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BIOACTIVE STEROIDS AND TRITERPENES FROM *Melilotus messanensis* AND THEIR ALLELOPATHIC POTENTIAL[1]

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Abstract—The aerial parts of *Melilotus messanensis* (sweet clover) afforded, from moderately and polar bioactive fractions, 11 triterpenes and five steroids. A series of aqueous solutions at 10^{-4}–10^{-9} M of five steroids and nine triterpenes was monitored to test their effects on germination and growth of the monocots *Hordeum vulgare* and *Allium cepa*, and the dicots *Lactuca sativa* (var. nigra and var. romana) and *Lycopersicon esculentum*. An important stimulatory effect on *H. vulgare* germination (between 40% and 80% for almost all tested compounds) was observed. Some considerations about the ecological role of triterpenes on *M. messanensis* are made. In addition to known lupane triterpenes (platanic acid and 3β,28,30-lup-20(29)-enetriol), oleanane triterpenes (soyasapogenol B, soyasapogenol G, and messagenolide), a gammacerane triterpene (gammacer-16-en-3-one), five new lupane triterpenes (messagenic acids A–E: (27-*cis*-4-hydroxycinnamoyloxy)-betulinic acid; 27-(*trans*-4-hydroxycinnamoyloxy)betulinic acid; 20(*S*)-3β-hydroxy-29-oxolupan-28-oic acid; 3β,30-dihydroxylup-20(29)-en-28-oic acid; and 3β,20-dihydroxylup-18(19)-en-28-oic acid, respectively), and sterols (β-sitosterol, ergosterol peroxide, 7α-hydroxysitosterol, 7β-hydroxysitosterol, and 7-oxositosterol) were isolated and chemically characterized. Their structures and stereochemistry were elucidated by spectroscopic methods (one- and two-dimensional ^{1}H and ^{13}C NMR, IR, MS).

Key Words—*Melilotus messanensis*, lupane triterpenes, messagenic acids; messagenolide, soyasapogenol G, gammacer-16-en-3-one, oleanane triterpenes, steroids, stigmasterols, ergosterol peroxide, allelopathy, *Lactuca sativa*, *Lycopersicon esculentum*, *Allium cepa*, *Hordeum vulgare*.

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[1] Part 5 in the series "Natural Products as Allelochemicals"; for Part 4 see Macías et al. (1996).

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INTRODUCTION

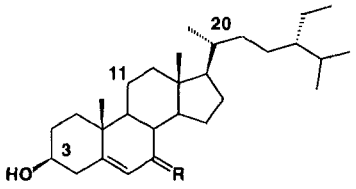
In continuation of our allelopathic studies in *Melilotus* species (Macías et al., 1994, 1996) we now report the isolation, structure elucidation, and biological activities of five known steroids and 10 triterpenes, five of which are first reported in the literature as natural products. Known steroids are β-sitosterol (**1**) [previously isolated from *M. caspius* (Rasulov and Belyi, 1985) and *M. indica* (Khafagy et al., 1980; Durrani and Ikram 1966)], ergosterol peroxide (**2**), 7β-hydroxysitosterol (**3**), 7α-hydroxysitosterol (**4**), and 7-oxositosterol (**10**) (Della Greca et al., 1990a; Guerriero et al., 1993; Gunatilaka et al., 1981). Known triterpenes are soyasapogenol B (**5**) (Baxter et al., 1990) previously isolated from *M. officinalis* (Kang et al., 1987), platanic acid (**9**) (Aplin et al., 1963; Fijioka et al., 1994), 3β,28,30-lup-20(29)-enetriol (**13**) Macías et al., 1994; González et al., 1992), soyasapogenol G (**14**), and gammacer-16-en-3-one (**15**) (Macías et al., 1996). The new triterpenes are messagenic acids A–E (**6–8**, **11**, **12**; Figure 1). All were identified by spectroscopic techniques (IR, MS, ^{1}H and ^{13}C 1D, 2D NMR experiments). Structure elucidation of the new lupane triterpenes (**6–8**, **11**, **12**) is described below.

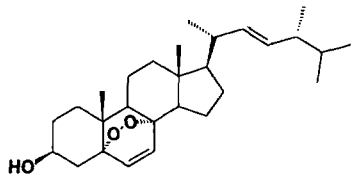
As part of our research on bioactive natural products (coumarins, terpenoids, lignans, phenolics), we are conducting a systematic study of their potential activity as allelopathic agents (Macías et al., 1993, 1994). Thus, we are evaluating the regulatory effects of compounds on *Lycopersicon esculentum* L. and *Lactuca sativa* L. (dicotyledon species), and *Hordeum vulgare* L. and *Allium cepa* L. (monocotyledon species), (Castellano, 1997). We have studied the effects of a series of aqueous solutions from 10^{-4} to 10^{-9} M of five steroids (**1–4**, **10**) and seven triterpenes (**5–7**, **9**, **11**, **14**, **15**) on germination, and on root and shoot lengths of the above-mentioned target species.

METHODS AND MATERIALS

Plant Material. Melilotus messanensis (L.) All was collected on April 11, and July 2, 1991, in Trebujena, Cádiz, Spain (voucher specimen is deposited at the University of Seville Herbarium, Spain, SEV-7992) during two different plant development stage periods: (a) plants at the beginning of flowering, and (b) plants with seeds. Period b was selected as it provided the more bioactive aqueous extract in the laboratory bioassay.

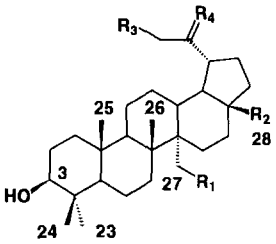
Extraction and Isolation. Fresh plants at stage b (382 g) were soaked in deionized H_2O (weight of plant: vol. solvent 1 : 3), for 24 hr at 25° in the dark. The H_2O extract was extracted 10× [v/v of 0.5 liters of CH_2Cl_2 (DCM) per 1.0 liter of H_2O], and the combined extracts were dried over Na_2SO_4 and evaporated in vacuo to yield 1.2 g of crude extract. Crude extract was separated by

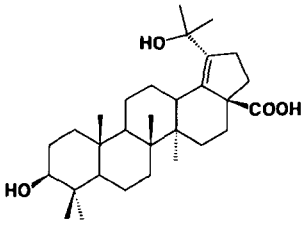




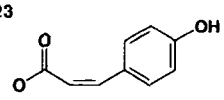
1 R = H; β-sitosterol
3 R = OHβ, H; 7β-hydroxy-sitosterol
4 R = OHα, H; 7α-hydroxy-sitosterol
10 R = O; 7-oxositosterol

2 (ergosterol peroxide)

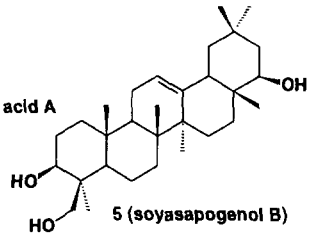




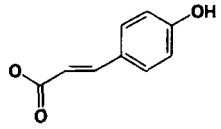
12 (messagenic acid E)

