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Phytotoxicity of constituents of glandular trichomes and the leaf surface of camphorweed, *Heterotheca subaxillaris*

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ABSTRACT

Camphorweed, *Heterotheca subaxillaris* (Lam.) Britt. & Rusby, has a camphor-like odor, and its leaf surfaces contain glandular trichomes of the type shown to contain high levels of isoprenoids in other species. Borneol (**1**), the phytotoxic calamenene-type sesquiterpenes (**2**–**5**, **9**–**11**), and methylated flavones (**12**–**15**) were isolated from the dichloromethane rinsate of camphorweed aerial tissues. The strongest plant growth inhibitor against *Agrostis stolonifera* and *Lactuca sativa* seedlings, as well as duckweed (*Lemna pausicostata*), was 2-methoxy-calamenene-14-carboxylic acid (**2**). Esterification of calamenene carboxylic acids decreased their biological activity.

1. Introduction

Phytotoxic natural products may be utilized either directly or as lead compounds for the development of herbicides (Duke et al., 2000a; Macías, 1995). For example, bialaphos was a commercial natural herbicide produced by fermentation of the producing microbe (Tachibana, 2003), and the allelochemical leptospermone was used as a lead compound for development of the triketone class of herbicides (Lee et al., 1997). Sesquiterpenes from plants are of interest as potential herbicide leads, because they often have plant growth inhibiting properties (*e.g.*, Duke et al., 1988; Cantrell et al., 2007; Macías et al., 2000; Morimoto and Komai, 2005). Duke et al. (1999) speculated that compounds sequestered in glandular trichomes for autotoxicity avoidance are more likely to possess high levels of phytotoxicity than phytochemicals from most other plant tissues.

Camphorweed, *Heterolheca subaxillaris* (Lam.) Britt. & Rusby (Asteraceae), is a common annual or biennial weed that grows in sandy fields throughout the USA. The shoot of this plant is covered with glandular trichomes, and the plant has a characteristic camphor-like odor. Several cadinene and calamenene-type sesquiterpenes were reported in chemotaxonomy studies of the *Heterotheca* genus (Bohlmann and Zdero, 1979). The volatile monoterpene constituents myrcene, limonene and borneol (**1**) were reported by Lincoln and Lawrence (1984) (Fig. 1). A species related to camphorweed, *H. inuiloides* Cass., is used as a medicinal plant in Mexico. A calamenene-type phytotoxic sesquiterpene, inuloidin, was isolated from this species (Kubo et al., 1995).

Soil nitrogen content can affect the quantity of monoterpenes and sesquiterpenes in camphorweed. Production of monoterpenes and sesquiterpenes is increased under poor nitrogen conditions (Mihaliak and Lincoln, 1989). Additionally, suppression of damage from Lepidopteran insect larvae is dependent on these terpenes (Charles et al., 1987). In this paper, we describe the phytotoxic activity of camphorweed leaf surface compounds and their derivatives, using a bioassay-guided isolation approach.

2. Results and discussion

2.1. The H. subaxillaris leaf surface

Glandular trichomes of camphorweed are present on both leaf surfaces. Viewed with light microscopy, each trichome appears to possess a terminal exudate droplet (Fig. 2A/B). Scanning electron microscopy (SEM) established the presence of an intact cuticular sac surrounding the apical region of each trichome (Fig. 2C). It is uncertain whether the secretory contents remain only within the subcuticular space, or if exudates are present outside the cuticular sac, as more volatile products would have been lost in the sample preparation process for SEM.

Material from the leaf surface and the trichomes were collected by rinsing with dichloromethane (DCM). This type of extraction has been previously found to almost completely extract the terpenoids

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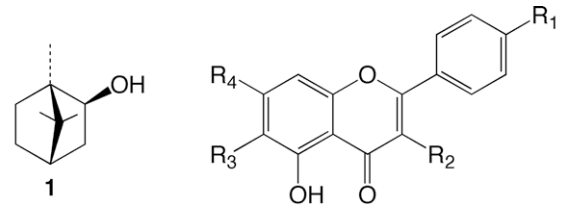


12 $R_1 = OCH_3$ $R_2 = OCH_3$ $R_3 = OCH_3$ $R_4 = OH$
13 $R_1 = OCH_3$ $R_2 = H$ $R_3 = OCH_3$ $R_4 = OH$
14 $R_1 = OH$ $R_2 = H$ $R_3 = OCH_3$ $R_4 = OH$
15 $R_1 = OH$ $R_2 = H$ $R_3 = OH$ $R_4 = OCH_3$

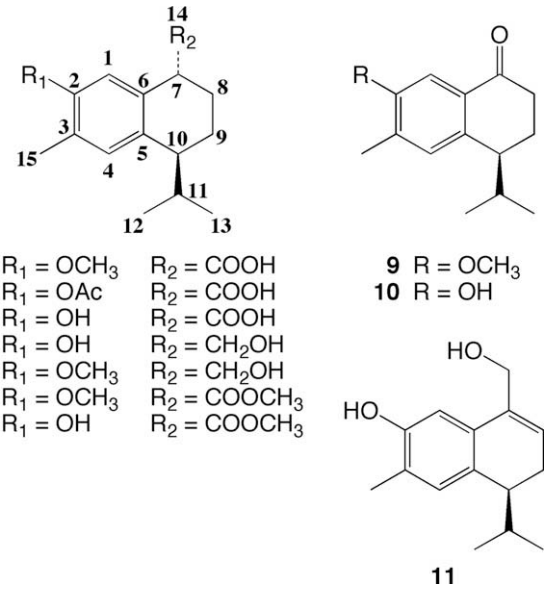
Fig. 1. Borneol and flavonoids as constituents of camphorweed exudates.

of the subcuticular storage space of similar glandular trichomes of the aerial parts *of Artemisia annua* L., without extracting other plant tissues (Duke et al., 1994, 1999; Tellez et al., 1999).

