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Bioactive phenolics and polar compounds from *Melilotus messanensis*☆

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Abstract

The methanolic extract from sweetclover, *Melilotus messanensis*, afforded the new coumestan melimessanol A, the 3-arylcoumarin melimessanol B and the 2-arylbenzofuran melimessanol C, which where identified on the basis of spectroscopic methods. The known isoflavones formononetin and cyclosin; the isoflavanone vestitone; the flavanone liquiritigenin; the pterocarpans (3*R*,4*R*)-medicarpin, 3-hydroxymedicarpin and melilotocarpane B; the coumestans coumestrol, 4′-*O*-methylcoumestrol and 7-hydroxy-4′,5′-dimethoxycoumestan; the 3-arylcoumarin 3-(4′-methoxy-2′-hydroxyphenyl)-7-hydroxycoumarin; the lignan pinoresinol; 3-hydroxycoumarin and the simple phenolics *p*-coumaric acid, vanillic acid, *p*-hydroxybenzoic acid, a diterpene, two loliolides, a cyclitol and two saponins have been also isolated and spectroscopically identified. Cyclosin, vestitone, 4′-*O*-methylcoumestrol, 7-hydroxy-4′,5′-dimethoxycoumestan, 3-(4′-methoxy-2′-hydroxyphenyl)-7-hydroxycoumarin and 3-hydroxycoumarin are first reported in the genus *Melilotus*. The effects of a series of aqueous solutions from 10^{-4}–10^{-9} M of twelve phenolics, two loliolides, a diterpene and a cyclitol on germination and growth of the dicotyledons *Lactuca sativa* cvs. Roman and Nigra and *Lycopersicum esculentum* and the monocotyledons *Allium cepa* and *Hordeum vulgare*, have been studied. The isoflavones formononetin and cyclosin and the pterocarpan medicarpin have been found to inhibit principally *A. cepa* germination. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Melilotus messanensis; Leguminosae; Sweetclover; Allelopathy; Phenolics; Flavonoids; Melimessanols A-C; Lignan; Coumarins; Diterpene; Saponins; Cyclitol; Loliolides

1. Introduction

The study of the phenolic content of *M. messanensis* (L.). All has been achieved attending to three main objectives: (a) to determine whether the levels of coumarins, coumestrol and related compounds are high enough to cause damage to herbivores (Benson, Casper & Johnson, 1981; Blackley, 1985; Casper, Alstad, Monson, & Johnson, 1982); (b) to establish the nature of the phytotoxins with the pterocarpan skeleton of *M. messanensis* in comparison with other species of *Melilotus*, which has been isolated previously as phytoalexins in this genus (Ingham, 1977; Ingham, 1976) and (c) to continue with the allelopathic studies on *M. messanensis* (Macías, Simonet & Esteban, 1994; Macías, Simonet & Galindo, 1995; Macías, Simonet, Esteban & Galindo, 1996; Macías, Simonet and Galindo, 1997; Macías et al., 1998).

Here we report the phenolic content of the methanolic extract of the fresh plant.

2. Results and discussion

Following the gel filtration technique with Sephadex LH-20 as stationary phase to obtain fractions enriched in phenolics and then combining HPLC with silica gel and RP-18 phase as stationary phases, we have isolated fourteen flavonoids, a lignan, a coumarin and three simple phenolics. The known isoflavones formononetin (**1**), previously isolated from *M. italica* (Ingham, 1977), and cyclosin (**2**); the isoflavanone vestitone (**3**); the pterocarpans (3*R*,4*R*)-medicarpin (**4**),

☆ Part 8 in the series "Natural Products as Allelochemicals"; for Part 7 see Macías *et al.* [Macías, F. A., Simonet, A. M., Galindo, J. C. G., Pacheco, P. C. and Sánchez, J. A., *Phytochemistry*, 1998. 149, 709].

