Mosquitocidal, Nematicidal, and Antifungal Compounds from *Apium graveolens* L. Seeds

Rafikali A. Momin and Muraleedharan G. Nair*

Bioactive Natural Products and Phytoceuticals, Department of Horticulture and National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan 48824

The methanolic extract of *Apium graveolens* seeds was investigated for bioactive compounds and resulted in the isolation and characterization of mosquitocidal, nematicidal, and antifungal compounds sedanolide (**1**), senkyunolide-N (**2**), and senkyunolide-J (**3**). Their structures were determined by ^{1}H and ^{13}C NMR spectral methods. Compounds **1**−**3** gave 100% mortality at 25, 100, and 100 μg mL^{-1}, respectively, on the nematode, *Panagrellus redivivus*. Compound **1** showed 100% mortality at 50 μg mL^{-1} on nematode, *Caenorhabditis elegans*, and fourth-instar mosquito larvae, *Aedes aegyptii*. Also, it inhibited the growth of *Candida albicans* and *Candida parapsilasis* at 100 μg mL^{-1}. Compounds **2** and **3** were isolated for the first time from *A. graveolens*. This is the first report of the mosquitocidal, nematicidal, and antifungal activities of compounds **1**−**3**.

Keywords: *Apium graveolens; Umbelliferae; celery; mosquitocidal; nematicidal; antifungal; sedanolide; senkyunolide*

INTRODUCTION

Apium graveolens L., celery, is widely grown in the temperate zone as a garden crop, and its leaf stalks are used as a popular vegetable. In the United States, celery fruit, commonly known as "celery seed", was largely imported from Europe, but since World War II, it has been supplied from domestic growers, especially in Michigan and Wisconsin. Celery seeds possess a characteristic aroma and pungent taste and are used as a condiment in the flavoring of food products. Celery can be classified both as a seasoning and as a vegetable. For seasoning purposes, the seeds and the plant, as well as the essential oil distilled from the seeds, are used (*1*). Celery seed extracts are extensively used as flavoring ingredients in many food products, including alcoholic and nonalcoholic beverages, frozen dairy desserts, candy, baked goods, gelatins, puddings, meat products, condiments and relishes, soups, gravies, snack foods, and others. Celery seeds are used for treating bronchitis, asthma, liver, and spleen diseases (*2*, *3*).

Sedanolide, considered to be one of the celery flavor compounds (*4*, *5*), was reported from celery seed oil in 1950 (*6*). Since the isolation of sedanolide, several other phthalides such as sedanenolide, 3-*n*-butylphthalide, cnidilide, neocnidilide, ligustilide, and 3-isobutylidene-3a,4-dihydrophthalide have been reported from celery seed oil (*4*, *7*, *8*). In this paper we report for the first time the mosquitocidal, nematicidal, and antifungal activities of compounds **1**−**3**.

MATERIALS AND METHODS

General Experimental Procedures. All NMR spectra (^{1}H and ^{13}C) were recorded on a Varian INOVA 300 MHz spectrometer. ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts were recorded in $CDCl_3$, and the values are in δ (parts per million) based on δ residual of $CHCl_3$ at 7.24 for ^{1}H NMR and of $CDCl_3$ at 77 ppm for ^{13}C NMR. Coupling constants, *J*, are in hertz. The silica gel used for VLC and MPLC was Merck silica gel 60 (30−70 μm particle size). For preparative HPLC (LC-20, Japan Analytical Industry Co., Tokyo) purification, two JAIGEL-ODS, A-343-10 (20 mm × 250 mm, 10 μm, Dychrom, Santa Clara, CA) columns were used in tandem. Peaks were detected using a UV detector equipped with a model D-2500 chromatointegrator (Hitachi, Tokyo). The CD analyses of compounds **2** and **3** were performed on a JASCO J-710 CD-ORD spectropolarimeter. Nitrogen was generated by a nitrogen generator model NG-150 at a rate of 40 L min^{-1}. Compounds **2** and **3** were dissolved in methanol separately, and the CD was determined at 185−400 nm. All solvents were of ACS reagent grade and were purchased from Aldrich Chemical Co., Inc.

