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Sesquiterpenes from the wood of *Juniperus lucayana*

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Abstract

Bioassay-guided fractionation of ethanolic extract from the wood of *Juniperus lucayana* afforded three sesquiterpenes named 3-hydroxypseudowiddran-6(7)-en-4-ol (**1**), 15-hydroxyallo-cedrol (**2**) and 12-hydroxywiddrol (**3**) together with six known sesquiterpenes (**4**–**9**) and two known flavonoids (**10** and **11**). Their structures were established on the basis of comprehensive spectroscopic analyses, including 2D NMR spectroscopy and mass spectrometry. The structures of compounds were identified as 1α,4β,11α,11β-tetramethylbicyclo[5,4,0]undec-6(7)-en-3α, 4α-diol (**1**), 4β-hydroxymethyl-5,5,9β-trimethyltricyclo[4.3.0.$2^{1,4}$]undecan-3α-ol (**2**) and 4β-hydroxymethyl-7α,11α,11β-trimethylbicyclo [5.4.0]undec-1-en-4α-ol (**3**). The major compounds isolated were evaluated for their antifungal activity against *Botrytis cinerea*. Widdrol (**7**) was the most active, reaching the 71% inhibition level on mycelial growth after 6 days.

Keywords: *Juniperus lucayana*; 3-Hydroxypseudowiddran-6(7)-en-4-ol; 15-Hydroxyallo-cedrol; 12-Hydroxywiddrol; Antifungal activity; *Botrytis cinerea*

1. Introduction

Juniperus lucayana Britton (sabina) belongs to the Cupressaceae family (Sabina section) and grows only in Cuba, Jamaica and the Bahama Islands. Aqueous extract from stems presents antimicrobial activity against *Staphylococcus aureus* (Martínez et al., 1996). This plant is also used in the treatment of urinary infections (Alakbarov, 2003). During our search for antifungal substances from Cuban plants, we found that the ethanolic extracts from the wood of *J. lucayana* exhibited good antifungal activity against the phytopathogen *Botrytis cinerea* (Ortiz et al., 2004). Studies focusing exclusively on the volatile leaf oils of specimens from the Cuban mainland and from the Isle of Pines, Cuba (Adams, 1987) and also from Jamaica and the Bahama Islands (Adams and Hogge, 1983) have been reported. These studies revealed that *J. lucayana* contains large amounts of α-pinene and limonene with considerable amounts of sabinene, as well as moderate amounts of myrcene, α-terpinene, γ-terpinene, terpinen-4-ol, bornyl acetate, β-cubebene, and various cadinenes. No significant differences in the composition of oils from Jamaica, Bahama Islands or Cuba were found. We report here on the bioassay-guided fractionation of the ethanolic extract from the wood of *J. lucayana* and its antifungal activity against the phytopathogenic fungus *B. cinerea*.

2. Results and discussion

J. lucayana wood was air dried, ground to a powder and thoroughly extracted with EtOH, which was then separated by column chromatography (Si gel) using mixtures of hexane, EtOAc, $CHCl_3$ and MeOH of increasing polarity giving 13 fractions. Fractions were tested against *B. cinerea*

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using fungal growth inhibition assays in accordance with the "*poisoned food technique*" (Soundharrajan et al., 2003). The fractions were dissolved in ethanol to give final concentration solutions of 500 mg l^{-1}. Of the 13 fractions tested, six significatively reduced fungus growth in comparison with the control. Fractions 5, 6 and 11 showed the highest growth inhibition percentage after 6 days. Fractions 1, 7, and 8 were considered actives with growth inhibition percentage from 70% to 85%, fraction 3 showed some weak activity and fractions 2, 4, 12 and 13 displayed no activity (Table 1).

