Bioactive Compounds and 1,3-Di[(*cis*)-9-octadecenoyl]-2-[(*cis,cis*)-9,12-octadecadienoyl]glycerol from *Apium Graveolens* L. Seeds

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Bioassay-directed isolation and purification of the hexane extract of *Apium graveolens* L. seeds led to the characterization of three compounds: β-selinene **(1)**, 3-*n*-butyl-4,5-dihydrophthalide **(2)** and 5-allyl-2-methoxyphenol **(3)**. The structures of these compounds were established by using ^{1}H and ^{13}C NMR spectral methods. Compounds, **1**−**3** demonstrated 100% mortality on fourth-instar *Aedes aegyptii* larvae at 50, 25, and 200 μg mL^{-1}, respectively, in 24 h. Also, **2** inhibited the growth of *Candida albicans* and *Candida kruseii* at 100 μg mL^{-1}. It inhibited both topoisomerase-I and -II enzyme activities at 100 μg mL^{-1}. Compound **2** displayed 100% mortality at 12.5 and 50 μg mL^{-1}, respectively, when tested on nematodes, *Panagrellus redivivus* and *Caenorhabditis elegans*. The triglyceride, 1,3-di[(*cis*)-9-octadecenoyl]-2-[(*cis,cis*)-9,12-octadecadienoyl]glycerol (**4**) and **3** were isolated for the first time from *A. graveolens* seeds, although **4** was not biologically active.

Keywords: *Apium graveolens, Umbelliferae, celery, triglyceride, β-selinene, sedanenolide, chavibetol, mosquitocidal, antimicrobial, nematicidal, topoisomerase*

INTRODUCTION

Apium graveolens L. (Umbelliferae), celery, is a hepaxanthic herb grown as a biennial and under certain conditions, as an annual. Celery is a native of Eurasia and is grown mainly in coastal regions. Celery is widely cultivated in the temperate zones as an important garden crop and the bleached leaf stalks are relished as a popular vegetable. Celery seeds are used in India to treat bronchitis, asthma, liver, and spleen diseases (Satyavati and Raina, 1976). *A. graveolens* is one of the ingredients in 8 of the 33 Indian polyherbal formulations with reputed life-protecting activity (Handa et al., 1986). Several components from celery seeds were also reported for their anticarcinogenic activity (Zheng et al., 1994).

Celery seed contains a variety of constituents. As a member of the class of bioactive natural products, phthalides occurs widely in umbelliferous plants (Bjeldanes and Kim, 1977; Chulia et al., 1986; Kaouadji et al., 1984; Kaouadji et al., 1986, Banerjee et al., 1982). Earlier studies of *A. graveolens* led to the isolation of limonene, *p*-mentha-2,8-dien-1-ol, *p*-mentha-8(9)-en-1,2-diol, 3-*n*-butylphthalide, sedanolide (Zheng et al., 1994), selinene (Semmler and Risse, 1912), selinenol (Ruzicka and Stoll, 1923) and seselin, isoimperatorin, osthenol, bergapten, isopimpinellin, apigravin, and apiumoside (Grag et al., 1978, 1979a,b).

Previous research on *A. graveolens* was focused on the isolation and structural identification of compounds for perfumery and culinary uses. In this paper, we report three biologically active compounds (**1**−**3**) and a triglyceride (**4**) from the hexane extract of celery seeds.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H and ^{13}C NMR, DEPT, arrayed decoupling, HMQC, and HMBC spectra were recorded on a Varian INOVA 300 MHz spectrometer. ^{13}C NMR and DEPT spectra were recorded at 75 MHz. Chemical shifts were recorded in $CDCl_3$, and the values are in δ (ppm) based on δ residual of $CHCl_3$ at 7.24 for ^{1}H NMR and $CDCl_3$ at 77 ppm for ^{13}C NMR. Coupling constants, *J*, are in Hz. The silica gel used for MPLC was Merck Silica gel 60 (30−70 μm particle size). FAB-MS were recorded at 70 eV. TLC plates (GF Uniplate, with binder, 250 μm, Analtech, Inc., Newark, DE) and preparative TLC plates (Analtech, Sigel, 20 × 20 cm, 250 and 500 μm), after developing, were viewed under UV light (254 and 366 nm). All organic solvents used were ACS reagent grade (Aldrich Chemical Co., Inc., Milwaukee, WI).