Plant Material. Celery seeds were donated by Asgrow Seed Co., Kalamazoo, MI, and stored at −20 °C until extraction.

Extraction and Isolation. The seeds (905 g) were milled using an industrial Waring blender and extracted with hexane (4 × 1.5 L, 48 h) to yield the hexane extract (85 g). The plant residue was then extracted with MeOH (3 × 1.5 L, 48 h) to yield the MeOH extract (20 g). The solvents were evaporated under reduced pressure at 40 °C to yield crude extracts. The MeOH extract (19 g) was stirred with hexane (2 × 400 mL, 15 min) to yield hexane soluble (7.4 g) and insoluble (11.5 g) fractions. The hexane soluble fraction (6 g) was further separated by vacuum liquid chromatography (VLC) followed by medium-pressure liquid chromatography (MPLC) on silica gel (Sanki Engineering Ltd., model LBP-V pump operating at 10−15 psi; Chemco MPLC glass column, 55 cm in length) using hexane with increasing amounts of acetone and finally with MeOH as the eluting solvents. The fractions collected were as follows: A, 404 mg, hexane/acetone, 4:1, 250 mL; B, 142 mg, hexane/acetone, 2:1, 80 mL; C, 483 mg, hexane/acetone, 2:1, 100 mL; D, 79 mg, hexane/acetone, 2:1, 120 mL; E, 61 mg, hexane/acetone, 1:1, 100 mL; F, 89 mg, hexane/acetone, 1:1, 200 mL; G, 35 mg, hexane/acetone, 1:1, 100 mL; H, 14 mg, 100% acetone, 20 mL; I, 33 mg, 100% acetone, 130 mL; and J, 8 mg, 100% MeOH, 250 mL. Mosquitocidal, nematicidal, and antifungal bioassays on these fractions revealed that fraction B was active. The bioactive fraction B (135 mg) was purified by preparative TLC (hexane/acetone, 20:1 × 3) to yield three

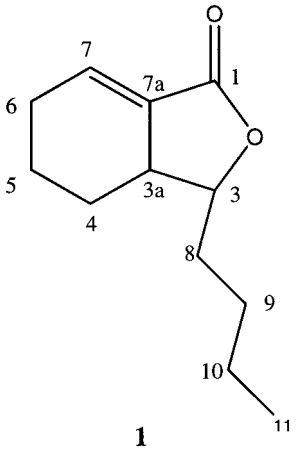
* Author to whom correspondence should be addressed [telephone (517) 353-2915; fax (517) 432-2310; e-mail nairm@msu.edu].

10.1021/jf001052a CCC: $20.00
Published on Web 11/28/2000

major bands: I, (48 mg, R_f 0.18; II, 65 mg, R_f 0.35; and III, 16 mg, R_f 0.50. Band II was biologically active and was further purified by repeated preparative TLC (hexane/ether 8:1 × 4 and 2:1 × 1) to yield a pure compound **1** (22 mg).

The hexane insoluble fraction (11 g) of MeOH extract was separated into chloroform soluble (2.1 g) and insoluble (8.2 g) fractions by stirring it with chloroform (2 × 50 mL, 15 min). The chloroform soluble fraction (2.1 g) was further separated into hexane soluble (607 mg) and insoluble (1.5 g) fractions. The bioactive hexane insoluble fraction was further separated into methanol/water, 75:25, soluble and insoluble fractions, and the precipitate was removed by centrifugation. The soluble fraction (684 mg) was separated into eight fractions by preparative HPLC using methanol/water, 75:25, as a mobile phase at flow rate of 3 mL min^{-1}. Fraction II (t_R = 48 min, 75 mg) was further purified by HPLC using methanol/water, 60:40, as a mobile phase at flow rate of 2 mL min^{-1} to yield a 1:1 mixture of compounds **2** and **3** (t_R = 65 min, 12 mg). This mixture was separated into pure compounds **2** and **3** by HPLC using H_2O/THF, 90:10, as a mobile phase at the flow rate of 4 mL min^{-1}.

