Antifungal Constituents of the Essential Oil Fraction of Artemisia dracunculus L. Var. dracunculus

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Antifungal Constituents of the Essential Oil Fraction of *Artemisia dracunculus* L. Var. *dracunculus*

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The isolation and structure elucidation of antifungal constituents of the steam-distilled essential oil fraction of *Artemisia dracunculus* are described. Antifungal activities of 5-phenyl-1,3-pentadiyne and capillarin against *Colletrotichum fragariae*, *Colletrotichum gloeosporioides*, and *Colletrotichum acutatum* are reported for the first time. The relative abundance of 5-phenyl-1,3-pentadiyne is about 11% of the steam-distilled oil, as determined by GC−MS. Methyleugenol was also isolated and identified as an antifungal constituent of the oil.

KEYWORDS: ***Artemisia dracunculus*; antifungal activity; 5-phenyl-1,3-pentadiyne; methyleugenol; capillarin; *Colletrotichum fragariae*; *Colletrotichum gloeosporioides*; *Colletrotichum acutatum*; *Botrytis cinerea***

INTRODUCTION

Anthracnose diseases of strawberries (*Fragaria x ananassa* Duch.) are serious problems related to fruit and plant production in many areas of the world (*1*, *2*). The pathogens, *Colletotrichum acutatum* J. H. Simmonds, *Colletrotichum gloeosporioides* (Penz.) Penz., and *Colletrotichum fragariae* A. N. Brooks, can occur singly or in combination, and can infect flowers, fruits, leaves, petioles, stolen, and crowns (*3*−*5*).

Botrytis fruit rot (gray mold) is among the most destructive diseases affecting strawberries. Effects of the disease appear chiefly on the fruit just before harvest, mainly when the plant experiences persistently wet conditions. Gray mold fungus, *Botrytis cinerea* Pers. Fr., which causes this type of fruit rot, also causes blossom blight and may infect strawberry leaves and petioles (*6*). Postharvest control of these fungi is achieved by physical and chemical methods. Storage of the strawberries at low temperature and exposure to CO_2 are some methods used to control the development of the fungi. The most effective way to reduce postharvest fungal diseases in strawberries so far has been to apply fungicides (*7*). However, the development of strains of fungal pathogens that are resistant to commercial fungicides, and consumer concerns about chemical residues in fresh produce, have drawn a lot of attention in recent years. Natural products offer diverse classes of chemicals which are often environmentally friendly and safer than the presently used synthetic-based fungicides (*8*). As part of our ongoing search for antifungal compounds of natural origin, we evaluated the steam-distilled oil fraction of aerial parts of *Artemisia dracunculus* L. var. *dracunculus*.

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A. dracunculus is a member of the Asteraceae. Plants in this family are rich sources of various types of biologically active compounds (*9*−*15*). Many *Artemisia* species possess allelopathic and antifungal chemicals (*16*−*19*). In this article we describe the isolation and identification of antifungal constituents of the steam distillate of the aerial parts of *A. dracunculus* and their activity against *C. fragariae*, *C. gloeosporioides*, *C. acutatum*, and *B. cinerea*.

MATERIALS AND METHODS

Plant Material. The steam-distilled fraction of the aerial parts of *A. dracunculus* was supplied by Aromagen in Albany, OR, and the oil sample was stored in a freezer at −25 °C until use.

General Experimental Procedures. Extracts were analyzed on silica gel TLC plates GF with fluorescent indicator (Analtech, Newark, DE). Iodine vapor, UV light, and anisaldehyde spray reagents were used for the detection of compounds. Fungicide standards vinclozolin, captan, and thiabendazole were purchased from Chem Service, West Chester, PA. Column chromatography was carried out with Kieselgel 60 (particle size 0.063−0.2 mm, Merck, Germany) with hexane and ethyl acetate in increasing amounts (0%−30%). All solvents used were reagent grade and were used without further purification. ^{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. GC−MS analysis was carried out on an HP5790 MSD spectrometer (Hewlett-Packard, Palo Alto, CA) equipped with a GC 5890 using a DB-1 column (20 m × 0.2 mm, 0.18 μm film thickness). The oven was temperature-programmed from 60 (5 min) to 280 °C (20 min) at 5 °C/min, with helium as the carrier gas.

