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# Phytotoxins from the Leaves of *Ruta graveolens*

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Bioassay-guided fractionation of the ethyl acetate extract of *Ruta graveolens* (common rue) leaves led to the isolation of the furanocoumarins 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), and the quinolone alkaloid graveoline as phytotoxic constituents. Graveoline and 8-MOP substantially inhibited growth of *Lactuca sativa* (lettuce) seedlings and reduced chlorophyll content at 100 $\mu$M; this effect was not due to a direct effect on chlorophyll synthesis. Radical growth of *L. sativa* was inhibited by 10 $\mu$M 8-MOP. Graveoline inhibited growth of *Lemna paucicostata* (duckweed) at 100 $\mu$M. This is the first report of the phytotoxic activity of graveoline. Growth of *Agrostis stolonifera* (bentgrass) was inhibited by 5-MOP at 30 $\mu$M. All three compounds substantially reduced cell division in *Allium cepa* (onion) at or below 100 $\mu$M. None of the compounds caused significant cellular leakage of *Cucumis sativus* (cucumber) cotyledon disks at 100 $\mu$M. All three compounds inhibit plant growth, at least partially through inhibition of cell division.

**KEYWORDS:** ***Ruta graveolens*; furanocoumarins; quinolone alkaloid; phytotoxin**

## INTRODUCTION

*Ruta graveolens* L. (Rutaceae), or common rue, originating in Southern Europe, is an evergreen shrub with bluish-green leaves that emit a powerful odor and have a bitter taste. The plant is cited in the ancient herbals and has deep roots in folklore, alchemy, and even demonology. It contains furanocoumarins, acridone and quinolone alkaloids, and flavonoids (*1*, *2*). These and other constituents of *R. graveolens* have significant biological activity. Extracts from *R. graveolens* have been used as an antidote for toxins such as snake and scorpion venoms (*3*), and to treat internal infections, inflammations, eczema, and external ulcers (*2*). In Germany, extracts of *R. graveolens* are marketed for relief of cramps and rheumatism (*4*). Synthetic quinolones have been used for over 40 years as antibacterial agents (*5*). Photochemotherapy with a constituent from *R. graveolens*, 8-methoxypsoralen (8-MOP), in combination with UVA (320−400 nm) radiation has been used to treat cutaneous T-cell lymphoma (*6*).

Fungicidal activity of the essential oil of *R. graveolens* and of 8-MOP has been reported (*7*, *8*). The essential oil of *R. graveolens* inhibits both germination and radical growth in *Raphanus sativus* (radish) (*9*). Elongation of roots of *Cucumis sativus* (cucumber), *Zea mays* (maize), and *Pisum sativum* (garden pea) is inhibited to different degrees by 8-MOP (*10*). 5-Methoxypsoralen (5-MOP) is phytotoxic to *R. sativus*, but 8-MOP has no activity (*11*).

Natural products may offer novel molecular target sites and mechanisms of action for new herbicides (*12*). The environmental half-life of many natural compounds is shorter, and they are generally less toxic to the environment than are many synthetic herbicides (*13*). Previous research has indicated that *R. graveolens* L. contains phytotoxic or allelopathic compounds (*11*, *14*), but bioassay-directed isolation of compounds from this source for this biological activity has not been conducted. This paper describes the bioassay-guided isolation and identification of the phytotoxic constituents of *R. graveolens* L. leaves and their inhibitory effects on the growth of the dicot *Lactuca sativa* L. (lettuce), the monocot *Agrostis stolonifera* L. (bentgrass), and the aquatic plant *Lemna paucicostata* L. (duckweed), as well as mitotic effects on *Allium cepa* L. (onion).

## MATERIALS AND METHODS

**Plant Material.** Fresh *R. graveolens* plants were purchased from Elixir Farm Botanicals, Brixey, MO. The leaves were separated from the flowers and stems. *L. sativa* cv. Burpee's Iceberg, *C. sativus* cv. Straight Eight, and *A. cepa* cv. Evergreen Long White Bunching seeds were purchased from W. Atlee Burpee & Co., Warminster, PA. *A. stolonifera* seeds were purchased from Tee-2-Green Corp., Hubbard, OR.