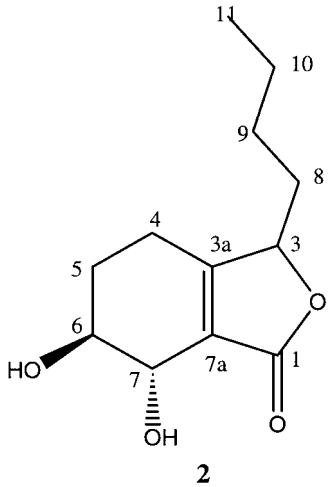
*Compound **1***: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83 (3H, t, J = 7.1 Hz, H-11), 1.00−2.20 (10H, m, H-4,5,8,9,10), 2.22 (1H, m, H-3a), 2.41 (2H, m, H-6), 3.87 (1H, m, H-3), 6.66 (1H, dd, J = 6.3, 3.0 Hz, H-7); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.87 (C-11),



1

20.74 (C-5), 22.50 (C-10), 24.96 (C-4), 25.34 (C-9), 27.49 (C-8), 34.30 (C-3a), 43.04 (C-6), 85.35 (C-3), 131.11 (C-7a), 135.20 (C-7), 170.24 (C-1). Compound **1** was identified as sedanolide. The spectral data of compound **1** were identical to the published values for sedanolide (*9*).

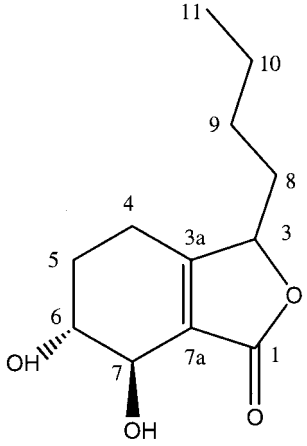
*Compound **2***: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.91 (3H, t, J = 7.2 Hz, H-11), 1.30−1.45 (4H, m, H-9,10), 1.50−2.21 (4H, m, H-5,8), 2.35−2.40 (2H, m, H-4), 3.91 (1H, ddd, J = 9.8, 6.1, 3.3 Hz, H-6), 4.41 (1H, dddd, J = 6.1, 2.5, 2.0, 1.9 Hz, H-7), 4.86 (1H, ddd, J = 8.1, 3.6, 2.4 Hz, H-3); ^{13}C NMR (75 MHz,



2

$CDCl_3$) δ 13.80 (C-11), 21.3 (C-4), 22.4 (C-10), 26.5 (C-5), 26.8 (C-9), 31.9 (C-8), 67.4 (C-7), 71.5 (C-6), 82.9 (C-3), 126.3 (C-7a), 166.7 (C-3a), 173.0 (C-1). Compound **2** was identified as senkyunolide-N by comparison of its ^{1}H and ^{13}C NMR spectral data with published values (*10*).

*Compound **3***: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.91 (3H, t, J = 7.2 Hz, H-11), 1.30−1.45 (4H, m, H-9,10), 1.50−2.21 (4H, m, H-5,8), 2.35−2.40 (2H, m, H-4), 3.91 (1H, ddd, J = 9.8, 6.1, 3.3 Hz, H-6), 4.41 (1H, dddd, J = 6.1, 2.5, 2.0, 1.9 Hz, H-7), 4.86 (1H, ddd, J = 8.1, 3.6, 2.4 Hz, H-3); ^{13}C NMR (75 MHz,



3

$CDCl_3$) δ 13.80 (C-11), 21.1 (C-4), 22.3 (C-10), 26.3 (C-5), 26.6 (C-9), 31.8 (C-8), 67.3 (C-7), 71.3 (C-6), 82.8 (C-3), 126.1 (C-7a), 166.5 (C-3a), 172.9 (C-1). Compound **3** was identified as senkyunolide-J by comparing its ^{1}H and ^{13}C NMR spectral data with published values (*10*).

Mosquitocidal Assay. Fourth-instar mosquito larvae, *Aedes aegyptii*, were reared from neonates in our laboratory. Ten to 15 larvae were placed in 980 μL of degassed distilled water, and 20 μL of DMSO or DMSO solution containing test extracts or pure compounds was added. Extracts were tested at 250 μg mL^{-1}, and pure compounds were tested at 1−200 μg mL^{-1} concentrations. There were three replicates per treatment. The number of dead larvae were recorded at 2-, 4-, 6-, and 24-h intervals. The control was prepared with 980 μL of degassed distilled water and 20 μL of DMSO solution to which larvae were added (*11*).