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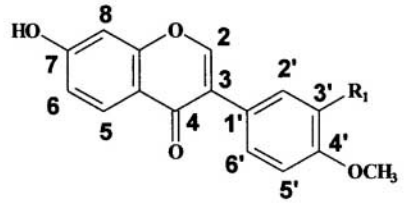
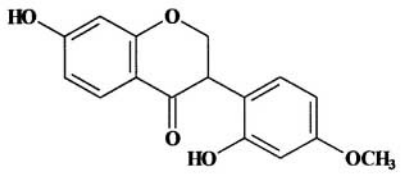
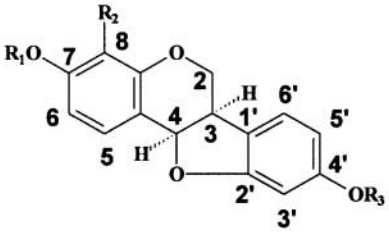
previously isolated in several species of *Melilotus* infected with the pathogen *Helminthosporium carbonum* (Ingham, 1977), melilotocarpane B (**5**), obtained also from *M. alba* (Miyase et al., 1982) and 3-hydroxymedicarpin (**6**), first reported from *M. alba* infected with the pathogen *Trifolium pratense* (Ingham, 1976); the coumestans coumestrol (**7**), 4′-*O*-methylcoumestrol (**8**); 7-hydroxy-4′,5′-dimethoxycoumestan (**9**); the 3-arylcoumarin 3-(4′-methoxy-2′-hydroxyphenyl)-7-hydroxycoumarin (**11**); the flavanone liquiritigenin (**14**), isolated from several species of *Melilotus* infected with *H. carbonum* (Ingham, 1976); the lignan pinoresinol (**15**); 3-hydroxycoumarin (**16**) and the simple phenolic acids *p*-coumaric (**17**), vanillic (**18**) and *p*-hydroxybenzoic (**19**), previously described for *M. officinalis* (Dombrowicz, Swiatek, Guryn & Zadernowski, 1991) have been isolated and identified. Compounds **2**, **3**, **7**, **8**, **9**, **11**, **15**, **16** are reported in the genus *Melilotus* for the first time. The coumestan melimessanol A (**10**), the 3-arylcoumarin melimessa-



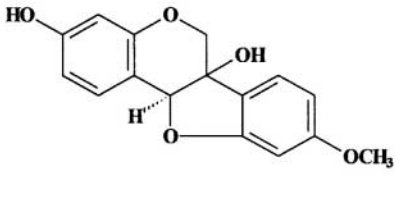
1: R_1=H, formononetin
2: R_1=OH, cyclosin



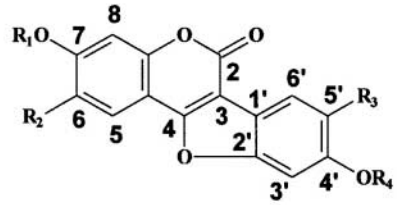
3: vestitone



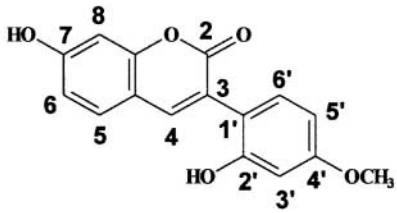
4: R_1=R_2=H; R_3=CH_3, medicarpin
5: R_1=CH_3; R_2=OH; R_3=H, melilotocarpane B



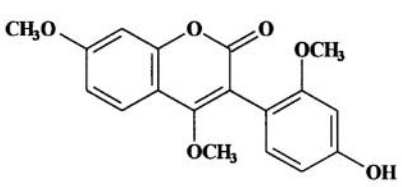
6: 3-hydroxymedicarpin



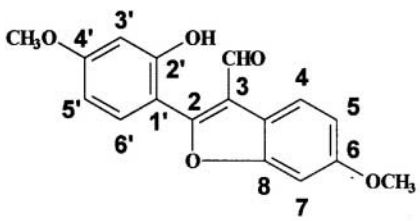
7: R_1=R_2=R_3=R_4=H, coumestrol
8: R_1=R_2=R_3=H; R_4=CH_3, 4′-*O*-methylcoumestrol
9: R_1=R_2=H; R_3=OCH_3; R_4=CH_3, 7-hydroxy-4′,5′-dimethylcoumestan
10: R_1=CH_3; R_2=OH; R_3=R_4=H, melimessanol A



11: 7,2′-dihydroxy-4′-methoxy-3-arylcoumarin



12: melimessanol B



13: melimessanol C

Fig. 1. Isolated compounds from *Melilotus messanensis*.