**General Chemical Methods.** Extracts were analyzed on precoated silica gel 60 $F_{254+365}$ TLC plates (250 $\mu$m thickness, Analtech, Newark, DE). Compounds on the TLC plates were detected by iodine vapor, UV light, or by spraying with anisaldehyde (prepared) or Dragendorff's reagent (Aldrich). Column chromatography was carried out with

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kieselgel 60 (particle size 0.063−0.2 mm, Merck, Germany) by eluting with hexane, EtOAc, and MeOH in varying amounts. All solvents were reagent grade and were used without further purification. Samples of 5-MOP and 8-MOP for spectroscopic comparison were purchased from Aldrich. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer operating at 300 MHz for $^1$H NMR and at 75 MHz for $^{13}$C NMR.

**Extraction and Isolation of Compounds.** Fresh leaves (1800 g) of *R. graveolens* were successively homogenized in a commercial blender and extracted successively with hexane (2 L), EtOAc (2 L), MeOH (2 L), and acetone/$H_2O$ (7:3) (2 L), respectively. The mixture was sonicated for 2 h, steeped overnight at room temperature, and filtered through filter paper (Whatman #1). Solvents were evaporated under reduced pressure. The EtOAc extract (17 g), which possessed the highest activity as a phytotoxin against *L. sativa* and *A. stolonifera* in the bioassays, was further fractionated by silica gel column chromatography with hexane and EtOAc in increasing amounts (0−100%), and then with MeOH in EtOAc (0−30%). A total of 120 fractions (250 mL each) were collected and combined into 43 fractions on the basis of similar TLC profiles. Fraction 12, eluted with hexane/EtOAc (15:85) (1 L), yielded white crystals of 5-MOP (96 mg), which were purified by recrystallization from a mixture of EtOAc and hexane. Phytotoxic fractions 13−18, eluted with hexane/EtOAc (1:4) (3 L), (25:75) (2 L), and (3:7) (2 L), were combined and yielded white crystals of 8-MOP (154 mg), which were further purified by recrystallization from a mixture of EtOAc and hexane. Phytotoxic fractions 33−38 were combined and concentrated under reduced pressure, rechromatographed on a silica gel column (50 × 450 mm), and eluted with a mixture of hexane and increasing amounts of EtOAc. Fraction 8 (700 mg), eluted from the column with EtOAc (1.2 L), was further purified by preparative TLC with acetone/hexane (45:55) to afford graveoline (217 mg).

**Bioassays in *L. sativa* and *A. stolonifera*.** Silica gel column chromatographic fractionation of the EtOAc extract of the leaves guided by *L. sativa* and *A. stolonifera* bioassays according to Dayan et al. (*15*) were used to identify and isolate the phytotoxic fractions. A filter paper (Whatman #1) and 5 *L. sativa* seeds or 10 mg of *A. stolonifera* seeds were placed in each well of 24-well multiwell plates (Corning Inc., Corning, NY). Test fractions were dissolved in acetone and mixed with distilled deionized (DDI) $H_2O$ such that the final concentration of acetone was 3%. To each test well, 250 $\mu$L of the DDI $H_2O$ mixture was added. Only acetone and DDI $H_2O$ were added to each control well. Plates were covered, sealed with Parafilm, and incubated at 26 °C in a Conviron growth chamber at 173 $\mu$mol s$^{-1}$ m$^{-2}$ continuous light intensity. Phytotoxicity was qualitatively evaluated by visually comparing the amount of germination of the seeds in each well with the untreated controls after 7 days for *L. sativa* and after 12 days for *A. stolonifera*. The qualitative estimate of phytotoxicity was evaluated by using a rating scale of 0−5, where 0 = no effect and 5 = no growth or no germination of the seeds. Each experiment was repeated in triplicate.

The quantitative measure of the phytotoxicity for *L. sativa* was also evaluated by weighing the plants from each well and measuring root lengths and chlorophyll content as compared to untreated controls.

