Phytotoxins from the Leaves of *Laggera decurrens*

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Upon biological screening of a series of African medicinal plants, substantial phytotoxic activity was found in the leaves of *Laggera decurrens* (Vahl.) Hepper & Wood (Asteraceae), using a *Lemna minor* bioassay. Bioassay-guided fractionation of the leaves led to the isolation of two physiologically active compounds: 3-hydroxythymoquinone and 5-acetoxy-2-hydroxythymol, causing death of *Lemna minor* in the 25−100 μM range. Symptoms were a rapidly developing chlorosis, followed by necrosis of fronds. The compounds also inhibited growth and germination of the grass weed *Agrostis capillaris* down to 250 μM. The mode of action of both compounds could not be elucidated, but they do not appear to be photosystem II inhibitors.

Keywords: *Laggera decurrens; Asteraceae; 3-hydroxythymoquinone; 5-acetoxy-2-hydroxythymol; phytotoxicity*

INTRODUCTION

Laggera decurrens (Vahl.) Hepper & Wood (Asteraceae), formerly known as *Blumea decurrens* (Vahl.) Merxm. or *B. gariepina* DC., is a halfshrub found in southern Africa (Anderberg, 1991) and is well-known for its use in traditional medicine. In Namibia, an extract of the leaves or the roots is drunk to relieve stomach pains and is also used against acne (Van den Eynden and Van Damme, 1993). Some attention has been devoted to the constituents of *L. decurrens*: β-isocomene, silphinene, modhephene, thymol, its acetate, thymoquinone, and the corresponding acetoxy derivative 2-acetoxythymoquinone as well as the phenolic compounds 5-acetoxy-2-hydroxythymol, 2-acetoxy-5-hydroxythymol, 5-acetoxy-2-hydroxythymol acetate, and a diol, 7β,12-dihydroxyhimachal-2-ene (Bohlmann et al., 1985). Upon biological screening of a series of African medicinal plants, phytotoxic activity was found in the leaves of *L. decurrens* using a *Lemna minor* bioassay. This paper describes the bioassay-guided isolation of the active principles and their phytotoxic properties.

MATERIALS AND METHODS

Plant Material. Leaves of *L. decurrens* were collected in the Namib desert in Namibia in February 1992. The plant was identified by one of the authors (P.V.D.), and a voucher specimen (VdE 2.4 e) was deposited at the National Herbarium of Namibia in Windhoek. The specimen was air-dried and ground to a powder.

Extraction and Isolation. Powdered leaves (1000 g) of *L. decurrens* were successively extracted until exhaustion with hexane, $CHCl_3$, EtOAc, MeOH, and H_2O and concentrated under reduced pressure. The hexane and the $CHCl_3$ extracts, which showed phytotoxic activity in the *L. minor* bioassay, were further fractionated.

The $CHCl_3$ fraction was extracted with MeOH/H_2O (1:1). The $CHCl_3$ phase was evaporated under reduced pressure to give a $CHCl_3$-1 extract (31.04 g; 3.1%) and the MeOH removed from the MeOH/H_2O extract. The H_2O phase was extracted with $CHCl_3$ to give a $CHCl_3$-2 extract (1.5 g; 0.15%) and with EtOAc (3.0 g; 0.3%). The phytotoxic $CHCl_3$-1 extract was chromatographed on silica gel (glash LC) (Merck, 230−400 mesh, 700 g) and eluted with hexane, hexane/EtOAc, and EtOAc/MeOH mixtures. A total of 160 fractions (100 mL each) were collected and combined into 16 fractions on the basis of similar TLC profiles. TLC analyses were carried out on TLC plates (Merck, silica gel 60 F_{254}) using UV and 10% H_2SO_4 (10 min at 105 °C) as developing agents. The phytotoxic fraction 3 (900 mg), eluted with hexane/EtOAc (7:3) (3700−4000 mL), was rechromatographed on silica gel (flash LC) and eluted with hexane/EtOAC (9:1) (1000 mL) and EtOAc (1000 mL). A total of 60 fractions (50 mL each) were collected and combined into 6 fractions. Fraction 2 (0.2 g; 0.02%), eluted with hexane/EtOAc (9:1) (600−800 mL), was finally purified on preparative TLC (Merck TLC plates, silica gel 60$F_{254+366}$, 2 mm thickness). Four elutions with hexane/EtOAc (9:1) afforded 3-hydroxythymoquinone (105 mg; 0.01%) (**1**).

