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Isolation and structure elucidation of three *neo*-clerodane diterpenes from *Teucrium fruticans* L. (LABIATAE)

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Dedicated to Prof. Francisco Camps on the occasion of his 70th birthday.

Abstract

Recently, the isolation from *Teucrium fruticans* of *neo*-clerodanes, namely 7β-hydroxyfruticolone, 11-hydroxyfruticolone, deacetylfruticolone and 6-acetyl-10-hydroxyteucjaponin B, in addition to fruticolone, isofruticolone and 8β-hydroxyfruticolone (three out of the four previously reported ones), and 6-acetylteucjaponin B (isolated from *T. scordium* and *T. grisebachii*) was reported. Minor compounds presumably of *neo*-clerodane nature were shown by HPLC analysis on a new extract. Three new compounds, difuranofruticol, deoxyfruticolone and 10-hydroxyteucjaponin B, and the known 7,8-didehydrofruticolone were unambiguously elucidated based on extensive NMR spectral studies (one- and two-dimensional experiments). The compounds were assayed for their antifeedant activity against *Spodoptera littoralis* and for their antifungal activity against *Rhizoctonia solani*. Compounds **9**–**11** showed low antifeedant activity and the feeding ratio of **12** was moderate-low. None of the tested compounds displayed significant activity against *R. solani*.

Keywords: *Teucrium fruticans*; Labiatae; Structure elucidation; NMR; *Neo*-clerodane diterpenes; Antifeedant activity; Antifungal activity

1. Introduction

The insect antifeedant property of clerodane diterpenes is the most extensively studied bioactivity of these compounds (Klein Gebbinck et al., 2002). *Scutellaria* and *Ajuga* genera (Labiatae) produce some of the most potent clerodane antifeedants. In *Scutellaria*, jodrellin B (occurring in *S. albida*, *S. galericulata*, *S. grossa*, *S. polyodon*, and *S. woronowii*) and scutecyprol B (found in *S. columnae*, *S. cypria*, *S. grossa* and *S. rubicunda*) show the highest antifeedant index against *Spodoptera littoralis* (Bruno et al., 2002). From *Ajuga pseudoiva* leaves, 14,15-dihydroajugapitin displayed the highest activity (AI > 90 even at 1 ppm dose) amongst several highly active compounds isolated (Ben Jannet et al., 2000). The genus *Teucrium* also belonging to the aforementioned family is one of the richest sources of clerodane diterpenes, and the new natural products are reviewed periodically (Piozzi et al., 1998). In early studies on *Teucrium fruticans*, diterpenes fruticolone (**1**), isofruticolone (**2**) and 8β-hydroxyfruticolone (**3**) were isolated (Savona et al., 1978a,b).

In addition to **1**–**3**, a dichloromethane extract of the aerial parts of this plant allowed recently the isolation of a new group of diterpenoids (Coll and Tandrón, 2004). The structures of the new isolates were: 7β-hydroxyfruticolone (**4**), 11-hydroxyfruticolone (**5**), deacetylfruticolone (**6**) and 6-acetyl-10-hydroxy-teucjaponin B (**7**). 6-Acetylteucjaponin B (**8**), previously reported as

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present in *T. scordium* and *T. grisebachii* (Jakupovic et al., 1985; Tonn et al., 1990), was also isolated from *T. fruticans*.

From plant collected in a different environment, new minor compounds, presumably of *neo*-clerodane nature, were shown by HPLC analysis. Their isolation and structural elucidation is presented in this report. The structures of difuranofruticol (**9**), 7,8-didehydrofruticolone (**10**), deoxyfruticolone (**11**) and 10-hydroxyteucjaponin B (**12**) were unambiguously elucidated based on NMR spectral studies (one- and two-dimensional experiments). Compound **10** has been reported to be formed by a spontaneous thermal dehydration process from **3** (Savona et al., 1978b; Fontana et al., 1999), but no previous antifeedancy index was established. The newly isolated compounds were assayed for their antifeedant activity against *S. littoralis* and for their antifungal activity against *R. solani*.