To measure chlorophyll content, the cotyledons of three plants from each well were removed, weighed, and placed in a test tube with 2 mL of DMSO. Test tubes were sealed and incubated at 60 °C for 2 h (*16*). After the test tubes were allowed to cool to room temperature, an additional 1 mL of DMSO was added to each test tube, and the tubes were vortexed. Absorbance was measured with a Shimadzu UV-3101 UV−Vis NIR scanning spectrophotometer at 645 and 663 nm using 1-cm, plastic cuvettes. The amount of chlorophyll (mg/L) was determined by the Nernst equation: Total chlorophyll mg of chl/L = $20.2A_{645} + 8.02A_{663}$, where $A_{645}$ = absorbance at 645 nm and $A_{663}$ = absorbance at 663 nm (*17*). Chlorophyll measurements were standardized by dividing by the weight of the cotyledons and multiplying by the average weight of the cotyledons from three plants.

Bioassays with *L. sativa* were repeated as before but using a final concentration of 1% acetone. Only control + solvent and concentrations of 100 $\mu$M were tested. Each plate was wrapped in foil and incubated at 26 °C for 7 days. The foil was removed, and each plate was exposed to 80 $\mu$mol s$^{-1}$ m$^{-2}$ PAR white light for 5 min. The plates were rewrapped with foil and incubated for 1 h. Ten cotyledon pairs were removed for each test concentration, weighed, and placed in test tubes with 2 mL of DMSO as before, but in the dark. Chlorophyll concentration was determined as discussed above.

**Bioassays in *L. paucicostata*.** *L. paucicostata* stocks were grown from a single colony consisting of a mother and two daughter fronds in a beaker on modified Hoagland media containing 1515 mg/L $KNO_3$, 680 mg/L $KH_2PO4$, 492 mg/L $MgSO_4 \cdot 7H_2O$, 20 mg/L $Na_2CO_3$, 1180 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mg/L $MnCl_2$, 0.025 mg/L $CoCl_2$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, and 18.355 mg/L Fe-EDTA. The media was adjusted to pH 5.5 with 1 M NaOH and filtered through a 0.2 $\mu$m filter. Each well of nonpyrogenic polystyrene sterile 6-well plates (CoStar 3506, Corning Inc., Corning, NY) was filled with 4950 $\mu$L of the Hoagland media mixed with 50 $\mu$L of DDI water, 50 $\mu$L of acetone with the appropriate concentration of 5-MOP or 8-MOP, or 50 $\mu$L of EtOH with the appropriate concentration of graveoline. The final concentration of acetone or EtOH was 1%. Two three-frond colonies from 4- to 5-day-old stock cultures were placed in each well. Total frond area per well was recorded by the image analysis system Scanalyzer (LemnaTec, Würselen, Germany) from days 0 to 7 (*18*).

**Mitotic Indexing.** Mitotic indexing was performed according to Armbruster et al. (*19*). A filter paper (Whatman #1) and 10 surface sterilized *A. cepa* seeds were placed in 6-cm Petri dishes. An appropriate amount of each compound, dissolved in acetone, was mixed with 1 mL of DDI $H_2O$ and was added to each dish for a final concentration of 100 $\mu$M. DDI $H_2O$ or a mixture of acetone and DDI $H_2O$ were added to each control dish. The final concentration of acetone in each dish was 0.05%. Dishes were covered, sealed with Parafilm, and incubated at 25 °C in a Conviron growth chamber at 173 $\mu$mol s$^{-1}$ m$^{-2}$ under a 16 h photoperiod. On the seventh day, root tips were fixed in glacial HOAc/absolute EtOH (1:3) for 30 min. The root tips were then hydrolyzed in 5 N HCl for 1 h at room temperature and then washed repeatedly with DDI $H_2O$ after hydrolysis. Root tips were stained in the dark for 45 min using Schiff's reagent (*20*) and then were fragmented with a glass rod in 45% glacial HOAc. Mitotic stages were observed in at least 1000 cells per slide at a magnification of 40× with an Olympus BX-60 microscope.