The hexane fraction was extracted with MeOH/H_2O (9:1). The hexane phase was evaporated under reduced pressure (25.6 g, 2.5%) and the MeOH removed from the MeOH/H_2O extract. The H_2O phase was extracted with $CHCl_3$ to give a $CHCl_3$ extract (15.0 g, 1.5%). This $CHCl_3$ extract was chromatographed on silica gel (flash LC) and eluted with hexane, hexane/EtOAC, and EtOAc/MeOH mixtures. A total of 200 fractions (50 mL each) were collected and combined into 13 fractions on the basis of similar TLC profiles. The active fraction 9 (1500 mg), eluted with hexane/EtOAc (60:40) (4500−5000 mL), was rechromatographed on silica gel and eluted with hexane, hexane/EtOAC, and EtOAc/MeOH mixtures. A total of 180 fractions (50 mL each) were collected and combined into 16 fractions. Fraction 8−10 (804 mg, 0.08%), eluted with hexane/EtOAc (70:30) (4200−5000 mL), afforded 5-acetoxy-2-hydroxythymol (216 mg; 0.02%) (**2**).

*3-Hydroxythymoquinone (**1**):* orange crystals ($CHCl_3$/hexane); mp 165.1−165.4 °C; IR (KBr) ν_{max} = 3280 (br, OH), 1610 (C=O) cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.24 (3H, d, J = 6.9 Hz, CH$\underline{Me}_2$), 2.05 (3H, d, J = 1.6 Hz, Me), 3.18 (1H, sept, J = 6.9 Hz, C$\underline{H}$Me$_2$), 6.47 (1H, q, J = 1.6 Hz, CH), 6.98 (1H, s, OH); ^{13}C N$\underline{M}$R (68 MHz, $CDCl_3$) δ 14.72 (Me), 19.84 (Me$_2$),

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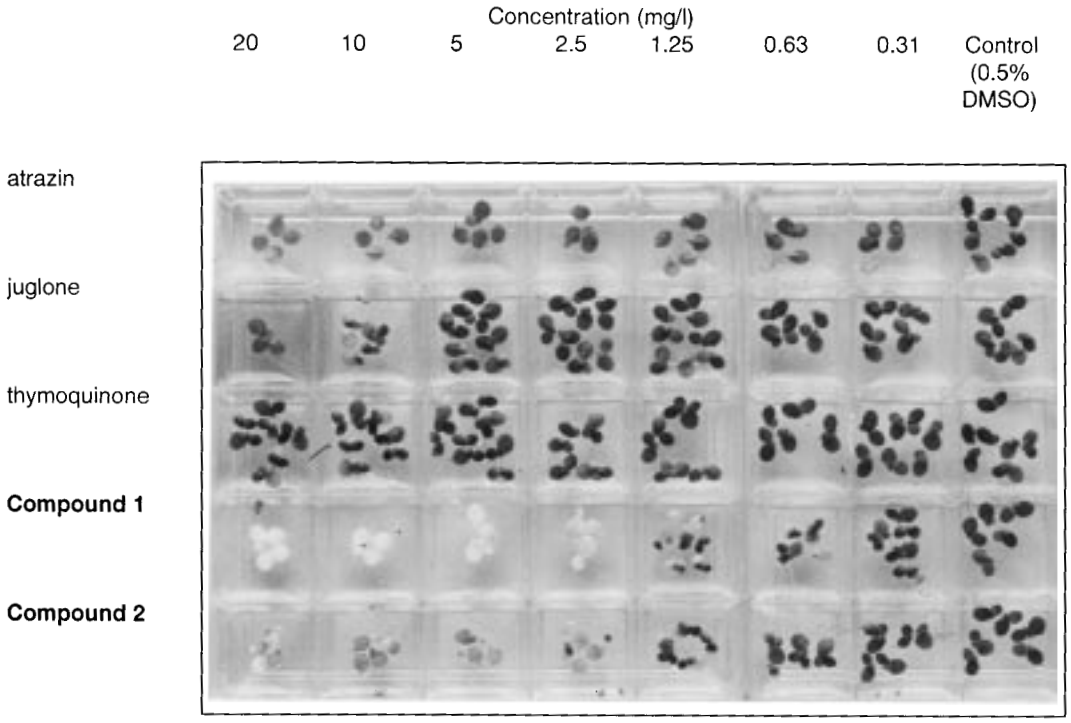
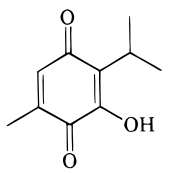


Figure 1. Activity of compounds **1** and **2** in the *L. minor* bioassay (representative experiment).