6 R1 = 

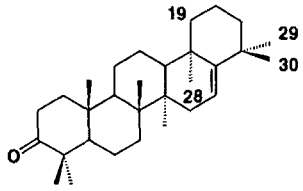
R2 = COOH; R3 = H; R4 = CH_2; messagenic acid A



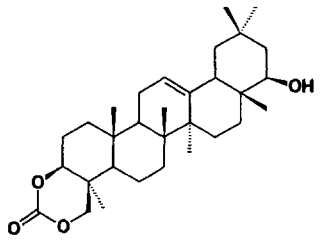
5 (soyasapogenol B)

7 R1 = 

R2 = COOH; R3 = H; R4 = CH_2; messagenic acid B
8 R1 = H; R2 = COOH; R3 = H; R4 = CHO, H; messagenic acid C
9 R1 = H; R2 = COOH; R3 = H; R4 = O; platanic acid
11 R1 = H; R2 = COOH; R3 = OH; R4= CH_2; messagenic acid D
13 R1 = H; R2 = CH_2OH; R3 = OH; R4 = CH_2



15 (gammacer-16-en-3-one)



14 (soyasapogenol G)

FIG. 1. Isolated compounds from *M. messanensis*.

column chromatography (CC) on silica gel using *n*-hexane–EtOAc mixtures of increasing polarity yielding 214 × 50-ml fractions, which were reduced to 13 fractions (A–M) after comparison by TLC.

Previously, we studied fractions C, D, G, and K (Macías et al., 1994, 1996). The current study of bioactive fractions E, I, L, and M provided 12 compounds. HPLC separation of fraction E (*n*-hexane–EtOAc, 4:1) afforded 30 mg of β-sitosterol (**1**). From fraction I, 1 mg of ergosterol peroxide (**2**) was obtained after CC on silica gel (DCM–EtOAc 98:2) and HPLC (DCM) purification.

Fraction L was chromatographed with 2-propanol on a Sephadex LH-20 column, providing four fractions. From the second fraction, two steroids were isolated after HPLC (*n*-hexane–EtOAc, 1:1) purification: 7β-hydroxysitosterol (**3**) (2 mg) and 7α-hydroxysitosterol (**4**) (5 mg). The third fraction afforded 10 mg of soyasapogenol B (**5**) under the same purification conditions.

Fraction M was chromatographed with 2-propanol on a Sephadex LH-20 column to provide four fractions. The third fraction was separated by CC on silica gel using DCM–EtOAc mixtures of increasing polarity yielding seven fractions (a–g). Fraction c, after HPLC purification (*n*-hexane–EtOAc 7:3) provided 2 mg of messagenic acid A (**6**); 1 mg of messagenic acid B (**7**); 5 mg of messagenic acid C (**8**); and 7 mg of platanic acid (**9**). Fraction d (HPLC, *n*-hexane–EtOAc, 3:2) afforded 3 mg of 7-oxositosterol (**10**). Chromatography of f by HPLC (*n*-hexane–EtOAc, 11:9) gave 4 mg of messagenic acid D (**11**), 3 mg of messagenic acid E (**12**), and 2 mg of 3β,28,30-lup-20(29)-enetriol (**13**).

Identification. Known compounds were identified by comparison of their physical and spectroscopic data (mp, [α], IR, MS, ^{1}H and ^{13}C NMR) with those previously reported in the literature.

Messagenic Acid A (6). $C_{39}H_{54}O_6$, oil; IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3353 (OH), 1722 (α,β-unsaturated ester), 1703 (CO-OH); EM (70 eV) *m/z* (rel. int.): 618 $[M]^+$ (1); 600 $[M\text{-}H_2O]^+$ (2); 557 (2) 454 (24); 439 (16); 436 (10); 393 (10); 278 (8); 233 (8); 207 (24); 189 (25); 147 (100). ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

Messagenic Acid B (7). $C_{39}H_{54}O_6$, oil; ^{1}H NMR: 7.60 (1H, d, J = 16 Hz, H-7′); 7.45 (2H, dd, J = 8 and 2 Hz, H-2′,6′); 6.84 (2H, dd, J = 8 and 2 Hz, H-3′,5′); 6.30 (1H, d, J = 16 Hz, H-8′); 4.75 (1H, m, H-29); 4.62 (1H, m, H-29); 4.62 (1H, d, J = 13 Hz, H-27); 4.43 (1H, d, J = 13 Hz, H-27); 3.19 (1H, dd, J = 11 and 5 Hz, H-3); 3.01 (1H, ddd, J = 10 Hz, H-19); 1.70 (3H, s, H-30); 0.99 (3H, s, H-26); 0.93 (3H, s, H-23); 0.85 (3H, s, H-25); 0.74 (3H, s, H-24).

Messagenic Acid C (8). $C_{30}H_{48}O_4$, amorphous solid; $[\alpha]_D^{25}$ = +8° ($CHCl_3$, c); IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3452 (OH), 2927, 1717 (COOH); 1684 (CHO); EM (70 eV) *m/z* (rel. int.): 472 $[M]^+$ (2); 454 (8); 439 (6); 414 (14); 207 (100); 189 (65). ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

TABLE 1. ^{1}H NMR OF TRITERPENES **6**, **8**, **9**, **11**, **12** (399.95 MHz, $CDCl_3$, SIGNAL OF RESIDUAL $CHCl_3$ CENTERED AT δ 7.25 PPM)[a]

H	6[b]	8	9	11	12
1a	0.92	0.92	0.89	0.89	0.91
e	1.73	1.69	1.64	1.65	1.68
2a	1.55	1.54	1.54	1.51	1.55
e	1.59	1.59	1.64	1.60	1.60
3	3.18 *dd*	3.19	3.19	3.18	3.18
5	0.68 *dd*	0.68	0.68	0.67	0.68
6a	1.33	1.37	1.37	1.37	1.37
e	1.46	1.52	1.49	1.51	1.51
7	1.23	1.21	1.54	1.48	
9		0.86			1.19
11a	1.29	1.30	1.25	1.21	1.58
e	1.20	1.21	1.66	1.45	1.34
12a	0.91	1.48	1.07	1.07	1.23
e	1.74	1.71	1.44	1.45	2.13
13	2.37 *ddd*	2.30	2.09	2.16	2.65
15a	1.75	1.48	1.48	1.50	1.44
e	1.22	1.20	1.20	1.20	1.18
16a	1.32	1.37	1.48	1.40	1.36
e	2.22 *ddd*	2.28	2.26	2.27	2.26
18	1.68	1.47	1.49	1.70	—
19	2.98 *ddd*	2.96 *dddd*	3.23 *ddd*	2.87	—
20a	—	2.63 *dq*	—	—	—
e					
21α	1.95	1.69	2.09	2.07	2.09
β	1.40	1.25	1.49	1.40	1.77
22α	1.98	1.92	1.97	1.95	1.95
β	1.41	1.33	1.59	1.51	1.43
23	0.93	0.96	0.95	0.95	0.96
24	0.74	0.75	0.74	0.74	0.75
25	0.83	0.83	0.81	0.81	0.83
26	0.96	0.96	0.90	0.92	0.92
27	4.59 *d*	0.94 *s*	0.99	0.97	0.96
	4.33 *d*				
29a	4.73 *s*	9.63 *s*	2.17	4.96 *s*	1.24 *s*
b	4.61 *s*			4.92 *s*	
30	1.68	1.00 *d*	—	4.12 *sbr*	1.23 *s*