**Cellular Leakage Test.** Using a modification of the method of Duke and Kenyon (*21*), C. *sativus* seeds were grown in flats with Miracle Grow potting soil in a Conviron growth chamber under 173 $\mu$mol s$^{-1}$ m$^{-2}$ continuous light intensity at 26 °C for 6 days. Disks 4 mm in diameter were cut from cotyledons of six-day-old plants with a cork borer under dim green light. Fifty disks were placed in polystyrene Petri dishes (6 cm in diameter) with 5 mL of 1 mM MES buffer [2-(4-morpholino)ethane sulfonic acid] supplemented with 2% sucrose (by weight) and were adjusted to pH 6.5 with 1 N NaOH. The MES buffer contained an appropriate amount of diluent or test compound. The test compound was dissolved in acetone and was added directly to the buffer. Compounds were tested at concentrations of 10, 33, and 100 $\mu$M.

Electrical conductivity readings of the bathing solutions were taken with a dip cell and a model 1056 digital conductivity meter (VWR Scientific) to measure cellular leakage. An initial 0 h conductivity reading was taken in the dark, and dishes were covered with aluminum foil and placed in the dark for 18 h. Another reading was taken in the dark before placing the dishes in the light (200 $\mu$mol s$^{-1}$ m$^{-2}$ PAR). Readings were taken at 0, 1, 2, 4, and 6 h, and the results were plotted as changes in conductivity based on the second "dark" reading or beginning of the light phase. The experiment was performed in triplicate.

## RESULTS AND DISCUSSION

Among sequentially extracted material of *R. graveolens* leaves with hexane, EtOAc, and MeOH, the EtOAc extract had the strongest growth inhibitory activity against *L. sativa* and *A. stolonifera*, causing phytotoxicity rankings of 3 and 4, respectively, at a concentration of 1 mg/mL. The EtOAc fraction was thus subjected to bioassay-guided fractionation using silica gel column chromatography. Bioassay-guided fractionation yielded three fractions which were phytotoxic to *L. sativa* and *A.*

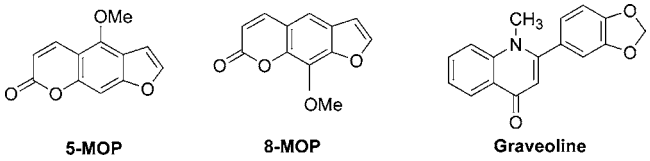


**Figure 1.** Structures of phytotoxic compounds isolated from *R. graveolens*.

*stolonifera*. The activity values of these crude fractions at 1 mg/mL for *L. sativa* and *A. stolonifera*, respectively, were 2 and 5 for fraction 12 (containing 5-MOP), 3−5 and 2−4 for fractions 13 through 18 (containing 8-MOP), and 3−4 and 3−4 for fractions 33 through 38 (containing graveoline). Further chromatographic purification of the active fractions, followed by recrystallization, yielded three compounds as white crystals which were identified as 5-MOP, 8-MOP, and graveoline (**Figure 1**) by spectroscopic comparison with authentic samples and with spectra reported in the literature (*22*).

Phytotoxic effects of the isolated compounds on *L. sativa* were evaluated in a dose−response manner (**Figure 2**). Graveoline and 8-MOP substantially reduced chlorophyll content and plant weight at 100 $\mu$M. Leaves of seedlings treated with these two compounds were chlorotic, indicating that these compounds affected chlorophyll accumulation. When plants were treated with the compounds in darkness for 7 days and then given a protochlorophyll(ide)-converting, 5 min exposure to white light (*23*), followed by a return to darkness for 1 h before extraction of newly formed chlorophyll, no effects of any of the compounds were found on chlorophyll content (data not shown). This indicates that chlorosis was not caused by a direct effect on chlorophyll synthesis. A 39% reduction in radical length was caused by 10 $\mu$M 8-MOP, and root tips were blunt and unbranched as compared to the branched roots on the controls. The 8-MOP analogue, 5-MOP, had a negligible affect on *L. sativa*. Cellular leakage tests on *C. sativus* were conducted (data not shown), but no significant leakage was observed for any of the compounds at 100 $\mu$M, over 18 h in darkness and 24 h in the light.