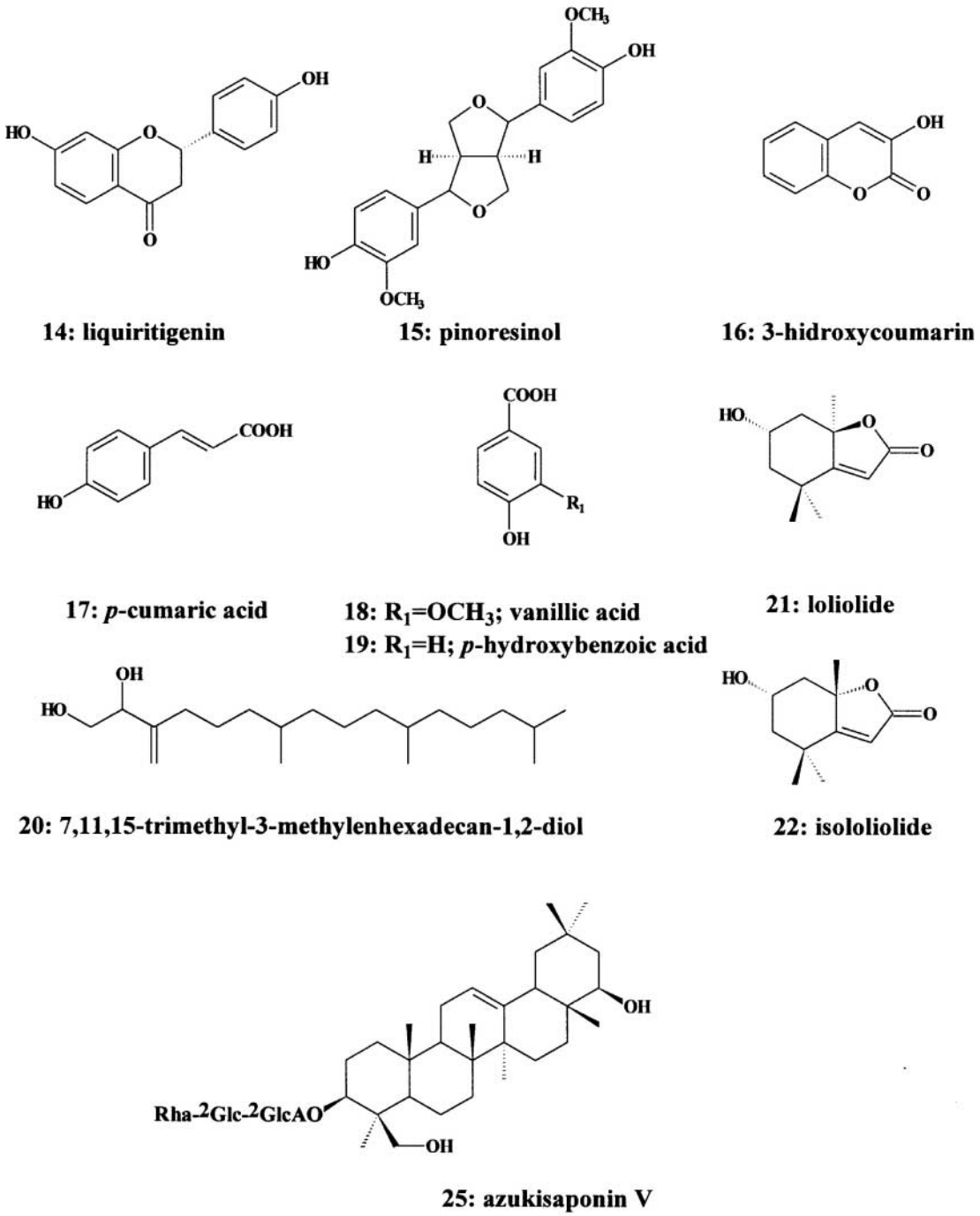


Fig. 2. Isolated compounds from *Melilotus messanensis* (continued).

nol B (**12**) and the 2-arylbenzofuran melimessanol C (**13**) are first reported as natural products (Figs. 1 and 2).