Similarly, the leaf surfaces of many other species contain sesquiterpene acids (*e.g.*, Ceccherelli et al., 1985) and diterpenes (*e.g.*, Urzúa et al., 1997b). These compounds are thought to function to protect plants from biotic threats (Duke et al., 2000b; Read and Menary, 2000). Only small amounts of phytotoxic calamenene carboxylic acids were found in a DCM extract of the entire leaf. This could mean that these compounds are generated by photo-oxidation (Simmonds et al., 2004) and/or microbial synthesis (Hashidoko et al., 1993) on the leaf surface. However, similar results with sesquiterpenes in *A. annua* clearly showed that synthesis of these compounds is confined to glandular trichomes, where the terpenes are stored in easily broken subcuticular compartments (Duke et al., 1994, 1999; Tellez et al., 1999).

2.2. Compounds from the leaf surface

In preliminary studies, we found that the DCM rinsate made from aerial parts of camphorweed had significant plant growth inhibitory activity against A *stolonifera* and *L. sativa* seedlings. Based on this result, we isolated phytotoxins from this rinsate by bioassay-guided fractionation. The calamenene-type sesquiterpene carboxylic acid (**2**) and alcohol (**5**), respectively, were obtained as active compounds (Fig. 3). These compounds had been isolated from the related species *H. grandiflora* Nutt. (Bohlmann et al., 1979). The acid **2** was a known compound having an optical rotation of $[\alpha]_D^{25}$ +8.5 (*c* 0.01, MeOH). Bohlmann et al. reported its optical rotation as $[\alpha]_D^{24}$ +6.9 (*c* 1.97, $CHCl_3$) therefore implying that both of the data were identical (Bohlmann and Zdero, 1979). Sufficient analogues were isolated for structure activity relationship (SAR) studies. Two analogues of (**2**), its acetate (**3**) and the demethyl derivative (**4**), were isolated from the same rinsate. Ketones (**9**, **10**) and a new dehydroderivative of **5** (**11**) were also isolated (see Fig. 3). The new compound **11** showed strong similarities in both NMR spectroscopic data and silica gel TLC Rf values to that of **5**. Compared with **5**, in the ^{1}H NMR spectroscopic data of **11**, a new olefinic proton signal at 5.87 ppm was evident and the methine proton assigned as C7 at 2.79 ppm of **5** was absent. Based on other spectroscopic data, this compound was identified as the 7,8-dehydroderivative of **5**. In an effort to understand the relationships between structure and activity, methylation of a few of the calamenene-type sesquiterpenes was performed. Similarly, in order to determine the requirement of the carboxyl group for plant growth inhibitory activity, corresponding esters were prepared using the BF_3–MeOH complex.



2 $R_1 = OCH_3$ $R_2 = COOH$
3 $R_1 = OAc$ $R_2 = COOH$
4 $R_1 = OH$ $R_2 = COOH$
5 $R_1 = OH$ $R_2 = CH_2OH$
6 $R_1 = OCH_3$ $R_2 = CH_2OH$
7 $R_1 = OCH_3$ $R_2 = COOCH_3$
8 $R_1 = OH$ $R_2 = COOCH_3$

9 R = OCH_3
10 R = OH

11

Fig. 3. Calamenene-type sesquiterpenes from camphorweed and their derivatives.

The monoterpene alcohol, borneol (**1**), and the methylated flavonoids, centaureidin (**12**) (Rashid et al., 1992), pectolinarigenin (**13**), hispidulin (**14**) (Liu et al., 1992) and sorbifolin (**15**) were isolated and identified as constituents of the rinsate (see Fig. 1). Three of these flavonoids were previously reported as constituents of *H. latifolia* Buckley (synonym of *H. subaxillaris*) (Rojo et al., 2004). There are reports of flavonoids, including hispidulin (**14**), on leaf surfaces of Asteraceae (Wollenweber et al., 1991), in the trichomes of *Dilosthepium* sp. (Urzúa et al., 1997a), and of polymethylated flavonoids in leaf surface exudates of Capparaceae (Wollenweber et al., 1989).