On the basis of their antifungal activity and their thin-layer chromatographies, fractions 5, 6, 8 and 9 were purified by a combination of column chromatography (Si gel, Sephadex LH-20) and HPLC affording the three new sesquiterpenes (**1**–**3**) along with six known sesquiterpenes and two known flavonoids. The structures of the known compounds were identified by physical and spectroscopic data measurement ($[\alpha]_D^{20}$, ^{1}H NMR, ^{13}C NMR, 2D NMR and MS) and by comparing the data obtained with published values, such as cedrol (8β-hydroxy-2,6,6,8-tetramethyltricyclo[4.3.1.1]undecane) (**4**) (Nathan et al., 1984), allo-cedrol (4β,5,5,9β-tetramethyltricyclo[4.3.0.$2^{1,4}$]undecan-3α-ol) (**5**) (Tomita and Hirose, 1973), α-bisabolol (2,10-bisaboladien-7-ol) (**6**) (Miyazawa et al., 1995), widdrol (4β,7α,11α,11β-tetramethylbicyclo[5.4.0]undec-1-en-4α-ol) (**7**) (Enzell, 1962), β-chamigrenic acid (11,11-dimethyl-7-methylenspiro[5.5]undec-2-eno-3-carboxylic acid) (**8**) (Kuo and Lin, 1980), 10,11-dihydroxy-β-bisabolene (**9**) (Bohlmann et al., 1983), naringenine (5,7,4′-trihydroxyflavanone) (**10**) (Levy et al., 1974; Agrawal, 1989) and aromadendrine (3,5,7,4′-tetrahydroxy flavanonol) (**11**) (Agrawal, 1989).

Compound **1** was obtained as a white solid with a m.p. 71–73 °C, with an HR-EIMS molecular ion peak at *m*/*z* 238.1917 consistent with molecular formula $C_{15}H_{26}O_2$ (calc. 238.1933). The IR spectrum showed an absorption band at ν_{max} 3389 cm^{-1} indicating the presence of the hydroxyl group. The ^{1}H NMR (Table 2) and ^{13}C NMR (Table 3) spectra signals at δ 3.84 ppm (1H, *br s*, H-3)/δ 72.2 (C-3) and 69.4 ppm (C-4) confirmed the presence of two hydroxyl groups. The ^{13}C NMR spectrum of **1** contained 15 carbons and its gradient-selected HSQC (gHSQC) with odd/even proton multiplicity editing (parameter mult = 2) spectrum showed the presence of four methyls, five methylenes, two methines and four quaternary carbons. The presence of a trisubstituted double bond was confirmed by its ^{1}H NMR spectra signal at δ 5.46 ppm (1H, *dd*, $J_1 = 5.3$ Hz, $J_2 = 1.51$ Hz, H-6).

Table 1
Antifungal activity of fractions of wood ethanolic extract at 500 ppm after 6 days

Treatment	Mean radial growth (mm)	Inhibition (%)
Control (medium)	$42.72^{a} \pm 0.8$	–
Control (ethanol)	$42.60^{a} \pm 0.7$	–
F-1	$17.80^{d} \pm 1.8$	69.30
F-2	$31.97^{bc} \pm 1.3$	25.56
F-3	$26.87^{c} \pm 1.6$	42.32
F-4	$35.90^{ab} \pm 0.9$	15.13
F-5	$9.03^{e} \pm 0.3$	93.36
F-6	$8.24^{e} \pm 0.4$	94.86
F-7	$12.68^{d} \pm 1.5$	84.49
F-8	$12.81^{d} \pm 2.2$	83.55
F-11	$9.50^{e} \pm 0.4$	91.74
F-12	$31.83^{bc} \pm 1.2$	29.34
F-13	$32.67^{bc} \pm 1.3$	31.10

F: fractions. Means followed by the same letters within a column after ± standard error values are not significantly different ($p \leqslant 0.05$).

The positions of the double bond and the hydroxyl groups were determined by gradient-selected heteronuclear connectivity experiments (gHSQC and gHMBC). The resulting gHMBC (Fig. 1) spectrum of **1** showed correlations between (i) H-6 and C-4, C-1, and (ii) H-12_{Me} and C-3, C-4, C-5.