Nematicidal Assay. Nematode cultures, *Panagrellus redivivus* and *Caenorhabditis elegans*, were maintained in our laboratory. *P. redivivus* was grown in axenic, liquid basal heme medium (5 mL) in scintillation vials. *C. elegans* was maintained on NG agar medium containing a strain of *Escherichia coli* in disposable Petri dishes wet with 2−4 mL of physiological saline solution. Cultures were stored at room temperature and subcultured prior to the assay. The assay was conducted in Corning polystyrene 96-well plates. Nematodes were added to 1 mL of physiological saline solution in a scintillation vial. This solution was diluted until the nematodes count were 15−20 in a 48-μL aliquot. This solution (48 μL) containing nematodes was delivered to each of three wells per treatment. Two microliters of DMSO (50%) or test compound in 50% DMSO was added to each well. The plate was covered, parafilmed, and kept in a humid chamber. The number of dead nematodes was recorded at 2-, 4-, 6-, 8-, and 24-h by observation under a microscope (*12*).

Antifungal Assay. Compounds **1**−**3** were evaluated for antifungal activity according to the reported procedure (*13*). The test organisms *Candida albicans* (MSU strain) and *Candida parapsilasis* (MSU strain) used for antifungal bioassays were cultured in Petri dishes containing YMG medium (20 mL). Physiological saline solution (2−3 mL) was added to fully grown plate of each organism, and then suspensions were diluted to obtain 5 × 10^6 colony-forming units (CFU)/mL. Bioassays were conducted by spreading 50 μL of the desired cell suspension on Petri dishes of the YMG medium. DMSO

or test compound dissolved in DMSO (20 μL) was spotted carefully on the bioassay plates at various concentrations. Plates were allowed to dry in a laminar flow hood and then incubated at 27 °C for 72 h. Zones of inhibition were measured in millimeter (*11*, *13*). Minimum inhibitory concentration (MIC) was determined for compounds **1**−**3** according to the published procedure (*12*).

RESULTS AND DISCUSSION

Celery seeds were milled and extracted sequentially with hexane and MeOH. The MeOH extract was partitioned into hexane soluble and insoluble fractions. Preliminary bioassays indicated that the hexane soluble fraction was active on *C. albicans*, *C. parapsilasis*, mosquito larvae, *A. aegyptii*, and nematodes, *C. elegans* and *P. redivivus*, whereas the hexane insoluble fraction was active on nematode, *P. redivivus*. The hexane soluble fraction was purified by VLC and MPLC on silica gel using hexane with increasing amounts of acetone and finally with MeOH as the eluting solvents. Ten fractions, A−J, were collected and tested for nematicidal activity. The active fraction B, eluting with hexane/acetone, was further purified by repeated preparative TLC to yield compound **1**.

The hexane insoluble fraction of the MeOH extract of celery seed was further partitioned to yield chloroform soluble and insoluble fractions. The chloroform soluble fraction was active against *P. redivivus*. This fraction was further fractionated into hexane soluble and insoluble fractions. The hexane insoluble fraction was dissolved in MeOH and precipitated with water and centrifuged. Purification of the MeOH/H_2O soluble fraction was carried out by HPLC on two JAIGEL-ODS columns using MeOH/H_2O. The fraction II (t_R = 48 min) was active on *P. redivivus* and was further purified by HPLC using MeOH/H_2O as the mobile phase to yield a mixture of compounds **2** and **3** (t_R = 65 min, 12.5 mg). This mixture was separated into compounds **2** and **3** by HPLC using H_2O/THF as the mobile phase.