24.08 (CH), 125.50 (CCHMe$_2$), 135.83 (CH), 140.52 (CMe), 150.76 (C−OH), 184.51 (C=O), 187.35 (C=O); EI-MS (70 eV), *m*/*z* (rel int %) 180 [M$^+$] (100), 165 [M$^+$ − Me] (28), 152 (23), 147 (25), 137 (30), 109 (21), 83 (30), 69 (28), 55 (26).



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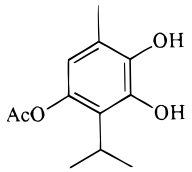

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*5-Acetoxy-2-hydroxythymol (**2**):* light brown crystals (CHCl$_3$/hexane); mp 113.9−114.5 °C; IR (KBr) ν_{max} = 3450 (OH), 1725 (C=O) cm^{-1}; ^{1}H NMR (270 MHz, CDCl$_3$) δ 1.29 (6H, d, J = 7.3 Hz, Me$_2$), 1.94 (3H, s, MeC=C), 2.31 (3H, s, MeC=O), 3.09 (1H, sept, J = 7.3 Hz, CHMe$_2$), 5.65 (1H, s, OH), 5.76 (1H, s, OH), 6.17 (1H, s, CH arom); ^{13}C NMR (68 MHz, CDCl$_3$) δ 15.23 (Me), 20.65 (Me$_2$), 21.08 (Me), 25.89 (CHMe$_2$), 114.43 (CH arom), 122.67 (C−Me), 123.50 (C−Me$_2$), 135.53 (C−OH), 141.65 (C−OAc), 144.32 (C−OH), 171.76 (C=O); EI-MS (70 eV), *m*/*z* (rel int %) 224 [M$^+$] (33), 182 [M − ketene]$^+$ (86), 167 [182 − Me]$^+$ (100).

Biological Assays. Fractionation was guided using a *L. minor* bioassay: an appropriate amount of the test fractions, dissolved in acetone, was added to 4 mL of Pirson and Seidel medium (Pirson and Seidal, 1950) in the wells of square 25-well multiwell plates. The maximum final acetone concentration in the medium was 1%. Each well was subsequently inoculated with one axenically cultured *L. minor* L. plant in the four-leaf stage. The plates were incubated in the laboratory at 27 °C under continuous cool white fluorescent illumination (90 μmol/m^2 s photosynthetically active radiation). The percentage of phytotoxicity (necrosis and growth inhibition), as compared to untreated controls, was evaluated visually after 2 weeks by estimating the well surface area covered by green, healthy leaf tissue. Acetone (1%) was used as a negative control. Juglone (5-hydroxy-1,4-naphthoquinone, 97% pure, Janssen Chimica, Geel, Belgium), thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone, Sigma), and atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-6-triazine, Janssen Chimica] were used as reference compounds. To obtain a quantitative measure of compound activity, the experiment was repeated in quadruplicate in standard 24-well multiwell plates, with purified samples of compounds **1** and **2** and thymoquinone and juglone as reference compounds, by determining fresh weight of the *Lemna* plants in each well after 2 weeks of incubation.