All of these data suggest that **1** possesses a pseudowiddrene skeleton. Relative stereochemistry was assigned by NOE experiments. The signal corresponding to H-3 (δ 3.84, *br s*) was enhanced upon irradiation at H-12β (δ 1.21), and it was therefore proposed that H-3 and H-12_{Me} were cis, which was the confirmed by Energy minimized molecular models (MOPAC). Compound **1** was assigned as: 3-hydroxypseudowiddran-6(7)-en-4-ol (1α,4β, 11α,11β-tetramethylbicyclo[5,4,0]undec-6(7)-en-3α, 4α-diol).

Compound **2**, obtained as a colorless oil, gave a molecular ion peak at *m*/*z* 238.1926 in its HR-EIMS corresponding to the molecular formula $C_{15}H_{26}O_2$ (calc. 238.1933). The IR band at 3394 cm^{-1} suggests a hydroxyl group. This was supported by its ^{1}H and ^{13}C NMR (Tables 2 and 3) and gHSQC spectra which showed signals at δ 4.37 (1H, *ddd*, $J_1 = 9.6$ Hz, $J_2 = 5.7$ Hz, $J_3 = 2.1$ Hz, H-3)/δ 71.7 (C quaternary, C-3); 3.82 (1H, *d*, $J = 11.3$ Hz, H-15a) and 3.48 (1H, *d*, $J = 11.3$ Hz, H-15b)/δ 65.9 (C methylenic, C-15). The ^{13}C NMR spectrum of **2** (Table 3) contained 15 carbons and its gHSQC spectrum showed the presence of three methyls, six methylenes, three methines and three quaternary carbons. The absence of a double bond and a carbonyl group in its IR and ^{13}C NMR spectra and its molecular ion indicated a possible tricyclic structure compound, typical of *Juniperus* genus. The spectroscopic data of compound **2** were similar to the known allo-cedrol sesquiterpenoid (4β,5,5,9β-tetramethyltricyclo[4.3.0.$2^{1,4}$]undecan-3α-ol) (**5**), which was isolated from various species of *Juniperus* genus (Tomita and Hirose, 1973), the only difference being the presence of another hydroxyl group in a methyl group. The hydroxymethyl group (δ 3.82 (*d*), 3.48 (*d*)), was connected to C-4 (δ 43.2) by their J^2 correlation in the gHMBC experiment, and C-4 showed J^3 correlation with Me-13/Me-14 (0.90(*s*)/0.96(*s*)).

These data and the gHMBC correlation between the proton at δ 0.81 (Me-12) with the C-1 (δ 41.9) and C-9 (δ 40.2) confirmed that **2** is a sesquiterpene analogous to allo-cedrol. Thus, the structure of **2** could be proposed as 4β-hydroxymethyl-5,5,9β-trimethyltricyclo[4.3.0.$2^{1,4}$]undecan-3α-ol, and the trivial name, 15-hydroxyallo-cedrol has been agreed on.

Table 2
^{1}H NMR spectroscopic data (δ, ppm) of compounds **1**–**3** and **7** in $CDCl_3$