From the non-phenolic fractions the diterpene **20**, loliolide (**21**) and isololiolide (**22**) has been isolated for the first time in the genus *Melilotus*, in addition to D-pinitol (**23**), 3-hydroxycoumarin (**16**), 3-*O*-*β*-glucopyranosylsitosterol (**24**), previously described for *Melilotus indica* (Durrani & Ikram, 1966; Khafagy, Sabri & Abou Donia, 1980), and azukisaponin V (**25**), also isolated from *Melilotus officinalis* (Kang, Lee and Lee, 1987) (Fig. 2).

The spectroscopic data of compounds vestitone (**3**, ^{1}H NMR), 3-hydroxymedicarpin (**6**, α, ^{1}H, ^{13}C NMR), 4′-*O*-methylcoumestrol (**8**, ^{1}H NMR), 7-hydroxy-4′,5′-dimethoxycoumestan (**9**, ^{1}H NMR), 3-(4′-methoxy-2′-

Table 1
Comparison between selected chemical shifts of coumestrol [1], 7,4′-di-*O*-dimethylcoumestrol, melimessanol A–C (399,95 MHz, $CDCl_3$, signal of residual $CHCl_3$ centred at δ 7.25 ppm)

H	Melimessanol A (**10**)	Coumestrol (**7**) (acetone-d_6)	7,4′-Di-*O*-dimethyl-coumestrol	Melimessanol B (**12**)	H	Melimessanol C (**13**) (CD_2Cl_2)
5	7.45 *s*	7.87 *d*	7.87 *d*	7.85 *d*	6′	7.44 *d*
6	–	7.00 *dd*	6.97 *dd*	6.95 *dd*	5′	6.66 *dd*
8	6.99 *s*	6.94 *d*	6.99 *d*	7.03 *d*	3′	6.72 *d*
3′	7.13 *d*	7.19 *d*	7.16 *d*	6.51 *d*	7	7.06 *d*
5′	6.94 *dd*	7.02 *dd*	7.04 *dd*	6.50 *dd*	5	6.95 *dd*
6′	7.92 *d*	7.78 *d*	7.95 *d*	7.40 *d*	4	8.02 *d*

hydroxyphenyl)-7-hydroxycoumarin (**11**, ^{1}H NMR) and 3-hydroxycoumarin (**16**, MS, ^{1}H, ^{13}C NMR) are reported for the first time in this paper.

Melimessanol A (**10**) has the structure 6,4′-dihydroxy-7-methoxycoumestan. Its EIMS presents a molecular ion as base peak at *m*/*z* 298, according with the molecular formula $C_{16}H_{10}O_6$ and main fragments at *m*/*z* 283 $[M-15]^+$ and *m*/*z* 255 $[M-43]^+$, typical of methylated coumestans. The UV spectrum is also in agreement with a coumestan skeleton, with maximum absorbances at 230, 314 and 353 nm and intense blue to violet fluorescence in the UV light (Williams & Harborne, 1989). The ^{1}H NMR spectrum presents two separate sets of signals, coupled in the ^{1}H NMR 2D COSY experiment, corresponding to two different aromatic systems: an ABX system and a ring with two hydrogens in *para* position. Signals at δ 7.13 (*d*, J = 2 Hz, H-3′), δ 6.94 (*dd*, J = 8 Hz, J = 2 Hz, H-5′) and δ 7.92 (*d*, J = 8 Hz, H-6′) are consistent with those reported for the B-ring of coumestrol (Koshino et al., 1993) (Table 1), while the two singlets at δ 7.45 (H-5) and δ 6.99 (H-8) are in agreement with an A-ring supporting two substituents at C-6 and C-7. The position of the methoxy substituent at C-7 (δ 4.00, *s*) is assigned through its positive NOE's effect with the signal corresponding to H-8.