Phytotoxic effects of the isolated compounds on the monocot *A. stolonifera* were determined at concentrations of 10, 33, 100, and 333 $\mu$M (**Figure 3**). There was little difference between the inhibitory effects of these compounds on this monocot, and all caused plant death at 333 $\mu$M.

Phytotoxic effects of the three compounds on *L. paucicostata* were evaluated in a dose−response manner. Graveoline substantially affected growth of *L. paucicostata* at 100 $\mu$M (**Figure 4**) and caused tissue degradation 250 $\mu$M and above, while the other compounds had a negligible effect (data not shown). Hormesis appeared to occur at low concentrations of graveoline. Other phytotoxins sometimes cause such a hormetic effect at concentrations below the concentrations at which phytotoxicity can be measured (*18*). Using the same bioassay, at least four commercial herbicides have $EC_{50}$ values similar to or higher than graveoline (*18*).

Phytotoxic effects of all compounds on mitotic indices of *A. cepa* root tips were determined at 100 $\mu$M, and 8-MOP was also tested at 33 $\mu$M (**Figure 5**). All compounds greatly inhibited cell division at 100 $\mu$M, with almost complete inhibition of cell division by 8-MOP at this concentration. Even at 33 $\mu$M, few cells were in prophase and metaphase, while no cells were in anaphase and telophase. Representative micrographs of controls and cells treated with each compound are shown in **Figure 6**.

In summary, the furanocoumarin, 5-MOP, had selective activity, only inhibiting growth of the monocot *A. stolonifera* and interfering with cell division in *A. cepa*, whereas 8-MOP had the strongest phytotoxicity toward *L. sativa*, causing a reduction in chlorophyll content and plant weight, and inhibition of radical elongation. 8-MOP also caused the greatest inhibition of cell division in *A. cepa*. Both 5-MOP and 8-MOP had a negligible effect on *L. paucicostata*. Since both 5-MOP and 8-MOP are known constituents of *R. graveolens* (*11*, *14*) and have previously been reported to be phytotoxic (*11*, *14*, *24*−*26*), it was not surprising for them to be found in a bioassay-directed isolation of phytotoxic compounds of *R. graveolens*.