Bioautography. Bioautography on silica gel TLC plates with *Colletotrichum* spp. was used to identify the antifungal activity according to the previously published method (*20*). Crude steam distillate of *A. dracinculus* was separated on silica TLC plates using 10% ethyl acetate and hexane and air-dried. Each plate was subsequently sprayed with a spore suspension (10^5 spores/mL) of the fungi

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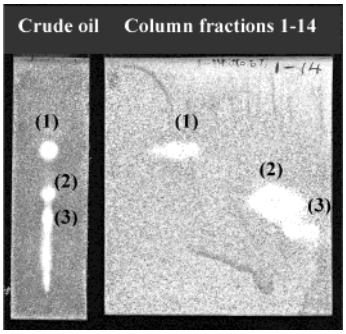


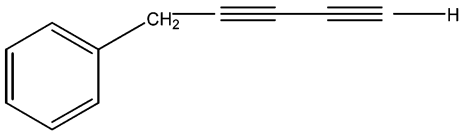
Figure 1. Bioautography of the crude oil of *A. dracunculus* and silica gel column fractions, showing clear zones where fungal growth inhibition occurs in response to antifungal constituents. Silica gel TLC plates were inoculated with spores of *C. fragariae*.

of interest and was incubated in a moisture chamber for 3 days at 26 °C with a 12-h photoperiod. Clear zones of fungal growth inhibition on the TLC plate indicated the presence of antifungal constituents.

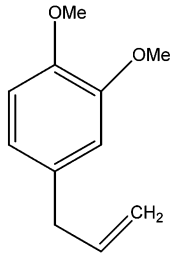
Microbioassay for Antifungal Activity. Purified active compounds were further evaluated for their quantitative antifungal activity using a 96-well microtiter plate according to the previously published methods (*21*). Compounds and standards were tested at 0.3, 3.0, and 30 μM concentrations against *B. cinerea*, *C. fragariae*, *C. gloeosporioides*, and *C. acutatum* in 96-well microtiter plates (Nunc Micro Well, untreated; Roskilde, Denmark). Microtiter plates were covered with a plastic lid and incubated in a growth chamber at 24 ± 1 °C and 12-h photoperiod under 60 ± 5 μmol·m^{-2}·s^{-1} light. Fungal growth was monitored spectrophotometrically by measuring absorbance at 620 nm at 24, 48, and 72 h. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 h. Analysis of variance of least-square means for percent inhibition of each fungus at each dose of test compound relative to the untreated controls was used to evaluate fungal growth inhibition. The experiment used a repeated measures design and was repeated once. Data presented were pooled from three independent experiments.

Isolation of the Antifungal Constituents. The steam-distilled oil (5 g) was column chromatographed using hexane and increasing amounts of ethyl acetate. Fractions of 100 mL were collected and concentrated at 40 °C, and the fractions with similar TLC profiles were combined to afford 28 fractions. Each fraction was tested by TLC bioautography to identify the antifungal fractions. Fractions that showed antifungal activity with the same R_f values on TLC were pooled and further purified by preparative layer and column chromatography. The active compounds were identified as 5-phenyl-1,3-pentadiyne (**1**), methyleugenol (**2**), and capillarin (**3**), respectively, by comparison of ^{1}H and ^{13}C NMR and GC−MS data with those reported in the literature (*22*, *23*).

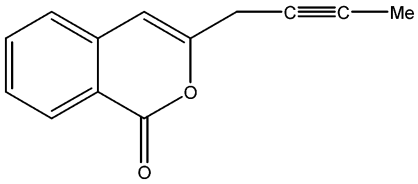
*5-Phenyl-1,3-pentadiyne (**1**).* Pale yellow oil (460 mg) eluted with hexane in fraction 5 of the column. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.06 (1H, t, J = 0.98 Hz, H-1), 3.70 (2H, s, H-5), 7.32 (5H, br s, aromatic protons). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 25.3 (C-1), 65.6 (C-2), 66.7 (C-3), 68.3 (C-4), 75.4 (C-5), 134.9 (C-1′), 127.9 (C-2′, C-6′), 128.6 (C-3′, C-5′), 126.9 (C-4′). GC−MS: M^+ = 140. The identity was confirmed by comparison of NMR and mass spectral data with those reported in the literature (*24*).