[a]Multiplicities are indicated when coupling constant can be measured and they are not repeated if identical with those in the preceeding column. *J*(Hz): **6**, **8**, **9**, **11**, **12**: 2a,3 = 11; 2e,3 = 5; 5,6a = 10; **6**, **8**, **9**, **11**: 18,19 = 19,21α = 11; 19,21β = 4; 22α,22β = 12; 22α,21β = 8; **6**, **11**, **12**: 12a,13 = 13,18 = 13; 12e,13 = 3; 16a,16e = 13; 16e,15a = 16e,15e = 3; **6**: 27a,27b = 13; 2′,3′ = 9; 2′,5′ = 2; 7′,8′ = 13; **8**: 19,20 = 3; 20,30 = 7.

[b]*cis*-4-Hydroxycinnamoyloxy (**6**): H-2′,6′ = 7.68, *dd*; H-3′,5′ = 6.82, *dd*; H-7′ = 6.85, *d*; H-8′ = 5.84, *d*.

TABLE 2. ^{13}C NMR OF TRITERPENES **6**, **8**, **11**, **12** (100.23 MHz, $CDCl_3$, SIGNAL OF RESIDUAL $CHCl_3$ CENTERED AT δ 77.00 PPM)

H	6[a]	8	11	12
1	38.9	38.7	38.8	38.7
2	27.3	27.3	27.2	27.4
3	78.7	78.9	78.9	79.0
4	38.7	38.8	38.6	39.0
5	55.3	55.2	55.3	55.3
6	18.2	18.2	18.2	18.3
7	35.2	34.3	34.3	34.3
8	41.5	40.6	40.6	40.7
9	51.8	50.1	50.5	50.5
10	37.4	37.1	37.1	37.2
11	20.9	20.7	20.9	20.9
12	25.2	26.6	26.7	26.8
13	38.8	38.2	38.2	49.9
14	45.2	42.6	42.3	42.5
15	30.3	29.5	29.7	32.0
16	32.5	31.9	32.3	32.3[b]
17	55.9	56.3	56.1	56.2
18	49.4	49.9	49.9	106.8
19	46.7	36.8	42.5	151.0
20	151.0	48.1	154.8	65.2
21	30.3	29.7	32.1	38.3
22	36.6	37.4	36.8	36.8
23	27.9	28.0	27.9	28.0
24	15.4	15.3	15.3	15.3
25	16.4	16.1	16.1	16.1
26	16.6	16.0	15.9	16.3
27	63.3	14.5	14.6	14.7
28	181.0	174.7	178.8	178.3
29	110.0	204.7	106.5	29.7[b]
30	19.5	24.3	65.1	29.7[b]

[a]**6**: $C_{1'}$: 131.1; $C_{2'}$–$C_{6'}$: 132.4; $C_{3'}$–$C_{5'}$: 115.0; $C_{4'}$: 157.9; $C_{7'}$: 143.7; $C_{8'}$: 117.4; $C_{9'}$: 166.7.
[b]These assignments may be interchanged.

Messagenic Acid D (11). $C_{30}H_{48}O_4$, white needles; mp: 258–262°C; IR $\nu_{max}^{KBr,\,neat}$ cm^{-1}: 3442 (OH), 1694 (COOH); EM (70 eV) *m/z* (rel. int.): 472 $[M]^+$ (3); 454 (9); 439 (6); 408 (5); 246 (8); 231 (14); 207 (40); 189 (100). ^1H NMR see Table 1; ^{13}C NMR see Table 2.

Messagenic Acid E (12). $C_{30}H_{48}O_4$, amorphous solid; EM (70 eV) *m/z* (rel. int.): 454 $[M\text{-}H_2O]^+$ (8); 439 (8); 414 (5); 411 (3); 246 (7); 217 (21); 207 (100); 203 (16); 189 (76). ^1H NMR see Table 1; ^{13}C NMR see Table 2.

Seed Germination Bioassays. Seeds of *Lactuca sativa* var. nigra and var. romana, *Lycopersicon esculentum* L., and *Allium cepa* L. were obtained from Fitó, S. L. (Barcelona, Spain). *Hordeum vulgare* L. was obtained from Rancho La Merced, Junta de Andalucía, Jerez, Cádiz, Spain. All undersized and damaged seeds were discarded and the assay seeds were selected for uniformity.

Germination bioassay of *L. sativa* var. nigra consisted of 25 seeds per dish, 5 ml test solution, 60 hr dark, 25°C, and four replicates of each concentration. Germination and growth bioassays for the other species tested were as follows: *L. sativa* var. romana, 25 seeds per dish, 5 ml test solution, four days dark, 25°C, and four replicates of each concentration; *L. esculentum*, 25 seeds per dish, 5 ml test solution, five days dark, 25°C, and four replicates of each concentration; *A. cepa*, 25 seeds per dish, 5 ml test solution, five days dark, 25°C, and four replicates of each concentration; *H. vulgare*, 25 seeds per dish, 10 ml test solution, five days dark, 25°C, and six replicates of each concentration (Castellano, 1997).

Test solutions (10^{-4} M) were prepared using H_2O–MES [2-(*N*-morpholino]ethanesulfonic acid, 10 mM], and test solutions of 10^{-5}–10^{-9} M were obtained by dilution. Parallel controls were performed. All pH values were adjusted to 6.0 before bioassay with MES. Osmotic pressure values were measured on a vapor pressure osmometer (Wescor 5500) and ranged between 30 and 38 mosm.