2.3. Plant growth inhibitory activity of rinsate constituents

The rinsate made from aerial tissues of camphorweed inhibited growth of both *A. stolonifera* and *L. saliva* seedlings. Compound **2** was highly toxic to *A. stolonifera* seedlings at 100 μg/ml, but its activ-

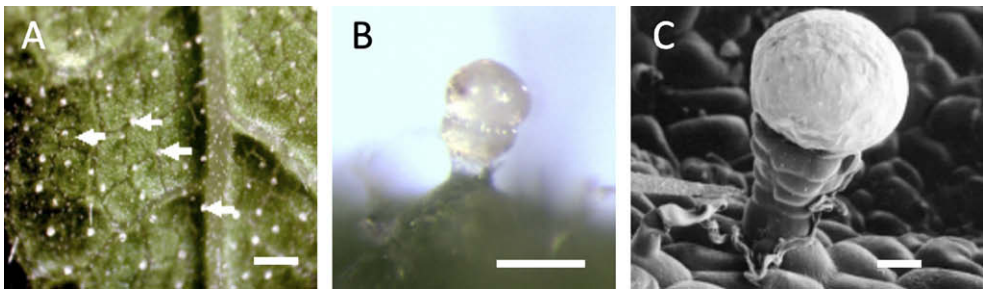


Fig. 2. Microscopic images of camphorweed leaf surface features. (A) Dissecting microscopic image of glandular trichomes indicated by arrows (bar = 1 mm): (B) Optical image of glandular trichome (bar = 50 μm); (C) SEM image of a glandular trichome (bar = 10 μm).

ity was much less against *L. sativa* seedlings at 250 μg/ml (Table 1). Two compounds (**2**, **5**) isolated by bioassay-guided fractionation, showed significant plant growth inhibitory activity, although alcohol **5** only showed significant plant growth inhibitory activity against *L. sativa*. Compound **2** was the strongest plant growth inhibitor of the isolated calamenene carboxylic acids. However, the acetate (**3**), and the demethyl derivatives of **2** (**4**), had plant growth inhibitory activity against *L. sativa* at 250 μg/ml (Table 1). Furthermore, the corresponding ketone (**9**) showed activity similar to that of **2**. The core structure of calamenene sesquiterpene carboxylic acids, **2**–**4** is the tetrahydro-α-naphthoic acid moiety. Its analogues have already been patented as herbicides (Takematsu et al., 1986). Additionally, this core structure is present in auxin-like molecules (Ferro et al., 2007). Interestingly, the order of plant growth inhibitory effects of **5** and **11** correlated with plant growth activity of hydro-1-naphthoic acid derivatives that 6-methyl-l,2,3,4-tetrahydro-l-naphthoic acid (minimum concentration causing a 10% elongation: 0.08 mM) and 6-methyl-3,4-dihydro-1-naphthoic acid (minimum concentration causing a 10% elongation: 0.5 mM) evaluated using the pea straight-growth test (Fujita et al., 1967) (Table 1).

Some of the phytotoxic calamenene-type carboxylic acids (**2**, **4**) and alcohols (**5**, **11**) caused hormesis in the duckweed test (see Fig. 4). Hormesis is the stimulatory effect of a toxicant at a low, non-toxic dose. It is commonly found in dose–response studies of phytotoxins, including allelochemicals (An, 2005; Belz et al., 2007; Duke et al., 2006). The strongest duckweed growth inhibitor was **2** with an IC_{50} of 37.5 μM (Fig. 4). The calamenene alcohols were less active than the acids. Eight of the 26 commercial herbicides tested with this bioassay were less active than **2** (Michel et al., 2004).

Table 1
Plant growth inhibitory activity of calamenene-type sesquiterpenes.

Compound	Conc. (μg/ml)	*Agrostis stolonigfera*	*Lactuca sativa*
2	100	4	1
	250	4	1
	500	4	2
3	100	1	1
	250	3	3
	500	4	4
4	100	2	1
	250	3	3
	500	4	4
5	100	nt	nt
	250	3	4
	500	4	5
6	100	1	2
	250	2	nt
	500	3	3
7	100	1	0
	250	2	0
	500	3	0
8	100	1	0
	250	2	1
	500	3	1
9	100	3	0
	250	4	0
	500	4	0
10	100	nt	nt
	250	3	0
	500	4	2
11	100	0	0
	250	1	0
	500	3	0
Rinsate	1000	5	4
DCM extract	1000	3	5

Values mean that plants were rated visually on a scale of 0–5 (no effect to 100% inhibition of growth) after a 7-day incubation. nt, not tested.

The monoterpene alcohol borneol (**1**), which causes the camphor-like odor from this plant, was not active in our bioassays. The dominant flavonoid in the rinsate, hispidulin (**14**), was reported as a phytotoxin against seedlings of onion, radish and cucumber at 500 μM (Baruah et al., 1994), but all flavonoids (**12**–**15**) isolated in our study showed no significant plant growth inhibitory activity.

2.4. SAR of calamenene-type sesquiterpenes

Esterification of calamenene carboxylic acids (**2**, **4**) decreased their activity against *A. stolonifera* seedlings, and activity almost disappeared against *L. sativa* (Table 1). This suggested that the appearance of this activity was dependent on the presence of a carboxyl group in the chemical structure. In a similar study, diterpene methyl esters decreased the phytotoxicity compared to their corresponding diterpene carboxylic acids (Sparapano et al., 2004). Finally, carboxyl, ketone, and alcohol substituents at C-7 are acceptable for maintaining plant growth inhibitory activity based on these results (Fig. 2).