5-Phenyl-1,3-pentadiyne (1)



Methyleugenol (2)



Capillarin (3)

*Methyleugenol (**2**).* Clear oil (170 mg) eluted with hexane/ethyl acetate (8:2) in fraction 12 of the column. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.33 (2H, d, J = 6.5 Hz, H-1′), 3.86 (3H, s, 3-OMe), 3.85 (3H, s, 4-OMe), 5.08 (2H, m, H-3′), 5.95 (1H, m, H-2′), 6.71−6.81 (3H, m, aromatic protons). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 39.7 (s, C-1′), 55.7 (s, 4-OMe), 55.8 (s, 3-OMe), 111.3 (s, C-5), 111.8 (s, C-2), 115.4 (s, C-3′), 120.3 (s, C-6), 132.6 (s, C-1), 137.6 (s, C-2′), 147.4 (s, C-4), 148.9 (s, C-3). GC−MS: M^+ = 178. The identity was confirmed by NMR and mass spectral data.

*Capillarin (**3**).* White solid (48 mg) eluted with hexane/ethyl acetate (80:20) in fractions 13 and 14 and purified by preparative layer chromatography using silica gel GF, 1000 μm, with fluorescent indicator (Analtech, Newark, DE), acetone/hexane (3:7). ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 1.85 (3H, t, J = 2.5 Hz, 4′-CH_3), 3.40 (2H, q, J = 1.2 Hz, 1′-CH_2), 7.37 (1H, d, J = 7.8 Hz, H-5), 7.42 (1H, dt, J = 7.4 and 1 Hz, H-7), 7.65 (1H, dt, J = 8 and 2 Hz, H-6), 8.2 (1H, br d, J = 8 Hz, H-8). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 3.9 (s, C-4′), 24.2 (s, C-1′), 72.2 (s, C-2′), 80.3 (s, C-3′), 103.5 (s, C-4), 120.5 (s, C-9), 125.8 (s, C-5), 128.3 (s, C-6), 129.9 (s, C-7), 135.2 (s, C-8), 137.6 (s, C-10), 154.1 (s, C-3), 162.8 (s, lactone carbonyl, C-1). GC−MS: M^+ = 198. The identity was confirmed by comparison of NMR and mass spectral data with those reported in the literature (*23*).

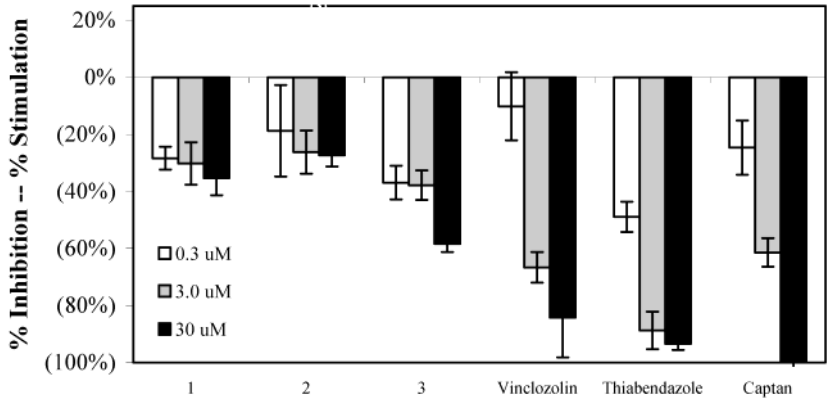


Figure 2. Growth inhibition of *B. cinerea* after 48 h using a 96-well microtiter format in a dose–response to 5-phenyl-1,3-pentadiyne (**1**), methyleugenol (**2**), and capillarin (**3**) and the commercial fungicide standards vinclozolin, thiabendazole, and captan. Means for percent growth inhibition were pooled from two experiments replicated in time.