Gas chromatography was performed using a HP 6890 gas chromatograph (Algilent Technology, Wilmington, Delaware). Samples (1 μL) were injected into a split injector at 200 °C and separated on an HP-5 capillary column (30 m × 0.25 mm i.d.) with helium carrier gas at a flow rate of 22 mL/min. Compounds were detected using flame ionization at 250 °C. The temperature profile was 150 (2 min) to 200 °C (1 min) at 10 °C min^{-1} and then to 250 °C (2 min) at 2 °C min^{-1}. Methyl esters of palmitate, linolenate, and γ-linolenate were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI.

Plant Material. Celery seeds were provided by Asgrow Seed Company, Kalamazoo, Michigan, and stored at −20 °C until extraction.

Extraction and Isolation. The seeds (100 g) were milled using an industrial Waring blender and extracted sequentially with 500 mL each of hexane, EtOAc, and MeOH over a period of 48 h. The hexane extraction afforded 17 g of residue upon removal of solvent. A portion of this hexane extract (5 g) was stirred with acetone to yield acetone-soluble (2.3 g) and -insoluble (2.6 g) portions. Fractionation of the bioactive acetone-soluble portion (1.6 g) was carried out by medium-pressure liquid chromatography (MPLC) on silica gel (Sanki Engineering Ltd., model LBP-V pump operating at 10−15 psi; Chemco MPLC tayperling type glass column, 55 cm in length) at 2 mL min^{-1} flow rates. The following fractions were collected: I (500 mL) eluted with 100% hexane, II and III with hexane/ether (10:1, 500 mL each), IV (500 mL) with 100% acetone, and V (500 mL) with 100% MeOH. Fractions I and IV were mosquitocidal, and fraction IV was antimicrobial, nematicidal, and inhibitory to Top-I and Top-II enzymes.

Fraction I was purified by repeated preparative TLC and

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yielded fractions A and B. Fraction A was further purified with hexane/ether (10:1) as the mobile phase to yield **4** (104 mg). Isolation of **1** (30 mg) from fraction B was accomplished by using 10:1 and 50:1 hexane/ether mobile phases in repeated preparative TLC. Fraction IV was purified by preparative TLC using hexane/ether (4:1) and yielded four fractions. The bioactive fraction was separated into hexane-soluble and -insoluble fractions. The hexane-soluble fraction was further purified by preparative TLC (pentane/ether, 4:1) and afforded pure **3** (6 mg). The band at $R_f = 0.45$ was eluted with MeOH on a C_{18}−Sep Pak cartridge and yielded **2** (26 mg).

Compound ***1***. ^{1}H NMR ($CDCl_3$) δ 0.71 (s, 3H, H-14), 0.80 (m, 1H, H-7), 1.2−1.6 [m, 10H, H-1,2,3,6,8) × 2], 1.7 (s, 3H, H-13), 1.9 (m, 2H, H-9), 2.3 (m, 1H, H-5), 4.4−4.7 [bs, 4H, H−(12,15)]; ^{13}C NMR ($CDCl_3$) δ 16.3 (C-14), 21.0 (C-13), 23.5, 26.8, 29.5, 36.9, 41.2, 41.9 (C-1,2,3,6,8,9), 36.0 (C-10), 45.9, 49.9 (C-5,7), 105.4, 108.1 (C-12,15), 150.8, 151.0 (C-4,11). ^{1}H NMR data for **1** were found to be in agreement with previously published data (Bowden et al., 1978).

Compound ***2***. ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3H, J = 7.2, H-4′), 1.2−1.8 [m, 6H, H-(1′,2′,3′)], 2.45 (m, 4H, H-4,5), 4.9 (m, 1H, H-3), 5.9 (m, 1H, H-6), 6.2 (d, 1H, J = 10, H-7); ^{13}C NMR ($CDCl_3$) δ 13.8−22.4 (C-1′,2′,3′,4′), 26.7−31.8 (C-4, 5), 82.5 (C-3), 116.8 (C-7), 128.3 (C-6), 124.5−135 (C-8, 9), 161.4 (C-1). The spectral data confirmed that **2** is 3-*n*-butyl-4,5-dihydrophthalide (Bjeldanes and Kim, 1976).