The structure of compound **1** was confirmed by ^1H and ^{13}C NMR spectral data and was identical to the published spectral data for sedanolide (*9*). Compounds **2** and **3** were identified as senkyunolide-N and senkyunolide-J, respectively, by comparison of their CD spectra. Also, both ^1H and ^{13}C NMR spectral data for compounds **2** and **3** were identical to the published data (*10*). The CD spectrum of compound **2** showed a positive Cotton effect at 194 nm, indicating that compound **2** has the 6*S* and 7*S* configuration, whereas compound **3** showed a negative Cotton effect at 194 nm, indicating that **3** has the 6*R* and 7*R* configuration (*10*). Thus, the structural identities and stereochemistries of compounds **2** and **3** were confirmed as senkyunolide-N and senkyunolide-J (*10*). Compounds **2** and **3** were previously reported from the dried rhizomes of *Ligusticum chuangxions* (*10*). This is the first report of the isolation of compounds **2** and **3** from *A. graveolens*.

Compound **1** exhibited 100% mortality (LD_{100}) at 25 and 50 μg mL^{-1} when tested against *P. redivivus* and *C. elegans*, respectively (Figures 1 and 2). At 100 μg mL^{-1}, compounds **2** and **3** showed 100% mortality when tested against *P. redivivus* (Figure 1). Also, compound **1** gave 100% mortality at 50 μg mL^{-1} when tested on fourth-instar *A. aegyptii* larvae. Compounds **2** and **3** were not active against fourth-instar *A. aegyptii* larvae. In the preliminary bioassay, only compound **1** gave zones of inhibition of 11 mm each for *C. albicans* and *C. parapsilasis* at 100 μg mL^{-1} on YMG culture plates. Also, it completely inhibited the growth of *C. albicans* and *C. parapsilasis* at 100 μg mL^{-1}.

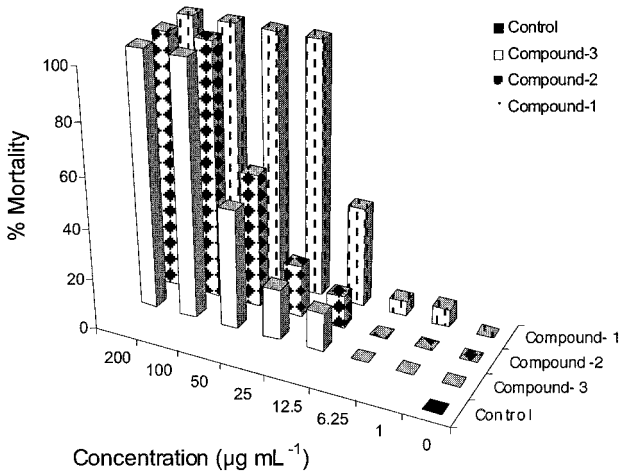


Figure 1. Percent nematicidal activity against *P. redivivus* for compounds **1**−**3** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$), and the means were compared by calculating the least significant difference (LSD).

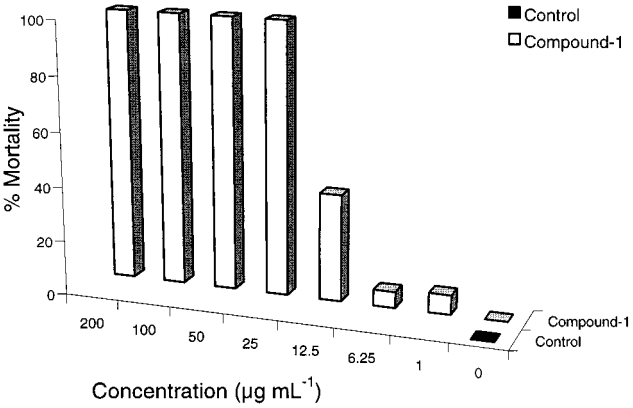


Figure 2. Percent nematicidal activity against *C. elegans* for compound **1** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$), and the means were compared by calculating the least significant difference (LSD).