Position	1	2	3	7
1	–	–	–	–
2	2.21–2.18 *m* 2.08–2.05 *m*	2.09 *ddd* (12.6, 9.6, 2.9) 1.10 *dd* (12.6, 5.7)	5.44 *dd* (8.7, 6.0)	5.49 *dd* (9.0, 6.0)
3	3.84 *br s*	4.37 *ddd* (9.6, 5.7, 2.1)	2.40 *dd* (14.0, 6.0) 2.15 *dd* (14.0, 8.7)	2.48 *dd* (13.6, 6.0) 1.98 *dd* (13.8, 9.0)
4	–	–	–	–
5	1.77 *dd* (13.0, 1.3) 1.56 *dd* (13.0, 5.5)	–	1.58–1.54 *m*	1.68–1.61 *m*
6	5.46 *dd* (5.3, 1.51)	1.18 *m*	1.51–1.34 *m* 1.78–1.70 *m*	1.68–1.61 *m* 1.51–1.34 *m*
7	–	1.35 *m* 1.29 *m*	–	–
8	2.16–2.09 *m* 1.45–1.40 *m*	1.94 *m* 0.98 *m*	1.51–1.34 *m* 1.27–1.23 *m*	1.51–1.34 *m* 1.28–1.21 *m*
9	1.65–1.59 *m*	1.48 *m*	1.51–1.34 *m* 1.78–1.70 *m*	1.68–1.61 *m* 1.51–1.34 *m*
10	1.65–1.59 *m* 1.48 *m*	1.45 *m* 1.20 *m*	1.51–1.34 *m* 1.27–1.23 *m*	1.51–1.34 *m* 1.28–1.21 *m*
11	–	1.84 *ddd* (13.8, 11.1, 8.3) 1.21 *m*	–	–
12	1.21 *s*	0.81 *d* (7.1)	3.45 *d* (11.0) 3.41 *d* (11.0)	1.20 *s*
13	0.99 *s*	0.90 *s*	1.21 *s*	1.18 *s*
14	0.78 *s*	0.96 *s*	1.07 *s*	1.07 *s*
15	1.0 *s*	3.82 *d* (11.3) 3.48 *d* (11.3)	1.04 *s*	1.06 *s*

Compound **3** was obtained as a white solid with a m.p. 107–109 °C with the molecular formula $C_{15}H_{26}O_2$ as deduced from its HR-EIMS (*m*/*z* 238.1931, calc. 238.1933). The IR spectrum showed absorption bands at ν_{max} 3448 and 1638 cm^{-1} indicating a hydroxyl group and a double bond, respectively. Its ^{1}H NMR spectrum was similar to that of widdrol **7** revealing, as in **7**, the presence of a trisubstituted double bond at δ 5.44 (1H, *dd*, $J_1 = 8.7$ Hz, $J_2 = 6.0$ Hz, H-2), and three methyl groups (δ 1.21, *s*, 3H, H-13; δ 1.07, *s*, 3H, H-14; δ 1.04, *s*, 3H, H-15). A new oxygenated methylene signal at δ 3.45 (1H, *d*, $J = 11.0$ Hz, H-12a) and δ 3.41 (1H, *d*, $J = 11.0$ Hz, H-12b) and the absence of a methyl signal were likewise observed. Comparison of ^{13}C NMR data of compounds **3** and **7** (Table 2), confirmed the presence of a trisubstituted olefinic carbon (δ 154.8 and δ 116.6) and two oxygenated carbons corresponding to C-4 and C-12 at δ 75.2 (C-quaternary) and δ 68.2 (C-methylene), respectively, in compound **3**. Similar values of ^{13}C NMR spectra signals were noted for both compounds.

Table 3
^{13}C NMR spectroscopic data (δ, ppm) of compounds **1**–**3** and **7** in $CDCl_3$

Position	1	2	3	7
1	50.9	41.9	154.8	154.2
2	24.7	41.0	116.6	117.7
3	72.2	71.7	33.7	39.8
4	69.4	43.2	75.2	73.0
5	33.1	34.7	32.7	38.0
6	122.1	53.4	38.3	39.4
7	147.6	24.0	39.8	39.8
8	36.6	35.4	41.6	41.7
9	19.1	40.2	18.6	18.6
10	40.4	30.7	40.1	40.0
11	43.7	19.9	36.9	36.8
12	24.9	17.8	68.2	28.5
13	22.8	21.4	27.1	26.7
14	26.2	26.4	31.7	32.0
15	24.6	65.9	33.0	32.9