Compound ***3***. ^{1}H NMR ($CDCl_3$) δ 3.32 (d, 2H, J = 7.0, H-7), 3.84 (s, 3H, $-OCH_3$), 5.08 (m, 2H, H-9), 5.45 (s, 1H, −OH), 5.90 (m, 1H, H-8), 6.66−6.69 (m, 2H, H-3,5), 6.83 (d, 1H, J = 8.5, H-6); ^{13}C NMR ($CDCl_3$) δ 39.8 (C-7), 55.8 ($-OCH_3$), 110.9 (C-9), 114.0 (C-8), 115.4 (C-6), 121.1 (C-5), 131.7 (C-4), 137.8 (C-3), 146.3 (C-2), 146.4 (C-1). The ^{1}H NMR data were found to be in agreement with previously published data for **3** (Kurihara and Kikuchi, 1979).

Compound ***4***. ^{1}H NMR ($CDCl_3$) δ 0.86 [t, 9H, J = 6.6, H-(18′ × 2), 18″], 1.24 [m, 54H, H-(4′-7′,12′-17′) × 2 and H-(4″-7″, 15″-17″)], 1.6 [m, 6H, H-(3′ × 2), 3″], 2.02 [m, 12H, H-(8′, 11′) × 2 and H-(8″,14″)], 2.3 [t, 6H, J = 7.2, H-(2′ × 2), 2″], 2.76 [dd, 2H, J = 6.1, 5.5, H-11″], 4.12 (dd, 2H, J = 18.0, 6.0, H-1a, 3a), 4.34 (dd, 2H, J = 16.2, 4.2, H-1b, 3b), 5.3−5.4 [m, 9H, H-(9′, 10′) × 2, H-(9″,10″,12″,13″) and H-2]; ^{13}C NMR ($CDCl_3$) δ 14.0 (C-18″); 14.1 (C-18′ × 2); 22.6, 22.7, 24.4, 24.8, 25.6, 26.8, 27.2, 27.3, 29.1−29.7 [C-(4′- 8′, 11′-17′) × 2, 4″-8″, 14″-17″]; 31.5 (C-3″); 31.9 (C-3′ × 2); 33.9 (C-2′ × 2); 34.0 (C-11″); 34.2 (C-2″); 62.1 (C-1, 3); 68.8 (C-2); 127.9−130.5 [C-(9′, 10′) ×2, C-(9″,10″,12″,13″)]; 172.8 (C-1″); 173.2 (C-1′ × 2). The ^{1}H and ^{13}C NMR data for **4** were identical to the published findings of Chandra and Nair (1993).

Saponification and Methylation of 4 and Standards. Compound **4** (7 mg) was stirred with 5% NaOH in MeOH (1 mL) for 5 min followed by acidification with 6 N HCl in MeOH. This solution containing free fatty acids was then dried under a stream of nitrogen. Diazomethane was prepared by reacting *N*-nitroso-*N*-methylurea with concentrated KOH solution in ether (Kelm and Nair, 1998). As the diazomethane product formed, it dissolved into the organic ether phase. This yellow ether solution containing the diazomethane product was collected and used to methylate the free fatty acids obtained in the previous step. Also, the diazomethane solution was used to methylate oleic, stearic, and linoleic acids separately. The methylated products were dissolved in hexane and filtered to remove any solids prior to GC analysis.

Mosquitocidal Assay. *A. aegyptii* larvae were reared in our laboratory from eggs. Eggs were hatched in 500 mL of distilled, degassed water prepared by sonication (30 min), and larvae were fed with approximately 5 mg of bovine liver powder. Ten to fifteen 4th instar larvae were placed in 980 μL of distilled water in test tubes, and 20 μL of DMSO or DMSO solutions containing the appropriate concentration of test compounds were added and left at room temperature. Initially, the crude extracts and pure compounds were tested at 250 and 100 ppm concentration, respectively. The test concentration for pure compounds were then serially diluted to 0.1 ppm as the final concentration. Pure DMSO was used as solvent control. The assay were conducted in triplicate, and the numbers of dead larvae were recorded at 2, 4, 12, and 24 h intervals (Roth et al., 1998; Ramsewak et al., 1999).