2. Results and discussion

2.1. Structural elucidation of isolated compounds

The ^{1}H NMR spectrum of difuranofruticol (**9**) (Table 1) showed the absence of either acetyl moieties, oxirane ring or C-19 AB system signals, whereas four furan absorptions pointed out the presence of two such rings [three α-furan protons at δ 7.40 (1H, apparent *t* (=*dd*), $J = 1.7$ Hz, H-15), 7.27 (1H, *dd*, $J = 1.5$, 1.0 Hz, H-16), 7.10 (1H, *m*, *br* $w_{1/2} = 3.4$ Hz, H-18) and one β-furan proton at δ 6.33 (1H, *dd*, $J = 2.0$, 1.0 Hz, H-14)]. One singlet and one doublet methyl groups were also present [δ 1.01 (3H, *d*, $J = 6{,}8$ Hz, H-17), 0.87 (3H, *s*, H-20)]. The ^{13}C NMR spectrum and DEPT experiments (Table 2) confirmed the presence of two furan rings [δ 125.3, C-13; 110.9, C-14; 142.9, C-15; 138.5, C-16; 135.7, C-18; 119.1, C-4; 115.9, C-5, 148.4, C-6] and two methyl signals (δ 14.7, C-17; 15.8, C-20). The second furan ring (with three quaternary carbons) appeared to involve C-6, C-5, C-4 and C-18 with loss of C-19, a structural feature present in montanin A (Malakov et al., 1978). The HMBC spectrum provided further support to the proposed structure (Fig. 1). The presence of just one-proton absorption in the δ 3–5 region (hydrogen on oxygen-substituted carbons) was confirmed in the ^{13}C NMR spectrum by the appearance of only one oxygen-bearing carbon resonance. From COSY and HSQC experiments it was possible to establish the following sequence –CH–CH(O)–CH_2–CH_2–(C10–C3) which was confirmed by a TOCSY experiment. This compound, difuranofruticol (**9**), was also obtained by

Table 1
^{1}H NMR spectral data of compounds **9**–**12** [in $CDCl_3$, at 499.81 MHz (**9**, **12**) and 299.94 (**11**)][A]

H	9	11	12
1a	4.44 *br* ($w_{1/2} = 9.4$)	1.63[B]	1.82[B]
1b	–[C]	1.80[B]	2.12[B]
2a	1.72 *dddd* (13.3, 11.8, 7.5, 1.2)	1.47[B]	1.86[B]
2b	2.11[D] *m*	2.01[B]	2.00[B]
3a	2.66[D] *(d)br d* [(16.0), 6.8]	1.04[B]	1.18[B]
3b	2.67[D] *(dd)dd* [(16.0, 12.0), 5.6, 1.7]	2.32[B]	2.34[B]
6	–[C]	–[C]	4.18 *dd* (11.3, 4.5)
7α	2.32 *ddd* (16.4, 11.2, 2.7)	2.16 *dd* (13.6, 3.7)	1.76[B]
7β	2.63 *ddd* (16.1, 4.9, 2.2)	2.78 *t*[E] (13.8)	2.20[B]
8	2.08[D] *m*	2.09[B]	2.18[B]
10	2.72 *q*[E] (2.0)	1.98[B]	–[C]
11a	1.79 *ddd* (14.4, 12.2, 4.9)	1.68[B]	2.15 *dd* (14.8, 9.5)
11b	1.98 *ddd* (14.4, 12.2, 5.4)	1.75[B]	2.94 *dd* (14.8, 8.5)
12a	2.42 *ddd* (15.0, 12.0, 4.6)	2.20[B]	5.32 *t* (8.6)
12b	2.46 *ddd* (15.0, 11.8, 5.4)	2.34[B]	–[C]
14	6.33 *dd* (2.0, 1.0)	6.26 *m*	6.39 *m*
15	7.40 *t*[E] (1.7)	7.37 *t*[E] (1.7)	7.45 *m*
16	7.27 *dd* (1.5, 1.0)	7.22 *m*	7.44 *m*
17	1.01 *d* (6.8)	0.94 *d* (6.6)	1.03 *d* (6.6)
18a	7.10 *br* ($w_{1/2} = 3.4$)	2.29 *d* (5.4)	2.64 *d* (3.5)
18b	–[C]	3.43 *dd* (5.4, 2.3)	3.35 *dd* (3.5, 2.0)
19a	–[C]	4.80 *d* (12.2)	4.67 *d* (13.0)
19b	–[C]	4.84 *d* (12.2)	5.13 *d* (13.0)
20	0.87 *s*	0.99 *s*	–[C]
19-Ac		2.10 *s*	2.10 *s*