These data suggest that compound **3** is similar to widdrol (**7**), differing only in the hydroxylation of a methyl group. The gHMBC correlations of H-12a, b with C-3 (δ 33.7) and C-4 (δ 75.2) allowed for the location of the hydroxyl group at C_{Me}-12. All of the ^{13}C and ^{1}H NMR spectroscopic data of **3** were assigned using the information provided by gHSQC and gHMBC experiments. The relative stereochemistry of C-4 was assigned by NOE and NOESY experiments and comparison with absolute configuration assigned to widdrol **7** (Enzell, 1962). The NOESY spectra exhibited correlations between Me-13 and Me-14 which confirming α disposition to be identical to that of widdrol **7**. Furthermore, in an NOE experiment, irradiation of the H_{Me}-15(β) resulted in enhancements of H-12a, b signals. Thus, the orientation of the hydroxymethyl group was determined to be β. The structure of **3** was assigned as 4β-hydroxymethyl-7α,11α,11β-trimethylbicyclo[5.4.0]undec-1-en-4α-ol.

Studies on the structure–activity relationships of the substrates that inhibited the growth of *B. cinerea* and their relationships to the botryanes, characteristic metabolites produced by the fungus, revealed structural similarities between them (Collado et al., 1998; Aleu et al., 1999). All of them possesses an hydrophobic geminal dimethyl group and an hydrophilic hydroxyl group. The distance between