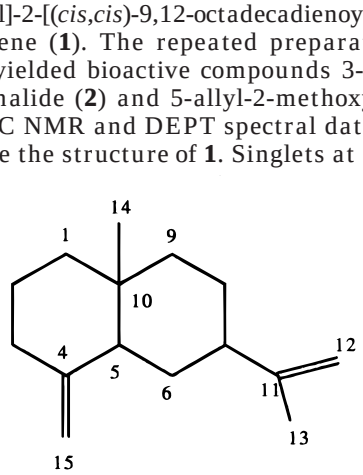
Nematicidal Assay. The nematode cultures, *C. elegans* and *P. redivivus*, were maintained in our laboratory. *C. elegans* was grown on NG agar media containing a strain of *Escherichia coli* in disposable Petri dishes wet with 2−4 mL of physiological saline solution. *P. redivivus* was maintained in axenic, liquid Basal Heme media (5 mL) in scintillation vials. The cultures were stored at room temperature and subcultured prior to the assay. The assay was conducted in Corning polystyrene 96-well plates. The 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. A solution (48 μL) containing nematodes was delivered to each of three wells per treatment. Two microliters of DMSO (50%) or DMSO (50%) and test compounds was added to each well. The plate was covered, parafilmed, and kept in a humid chamber. The number of dead nematodes was recorded every at 2, 4, 6, 8, and 24 h by observing under a microscope (Nair et al., 1989).

Antimicrobial and Topoisomerase-I and -II Inhibitory Assays. The test organisms *Candida kruseii* (MSU strain) and *Candida albicans* (MSU strain) used for antimicrobial bioassays were cultured in Petri dishes containing YMG media (20 mL) and *Saccharomyces cerevisiae* mutant cell cultures, JN394, JN394 t_{-1}, and JN394 t_{-2-5}, used for the topoisomerase assay, were cultured in Petri dishes containing YPDA 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 CFU/mL. A 50 μL aliquot of this suspension was then used to inoculate culture tubes containing the corresponding media (930 μL). DMSO or test compounds dissolved in DMSO were added to the inoculated tubes (20 μL) at concentrations ranging from 100 to 0.1 ppm. The tubes containing cell cultures and compounds were incubated at 27 °C on a rotary orbital shaker at 120 rpm for 72 h. MIC_{100} values for the test compounds were recorded for each test organism at the end of incubation period (Chang et al., 1995; Roth et al., 1998; Ramsewak et al., 1999).

RESULTS AND DISCUSSION

Seeds of *A. graveolens* extracted with hexane, successive silica gel MPLC, and preparative TLC gave four compounds, **1**−**4**. The hexane extract was fractionated into acetone-soluble and -insoluble fractions. The bioactive acetone-soluble fraction was subjected to medium-pressure liquid chromatography. Fraction A eluting with 100% hexane and fraction D eluting with hexane/ether (10:1) were found to be active. Fraction A was purified by repeated preparative TLC to yield 1,3-di[(*cis*)-9-octadecenoyl]-2-[(*cis*,*cis*)-9,12-octadecadienoyl]glycerol (**4**) and β-selinene (**1**). The repeated preparative TLC of fraction D yielded bioactive compounds 3-*n*-butyl 4,5-dihydrophthalide (**2**) and 5-allyl-2-methoxyphenol (**3**).

^{1}H and ^{13}C NMR and DEPT spectral data were used to determine the structure of **1**. Singlets at 1.7 and 0.71

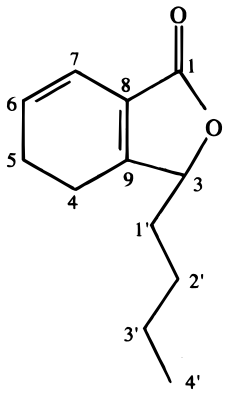


1

ppm, each integrated for three protons, indicated the presence of two methyl groups (C-13 and C-14) attached

to olefinic and quaternary carbons, respectively. The broad singlet at 4.4−4.7 ppm indicated the presence of vinyl protons at C-12 and C-15 in support of the presence of ^{13}C NMR signals at 105.4 and 108.1 ppm. The DEPT spectrum supported the ^{1}H NMR and ^{13}C NMR data. The ^{1}H NMR spectrum of **1** was identical to the published finding of Bowden et al. (1978).