2.5. Conclusions

In summary, phytotoxicity bioassay-guided fraction of the DCM rinsate of *H. suhaxillaris* aerial tissues provided the highly active calamenene-type sesquiterpenes. The strongest plant growth inhibitor against A *stolonifera* and *L. sativa* seedlings, as well as duckweed (*L. pausicostata*), was compound (**2**) with an IC_{50} of 37.5 μM against duckweed. The non-methoxylated isomer of **2**, compound **4**, was also quite active against duckweed with an IC_{50} of 62.0 μM. This level of activity is in the range of commercial herbcides evaluated with the same bioassay in our laboratory (Michel et al., 2004). In that study of 26 commercial herbicides with 19 different modes of action, the IC_{50} values ranged from 0.003 μM for sulcotrione to 407 μM for asulam. We have also found more active natural compounds than **2** in our studies. For example, the sesquiterpene 6β-angeloyloxy-10β-hydroxyfuranoeremophilane, from the herbaceous plant *Ligularia macrophylla*, had an IC_{50} of 2.94 μM against duckweed (Cantrell et al., 2007). The activity of **2** is sufficient to warrant further study of its mode of action and, perhaps, its role in chemical ecology, including allelopathy.

3. Experimental

3.1. General

Laboratory grade reagents and solvents were purchased locally. Analtech (Newark, DE) TLC plates (Uniplate, 250 μm) were used for methods development and chromatographic evaluations; spots were detected either by UV or by spraying with a vanillin in EtOH solution followed by heating. The optical microscope and SEM were Nikon SMZU and JEOL JSM 840, respectively. The ^{1}H and ^{13}C NMR spectra were recorded in $CDC1_3$ on a Bruker Avance 400 MHz spectrometer. The HPLC method development work was performed using an Agilent 1100 system equipped with a quaternary pump, autosampler, diode-array detector, and vacuum degasser. High resolution mass spectra were recorded on a JEOL Accu TOF, JMS T100LC. Optical rotations were measured with Horiba, high sensitivity polarimeter SEPA 300.

3.2. Plant material, extraction and isolation

H. subaxillaris raw material was collected from sandy fields located in Lafayette County, Mississippi, USA (N 34°21′57.967″, W

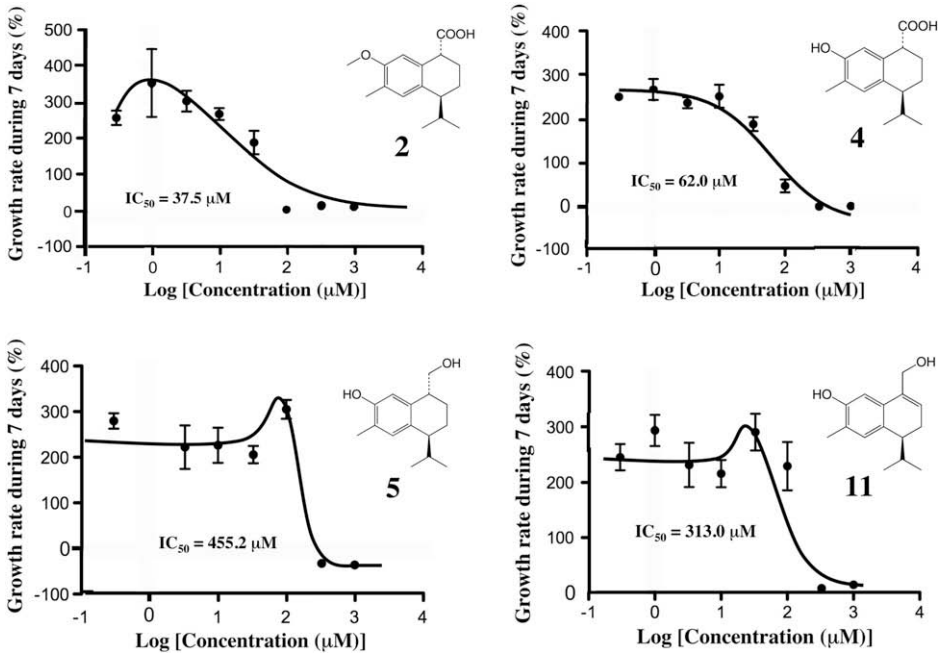


Fig. 4. Plant growth inhibitory activity of calamenene carboxylic acids (**2**, **4**) and alcohols (**5**, **11**) against duckweed at 7days after treatment.