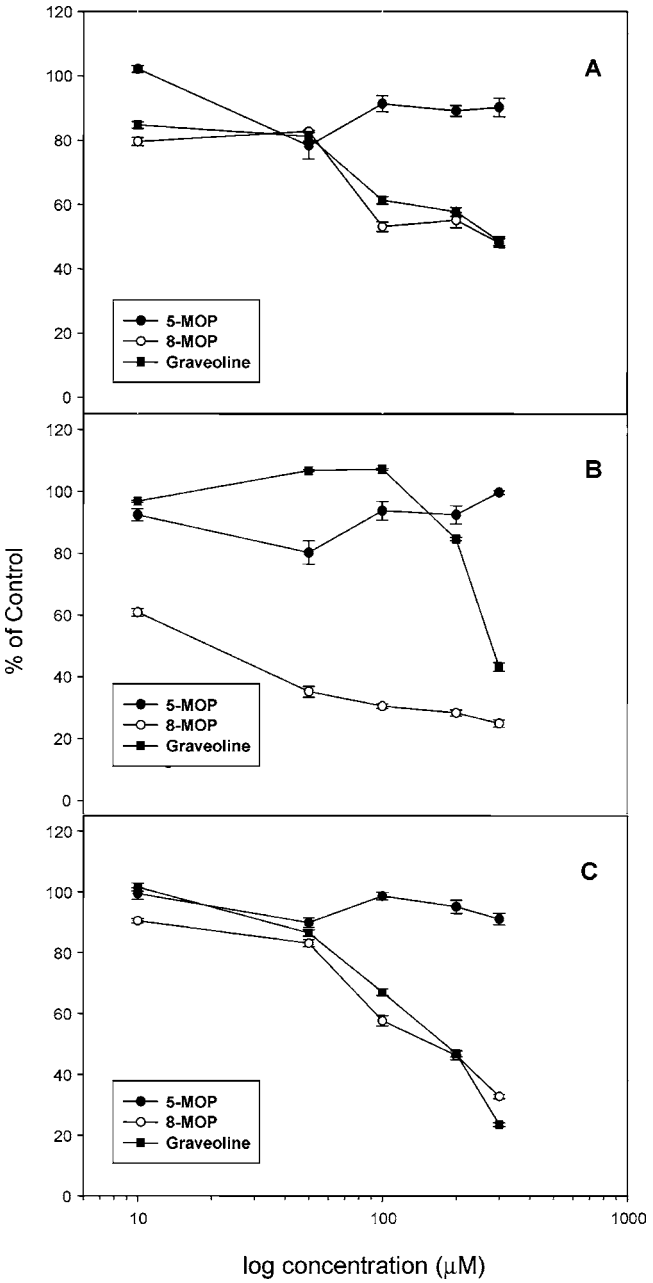


**Figure 2.** Effects of compounds isolated from *R. graveolens* on (**A**) plant weight, (**B**) radical length, and (**C**) chlorophyll content of *L. sativa* at 7 days. Bars represent the standard error of each mean.

Finding graveoline, another known component of *R. graveolens* (*27*), to be a phytotoxin was a new finding. Graveoline caused a reduction in both plant weight and chlorophyll content in *L. sativa*, but no substantial reduction in root length below 200 $\mu$M, suggesting that the mechanisms of action for 8-MOP and graveoline are probably different. Graveoline also affected growth of *L. paucicostata* at low concentrations and substantially affected cell division in *A. cepa*.

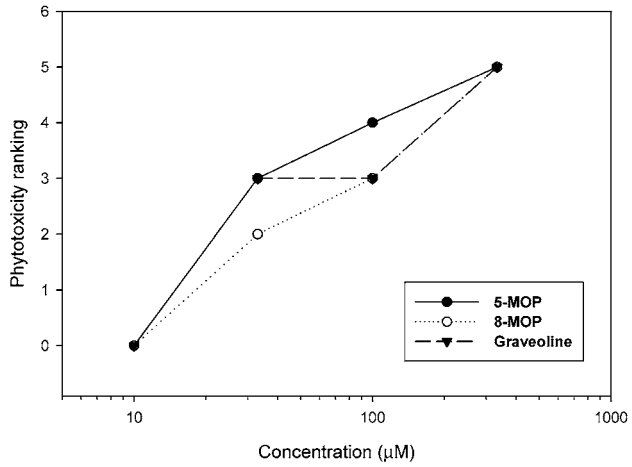


**Figure 3.** Effects of compounds isolated from *R. graveolens* on *A. stolonifera* at 7 days, where 0 = no effect and 5 = no germination or no growth.

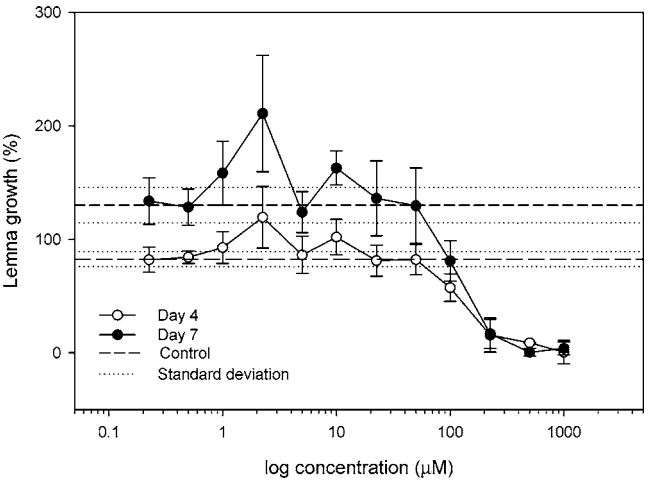


**Figure 4.** Effect of graveoline on growth of *L. paucicostata* at 4 and 7 days. Bars represent the standard error of each mean. Bold dashed lines represent the mean values of untreated plants, and dotted lines represent the standard errors of the means of these control values.