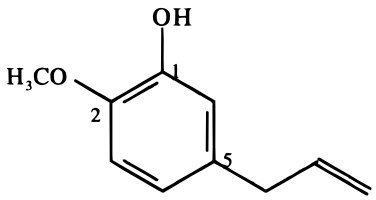
The structure of **2** was determined by using ^{1}H, ^{13}C



2

NMR, and DEPT spectral data. The two-dimensional HMQC proton-carbon correlations, HMBC proton-carbon long-range coupling, and arrayed-decoupling experiments facilitated further evidence to support the 1-D NMR experiments. The ^{1}H NMR spectrum displays a doublet at 6.2 ppm (1H, $J = 10$ Hz) and a multiplet at 5.9 ppm (1H) for the vinyl protons, H-7 and H-6, respectively; as well as a multiplet at 4.9 ppm for H-3. The ^{1}H NMR spectral data of **2** were identical to previously published data (Bjeldanes and Kim, 1976).

The structure of **3** was determined by using ^{1}H, ^{13}C



3

NMR, and DEPT spectral data. A singlet at 3.84 ppm corresponded to methoxy protons. A singlet at 5.45 ppm was determined to be the hydroxy group as indicated by the disappearance of this peak following a D_2O shake in the ^{1}H NMR spectrum. The presence of two peaks, at aromatic region (6.66−6.83 ppm), integrated for three protons, indicated a trisubstituted aromatic ring. This proton data were found to be in agreement with those previously published (Kurihara and Kikuchi, 1979).

The structure of **4** was determined by using ^{1}H and ^{13}C NMR and MS spectroscopy. The ^{1}H NMR signals at δ 4.12 and 4.34 corresponded to 2 × CH_2− methylene protons of a glycerol backbone. The overlapping multiplets at δ 5.3 for eight protons correlated with unsaturated carbons at δ 127.9−130.5 indicated the presence of four double bonds in this molecule. MS peaks at *m/z* 883 $[C_{57}H_{102}O_6 + H^+]$, 617 $[C_{57}H_{102}O_6 - C_{18}H_{31}O]^+$, 263 $[C_{18}H_{31}O]^+$, 265 $[C_{18}H_{33}O]^+$ supported the presence of oleic and linoleic acids. The GC profile of methylated hydrolysis product of this molecule confirmed the presence of the methyl esters of oleic and linoleic acids with a ratio of 2:1, respectively. The retention times for both methyl esters were identical to those of authentic samples of oleic and linoleic acid methyl esters analyzed under the same conditions. The spectral data of **4** were identical to the published finding by Chandra and Nair, 1993.

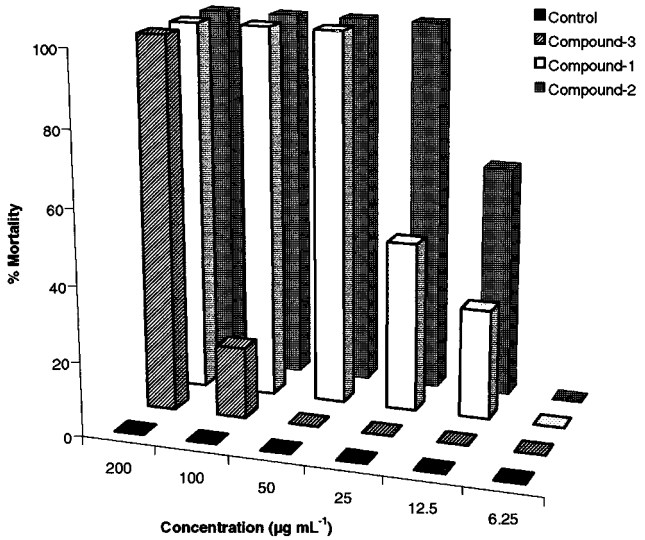


Figure 1. Percent mortality of 4th instar *A. aegyptii* larvae for **1**−**3** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$), and the means were compared by calculating least significant difference (LSD).