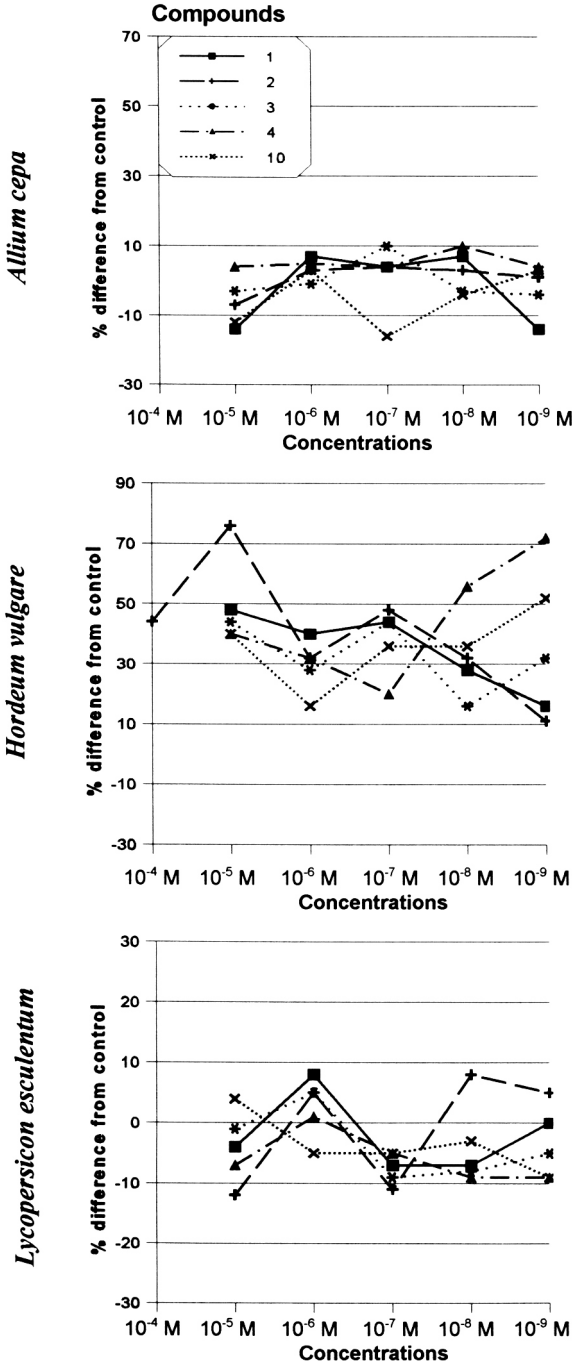
Data are presented as percent differences from control in graphics and tables (Figures 2–6 and Tables 3–6). Thus, zero represents the control; positive values represent stimulation of the studied variable and negative values represent inhibition.

Statistical Treatment. Germination and root and shoot length values were tested by the Mann-Whitney test; differences between experimentals and controls were significant ($P = 0.01$) (Tables 3–6).

RESULTS AND DISCUSSION

Chromatography of moderately bioactive and polar fractions of *M. messanensis* revealed the steroids β-sitosterol (**1**), ergosterol peroxide (**2**), 7β-hydroxysitosterol (**3**), 7α-hydroxysitosterol (**4**), and 7-oxositosterol (**10**); the oleanane triterpenes soyasapogenol B (**5**) and G (**14**); gammacer-16-en-3-one (**15**); the lupanic triterpenes messagenic acids A–E (**6–8**, **11**, **12**); platanic acid (**9**); and 3β,28,30-lup-20(29)-enetriol (**13**). Compounds **1–5**, **9**, **10**, **13–15** have been previously reported.

Chemical Data. Platanic acid (**9**) was characterized by comparison of physical data with those reported in the literature (Aplin et al., 1963; Fijioka et al., 1994) and ^{1}H NMR data are given in Table 1 since they include new and unambiguous assignments.

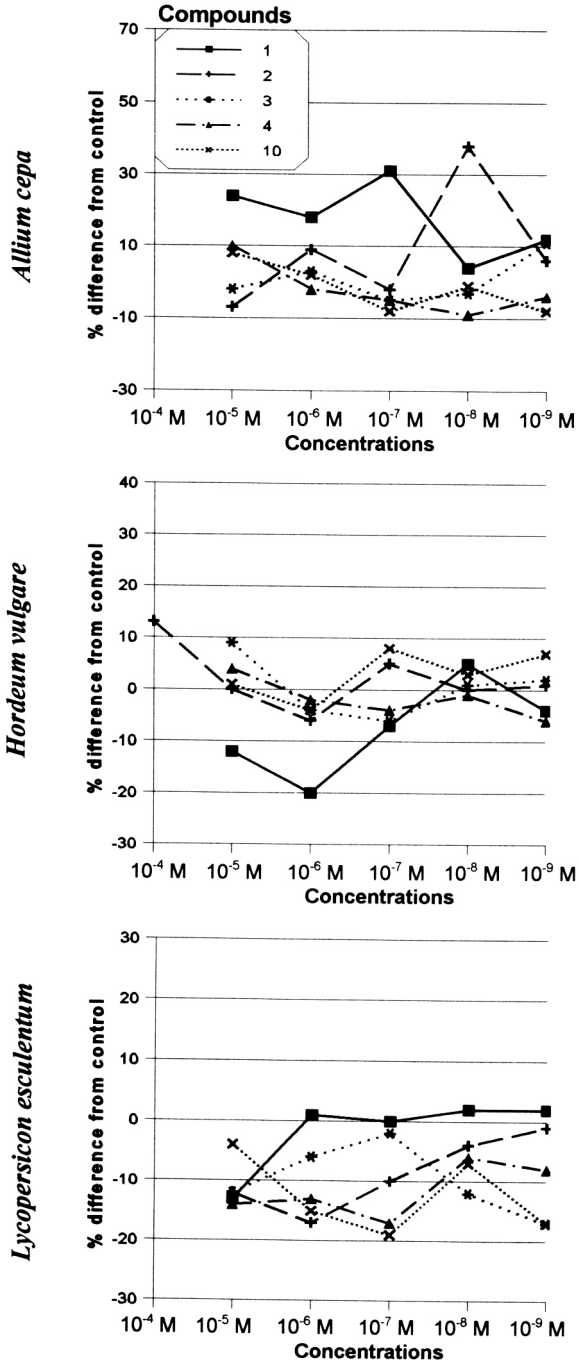

Compounds
1
2
3
4
10
Allium cepa
Hordeum vulgare
Lycopersicon esculentum
% difference from control
Concentrations
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M