Melimessanol B (**12**) has the structure of 3-(4′-methoxy-2′-hydroxyphenyl)-7-hydroxycoumarin. Its EIMS presents a molecular ion as base peak at *m*/*z* 328, corresponding to the molecular formula $C_{18}H_{16}O_6$. The intense blue to violet fluorescence in the UV light, typical of a coumarin nucleus (Murray, Méndez & Brown, 1982), along with the lack of typical fragments related with the break of A- and B-rings in the EIMS (typical of a flavonol skeleton), allow us to confirm the structure of 4-methoxy-3-arylcoumarin, similar to the other isolated compounds from *M. messanensis*. The ^{1}H NMR spectrum presents signals corresponding to three methoxy substituents in an aromatic system at δ 3.86 (*s*), δ 3.78 (*s*) and δ 3.82 (*s*), as well as two ABX systems, correlated in the ^{1}H NMR 2D COSY experiment. Signals corresponding to the A ring are those at δ 7.85 (*d*, J = 9 Hz, H-5), δ 6.95 (*dd*, J = 9 Hz, J = 2 Hz, H-6) and δ 7.03 (*d*, J = 2 Hz, H-8), while those of B ring are at δ 6.51 (*d*, H-3′), δ 6.50 (*dd*, H-5′), and δ 7.40 (*d*, J = 9 Hz, H – 6′). The position of the hydroxy substituent is assigned at C-4′ due to the deshielding effect observed in the acetate derivative for the signals of H-3′ (δ 6.77, *d*, J = 2 Hz) and H-5′ (δ 6.83, *dd*, J = 8 Hz, J = 2 Hz). Comparison between the signals of **12** and the methylated derivative of coumestrol (Table 1) shows the great similarities of those corresponding to the A-ring, while those of B-ring appear upfield due to the shielding effect obtained when the conjugation of the B-ring with the rest of the molecule is lost.

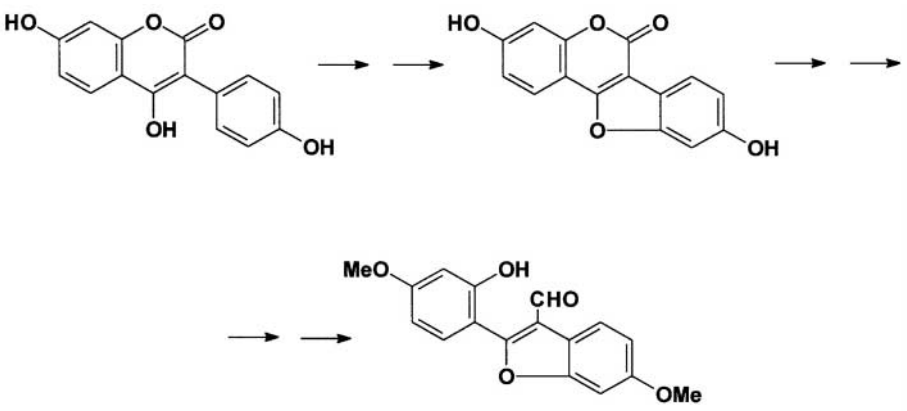


Fig. 3. Proposed biogenetic hypothesis for the formation of **13**.

Melimessanol C (**13**) has the structure 6-methoxy-2-(4′-methoxy-2′-hydroxyphenyl)-benzofuran-3-carbaldehyde and was isolated as its *tert*-butyl-dimethylsilane derivative due to the extreme complexity of the original fraction, which was entirely derivatized. Its ^{1}H NMR spectrum presents a signal at δ 10.06 (*s*) which remains if the spectrum is recorded with a drop of D_2O, corresponding to the carbaldehyde moiety, two methoxy signals at δ 3.82 (*s*) and δ 3.83 (*s*) and two ABX systems correlated in the ^{1}H NMR 2D COSY experiment. Comparison between the signals of **13** and 7,4′-di-*O*-dimethylcoumestrol (Table 1) reveals a shielding effect of the A-ring signals in **13**, due to the lost of the conjugation that occurs when the C-ring of coumestrol is not present. This effect is similar to that observed for **12** in the B − ring.