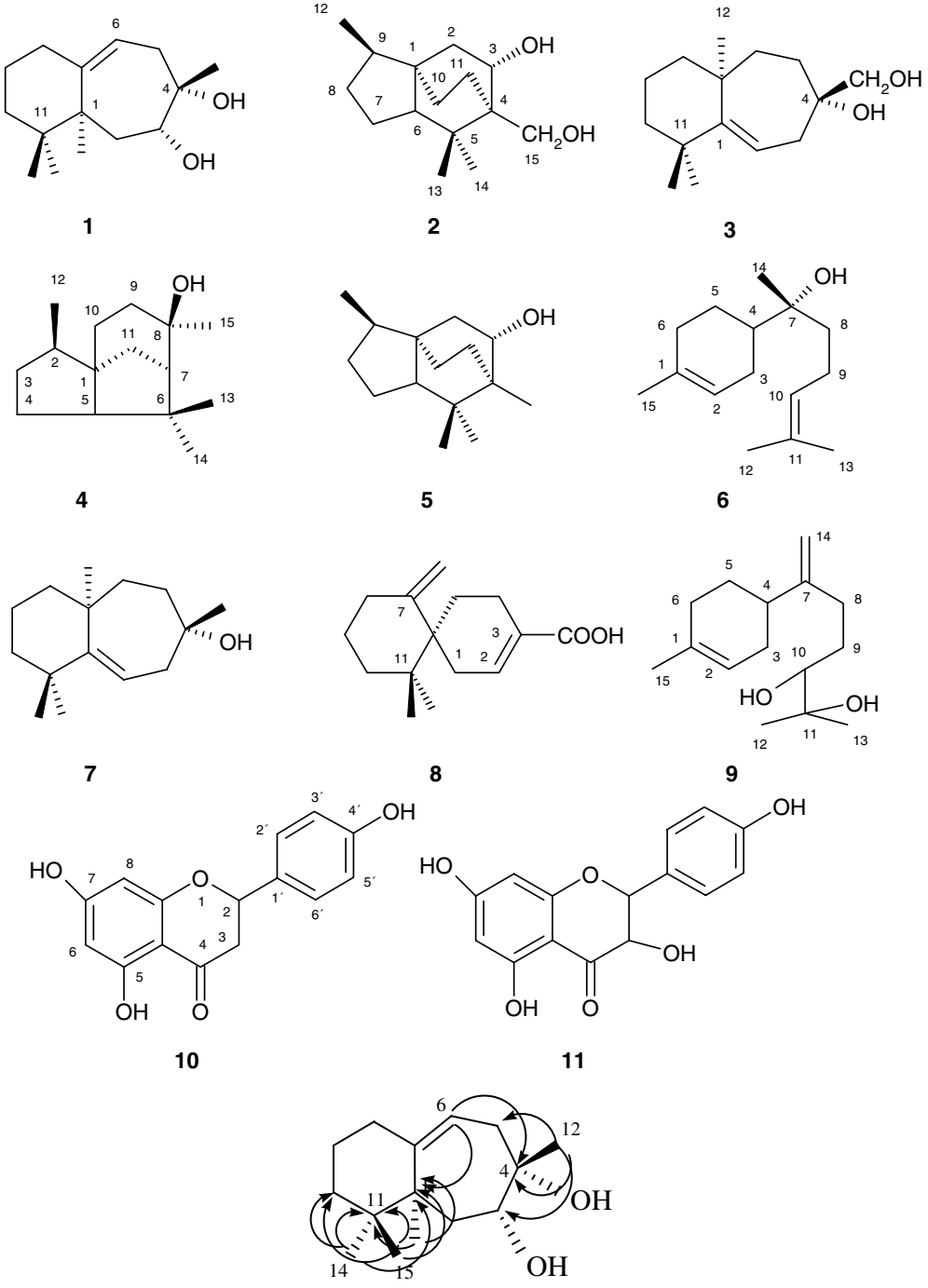

Fig. 1. Selected HMBC correlations of compound **1**.

them seems to mimic the analogous distances found in some botryanes (Collado et al., 1998). There two key distances, 1,3 found between the carbon bound to the dimethyl group and the tertiary hydroxyl group on C-9 of botryane skeleton and 1,5- between the gemdimethyl group and the hydroxyl group on C-10. Compounds **2**, **3**, **4** and **5** possess the 1,3-distance and **6** and **7** the 1,5-distance. Unfortunately compounds **2** and **5** were not isolated in enough amounts to test its antifungal properties.

The antifungal properties of the compounds **3**, **4**, **6** and **7** were examined using fungal growth inhibition assays in accordance with the "*poisoned food technique*" (Soundharrajan et al., 2003). Compounds **4**, **6** and **7** exhibited moderate levels of antifungal activity against *B. cinerea* (Fig. 2), whereas compound **3** was found to be weakly active. As can be seem in Fig. 2, widdrol (**7**) was the most active compound, showing mycelial growth inhibition from 50 ppm, retaining an inhibition percentage of 58% and 71% at 100 and 200 ppm, respectively, after 6 days. In particular, widdrol (**7**) can be used as a lead compound in the future development of semisynthetic derivatives with improved activity against commercial pathogenic fungi.