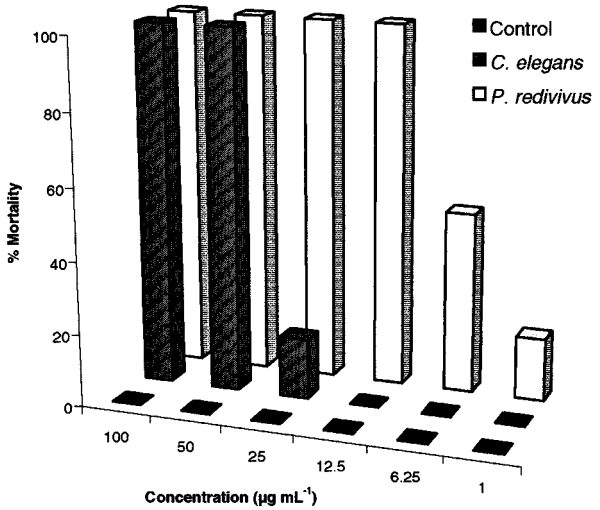
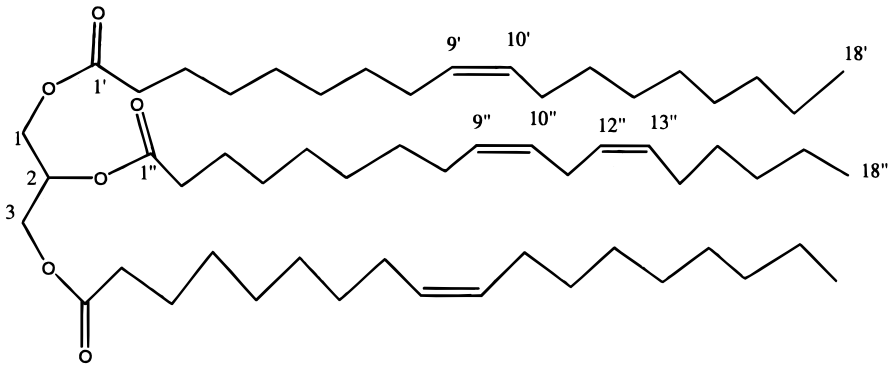


Figure 2. Percent mortality of *C. elegans* and *P. redivivus* nematodes for **2** at 24 h. Statistical analysis was done using ANOVA ($p \leq 0.01$), and the means were compared by calculating least significant difference (LSD).

Mosquitocidal assays using *Aedes aegyptii* on **1**−**4** indicated that **1**−**3** were active. Compounds **1**−**3** had LD_{100}'s (24 h) at 50, 25, and 200 μg mL^{-1}, respectively (Figure 1). Compound **2** displayed nematicidal activity on *P. redivivus* and *C. elegans*. LD_{100} values in 24 h were 12.5 and 50 μg $mL^{-1}$for *P. redivivus* and *C. elegans*, respectively (Figure 2). Compound **2** showed topoisomerase-I and -II inhibitory activity when tested on *S. cerevisiae* mutant strains. JN394 is hypersensitive to both topoisomerase-I and -II poisons, whereas JN394 t_{-1} lacks the top-I gene and therefore shows a lack of response to topoisomerase-I poisons. JN394 t_{-2-5} carries the top-II gene, which is resistant to topoisomerase-II poisons but responds to topoisomerase-I poisons. MIC_{100} determination of **3** indicated that it completely inhibited



4

topoisomerase-I and -II activity at 100 μg mL^{-1}. The zones of inhibition at 50 μg mL^{-1} of **2** on YPDA plate were 1.1, 1.1, and 1.2 cm for JN394, JN394 t_{-1}, and JN394 t_{-2-5}, respectively. Antimicrobial assays showed that **2** was active when tested on *C. albicans* and *C. kruseii* (MSU strains). The inhibition zones for *C. albicans* and *C. kruseii* were found to be 1.1 and 1.0 cm at the concentration of 50 μg mL^{-1}, respectively. Compound **2** had an MIC_{100} at 100 μg mL^{-1}.

The flavor of celery seed oil is mainly due to β-selinene and phthalides (Lund, 1978). Terpenes, terpenoids, and phthalides have been implicated as being natural insecticides, insect pheromones, and mammalian sex hormones, and they have been used since antiquity as ingredients of flavors, preservatives, perfumes, medicines, narcotics, and pigments. Present study supports that the observed biological activity of celery seeds is partly due to the presence of terpenes and phthalides. To the best of our knowledge, this is the first report of **3** and **4** from *A. graveolens* seeds, although **4** is not biologically active. Till now, there are no reports on the biological activity of **1**−**3,** although **2** was used as one of the component of perfume.

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