[A] δ in ppm; (J or $w_{1/2}$) in Hz.
[B] Chemical shift derived from HETCOR spectrum.
[C] No protons.
[D] Overlapped signal.
[E] Apparent multiplicity: ($t = dd$), $q = ddd$.

Table 2
^{13}C NMR spectral data for compounds **9**–**12** [in $CDCl_3$, at 125.69 MHz (**9**) and 75.43 MHz (**11**–**12**)]

C	9	11	12
1	66.2	21.5	29.5
2	31.5	24.8	19.5
3	14.0	33.3	30.2
4	119.1	61.7	64.4
5	115.9	54.3	49.2
6	148.4	207.5	69.0
7	29.5	44.6	33.7
8	36.3	38.0	33.9
9	38.8	39.1	57.5
10	42.2	50.9	80.4
11	36.2	38.4	38.6
12	18.2	18.0	71.6
13	125.3	124.5	125.1
14	110.9	110.7	108.0
15	142.9	143.0	144.2
16	138.5	138.5	139.6
17	14.7	15.8	16.6
18	135.7	49.0	50.4
19	–	62.9	62.9
20	15.8	18.4	174.5
19-1	–	171.2	170.5
19-2	–	20.9	21.3

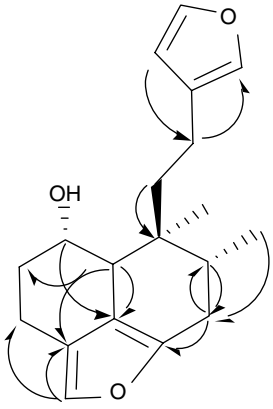


Fig. 1. Selected HMBC correlations for compound **9**.

treatment of fruticolone (**1**) with methanolic KOH at room temperature and subsequent isolation from silica gel column chromatography, as described for the conversion of 19-acetylgnaphalin to montanin A (Savona et al., 1979).

The ^1H NMR spectrum of 7,8-didehydrofruticolone (**10**), was in perfect agreement with data reported for the unsaturated ketone obtained as a dehydration product of 8β-hydroxyfruticolone (Savona et al., 1978b). For the complete ^1H NMR spectrum assignment of **10** and corrected assignments of ^{13}C NMR data see Fontana et al., 1999.

Several of the described signals of fruticolone (Savona et al., 1978a), were observed in the ^1H NMR spectrum of deoxyfruticolone (**11**) with some shift changes. The ^{13}C NMR spectrum confirmed the presence of a furan ring [δ 124.5, C-13; 110.7, C-14; 143.0, C-15 and 138.5, C-16], a carbonyl group (δ 207.5, C-6), an acetate (δ 171.2, O*C*OMe; 20.9, OCO*Me*), two methyl signals (δ 15.8, C-17; 18.4 C-20) and the CH_2-O for the oxirane ring and C-19 (δ 49.0, C-18; 62.9, C-19). The disappearance of the corresponding absorption for H-1 and C-1 in the oxygen-substituted carbon region in proton and carbon spectra with respect to fruticolone were the more significant features in the NMR spectra, pointing out the absence of hydroxyl group in that position (Tables 1 and 2).