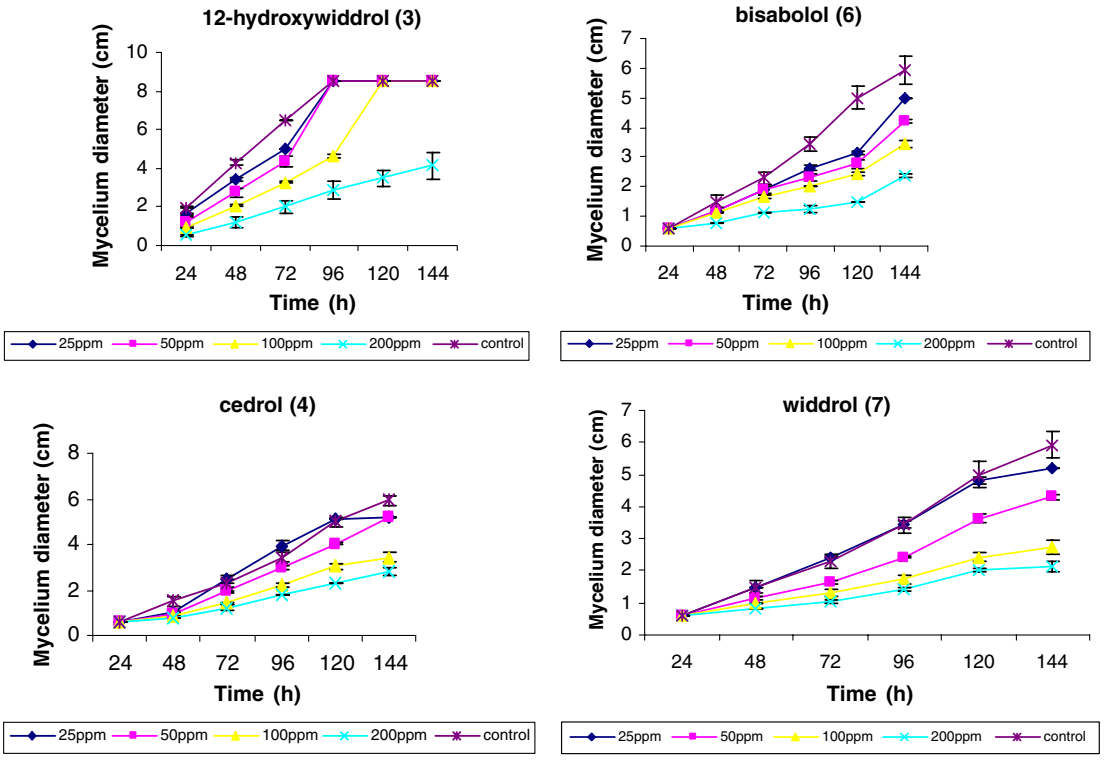

Fig. 2. Antifungal effect of **3**, **4**, **6** and **7** on *B. cinerea*. Values represent means of two independent experiments with three replicates in each experiment. Bars represent SD of the mean.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined with a Perkin–Elmer 241 polarimeter. IR spectra were recorded on a Mattson Genesis spectrophotometer, series FT-IR. ^{1}H and ^{13}C NMR measurements were obtained on Varian Inova 400 NMR and 600 MHz spectrometers with $SiMe_4$ as the internal reference. Mass spectra were recorded on GC/MS Agilent-LR and Finnigan MAT95 S instruments. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with an UV/Vis detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F_{254}, 0.2 mm thick. Silica gel (Merck) was used for column chromatography. Purification by means of HPLC was conducted using a silica gel column Lichrospher Si-60 column (10 μm, 1 cm wide, 25 cm long) and Lichrospher Si-60 column (5 μm, 0.5 cm wide, 25 cm long) for preparative and analytical HPLC, respectively.

3.2. Plant material

J. lucayana was collected at the Institute of Fundamental Research on Tropical Agriculture "Alexander von Humboldt" in Santiago de las Vegas, Cuba, in March, 2003 and identified by Dr. Pedro Sánchez. A specimen of this plant is deposited in the herbarium of the "Instituto de Ecología y Sistemática de las plantas en Cuba" with the number HAC-42498.

3.3. Extraction and isolation

Air-dried powdered wood (870 g) of *J. lucayana* was extracted with ethanol in a Soxhlet apparatus for 8 h. The solvent was evaporated under reduced pressure, obtaining an ethanolic crude extract (52.8 g). This extract was purified by column chromatography using mixtures of *n*-hexane, EtOAc, $CHCl_3$ and MeOH of increasing polarity to obtain 13 fractions. On the basis of the antifungal activity of these fractions, fraction 5 (5.0 g) was rechromatographed by column over Si gel (*n*-hexane/EtOAc with increasing polarity), then the white solid was further purified by HPLC on a semi-preparative column to give **4** (863.7 mg), **5** (4.3 mg) and **6** (66.1 mg). Fraction 6 (9.0 g) was subjected to repeated semi-preparative HPLC separation (EtOAc/*n*-hexane, 10% and CH_2Cl_2/*n*-hexane, 70%) to give **7** (654.1 mg). Fraction 8 (2.0 g) was purified by semi-preparative HPLC (acetone/*n*-hexane and EtOAc/*n* – hexane) to give **8** (42.9 mg), **1** (5.6 mg), **9** (9.4 mg), **2** (2.7 mg) and **10** (39.7 mg). Fraction 9 (1.2 g) was subjected to column chromatography over Si gel (EtOAc/*n*-hexane, 10–15%), then further purified by Sephadex LH-20 (*n*-hexane/$CHCl_3$/MeOH 2:1:1) to give **3** (520.5 mg) and **11** (29.3 mg).