The more polar fraction (**M**) afforded, after separation, five new lupane triterpenes of increasing polarity: messagenic acids A–E (**6–8**, **11**, **12**). Messagenic acid A (**6**) and B (**7**) were easily interconverted by light. Only a careful HPLC separation in the dark allowed the isolation of both compounds. Messagenic acid A (**6**) shows a $[M]^+$ peak at *m/z* 618, allowing the molecular formula $C_{39}H_{54}O_6$. The ^1H NMR spectrum (Table 1) of **6** exhibits resonance for two sets of AB doublets at δ 5.84 (1H, d, J = 13 Hz) and 6.85 (1H, d, J = 13 Hz) and δ 6.82 (2H, d, J = 9 Hz) and 7.68 (2H, d, J = 9 Hz), consistent with a *p-cis*-coumaroyloxy substituent (Kashiwada et al., 1993). This is further supported by mass spectral fragments at *m/z* 164 $[C_9H_8O_3]^+$ and 147 $[C_9H_7O_2]^+$. Furthermore, the ^1H NMR (Table 1) spectrum of **6** shows signals of five methyl groups, singlets at δ 0.74, 0.83, 0.93, 0.96, and 1.68; a methylene group attached to the ester side chain [δ 4.59 (1H, d, J = 13 Hz), 4.33 (1H, d, J = 13 Hz)]; a hydroxymethine group [δ 3.18 (1H, dd, J = 11 and 5 Hz)]; and an *exo*-methylene [δ 4.73, 4.61 (bs)]. These data and the presence of fragments at *m/z* 207 $[C_{14}H_{23}O]^+$ and 189 $[C_{14}H_{21}]^+$ (base peak) in the mass spectrum are indicative of pentacyclic lupane triterpenes with a hydroxyl group at C-3 (Macías et al., 1994). The IR spectrum shows two carbonyl signals at 1722 (α,β-unsaturated ester) and 1703 cm^{-1}, corresponding to an additional carboxylic acid. The mass spectrum fragments suggest allocation of the substituents (—COOH and *p-cis*-coumaroyloxy) at C, D, or E rings (Budziekiewicz et al., 1963). The presence of a methyl attached to a double bond at δ 1.68 (C-30) and the peak ions at *m/z* 246 $[C_{16}H_{22}O_2]^+$ and 201 $[C_{15}H_{21}]^+$ are in agreement with two modified methyls at C-14 and C-17 (Siddiqui et al., 1992). The downfield chemical shift showed by H-19 (δ 2.98) indicates the presence of a carboxylic group at C-17 corresponding to betulinic acid (Macías et al., 1994). Consequently, the structure of messagenic acid A (**6**) is established as 27-(*cis*-4-hydroxycinnamoyloxy)betulinic acid.

The complete ^{13}C NMR (Table 2) assignment of messagenic acid A (**6**) was established by comparison with methylated betulinic acid data (Macías et al., 1994) and the diacetylated derivative of 3β,27-dihydroxylup-20(29)-en-28-oic acid (cylicodiscic acid) (Tchivounda et al., 1990).

The mass spectrum of messagenic acid B (**7**) is identical to **6**, and their ^1H NMR spectra showed great similarity. The only difference appeared downfield, where two sets of AB doublets at δ 7.60 (1H, J = 16 Hz), δ 6.30 (1H, J = 16 Hz) and δ 7.45 (2H, J = 8 Hz), δ 6.84 (2H, J = 8 Hz) correspond to a *p-trans*-coumaroyloxy substituent (Wang and Fujimoto, 1993). Thus, the structure of messagenic acid B (**7**) is established as 27-(*trans*-4-hydroxycinnamoyloxy)betulinic acid.

FIG. 2. Germination activity of steroids **1–4**, and **10**. Data are presented as the percentage difference from control (0 value).


Allium cepa
Compounds
1
2
3
4
10
% difference from control
70
50
30
10
-10
-30
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations
Hordeum vulgare
% difference from control
40
30
20
10
0
-10
-20
-30
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations
Lycopersicon esculentum
% difference from control
30
20
10
0
-10
-20
-30
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations

Messagenic acid C (**8**) was the third compound, in increasing order of polarity, isolated from fraction M. Mass spectrum shows a molecular ion at *m/z* 472 ($C_{30}H_{48}O_4$) and fragment ions involving A and B rings at *m/z* 207 and 189, indicative of a lupane triterpene skeleton with a C-3 hydroxyl group. The ^{1}H NMR spectrum (Table 1) presents five singlet methyl signals at δ 0.75, 0.83, 0.94, and 0.96 (6H); a methyl group at δ 1.00 ppm (3H, d, J = 7 Hz); a hydroxymethine group (δ 3.19, dd, J = 11 and 5 Hz, H-3); and an aldehyde group (δ 9.63, s). Its IR shows two absorption signals according to the presence of a carboxylic and an aldehyde group. The absence of olefinic protons and the existence of a methyl doublet suggests that the isopropyl group is modified as CH_3RCH- (δ 2.63, dq, J = 7 and 2 Hz, H-20). All these data suggest a lupane triterpene with a hydroxyl group at the C-3, and an aldehyde and a carboxylic group attached to the C-17 and C-20, or vice versa, based on the chemical shift of H-19.

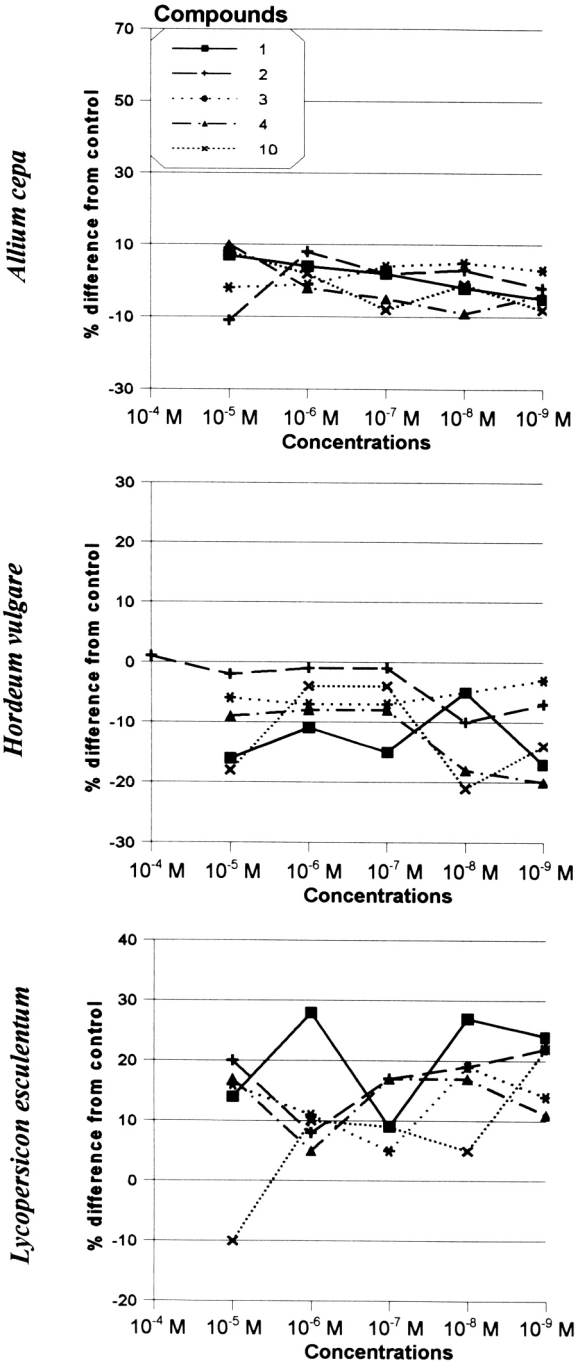
The position of the aldehyde and carboxylic groups at C-17 or C-20 is established by comparison of ^{13}C NMR data of **8** with the acetate derivative of betulinic acid (Macías et al., 1994) and betulinaldehyde (Pathak et al., 1988). The coincidence between C-17 signals of **8** (δ 56.3 ppm) and the acetate derivative of betulinic acid (δ 56.3 ppm), rather than with the corresponding one of betulinaldehyde (δ 59.3 ppm), allowed us to assign the aldehyde group at C-20. Positive NOE effects between H-29, H-19, and H-20 further substantiated the proposed structure. The absolute configuration at C-20 was established by correlation with the 3β,28-acetoxy-29-lupanal epimers (Vystrcil et al., 1973) (20*R*, $[\alpha]^D = -103°$; 20*S*, $[\alpha]^D = +70°$). Compound **8** shows $[\alpha]^D = +8°$, so a structure of (20 *S*)-3β-hydroxy-29-oxolupan-28-oic acid is proposed for messagenic acid C (**8**).