89° 33′ 32.523″; WGS84), and a voucher specimen (KYO73385) has been deposited in the Kyoto University museum herbarium in Kyoto, Japan. For extraction purposes, fresh aerial tissues (2 kg) were rinsed with CH_2CI_2 (1 L) with the resulting solution filtered using a Buchner funnel. The solvent was removed via a rotary evaporator under reduced pressure to obtain a brown oil (16.4 g, yield 0.82%) as the rinsate. A portion of this rinsate (2.9 g) was next partially dissolved using MeOH (50 ml) and filtered using a Buchner funnel providing **1** (257 mg) as a white gummy solid. The filtrate was concentrated producing a brown oil (2.4 g) which was separated using a silica gel flash CC system operating at 40 mL/min (SP1, Biotage, 40 + M) and with elution using a step gradient of hexane and EtOAc [step 1 was hexane (500 ml); step 2 was a linear gradient from 0% EtOAc to 100% EtOAc (2544 ml)] to obtain fr. 1–9. From a portion of fr.6 (397 mg), compounds **5** (79.7 mg) and **11** (25.2 mg) were isolated by silica gel (Sorbent Technologies, 40–63 μm particle, 230 × 400 mesh) CC purification eluted with $CHCl_3$ and MeOH (20:1). Following removal of borneol (**1**) from fr.3 (764.4 mg), in a manner similar to that described above, compound **9** (3.7 mg) was isolated using a silica gel flash CC system operating at 40 mL/min (SP1, Biotage, 40 + M) with elution using a step gradient of hexane and EtOAc [step 1 was hexane (400 ml); step 2 was a linear gradient from 0% EtOAc to 40% EtOAc (2544 ml)], followed by preparative Si-gel TLC developed with CHCI3. From fr.4 (752 mg), compound **10** (2.3 mg) was isolated by silica gel (Sorbent Technologies, 40–63 μm particle, 230 × 400 mesh, 35 g) CC purification eluted with $CHCl_3$, followed by purification using a FlashTube 2002 chromatography column (Trikonex AB, Sweden) eluted with $CHCl_3$.

For the acids, the rinsate (16.4 g) was separated by liquid–liquid partitioning between EtOAc (100 ml) and saturated $NaHCO_3$ (100 ml) in triplicate. Following acidification of this aqueous solution with a dilute aqueous H_2SO_4 solution to pH 4, this liquid was extracted using EtOAc (200 ml) to obtain a sesquiterpene carboxylic acid fraction (1.057 g). A portion of this acid fraction (321 mg) was separated by ODS-HPLC (Imtakt, Unison US-C18, 5 μm, 150 × 20 mm) eluted using CH_3CN–H_2O (1:1, v/v) at 10 mL/min to give **2** (22.8 mg), **3** (14.7 mg), and **4** (45.7 mg) (Bohlmann and Zdero, 1979).

Methylated flavonoids (**12**–**15**) were isolated from each fraction as described below. Fr.5 (1 g) was separated using silica gel (Sorbent Technologies, 40–63 μm particle, 230 × 400 mesh, 100 g) CC with elution using $CHCl_3$:MeOH (100:1) to give 5 subfractions of which compound **12** (5.0 mg) was purified by recrystallization from subfraction 2 (154 mg). Also from subfraction 2, flavonoid **13** (2.5 mg) was isolated with FlashTube 2002 chromatography (Trikonex AB, Sweden) eluted with $CHCl_3$:MeOH (20:1, v/v). Following storage of the remaining portion of fr.6 at −4 °C, yellow crystals were produced. The crystals were washed with EtOAc followed by partially dissolving with MeOH providing MeOH dissolved and insoluble parts. The flavonoids **14** (112 mg) and **15** (26.3 mg) were isolated from the MeOH insoluble part (200 mg) with silica gel (Sorbent Technologies, 40–63 μm particle, 230 × 400 mesh, 140 g) CC elution using $CHCl_3$–Me0H (19:1, v/v). These isolated compounds were analyzed and characterized by their spectroscopic data.

3.2.1. 2-Methoxy-calamenene-l4-carboxylic acid (*2*)

Colorless crystals, m.p. 96 °C, $[\alpha]_D^{25}$ +8.5 (*c* 0.01, MeOH); UV λ_{max}, nm (log ε MeOH): 220 (4.2), 279.5 (3.6), 284.5 (3.6); ^{1}H NMR spectroscopic data in complete agreement with that previously reported (Bohlmann and Zdero, 1979); ^{13}C NMR (400 MHz, $CDC1_3$): δ 176.5 (H-14), 152.3 (C-2), 132.7 (C-5), 132.6 (C-6), 131.4 (C-4), 123.5 (C-3), 115.9 (C-l), 52.6 (CH_3–O–), 45.3 (C-7), 43.0 (C-10), 31.7 (C-ll), 24.8 (C-8), 21.7 (C-12), 21.1 (C-9), 17.8 (C-13), 16.2 (C-15); HR-ESI-TOF-MS, found *m*/*z* 285.14478 (M+Na) ($C_6H_{22}NaO_3$), calcd. 285.14666, 261.14941 (M−l) ($C_{16}H_{21}O_3$), calcd. 261.14907.

3.2.2. 2-Acetoxy-calamenene-14-carboxylic acid (*3*)

Colorless oil, $[\alpha]_D^{25}$ +12.1 (*c* 0.049, EtOH); UV λ_{max}, nm (log ε EtOH):220 (3.9), 269.0 (2.9), 277.0 (2.9); ^{1}H NMR spectroscopic data in complete agreement with that previously reported (Bohlmann and Zdero, 1979); ^{13}C NMR (400 MHz, $CDC1_3$): δ 180.7 (C-14), 169.7 (CH_3–CO–), 147.1 (C-2), 138.6 (C-5), 132.2 (C-6), 131.2 (C-4), 128.8 (C-3), 123.0 (C-l), 44.4 (C-7), 43.0 (C-10), 31.1 (C-ll), 24.0 (C-8), 21.4 (C-12), 21.0 (C-9), 20.4 (CH_3–CO–), 17.5 (C-l3),

16.3 (C-l5); HR- ESI-TOF-MS, found *m/z* 313.14186 (M+Na) ($C,7H_{22}NaO_4$), calcd. 313.14158, 289.11473 (M−l) ($C_{17}H_{21}O_4$), calcd. 247.14398.