For further study of the phytotoxic properties of compounds **1** and **2**, selected agar-grown weed species were used. Compounds **1** and **2** were dissolved at 0.2 M in DMSO and added in appropriate amounts to liquid Hoagland's mineral nutrient medium containing 0.8% of agar (Hoagland and Arnon, 1950). The maximum final DMSO concentration in the medium was 0.5%. The medium was allowed to solidify in standard 24-well multiwell plates, and the wells were subsequently seeded with *Arabidopsis thaliana* (L.) Heynh., *Solanum nigrum* L., *Agrostis capillaris* L., or *Poa annua* L. Wells were then incubated in a Weiss climate room with a 16 h day at 22 °C and an 8 h night at 20 °C. Light intensity was 8 μmol/m^2 s photosynthetically active radiation during the first 5 days (germination) and 90 μmol/m^2 s photosynthetically active radiation thereafter. Evaluation was performed 1 week after sowing by visually scoring plant injury and death using a linear, 11-stage rating scale as follows: 0 = no growth, 1 = 1−10%, 2 = 11−20%, 3 = 21−30%, 4 = 31−40%, 5 = 41−50%, 6 = 51−60%, 7 = 61−70%, 8 = 71−80%, 9 = 81−90%, 10 = 91−100% healthy green leaf tissue as compared to controls. Experiments were repeated four times. DMSO (0.5%) was used as a negative control. Juglone and thymoquinone were used as reference compounds.

To evaluate effects on germination, compounds **1** and **2** were dissolved at 0.2 M in DMSO and added in appropriate amounts to deionized water. Six layers of filter paper (Schleicher and Schuell 589[2] ashless "white ribbon") were placed on the bottom of the wells of standard multiwell plates, and 0.25 mL of deionized water containing the test compounds was added to them. Each well was subsequently seeded with 20 *A. capillaris* seeds. The multiwell plates were then incubated in a Weiss climate room, as described above. Evaluation was performed after 2 weeks by counting the number of germinated seeds in each well and calculating percentage germination. Experiments were repeated four times. DMSO (0.5%) was used as a negative control. Juglone and thymoquinone were used as reference compounds.

Fluorescence transients from microwell-grown *A. capillaris* plants were recorded using a Hansatech modulated fluorescence measurement system (Ögren and Baker, 1985), by positioning single 16 mm diameter wells under the hole in the instrument's leaf clip. Atrazine was used as a reference compound.

RESULTS AND DISCUSSION

Bioassay-guided fractionation of the hexane and chloroform extracts of the leaves of *L. decurrens* led to the isolation of two phytotoxic compounds, **1** and **2**.

The structure of 3-hydroxythymoquinone (**1**) was deduced from the spectrometric data. The ^{1}H NMR revealed the presence of a methyl, an isopropyl, a hydroxy group (also visible in the IR spectrum), and an aromatic proton. Because of the allylic coupling of the methyl group with the olefinic proton, the methyl group is located at the ortho position of the olefinic proton. The ^{13}C NMR data showed two carbonyl signals together with three quaternary carbons (125−150 ppm) and a CH signal at 135 ppm, characteristic of a quinone moiety. The mass spectral analysis (molecular weight = 180) also confirmed the presence of the substituents mentioned above. 2D-HETCOR and COSY measurements were also in agreement with the structure of 3-hydroxythymoquinone, which was synthesized (Jozeph-Nathan et al., 1987) and isolated before from *Antiphiona pinnatisecta* (Zdero and Bohlmann, 1989). These chemical findings allow one to accept the relative positions of the methyl and isopropyl groups in 3-hydroxythymoquinone.

The structure of 5-acetoxy-2-hydroxythymol (**2**) could also be deduced from the spectroscopic data, although more advanced NMR techniques had to be used to distinguish between the different isomers. The substituents on the aromatic ring were easily found from the ^{1}H NMR, that is, a methyl, an isopropyl, two hydroxy groups, and an acetoxy group (confirmed by a carbonyl absorption in the IR and a carbonyl signal in the ^{13}C NMR). The structure of the pentasubstituted aromatic ring was also confirmed by the mass spectral analysis, which showed a molecular ion at *m*/*z* 224. As mentioned before, the relative position of the substituents on the aromatic ring had to be determined by ECHO−INADEQUATE and NOESEL experiments. ECHO−INADEQUATE measurements revealed cross-peaks between 171.76 and 141.65 ppm and between 15.23 and 139.33 ppm, proving that the methyl group is located at the ortho position of a hydroxy group and that the acetoxy group is attached to the carbon atom resonating at 141.65 ppm. A NOESEL experiment with irradiation at δ 6.17 led to a considerable reduction of the complexity of the signals at 141.65 ppm (the signal becomes a doublet), proving that the acetoxy group is located at the ortho position of the aromatic proton. Combining this information led to the conclusion that the active compound was 5-acetoxy-2-hydroxythymol (**2**). The positioning of the substituents is in agreement with some monoterpene analogues isolated from *Relhania* species (Tsichritzis and Jakupovic, 1990), and compound **2** was also isolated from *Blumea alata* (3) and *Antiphiona* species (Zdero and Bohlmann, 1989) (no ^{13}C spectral data given). Compound **2** was obtained as a crystalline product, although it has been reported as an oil by Bohlmann and co-workers (Bohlmann et al., 1985; Zdero and Bohlmann, 1989).