Compound **11** (fraction M) shows a $[M]^+$ at *m/z* 472 and a fragmentation pattern similar to that of **8**, indicative of a lupane triterpene skeleton with a C-3 hydroxyl group. The ^{1}H NMR (Table 1) spectrum is similar to 3β,28,30-lup-20(29)-enetriol (**13**) (Macías et al., 1994). Signals of the H-28 methylene group do not appear, and the H-19 signal appears 0.6 ppm downfield (δ 21.87 ppm) in comparison with the corresponding H-19 signal of **13**. These data confirm the structure of **11** as 3β,30-dihydroxylup-20(29)-en-28-oic acid.

Compound **11** was first isolated as a methyl ester from *Relhania genistifolia* (Tsichritzis and Jakupovic, 1990). This is the first report of the isolation and spectroscopic data of the natural alcohol.

Compound **12** was obtained from a mixture of platanic acid (**9**), which easily degraded. The ^{1}H NMR spectrum shows signals for seven C-Me singlets at δ 1.24, 1.23, 0.96 (6H), 0.92, 0.83, and 0.75; a hydroxymethine group

FIG. 3. Radicle length activity of steroids **1–4**, and **10**. Data are presented as the percentage difference from control (0 value).


Allium cepa
Compounds
1
2
3
4
10
% difference from control
70
50
30
10
-10
-30
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations
Hordeum vulgare
% difference from control
30
20
10
0
-10
-20
-30
Concentrations
Lycopersicon esculentum
% difference from control
40
30
20
10
0
-10
-20
Concentrations

(δ 3.18, dd, J = 11 and 5 Hz); and a signal at δ 2.65 (dd, J = 11 and 5 Hz). The mass fragmentation pattern shows major peaks at m/z 207 (base peak) and m/z 189. The ^{13}C NMR spectrum (Table 2) shows signals of a carboxylic group, a tetrasubstituted double bond, and two hydroxyl groups. All data suggest a lupane triterpenic structure with a secondary hydroxyl group placed at C-3, a modified methyl group (COOH), a double-bond fully substituted group, and a tertiary hydroxyl group, corresponding to a molecular formula $C_{30}H_{48}O_4$ (mass spectrum, 454 $[M\text{-}H_2O]^+$).

The deshielding effect showed by C-29 and C-30 methyls (δ 1.24 and 1.23 ppm) is in good agreement with the existence of a tertiary hydroxyl group at C-20, as C-20 and C-30 methyl signals in 3β,20-lupanediol appear at δ 1.12 ppm and δ 1.22 ppm (Hui and Li, 1977). The difference between them can be explained by the presence of a tetrasubstituted double bond at C-18(19). The lack of the C-18 proton induces a displacement of the methyl groups, in good agreement with ^1H NMR (Table 1) and ^{13}C NMR data (Table 2) (Wenkert et al., 1978). Thus, the structure is proposed as 3β,20-dihydroxylup-18(19)-en-28-oic acid.

Bioassay Data. Only limited data are available concerning triterpene and steroid activity and growth development. Previous work in our laboratory shows that lupanic triterpenes stimulate germination of *Lactuca sativa* and, in certain cases, *Hordeum vulgare* (Macías et al., 1994). Some steroids, such as chondrillasterol or amasterol, are reported to affect germination (Bradow, 1985; Della Greca et al., 1990b). The only steroids that have been reported with potent plant growth promoter effects at levels as low as 1 ng (Maugh, 1981; Mandava et al., 1981) are the brassinosteroids, acting in many cases in a synergistic manner with auxin (Yopp et al., 1981). Saponins are another group of closely related compounds that have exhibited important inhibitory activities, e.g., several isolated from alfalfa roots belonging to the medicagenic acid, hederagenin, lucernic acid, zhanic acid, and soyasapogenol B types have been tested and proved to be active to some weeds and wheat (Waller et al., 1993). The effects produced by our tested triterpenes and steroids (Figure 1) on germination and growth are presented in Tables 3–6 and Figures 2–6 and show similar patterns. The overall effect on *Lactuca sativa* germination and growth falls below a 10% difference from controls in all cases. It is clear that none of these compounds exerts any significant effect on *L. sativa*. The other dicotyledon tested species, *Lycopersicon esculentum*, is slightly more sensitive than *L. sativa*, especially on growth development. Although the values obtained are low, in all concentrations tested, the inhibitory effect on germination is slightly greater with triterpenes than with steroids.

FIG. 4. Shoot length activity of steroids **1–4**, and **10**. Data are presented as the percentage difference from control (0 value).

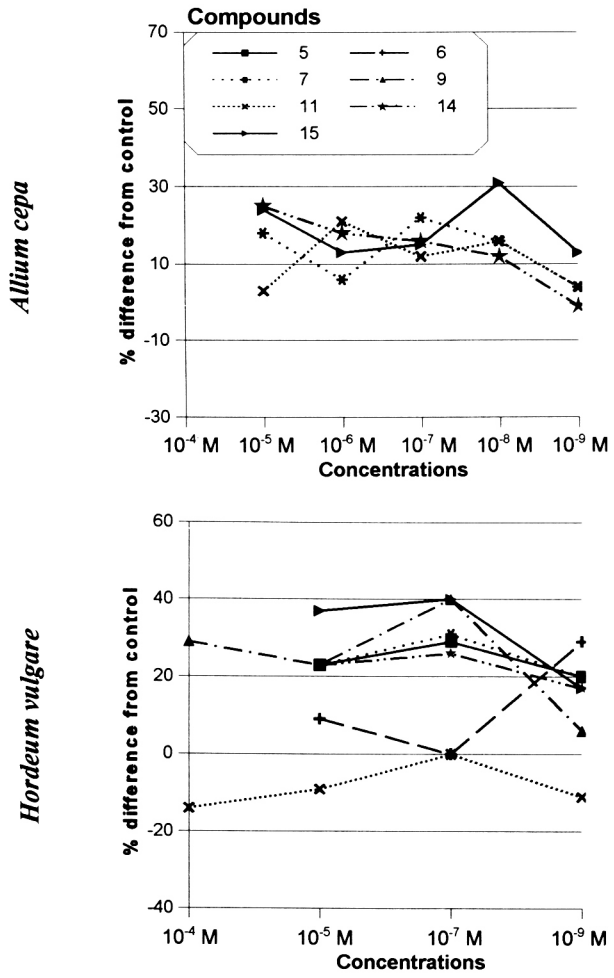


FIG. 5. Germination activity of triterpenes **5**–**7**, **9**, **11**, **14**, and **15**. Data are presented as the percentage difference from control (0 value).