The ^1H NMR spectrum of 10-hydroxyteucjaponin B (**12**) showed the expected structural relationship with 6-acetylteucjaponin B (Miyase et al., 1981), isolated from this plant and with the parent teucjaponin B (Sosa et al., 1994) [furan: δ 6.39, 7.45 and 7.44 (1H each, *m*, H-14, H-15 and H-16, respectively); CH_2O AB systems: δ 2.64 and 3.35 (1H each, *d*, $J = 3.5$; *dd*, $J = 3.5$ and 2.0, respectively) for H-18 and δ 4.67 and 5.13 (1H each, *d*, $J = 13.0$) for acetoxy substituted H-19; and δ 5.32 (1H, *t*, $J = 8.6$) for an H-12 in a γ-lactone ring]. The more significant differences were the presence of only one acetate group [δ 2.10 ppm (3H, s)], the deshielded resonance of one of the C-11 methylene protons [δ 2.94 ppm] and the upfield shift of the H-6 signal [δ 4.18 (1H, *dd*, $J = 11.3$, 4.5)] due to the presence of an hydroxyl group instead of an acetoxy group (Tables 1 and 2).

Once purified and identified, the retention time of all isolated compounds was established (Table 3).

2.2. Antifeedant activity bioassay

The results of the antifeedant bioassay against *S. littoralis* are reported in Table 4. In this bioassay, compounds **9**–**11** showed low antifeedant activity, close to the values previously found for **1**–**5** (Coll and Tandrón, 2004). The low FR_{50} value for **10** makes little difference with those for **1** or **3.** No antifeedant activity has been found for a compound closely related to **9**, as this kind

Table 3
HPLC retention time of isolated compounds from *Teucrium fruticans* (see Section 3 for conditions)

Compound	RT (min)
Deoxyfruticolone (**11**)	19.10
Difuranofruticol (**9**)	13.15
Fruticolone (**1**)	12.03
Isofruticolone (**2**)	12.03
Deacetylfruticolone (**6**)	9.80
6-Acetylteucjaponin B (**8**)	8.83
7,8-Didehydrofruticolone (**10**)	8.00
6-Acetyl-10-hydroxyteucjaponin B (**7**)	7.63
7β-Hydroxyfruticolone (**4**)	7.12
8β-Hydroxyfruticolone (**3**)	5.89
10-Hydroxyteucjaponin B (**12**)	4.54
11-Hydroxyfruticolone (**5**)	4.50

Table 4
Antifeedant activity of compounds **9**–**12** against *S. littoralis*

Compound	Dosis (μg/cm^2)	*N*	$FR_{50} \pm SEM$[a]
9	10	8	0.67 ± 0.07
10	10	5	0.50 ± 0.05
11	10	5	0.75 ± 0.08
12	10	5	0.42 ± 0.03

[a] Feeding ratio when the consumed area of control disc (CCD) is 50%, [FR = CTD (consumed area of treated disc)/CCD]; SEM, standard error of the mean.

of structure with no substitution in the side-chain attached to C-9 and a furan ring involving C-4, C-5, C-6, and C-18, has not been either naturally found or synthetically obtained. At least against *S. littoralis* it shows low activity. A high antifeedant activity for compound **12** was anticipated, considering that in the previous work (Coll and Tandrón, 2004) the highest ratio was displayed by **8** and **7**. Furthermore, **8** was previously reported to show potent antifeedant activity against *Tenebrio molitor* larvae (Sosa et al., 1994), and the C-10 hydroxy derivative **7**, showed a moderate activity even at lower dosage. Instead, the feeding ratio of **12** was moderate-low. This fact points out again that small changes in the structure mean sometimes a big change in the activity and vice versa (Klein Gebbinck et al., 2002).

2.3. Antifungal activity bioassay

There is a large number of reports about the antifungal activity of *neo*-clerodane diterpenoids towards human pathogenic fungi but, to our knowledge, only one on the effects on plant pathogenic fungi (Cole et al., 1991). Other works related with antifungal activity of diterpenoids have been reported recently, (Koga et al., 1995; Kofujita et al., 2002; Salah et al., 2003; Cotoras et al., 2004).