Bioassay-guided fractionation of the extracts using *L. minor* showed that compounds **1** and **2** completely killed the indicator organism down to 2.5 mg/L (14 μM) and 5 mg/L (22 μM), respectively, as can be seen in a photograph of a representative experiment (Figure 1). The related compound thymoquinone was inactive at the concentrations tested (0.3−20 mg/L), and the allelopathic quinone compound juglone, which was tested for comparison, caused complete necrosis at 20 mg/L (115 μM) only. In a separate experiment, the effects of compounds **1** and **2** and thymoquinone and juglone on *L. minor* fresh weight accumulation were compared at five doses, ranging from 100 to 0.39 μM. As can be seen in Table 1, only compounds **1** and **2** significantly inhibited fresh weight accumulation in the test plants within this dose range, at concentrations down to 25 and 100 μM, respectively. The higher doses of **1**, **2**, or juglone caused a rapidly (within 48 h) developing chlorosis in *L. minor* plants, followed by complete or partial necrosis. The symptomology of plant injury was markedly different from what could be observed with a photosystem II inhibiting herbicidal compound such as atrazine, which caused, down to 2.5 mg/L (12 μM), a slowly developing necrosis that took >1 week to show its full effect.

The phytotoxic properties of compounds **1** and **2** were further studied on agar-grown seedlings of the weeds *A. thaliana*, *S. nigrum*, *A. capillaris*, and *P. annua*. Compounds **1** and **2** were mainly active on the grasses

Table 1. Effect of 1 and 2 on Fresh Weight Accumulation of *L. minor* Plants As Compared to Juglone and Thymoquinone[a]

test compd	concentration ($\times 10^{-6}$ M)				
	100	25	6.25	1.56	0.39
1	7.6* (4.3)	34.9* (9.3)	60.7 (3.0)	66.0 (9.6)	67.2 (4.3)
2	6.9* (1.8)	42.8 (11.3)	72.5 (13.4)	66.1 (9.1)	99.3 (22.1)
juglone	101.8 (3.9)	98.2 (6.4)	102.8 (5.7)	117.0 (23.2)	100.8 (9.3)
thymo-quinone	96.4 (8.6)	104.9 (11.3)	98.7 (7.5)	96.0 (4.6)	103.4 (7.7)

[a] Growth is expressed as milligrams of fresh weight per treatment after 2 weeks of incubation, mean of four replicates. Standard deviation shown in parentheses. Control = 102.8 (13.5). Asterisks indicate significant difference from control at 1% level using Student's test.

Table 2. Effect of 1 and 2 on Agar-Grown *A. capillaris* Plants As Compared to Juglone and Thymoquinone[a]

test compound	concentration ($\times 10^{-6}$ M)				
	1000	500	250	125	63
1	0* (0)	1* (1)	4* (1)	8 (1)	10 (0)
2	0* (0)	0* (0)	1* (1)	8 (1)	10 (1)
juglone	0* (0)	1* (1)	2* (1)	8 (2)	10 (1)
thymoquinone	4 (2)	9 (1)	10 (0)	10 (0)	10 (1)

[a] Plant injury is expressed as a score from a 0−10 rating scale, mean of four replicates. Standard deviation shown in parentheses. Control = 10 (1). Asterisks indicate significant difference from control at 1% level using Student's test.