Root growth responds differently, depending upon the substance tested: while triterpenes are clearly stimulatory (with values ranging between 20% and 40%), steroids present a low and homogeneous inhibitory activity profile, pointing out **10** (-15%, 10^{-6} M; -19%, 10^{-7} M; -17%, 10^{-9} M) and **4** (-14%, 10^{-5} M; -17%, 10^{-7} M). Compounds **5** and **11** have a promising profile of activity: from insignificant negative values at 10^{-5} M to a stimulatory effect as the concentration falls (**5**: 29%, 10^{-6} M; 24%, 10^{-8} M; **11**: 27%, 10^{-6} M,

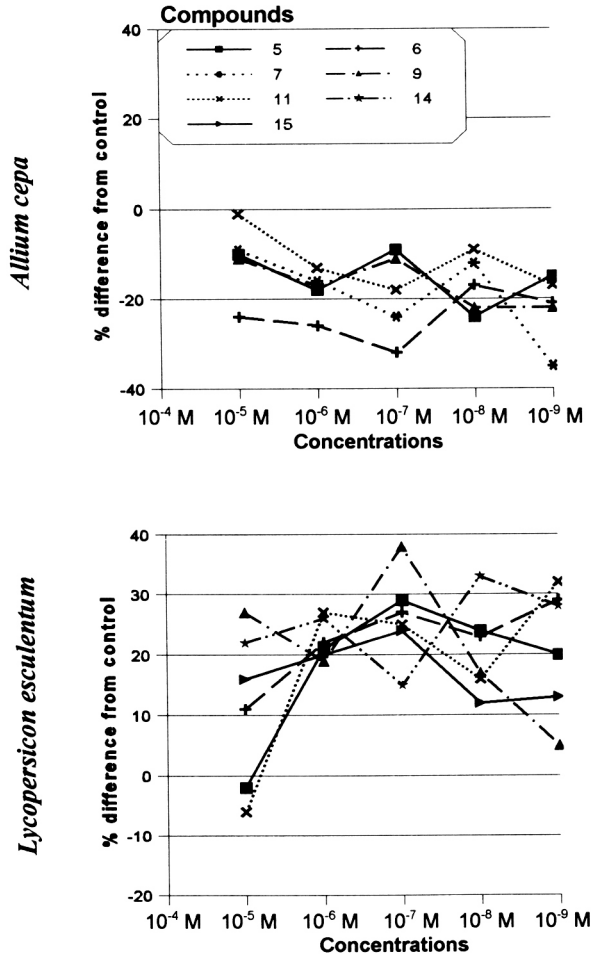


FIG. 6. Radicle length activity of triterpenes **5–7**, **9**, **11**, **14**, and **15**. Data are presented as percentage difference from control (0 value).

32%, 10^{-9} M). The small quantities of product obtained did not allow us to test higher concentrations, but negative effects at higher concentrations would be expected.

Shoot growth behaves in the opposite manner: stimulated by steroids, but showing no general trend with triterpenes. Compound **1** shows stimulatory activity (14%, 10^{-5} M; 28%, 10^{-6} M; 27%, 10^{-8} M; 24%, 10^{-9} M). Among triterpenes, although all values are low, the behavior of **6** and **11** at the highest

TABLE 3. GERMINATION AND GROWTH ACTIVITY OF TRITERPENES **5–7**, **9**, **11**, **14**, **15** ON DICOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Lactuca sativa (var. nigra and var. romana)																		
5	−6	0	−2	−1	3a	0	12	0	−1	0	6a	−4	10	8	9	5	−1	9
6	—	3	−3	2	−8	−4	—	7	−1	3	5	11	—	4	7a	5	3	−4
7	—	−8	3	4	−11a	−14	—	4	8	−5	7a	5	—	−3	8b	−3	−5b	−6b
9	1b	−8	−1	−2	−4	0	−7b	0	−11	0	2	2	4	13b	−5	−4	15b	−15
11	0	2	−1a	−3	−2	−2	−18	0	10a	−6	−5	−2	−15b	−19a	9	18a	5	26a
14	—	22	−2	−4	−1	0	—	8	7	−9	1	−6b	—	13b	19a	8	11a	7
15	—	0	−2a	−1	−1	2	—	10b	8a	1	−5	8	—	5	11b	8	−7	−1
Lycopersicon esculentum L.																		
5	—	−4a	−6b	−2b	−4a	−5	—	−2	21b	29b	24b	20	—	8	0	7	9	4
6	—	−16	−5	−12a	−15	−16	—	11	22b	27a	23	29	—	14b	23b	16b	7	6
7	—	−11	−19	9	8b	−7b	—	13	22b	2	24b	23b	—	−7	−4	1	−12	−8
9	—	−2b	−5	9a	−2	0	—	27b	19	38a	17	5	—	12	7	−4	−1	−1
11	—	6	−16b	−5b	−4	−8a	—	−6	27b	25b	16	32a	—	−12	−19b	−6	−8	−1
14	—	2b	−5b	−15	−1b	0	—	22b	26b	15	33b	28	—	9	10	8	8	8
15	—	−5	−4	1b	1	−15	—	16	20	24b	12	13	—	10	14	10	4	19

[a] Values are presented as percentage differences from the control (e.g., +16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

TABLE 4. GERMINATION AND GROWTH ACTIVITY OF TRITERPENES **5-7, 9, 11, 14, 15** ON MONOCOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Hordeum vulgare L.																		
5	—	23	—	29	—	20	—	−17	—	−4	—	−17b	—	−13	—	−7	—	−23b
6	—	9	—	0	—	29	—	8	—	−3	—	−9	—	−7	—	−11	—	−9
7	—	23	—	31	—	20	—	12	—	12	—	4	—	−5	—	−3	—	−5
9	29	23	—	40b	—	6	−4	−4	—	−6	—	17	8	−4	—	1	—	6
11	−14	−9	—	0	—	−11	−3	2	—	3	—	−6	−3	5	—	5	—	−16
14	—	23	—	26	—	17	—	8	—	0	—	−3	—	−5	—	−23b	—	−11
15	—	37	—	40b	—	17	—	4	—	−9	—	3	—	−9	—	−11	—	5
Allium cepa L.																		
5	—	−9b	−10b	9	16	16	—	−10b	−18b	−9	−24	−15	—	24b	−10	1	16	13
6	—	18a	13b	−22b	3b	15	—	−24a	−26b	−32b	−17b	−21	—	10	14	−3	13	2
7	—	18	6	22	16	4a	—	−9	−16	−24	−12	−35a	—	7	0	−3	13	2
9	—	18	−12	−1	−13	13b	—	−11	−17	−11	−22	−22b	—	19b	16	−3	−1	−7
11	—	3	21b	12	16	4a	—	−1	−13b	−18	−9	−17a	—	12	8	9	11	−3
14	—	25	18	16	12	−1	—	−9	−23	−17	−11	−19	—	13	8	8	14	11
15	—	24b	13	15b	31	13	—	−14	−18	−13b	5	−16	—	4	8	9	11	22b