Phthalides are bioactive natural products and occur widely in umbelliferous plants. Besides its role in the characteristic odor of celery, sedanolide was previously reported as a tumor-inhibitory compound in the forestomach of female A/J mice by inducing the glutathione *S*-transferase enzyme activity (*9*). The Chinese traditional plant, *Ligusticum chuangxiong*, has been used to treat headache, anemia, feeling of cold, and irregularity of menstruation. Senkyunolide-N and -J were reported from the rhizomes of *L. chuangxiong* (*10*). These results support the notion that phthalides are biologically active. The five-membered lactone ring along with the butyl side chain in phthalides may be important for observed biological activities. Zheng et al. (*9*) reported that the five-membered lactone ring in phthalides is important for the high glutathione *S*-transferase activity. Celery seed extracts and oil, which are used in perfumery, for flavoring and seasoning of food, and in pharmaceutical preparations, have also the potential to be used in agriculture pest management, if toxicity and side effects are minimal. The nematicidal and mosquitocidal activities of compounds **1**−**3** suggest that further examination is required for their potential toxicity and side effects.

ACKNOWLEDGMENT

A. aegyptii neonates were supplied by Drs. A. Raikhel and A. Hays, Department of Botany and Plant Pathology, Michigan State University. *S. cerevisiae* strains were provided by Dr. John L. Nitiss, St. Jude Children's Research Hospital, North Lauderdale, Memphis, TN.

LITERATURE CITED

(1) Salzer, U. On the fatty acid composition of the nonvolatile lipoids of some spices. *Fette Seifen Anstrichm.* **1975**, 446.
(2) Chopra, R. N.; Chopra, I. C.; Handa, K. L.; Kapur, L. D. In *Chopra's Indigenous Drugs of India*; Dhur and Sons Publication: Calcutta, India, 1958; p 495.
(3) Satyavati, G. V.; Raina, M. K. In *Medicinal Plants of India*; Indian Council of Medical Research: New Delhi, India, 1976; Vol. I, pp 80, 107.
(4) Bjeldanes, L. F.; Kim, I. S. Phthalide components of celery essential oil. *J. Org. Chem.* **1977**, *42*, 2333−2335.
(5) Tang, J.; Zhang, Y.; Hartman, T. G.; Rosen, R. T.; Ho, C. T. Free and glycosidically bound volatile compounds in fresh celery (*Apium graveolens* L.). *J. Agric. Food Chem.* **1990**, *38*, 1937−1940.
(6) Guenther, E. In *The Essential Oils*; Van Nostrand: New York, 1950; Vol. 4, pp 591−602.
(7) Fehr, D. Essential leaves of celery (*Apium graveolens*). *Pharmazie* **1974**, *29* (5), 349.
(8) Uhlig, J. W.; Chang, A.; Jen, J. J. Effect of phthalides on celery flavor. *J. Food Sci.* **1987**, *52*, 658−660.
(9) Zheng, G. Q.; Zhang, J.; Kenney, P. M.; Lam, L. K. T. Chemoprevention of benzo[a]pyrene induced forestomach cancer in mice by natural phthalides from celery seed oil. *Nutr. Cancer* **1993**, *19*, 77−86.
(10) Naito, T.; Katsuhara, T.; Nitsu, K.; Ikeya, Y.; Okada, M.; Mitsuhashi, H. Two phthalides from *Ligusticum chuangxiong*. *Phytochemistry* **1992**, *13* (2), 639−642.
(11) Roth, G. N.; Chandra, A.; Nair, M. G. Novel bioactivities of *Curcuma longa* constituents. *J. Nat. Prod.* **1998**, *61*, 542−545.
(12) Nair, M. G.; Putnam, A. R.; Mishra, S. K.; Mulks, M. H.; Taft, W. H.; Keller, J. E.; Miller, J. R.; Zhu, P.-P.; Meinhart, J. D.; Lynn, D. G. Faeriefungin: A new broad-spectrum antibiotic from *Streptomyces griseus* var. *autotrophicus*. *J. Nat. Prod.* **1989**, *52*, 797−809.
(13) Chang, Y.-C.; Nair, M. G.; Nitiss, J. L. Metabolites of daidzein and genistein and their biological activities. *J. Nat. Prod.* **1995**, *58*, 1901−1905.

Received for review August 25, 2000. Revised manuscript received October 23, 2000. Accepted October 25, 2000. R.A.M. thanks the Aga Khan Foundation, Geneva, Switzerland, for financial support. NMR data were obtained on instrumentation that was purchased, in part, with funds from NIH Grant 1-S10-RR04750, NSF Grant CHE-88-00770, and NSF Grant CHE-92-13241.

JF001052A