*3.2.3. 2-Hydroxy-calamenene-l4-carboxylic acid (**4**)*

Colorless oil, $[\alpha]_D^{25}$ +16.0 (*c* 0.01, MeOH); UV λ_{max}, nm (log ε MeOH): 224.0 (2.9), 283.0 (3.5); ^{1}H NMR spectroscopic data in complete agreement with that previously reported (Bohlmann and Zdero, 1979); ^{13}C NMR (400 MHz, $CDC1_3$): δ 181.6 (C-14), 152.1 (C-2), 133.0 (C-5), 132.0 (C-6), 131.5 (C-4), 123.8 (C-3), 116.0 (C-l), 45.0 (C-7), 43.1 (C-10), 31.6 (C-ll), 24.5 (C-8), 21.8 (C-12), 21.2 (C-9), 18.0 (C-13), 15.7 (C-15); HR-TOF-MS, found *m/z* 271.13040 (M+Na) ($C_{15}H_{20}NaO_3$), calcd. 271.13101, 247.08867 (M−l) ($C_{15}H_{19}O_3$), calcd. 247.13342.

*3.2.4. 2-Hydroxy-calamenene-14-ol (**5**)*

Orange oil, $[\alpha]_D^{25}$ +25.9 (*c* 0.047, EtOH), UV λ_{max}, nm (log ε EtOH): 220.0sh (3.9), 283.0 (3.3); ^{1}H NMR spectroscopic data in complete agreement with that previously reported (Bohlmann and Zdero, 1979); ^{13}C NMR (400 MHz, $CDC1_3$): δ 151.9 (C-2), 136.2(C-6), 133.2 (C-5), 131.3 (C-4), 121.9 (C-3), 113.7 (C-l), 67.4 (C-14), 42.8 (C-10), 40.3 (C-7), 32.0 (C-ll), 24.1 (C-8), 21.6 (C-12), 21.4 (C-12), 21.1 (C-9), 18.0 (C-13), 15.8 (C-15); HR-ESI-TOF-MS, found *m/z* 235.16743 (M+H) ($C_{15}H_{23}O_2$), calcd. 235.16980, 233.15481 (M−l) ($C_{15}H_{21}O_2$), calcd. 233.15415.

*3.2.5. 2-Methoxy-calamenene-14-ol (**6**)*

Compound **5** (17.3 mg, 0.073 mmol) was dissolved in acetone (1 ml) with K_2CO_3(100 mg) and methyl iodide (0.1 ml, l mmol) added to this solution. This solution was kept at room temperature for 68 h. After methylation, H_2O (ca. lml) was poured into this solution and the product was extracted with EtOAc (2 ml each, three times). After purification by silica gel CC, 2-methoxy-calamenene-14-ol (**6**, 9.3 mg, 0.375 mmol, 51.4% yield) was obtained. Colorless oil, $[\alpha]_D^{25}$ +28.4 (*c* 0.028, EtOH), λ_{max}, nm (log ε EtOH): 219.0sh (4.0), 279.5 (3.4), 285.5 (3.4); ^{1}H NMR (400 MHz, $CDC1_3$): δ 6.97 (1H, s, H-4), 6.70 (1H, s, H-l), 3.79 (3H, s, CH_3–O–), 3.43-3.74 (2H, br s, H-14), 2.88 (1H, ddd, *J* = 11.7, 6.32, 5.37 Hz, H-7), 2.46–2.59 (1H, m, H-9), 2.17 (3H, s, H-15), 2.09–2.16 (2H, m, H-10, H-11), 1.88–2.03 (1H, m, H-8), 1.75–1.85 (1H, m, H-9), 1.56–1.75 (1H, m, H-8), 0.99 (3H, d, *J* = 6.5 Hz, H-12 or H-13), 0.75 (3H,d, *J* = 6.5 Hz, H-12 or H-13); ^{13}C NMR (400 MHz, $CDC1_3$): δ 135.7 (C-6), 132.9 (C-5), 131.1 (C-4), 124.3 (C-3), 109.2 (C-l), 67.5 (C-14), 55.4 (CH_3-O-), 42.8 (C-10), 40.8 (C-7), 32.1 (C-ll), 23.7 (C-8), 21.7 (C-l2), 20.9 (C-9), 18.2 (C-l3), 16.2 (C-15).