The fungitoxic effect of the *neo*-clerodane diterpenes isolated from *T. fruticans* plants were evaluated against the phytopathogenic fungi *R. solani* which is a very common soil-borne pathogen with a great diversity of host plants and causative agent of root rot disease (Adam, 1988). The results of the antifungal activity bioassay against *R. solani* are reported in Table 5. None of the tested compounds displayed significant activity and thus, it does not appear that these compounds may contribute to the antifungal defence in this plant against *R. solani*.

Table 5
Antifungal activity of compounds **1**–**9** against *Rhizoctonia solani* after 48 h of incubation

Compound	Concentration (M)	(% Inhibition)[a]
1	5×10^{-4}	NI[b]
	2.5×10^{-4}	NI
2	5×10^{-4}	8 ± 2
	2.5×10^{-4}	NI
3	5×10^{-4}	9 ± 3
	2.5×10^{-4}	8 ± 3
4	5×10^{-4}	5 ± 1
	2.5×10^{-4}	9 ± 3
5	5×10^{-4}	8 ± 3
	2.5×10^{-4}	NI
6	5×10^{-4}	9 ± 2
	2.5×10^{-4}	NI
7	5×10^{-4}	10 ± 3
	2.5×10^{-4}	NI
8	5×10^{-4}	8 ± 3
	2.5×10^{-4}	6 ± 3
9	5×10^{-4}	12 ± 5
	2.5×10^{-4}	NI

[a] % Inhibition = 100 − [(growth on treated/growth in control) × 100] ± SD.
[b] NI, no inhibition. All compounds showed no inhibition at 1.25×10^{-4} M concentration.

3. Experimental

3.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded as $CDCl_3$ solutions on Varian Unity 300/Inova 500 spectrometers (operating at 299.94/499.81 MHz for ^{1}H and 75.43/125.69 MHz for ^{13}C) under standard conditions and pulse sequences (s2pul, gDQCOSY, gHSQC, gHMBC, TOCSY, NOEdiff). Chemical shifts are given in ppm referred to the residual C**H**Cl_3 signal set at δ 7.27 or **C**DCl_3 middle band set at δ 77.0 ppm; coupling constants are given in Hz. EIMS in positive ion mode were obtained by flow injection analysis (FIA) with an HPLC apparatus (HP 1100 instrument from Agilent MeCN: H_2O 50:50 at 1 ml/min as mobile phase) coupled to an MS detector with an APCI interface. The IR spectra were recorded on a BOMEM MB-120 apparatus as films over KBr. Silica gel 60 F_{254} plates (Merck) were used for analytical and preparative TLC. Silica gel 60 H (Merck; 5–40 μm particle size) was used for vacuum liquid chromatography (VLC). ISOLUTE® C-18 column (IST; 10 g) was used for solid phase extraction (SPE) procedures. HPLC work was performed using a Waters Alliance 2695 apparatus with a 996 UV photodiode array (PDA) detector together with a Polymer Laboratories Inc PL-ELS 1000 evaporative light scattering (ELS) detector. A C18 guard column was coupled to protect the integrity of the columns. The analytical column used was a LiChrospher® 100 RP-18 (5 μm) in LiChroCART® 125-4; mobile phase water:methanol (50:50–30:70, 0–20 min; 30:70–20:80, 20–25 min; 20:80, 25–40 min; 20:80–50:50, 40–45 min; 50:50, 45–50 min), 1 ml/min, 25 °C. Solvents used for extraction and chromatographic procedures were HPLC grade (Merck). Water used in HPLC mobile phase mixtures was Milli Q (Millipore).

3.2. Plant material

Teucrium fruticans aerial parts (leaves and stems) were collected from a private garden in Barcelona in March 2003 and a voucher specimen has been deposited in the herbarium of the Institut Botànic de Barcelona (number BC 831500).