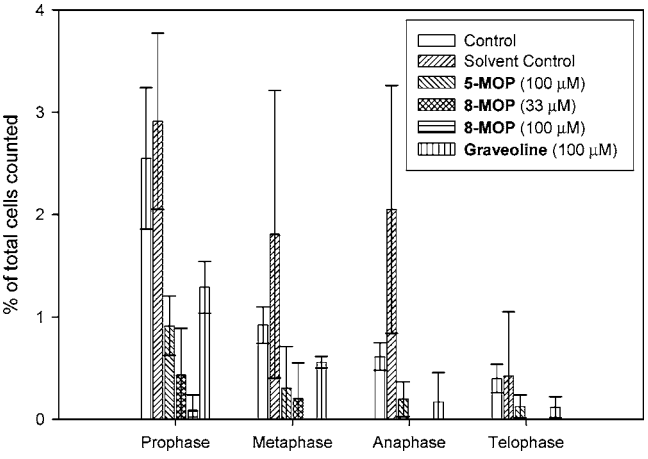


**Figure 5.** Effects of compounds isolated from *R. graveolens* on mitotic indices of *A. cepa*. Bars represent the standard error of each mean.

Cellular leakage results on *C. sativus* cotyledons indicated no significant effects of any of the compounds; hence, the phytotoxic mode of action of these compounds is unlikely to be related a direct or indirect effect on plasma membrane function. Binding of furanocoumarins to proteins and nucleic acids has been observed (*28*), providing a probable mechanism for inhibition of mitosis. The structural difference between 5-MOP and 8-MOP is limited to the position of the methoxy group, but there is a significant difference in their phytotoxicity. Graveoline has been reported to possess antifungal activity (*8*), but this is the first report of its phytotoxic activity. The putative mode of action of the furanocoumarins found to be phytotoxic in this study would preclude them from use as commercial herbicides; however, the graveoline structure could be a template for further discovery efforts. Graveoline is cytotoxic to human cancer cell lines (*27*), but such an activity would not preclude it from consideration as an agrochemical.

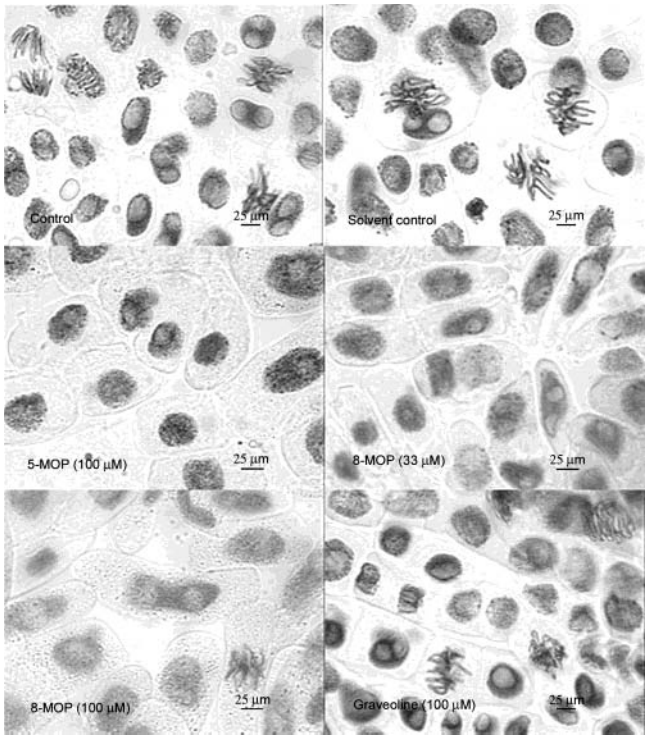


**Figure 6.** Representative light micrographs of cell division in *A. cepa* comparing controls with cells treated with compounds isolated from *R. graveolens*. Bars represent 25 $\mu$m.

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