Compounds **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11** are all known: cedrol, allo-cedrol, α-bisabolol, widdrol, β-chamigrenic acid, 10,11-dihydroxy-β-bisabolene, naringenine, aromadendrine.

3.4. 3-Hydroxypseudowiddran-6(7)-en-4-ol (*1*)

White solid, m.p. 71–73 °C; $[\alpha]_D^{20}$ +29.8° ($CHCl_3$, *c* 0.058); IR ν_{max}^{KBr} cm^{-1}: 3389, 2959, 2931, 2873, 1711, 1462, 1030, 877; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 2 and 3; EIMS 70 eV *m/z* (rel. int.): 238 $[M]^+$ (0.66), 220 $[M-H_2O]^+$ (6), 205 $[M-H_2O-CH_3]^+$ (4), 151 (53), 111 (72), 109 (73), 95 (78), 55 (51), 43 (100); HR-EIMS *m/z* 238.1917 $[M]^+$, calc. for $C_{15}H_{26}0_2$, 238.1933.

3.5. 15-Hydroxyallo-cedrol (*2*)

Colorless oil, $[\alpha]_D^{20}$ +496.2° ($CHCl_3$, *c* 0.027); IR ν_{max}^{KBr} cm^{-1}: 3394, 2929, 2869, 1458, 1032, 718; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 2 and 3; EIMS 70 eV *m/z* (rel. int.): 238 $[M]^+$ (18), 220 $[M-H_2O]^+$ (17), 207 (10), 176 (100), 161 (68), 149 (44), 121 (60), 107 (57), 95 (62), 81 (54); HR-EIMS *m/z* 238.1926 $[M]^+$, calc. for $C_{15}H_{26}0_2$, 238.1933.

3.6. 12-Hydroxywiddrol (*3*)

White solid, m.p. 107–109 °C; $[\alpha]_D^{20}$ +173.4° ($CHCl_3$, *c* 0.244); IR ν_{max}^{KBr} cm^{-1}: 3448, 2928, 1638, 1459, 1073, 903, 668; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 2 and 3; EIMS 70 eV *m/z* (rel. int.): 238 $[M]^+$ (14), 220 $[M-H_2O]^+$ (10), 207 (29), 189 (21), 151 (100), 109 (49), 95 (79), 81 (48), 69 (54), 55 (46), 41 (44); HR-EIMS *m/z* 238.1931 $[M]^+$, calc. for $C_{15}H_{26}0_2$, 238.1933.

3.7. Microorganism

B. cinerea culture used in this work, *Bc* 2100, was obtained from the "Colección Española de Cultivos Tipos (CECT)", Universidad de Valencia, Facultad de Biología, Spain, where a culture of this strain is on deposit.

3.8. Antifungal assays. Poison food technique

Fractions or pure compounds (**3**, **4**, **6** and **7**) were dissolved in ethanol to give final of 500 mg/l for fractions and concentrations ranging from 25 to 200 mg/l for pure compounds. Antifungal assays were then carried out in accordance with the poison food technique (Soundharrajan et al., 2003). Solutions of the tested compounds were added to a glucose–malt–peptone–agar (61 g/l of glucose (20 g)–malt (20 g)–peptone (1 g)–agar (20 g), pH 6.5–7.0). The final ethanol concentration was identical in both control and treated cultures. The medium was poured into sterile plastic Petri dishes measuring 9 cm in diameter and 1.0-cm diameter mycelial discs of fungus cut from an actively growing culture were placed at the center of the agar plates. Inhibition of radial growth was measured during 6 days. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the negative control. Two independent assays were conducted, each in triplicate. The results are shown as mean values of colony diameters; (±SD). Inhibition of radial growth was measured for 8 days.

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