[a]Values are presented as percentage differences from the control (e.g., +16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

TABLE 5. GERMINATION AND GROWTH ACTIVITY OF STEROIDS **1–4** AND **10** ON DICOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Lactuca sativa (var. nigra and var. romana)																		
1	−1a	4	0	2	−2	−2	16a	3	3	5	−8	−8	7	10	2	4	−3	−3
2	—	4	0	−1	4	3	1	−3	4	−4	−7	2	4	−2	1	10	8	6
3	—	2	1	−2	−1	2	—	−3	−10	−9	−8	−7	—	13b	6	−2	10	9
4	—	3b	−1	−3	4b	−1	—	−12b	0	−7	−15b	−9	—	19a	8	15	6	4
10	6a	7	−1	6	1	1	20a	0	4	6	−3	−5	5	6	7	5	4	0
Lycopersicon esculentum L.																		
1	—	−4b	8	−7	−7	0	—	−13b	1	0	2	2	—	14a	28a	9	27a	24a
2	—	−12	5	−11	8	5	—	−12	−17b	−10a	−4	−1	—	20a	8	17	19a	22b
3	—	−1	5	−9	−8	−5a	—	−12	−6	−4	−12	−17a	—	16	11b	5	19a	14a
4	—	−7b	1	−5b	−9	−9	—	−14b	−13	−17b	−6	−8	—	17	5	17b	17a	11
10	—	4	−5b	−5b	−3	−9b	—	−4	−15b	−19b	−7	−17b	—	−10b	10b	9	5	22

[a] Values are presented as percentage differences from the control (e.g., + 16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

TABLE 6. GERMINATION AND GROWTH ACTIVITY OF STEROIDS **1–4** AND **10** ON MONOCOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Hordeum vulgare L.																		
1	—	48	40	44	28	16	—	−12b	−20	−7	5	−4	—	−16	−11	−15	−5	−17
2	44	76	32	48	32	11	13	0	−6	5	0	1	1	−2	−1	−1	−10	−7
3	—	44	28	44	16	32	—	9	−4	−6	1	2	—	−6	−7	−7	−5	−3
4	—	40	32	20	56	72	—	4	−2	−4	−1	−6	—	−9	−8	−8	−18	−20
10	—	40	16	36	36	52	—	1	−4	8	3	7	—	−18	−4	−4	−21	−14
Allium cepa L.																		
1	—	−14b	7	4a	7	−14	—	24b	18	31a	4	12	—	7	4	2	−2	−5
2	—	−7	3	4	3b	1	—	−7	9	−2	38b	6	—	−11	8	2	3	−2
3	—	−3	−1	10	−3	−4a	—	−2	3	−5	−3	11	—	−2	−1	4	5	3
4	—	4	5	4	10	4	—	10	−2	−5	−9	−4	—	7	−1	4	−1	14
10	—	−12	3	−16	−4	3	—	8	2	−8	−1	−8	—	5	2	0	−4	4

[a]Values are presented as percentage difference from the control (e.g., +16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

concentrations (**6**: 14%, 10^{-5} M; 23%, 10^{-6} M; 16%, 10^{-7} M; **11**: −12%, 10^{-5} M, −19%, 10^{-6} M) should be pointed out.

H. vulgare is the only bioassay where significant values for the majority of compounds were obtained. Triterpenes, as well as steroids, exert a stimulatory effect on germination, steroids showing higher values (Figures 2 and 5). The most active steroids are **1** and **2**, with similar profiles: high levels of stimulatory activity at higher concentrations (**1**: 48%, 10^{-5} M; 40%, 10^{-6} M, 44%, 10^{-7} M; **2**: 44%, 10^{-4} M; 76%, 10^{-5} M; 48%, 10^{-7} M). Compounds **4** and **10** are the most active at lower dilutions, being less active at higher concentrations (**4**: 32%, 10^{-6} M; 72%, 10^{-9} M; **10**: 16%, 10^{-6} M; 52%, 10^{-9} M). Triterpenes can be divided into two groups: those showing a good level of stimulatory activity within all ranges of concentration (compounds **5**, **7**, **9**, **14**, **15**), and those with no significant activity (compounds **6** and **11**). The only difference in the stereochemistry of compounds **6** and **7** is the double bond in the cinnamoyloxy moiety. The *E* isomer (**7**) behaves as a growth promoter at all concentrations, while the *Z* isomer (**6**) has no activity, except at the lower concentration. No significant effects or general trends were shown on root or shoot length.

Allium cepa germination values are either low or insignificant, e.g., the triterpene **15** has moderate stimulatory activity (24%, 10^{-4} M; 15%, 10^{-7} M; 31%, 10^{-8} M). Root length is clearly inhibited by triterpenes but not by steroids, except **1**, which stimulates root development. The *Z* and *E* isomers **6** and **7** both show inhibition, with the *Z* isomer slightly more active. No significant activity was observed on shoot growth.

In attempting to elucidate roles for these compounds in plants, there are at least three aspects that need further study: (1) The compounds may act as self-germination controllers, since they stimulate germination at low concentrations. Germination data are significant with almost all compounds tested in three of the four target species. (2) They may form micromicelles that facilitate transport of low polar terpenes through cell membranes. Ursolic acid is present in high quantities in the leaves of some allelopathic shrubs of the Florida scrub community (Weidenhamer et al., 1993) and has been proved to enhance monoterpene phytotoxicity. Betulinic acid is a major constituent of *M. messanensis*, and it is the precursor of many other terpenoid acids; thus, it is possible that it or its products may play a role as natural detergents that facilitate transport of low polar compounds towards cell membranes of competitor species. (3) Saponins are known to be growth regulators and have been associated with the allelopathic potential of alfalfa (*Medicago sativa*) (Oleszek, 1993; Waller et al., 1993; Wyman-Simpson et al., 1991). Our previous results (unpublished) revealed a high saponin content in the polar fraction that requires further study to establish the structure–activity relationship. If the saponins exhibit reasonable plant growth regulatory activity, their acidic precursors will also be examined for activity in the same systems.

This paper constitutes a first approach to understanding the roles that triterpenes play in *M. messanensis* L. Further study will establish if any of the above-mentioned hypotheses are true. Whether they are involved in internal regulation of germination or act as plant allelopathic agents, it is clear that they are involved with germination processes. Finally, another point needing further work is whether synergistic effects occur in mixtures. There are many examples where mixtures are much more active than single compounds, e.g., phenolics (Einhellig, 1986, 1987). At present the amounts of isolated compounds available do not allow us to check this possibility.

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