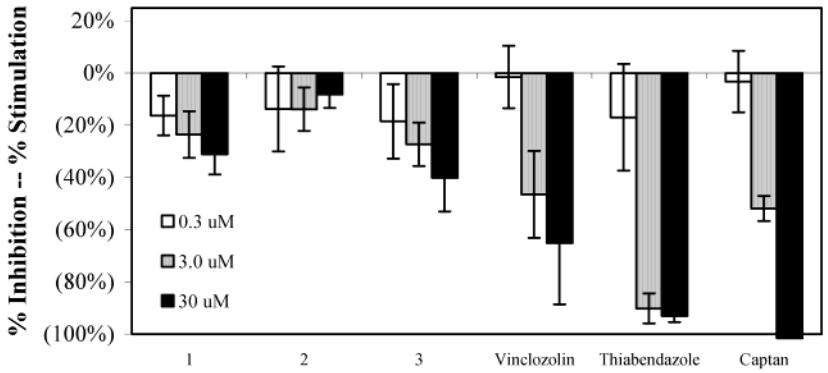


Figure 3. Growth inhibition of *C. fragariae* after 48 h using a 96-well microtiter format in a dose–response to 5-phenyl-1,3-pentadiyne (**1**), methyleugenol (**2**), and capillarin (**3**) and the commercial fungicide standards vinclozolin, thiabendazole, and captan. Means for percent growth inhibition were pooled from two experiments replicated in time.

RESULTS AND DISCUSSION

Preliminary bioautography of *A. dracunculus* steam distillate on TLC plates revealed antifungal activity against *C. fragariae* (**Figure 1**). The occurrence of antifungal compounds was evidenced by the presence of clear zones on a dark background on the TLC plate (**Figure 1**). The antifungal compounds **1**−**3** were isolated by bioassay-guided fractionation and identified by spectroscopic methods as 5-phenyl-1,3-pentadiyne, methyleugenol, and capillarin, respectively. This is the first report of the antifungal activity of **1** and **3** against *C. fragariae*, *C. gloeosporioides*, *C. acutatum*, and *B. cinerea*. The relative abundance of **1**, **2**, and **3** in the steam-distilled oil of *A. dracunculus* was determined by GC−MS to be ca. 11%, 9%, and 4%, respectively. According to bioautography results, more than three antifungal components were present in the crude oil, but we were unable to isolate these compounds in pure form due to rapid degradation yielding complex mixtures. Compounds **1**−**3** were also tested for phytotoxic activity on lettuce (*Lactuca sativa* cv. Ice berg) and bent grass (*Agrostis stolonifera* cv. Pencross). Compounds **1** and **3** showed mild phytotoxicity.

Microbioassay for fungicidal activity of the compounds indicated that capillarin (**3**) was the most effective of the three compounds against *B. cinerea*. Compound **3** at 30 μM concentration showed ca. 60% growth inhibition of *B. cinerea*, whereas **1** and **2** effected ca. 30% inhibition at the same concentration (**Figure 2**). Microbioassay also indicated that *C. gloeosporioides*, *C. fragariae*, *and C. acutatum* were sensitive to all three compounds at micromolar concentrations (only data for *C. fragariae* are shown) (**Figure 3**). Results indicated that at 30 μM concentration, **3** had about 30% growth inhibition of *C. fragariae*, whereas **1** and **2** had marginal activity after 48 h. These results and the volatile nature of **1**−**3** suggest that these compounds may have potential as fumigants in controlling postharvest fungi.

Antifeedant activity has been reported for **1**−**3** previously isolated from *Artemisia capillaris* against cabbage butterfly larva *Pieris rapae crucivora* by the leaf disk assay (*22*, *24*). This is the first report of antifungal activity of **1**−**3** against plant pathogenic fungi. The occurrence of antifungal and antifeedant compounds in *A. dracunculus* indicates the ecological impact of these compounds in the plant. Many *Artemisia* species exist as weeds in nature, and they produce defense compounds against pathogens and insects to survive in the ecosystem.

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