*3.2.6. 2-Methoxy-calamenene-l 4-carboxylic acid methyl ester (**7**)*

The crude calamenene carboxylic acid fraction (160 mg) was dissolved in MeOH (1 ml) and a 50–52% BF_3-MeOH complex in MeOH (1 ml, 5 mM BF_3–MeOH complex) was added to this solution. This solution reacted a few minutes at 60 °C, after which H_2O (*ca.* 2 ml) was added and the crude product was extracted with hexane (2 ml × 3). The hexane layer was washed by saturated $NaHCO_3$ aqueous solution in order to remove any unesterified acids. The crude reaction mixture was purified by silica gel CC eluted with a mixture of hexane and EtOAc (10:1, v/v ratio), providing 2-methoxy-calamenene-14-carboxylic acid methyl ester (7, 7.5 mg, 4.7%). Other acids were not esterified under these conditions. Colorless oil, $[\alpha]_D^{25}$ +12.1 (*c* 0.0065, EtOH); λ_{max}, nm (log ε EtOH): 217.5sh (4.0), 279.5 (3.2), 286.5 (3.2); ^{1}H NMR (400 MHz, $CDC1_3$): δ 6.99 (1H, s, H-4), 6.58 (1H, s, H-l), 3.77 (3H, s, CH_3–O–), 3.73 (1H, t. *J* = 5.27 Hz, H-7), 3.68 (3H, s, CH_3–O–CO), 2.58 (1H, dd, *J* = 13.5, 6.3 Hz, H-10), 2.20–2.30 (2H, m, H-8, H-l1), 2.17 (3H, s, H-15), 1.79–1.88 (1H, m, H-7), 1.72–1.79 (1H, m, H-8), 1.63–1.72 (1H, m, H-9), 0.99 (3H, d, *J* = 6.8 Hz, H-12 or H-13), 0.71 (3H, d, *J* = 6.8, H-12 or H-13); ^{13}C NMR (400 MHz, $CDC1_3$): δ 175.5 (C-14), 155.6 (C-2), 132.31 (C-5), 132.29 (C-6), 130.7 (C-4), 125.5 (C-3), 110.9 (C-l), 55.4 (CH_3-O-), 52.1 (CH3-O-CO), 45.3 (C-7), 42.7 (C-10), 31.3 (C-l1), 24.7 (C-8), 21.4 (C-l2), 20.6 (C-9), 17.4 (C-13), 16.3 (C-15); HR-ESI-TOF-MS, found *m/z* 299.16019 (M + Na) ($C_{17}H_{24}NaO_3$), calcd. 299.16231.

*3.2.7. 2-Hydroxy-calamenene-14-carboxylic acid methyl ester (**8**)*

The crude recovered calamenene carboxylic acid fraction (62.5 mg), from preparation of 7, was dissolved in MeOH (1 ml) and a 50–52% BF_3–MeOH complex in MeOH (1 ml, 5 mM BF_3–MeOH complex). This solution was allowed to react for 1 h at 80 °C followed by addition of H_2O (*ca.* 2 ml) with the whole then extracted with hexane (3 × 2 ml). The combined hexane soluble were washed using satd. $NaHCO_3$ aqueous solution in order to remove acids. The crude reaction mixture was purified by silica gel CC eluted with a mixture of hexane and EtOAc (5:1, v/v) to afford 2-hydroxy-calamenene-14-carboxylic acid methyl ester (**8,** 23.7 mg). Colorless oil, $[\alpha]_D^{25}$ +18.8 (*c* 0.024, EtOH); λ_{max}, nm (log ε EtOH): 218.5sh (4.1), 284.5 (3.5), 286.5 (3.5) nm (EtOH); ^{1}H NMR (400 MHz, $CDC1_3$): δ 6.97(1H, s, H-4), 6.53 (1H, s, H-l), 5.37-5.73 (1H, br s, ArOH), 3.69 (3H, s, CH_3–O–), 2.55 (1H, dd, *J* = 12.9, 6.1 Hz, H-10), 2.17–2.27 (2H, m, H-8, H-11), 2.17 (3H, s, H-15), 1.79–1.88 (1H, m, H-7), 1.71–1.79 (1H, m, H-8), 1.62–1.71 (1H, m, H-9), 0.99 (3H, d, *J* = 6.8 Hz, H-12 or H-l3), 0.74 (3 H, d, *J* = 6.8, H-12 or H-13); ^{I3}C NMR (400 MHz, $CDCl_3$): δ 176.2 (C-14), 152.0 (C-2), 132.4 (C-5), 132.3 (C-6), 131.1 (C-4), 123.2 (C-3), 115.6 (C-l), 52.3 (CH_3–O–CO), 45.0 (C-7), 42.7 (C-10), 31.4(C-11), 24.5(C-8), 21.4 (C-12), 20.8 (C-9), 17.5 (C-13), 15.9 (C-l5); HR-ESI-TOF-MS, found *m/z* 285.14400 (M+Na) ($C_{16}H_{22}NaO_3$), calcd. 285.14666, 261.14741 (M−l) ($C_{16}H_{21}O_3$), calcd. 261.14907.

*3.2.8. 2-Methoxy-14-calamenenone (**9**)*

Orange oil, $[\alpha]_D^{25}$ −41.5 (*c* 0.006, EtOH); λ_{max}, nm (log ε EtOH): 226.0 (4.1), 263.0 (3.8), 320.0 (3.3); ^{1}H NMR spectroscopic data in complete agreement with that previously reported (Bohlmann and Zdero, 1979); ^{13}C NMR (400 MHz, $CDC1_3$): δ 198.8 (C-7), 156.7 (C-2), 140.3 (C-6), 133.2 (C-3), 131.4 (C-5), 131.2 (C-4), 107.2 (C-l), 55.7 (CH_3-O-), 44.2 (C-10), 35.4 (C-8), 30.5 (C-ll), 24.6 (C-9), 21.8 (C-12), 20.0 (C-l3), 17.0 (C-l5); HR-ES1-TOF-MS, found *m/z* 255.12632 (M+Na) ($C_{15}H_{20}NaO_2$), calcd. 255.13610.

