cool-white fluorescent lights ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16 h daily light period at 25°C. After this period the fresh weight of each plant was recorded and the percentage of the fresh weight of the plants was determined by reference to the fresh weight of control plants.

The statistical significance of plant growth inhibition relative to controls between extracts, fractions and pure compounds was determined by a Student's t-test, calculating the mean values and their population variance. The level of significance was $p < 0.05$.

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