Table 3. Effect of 1 and 2 on Germination of *A. capillaris* Seeds As Compared to Juglone and Thymoquinone[a]

test compound	concentration ($\times 10^{-6}$ M)				
	400	200	100	50	25
1	5* (4)	25 (14)	58 (3)	54 (3)	63 (10)
2	8* (6)	15 (8)	54 (8)	63 (12)	59 (19)
juglone	1* (3)	15 (11)	31 (3)	44 (24)	51 (18)
thymoquinone	40 (11)	43 (10)	58 (13)	54 (5)	60 (4)

[a] Percentage germination, 2 weeks after sowing, mean of four replicates. Standard deviation shown in parentheses. Control = 64 (10). Asterisks indicate significant difference from control at 1% level using Student's test.

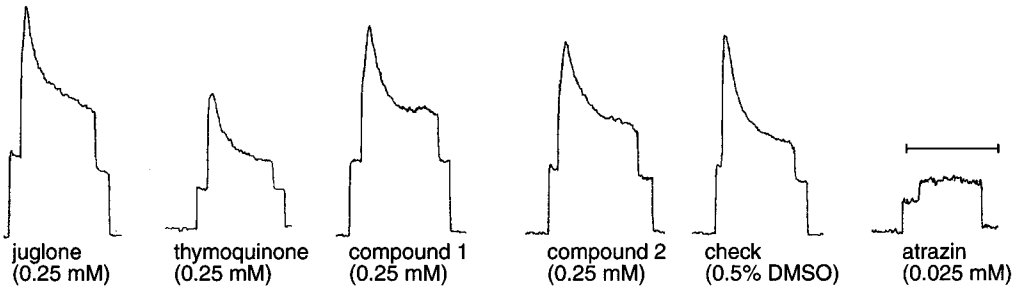

Figure 2. Fluorescence induction curves of treated agar-grown *A. capillaris* plants.

and weak on the two dicotyledonous plants (data not shown). This suggests a narrower spectrum than that of juglone, which completely inhibited growth of all four weeds down to 500 μM. Subsequently, an experiment on agar-grown *A. capillaris*, comparing compounds **1** and **2** with thymoquinone and juglone, was performed. The results are summarized in Table 2.

Compounds **1** and **2** and juglone significantly inhibited growth of *A. capillaris* down to 250 μM in this experiment, whereas thymoquinone was inactive. The affected plants were stunted, but no obvious chlorosis could be observed, in contrast to the effects on *L. minor*.

Because the phytotoxicity observed appeared to be a combination of growth retardation and germination inhibition, a germination experiment with *A. capillaris* seeds was also performed. The results are summarized in Table 3. As it turned out, compounds **1** and **2** and juglone inhibited *A. capillaris* germination, but results were only significant at 400 μM.

Interaction with photosystem II and inhibition of photosynthetic light reactions have been described for various types of quinones (Pfister et al., 1981; Renger et al., 1988; Nimbal et al., 1996; Gonzales et al., 1997; Rimando et al., 1998). Moreover, inhibition of the photosynthesis by the well-known allelopathic quinone compound juglone has been reported (Hejl et al., 1993), although the main mode of action of this compound appears to be inhibition of respiration (Perry et al., 1967; Koeppe, 1972). To check for photosynthetic electron transport inhibition as a possible mode of action for compounds **1** and **2**, fluorescence transients from microwell-grown *A. capillaris* plants were recorded using a Hansatech modulated fluorescence measurement system (Ögren and Baker, 1985). The measurements revealed normal fluorescence decay curves in plants treated with **1**, **2**, thymoquinone, and juglone, as compared to the typically flat response curve in atrazine-treated plants. This rules out photosystem II inhibition as a possible mode of action for compounds **1** and **2** (Figure 2).

To conclude, the *L. minor* bioassay proved to be useful in guiding the isolation of the two phytotoxic compounds 3-hydroxythymoquinone (**1**) and 5-acetoxy-2-hydroxythymol (**2**) from the leaves of *L. decurrens*. The phytotoxic properties of compounds **1** and **2** have not been reported before. The compounds are more potent than the well-known allelopathic compound juglone on *L. minor* but significantly weaker on the dicotyledonous weeds tested. For the grass species *A. capillaris*, on the other hand, the inhibitory effect of compound **2** is comparable to that of juglone, whereas compound **1** is slightly weaker. Compounds **1** and **2** and juglone cause growth retardation as well as germination inhibition in *A. capillaris*. Results from variable fluorescence measurements rule out photosystem II as a possible mode of action. The nonhydroxylated analogue thymoquinone was nearly inactive in all assays performed.

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