3.3. Extraction and isolation of neo-clerodane diterpenes

Finely powdered plant material (20 g) was extracted with CH_2Cl_2 (1 l) for 1 week at room temperature. After filtration and solvent removal, the residue (0.9 g) was digested in H_2O: MeOH (2:3, 10 ml; 10 min sonication) and centrifuged. The treatment was repeated twice with the pellet and the pooled solution was filtered through a conditioned SPE column (60F fraction) and sequentially eluted with H_2O: MeOH mixtures (2:3, 30 ml, 60E fraction; 1:9, 30 ml, 90E fraction). The residue from fraction 90E (30 mg) was subjected to VLC (silicagel 0.6 g). Elution with Hx:TBME (1:1 → 3:7) afforded deoxyfruticolone (**11**) (1.4 mg, fr. 4–6). After HPLC analysis the residue from fractions 60F/60E (212 mg) was treated in a similar way (silicagel 2.0 g; Hx:TBME: 7:3 frs 1–13; 3:2 frs 14–81; 1:1 frs 82–86; 2:3 frs 87–95; 3:7 frs 96–120; 1:4 frs 121–130). Difuranofruticol (**9**) (2.3 mg) was isolated as fr 2, 7,8-didehydrofruticolone (**10**) (3.7 mg) as frs 57–59, and 10-hydroxyteucjaponin B (**12**) (1.1 mg) as frs 111–115.

3.3.1. Difuranofruticol (*9*)

White amorphous solid. IR ν_{max} cm^{-1}: 3433, 2929, 1744, 1453, 1387, 1168, 1056, 737. ^1H NMR data: Table 1; ^{13}C NMR data: Table 2. EIMS *m/z*: 301.1 $[M+1]^+$.

3.3.2. Didehydrofruticolone (*10*)

White amorphous solid. IR ν_{max} cm^{-1}: 2931, 1734, 1655, 1376, 1030, 756, 492. EIMS *m/z*: 799.6 $[2M+23]^+$, 411.3 $[M+23]^+$, 389.3 $[M+1]^+$, 329.3 $[M-59]^+$, 299.2 $[M-89]^+$. ^1H NMR: δ 1.47 (3H, *s*, H-20); 1.94 (3H, *d*, $J=1.2$, H-17); 2.04 (3H, *s*, OAc); 2.47 and 3.00 (1H each, *d* $J=5.1$ Hz and *dd*, $J=5.1$ and 1.7 Hz, respectively, H-18); 4.46 (1H, *m*, H-1); 4.82 and 5.05 (1H each, *d*, $J=11.4$, H-19); 5.91 (1H, *br s*, H-7); 6.25 (1H, *dd*, $J=1.7$ and 1.0 Hz, H-14), 7.21 (1H, *m*, H-16) and 7.38 (1H, apparent *t*, $J=1.7$, H-15).

3.3.3. Deoxyfruticolone (*11*)

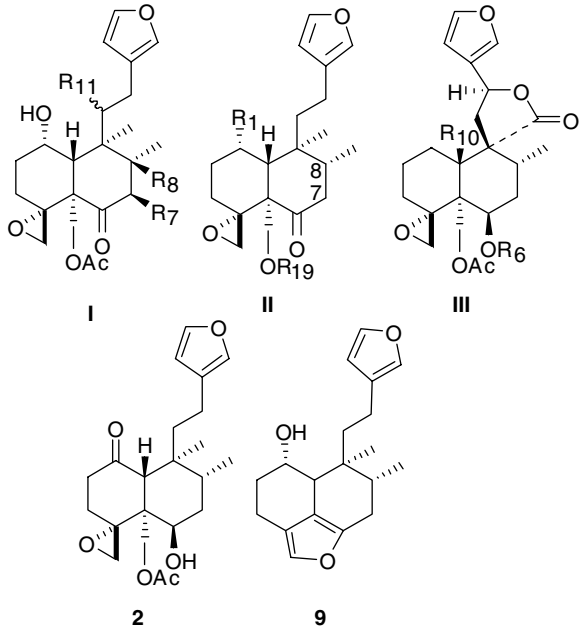
White amorphous solid. IR ν_{max} cm^{-1}: 2948, 1738, 1716, 1376, 1041, 755. ^1H NMR data: Table 1; ^{13}C NMR data: Table 2; EIMS *m/z*: 771.7 $[2M+23]^+$, 407.3 $[2M+23-364.4]^+$,376.3 $[M+2]^+$, 375.3 $[M+1]^+$, 365.2 $[M+23-32]^+$, 347.3 $[M-27]^+$, 315.3 $[M-59]^+$, 285.2 $[M-89]^+$.