*3.2.9. 2-Hydroxy-14-calamenenone (**10**)*

Dark orange oil, $[\alpha]_D^{25}$ 0.0 (*c* 0.01, EtOH); λ_{max}, nm (log ε EtOH): 220.5 (3.9), 299.0 (3.2), 330.0 (3.3); ^{1}H NMR spectroscopic data in complete agreement with that previously reported (Bohlmann and Zdero, 1979); ^{13}C NMR (400 MHz, $CDC1_3$): δ 200.0 (C-7), 153.4 (C-2), 140.3 (C-6), 131.7 (C-3), 131.6 (C-4), 131.3 (C-5), 112.5 (C-l), 44.1 (C-10), 35.5 (C-8), 30.4 (C-ll), 24.6 (C-9), 21.8 (C-12), 20.0 (C-13), 16.7 (C-15); HR-ESI-TOF-MS, found *m/z* 241.11976 (M+Na) ($C_{14}H_{18}NaO_2$), calcd. 241.12045, 217.12314 (M−l) ($C_{I4}H_{17}O_2$), calcd. 217.12285.

*3.2.10. 7,8-Dehydro-2-Hydroxy-calamenene-14-ol (**11**)*

Pale orange crystrals, m.p. 128-131°C, $[\alpha]_D^{25}$ −35.3 (*c* 0.01, $CHCl_3$); λ_{max}, nm (log ε $CHCl_3$): 241.5 (4.1), 266.5 (3.7), 300.5 (3.5); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (2H, s, H-l,H-4), 5.87(1H, s, H-8), 4.36 (1H, d, *J* = 12.0 Hz, H-14), 4.29 (1H d, *J* = 12.0 Hz, H-14), 2.36 (2H, br s, H-9, H-9), 2.26–2.32 (1H, br. m, H-10), 2.21(3H, s, H-15), 2.02 (1H, s, *J* = 6.8 Hz, H-11), 0.76 (3H, d, *J* = 6.7 Hz, H-12 or H-13), 0.93 (3H, d, *J* = 6.6 Hz, H-12 or H-13); ^{13}C NMR (400 MHz, $CDC1_3$): δ 152.7 (C-2), 134.9 (C-6), 131.7 (C-4), 131.4 (C-7), 131.2 (C-5), 127.1 (C-8), 122.6 (C-3), 110.0 (C-l), 64.5 (C-14), 43.3 (C-10), 30.7 (C-ll), 25.8 (C-9), 21.5 (C-12), 20.4 (C-13), 16.0 (C-15); HR-ESI-TOF-MS, found *m/z* 255.14060 (M+Na) ($C_{15}H_{20}NaO_2$), calcd. 255.13610, 231.13864 (M−l) ($C_{15}H_{19}O_2$), calcd. 231.13850.

3.3. Phytotoxicity bioassay

Phytotoxicity of the extract, fractions and sesquiterpenes were obtained for both lettuce (*Lactuca sativa* cv. Iceberg) and bentgrass (*Agrostis stolonifera* cv. penncross) using a 24-well plate bioassay (Dayan et al., 1999). Each sample was dissolved in acetone to make a stock solution which was diluted to the test concentrations in the wells. All wells, including control treatments, received the same amount of acetone (1% v/v). Plates were incubated at 25 ± 2 °C under fluorescent lights with a 16-h photoperiod at 400 μmol m^{-2} s^{-1} photosynthetically active radiation. Plants were rated visually on a scale of 0–5 (no effect to 100% inhibition of growth) after a 7-day incubation. The activity was tested in the range of 0.01–1 mg/ml and by three replicates.

A duckweed bioassay was performed with phytotoxic sesquiterpenes (Tanaka et al., 1993; Michel et al., 2004). Plants of duckweed (*Lemna pausicostata*) were incubated with growth medium (pH 6.4) in a conical flask. Plants were grown autotrophically for a week in a growth chamber in continuous illumination (105 μmol m^{-2} s^{-1}) at 25 ± 2 °C. Fifty colonies of duckweed from incubation colony placed in 6-cm diam. polystyrene Petri dishes with 5 ml of nutrient medium containing test compound and 0.5% DMSO. This level of DMSO had no effect on duckweed growth. The activity was tested in the range of 0.3–1000 μM and by three replicates.

3.4. Scanning electron microscopy (SEM)

Leaf material, taken from leaves three nodes below the apical meristem, were cut into segments <0.5 cm and fixed 24 h in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) solution. Material was then rinsed in 0.1 M cacodylate buffer (pH 7.2) buffer, dehydrated in a graded ethanol series and dried in a critical point drier (Balzers CPD030). The dried tissue was then mounted onto aluminum specimen stubs with double-sided carbon coated adhesive discs and coated with 20 nm of gold–palladium in a sputter coater (Hummer X, Anatech LTD). After coating, specimens were examined by SEM, using a JEOL JSM 840 instrument.

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