3.3.4. 10-Hydroxyteucjaponin B (*12*)

White amorphous solid. IR ν_{max} cm^{-1}: 3000, 1738, 1457, 1241, 672. ^1H NMR data: Table 1; ^{13}C NMR data: Table 2. EI-MS *m/z*: 863.6 $[2M+23]^+$, 443.3 $[M+23]^+$, 429.3 $[M+23-14]$, 411.3 $[M+23-32]^+$, 361.3 $[M-59]^+$, 343.2 $[M-77]^+$, 325.2 $[M-95]^+$, 297.1 $[M-123]^+$.

3.4. Antifeedant activity bioassay

A binary choice feeding bioassay using leaf disks of lettuce, *Lactuca sativa*, with an area of 1 cm^2 was used to evaluate the activity of the compounds against fifth instar larvae of *S. littoralis* (Bellés et al., 1985). Compounds to be tested were uniformly distributed on the upper surface of the disk by application of 10 µl acetone solutions (treated disks, TD). Control disks were analogously treated with acetone (CD). In each replicate, four treated and four control disks were alternatively placed in a covered polyethylene Petri dish (8.5 cm diameter) in the presence of five larvae. Experiments were performed



I		R_7	R_8	R_{11}
Fruticolone	**1**	H	H	H
8β - Hydroxyfruticolone	**3**	H	OH	H
7β - Hydroxyfruticolone	**4**	OH	H	H
11 - Hydroxyfruticolone	**5**	H	H	OH
II		**R_1**	**R_{19}**	**7,8**
Deacetylfruticolone	**6**	OH	H	Single
7,8-Didehydrofruticolone	**10**	OH	Ac	Double
Deoxyfruticolone	**11**	H	Ac	Single
III			**R_6**	**R_{10}**
6-Acetyl-10-hydroxy-teucjaponin B	**7**	Ac		OH
6-Acetylteucjaponin B	**8**	Ac		H
10-HydroxyteucjaponinB	**12**	H		OH

Scheme 1. Structures of compounds **1**–**12**.

under the same temperature and humidity conditions of the laboratory culture, but in constant darkness.

3.5. Antifungal activity bioassay

Rhizoctonia solani AG 2-1 was obtained from Agriculture and Agri-Food Canada Research Station, Saskatoon, Saskatchewan. The fungus was grown on potato dextrose agar (PDA) plates at 24 ± 2 °C, under constant light. Solid cultures were started by placing a plug of mycelium (6 mm diameter) at the centre of the plate containing the agar. A radial growth bioassay was used to investigate the antifungal activity of compounds **1**–**9**. A stock solution of each compound in DMSO (5×10^{-2} M) was used to prepare assay solutions (5×10^{-4}, 2.5×10^{-4}, 1.5×10^{-4} M) in PDB by serial dilution. Control solutions contained 1% DMSO. Sterile tissue culture 6-well plates were inoculated with mycelium plugs placed upside down on the centre of each well (5 mm cut from 5-days-old PDA plates of *R. solani*). After inoculation, test solutions were added (2 ml) to each well and the plates were sealed with parafilm and incubated under constant light at 24 ± 2 °C. Control plates containing only DMSO and PDB were also prepared and incubated with plugs of *R. solani*, similarly. Measurement of the mycelium radial growth was carried out every 24 h up to five days. Each assay was conducted in triplicate (Scheme 1).

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