Positive NOE's effects obtained between the signals corresponding to C-4′ methoxy group, H-3′ and H-5′; and C-6 methoxy group, H-5 and H-7 allow us to establish the proposed structure for **13**, this being the second example of 2-aryl-3-carbaldehydebenzofuran isolated as a natural product (Ferreira, Moir & Thomson, 1974). Its biogenesis should come through the C ring opening in a coumestan precursor, since the other possibility should imply the formation of the D-ring from a 3-arylcoumarin derivative, which has been discarded in previous studies (Fig. 3) (Dewick, Barz & Grisebach, 1970).

2.1. Bioassay data discussion

A summary of the results of the bioassay is presented in Tables 2 and 3 and Figs. 4 and 5, where data are presented as percentages from the control. Positive values represent stimulation and negative values represent inhibition of the parameters studied. The discussion is presented based on the observed germination and growth effects, separately.

The overall effect of tested phenolics is low, excepting the most concentrated solutions of vanillic **18**, *p*-hydroxybenzoic **19** and *p*-coumaric **17** acids, well known as allelopathic agents with several reported effects at macroscopic level and cellular metabolism (Kuiters, 1989; Einhellig & Rasmussen, 1979; Lee, Starratt & Jevnikar, 1982). Thus, they will no longer be discussed.

Previously, the inhibitory activity of the dihydroactinidiolide (a related compound of loliolides), isolated from *Eleocharis coloradoensis* (Stevens & Merril, 1981),

Table 2
Germination and growth effects of active compounds on dicotyledons

	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
Lycopersicum esculentum L.																		
1	9	25	23	17	6	8	21	14	28	2	2	−9	25	11	30	−5	5	5
2		19	26	6	26	11		21	17	6	8	−15		29[b]	12	10	1	−6
4	4	11	21	36	21	4	9	4	4	15	8	−10	−9	8	7	7	19	−9
6		−2	0	−4	23	13		19	−2	−7	9	13		28[b]	16	−9	11	10
14		19	−13	21	8	9		19	7	18	12	15		24	12	6	9	12
15	2	11	−9	2	−8	−4	17	−25	−27	4	−11	−17	8	−14	−3	10	0	0
16	2	−2	0	−1	9	9	0	−6	2	−5	3	3	−16[b]	−14[b]	−6[b]	−22	−10	−8
Lactuca sativa cv. Nigra																		
2		3	11	22	8[b]	4		−12	−18	−10	−15[b]	−10		0	−2	8	−9	−8
4	6	15	4	1	3	0	−6	2	0	−5	−6	0	−20	6	−6	−1	1	4
6		−3	1	8	3	−3[b]		−1	−6	−11	−9	−21[b]		−1	5	−5	12	9
14		−17	−33	−22	0	13		−4	3	−15	−21[b]	−17		10	18	−1	−6	−19
16	−23	−2	−5	−14	−8	0	−13	8	−1	10	9	0	−11	2	−5	11	0	9
Lactuca sativa cv. Roman																		
1	4	3	−15	−10	8	−4	38	23	27	24	23	31	16	−1	15	1	9	3
2		−19	5	−1	−10	−22		36	5	7	3	17		41[b]	9	15	15	30[b]
4	−9	−1	−3	−13	−12	−22[b]	36	23	4	25	39	41[b]	−15	17	9	21[b]	29	41[b]
7	4	−3	1	3	22	18	−10	−11	13	−4	−8	8	−3	9	27	12	2	15
11		7	12	18	7	20		−12	−7	−3	2	11		−5	−43	−6	21	14
15	−14b	−22	−4	−12	−6	−1	60[b]	3	3	−6	5	−17	24[b]	18	3	−1	0	3
16	−14	−8	−12	8	0	−12	−7	2	2	−1	−18	5	−6	−5	8	16	−1	22

Values presented as percentage differences from the control (e.g. 16% means 116% compared with the control).Values are significative different from the control with $P > 0.05$ for the Welch's test.

[a] $P < 0.01$.

[b] $0.01 < P < 0.05$.

Table 3
Germination and growth effects of active compounds on monocytoledons

	Germination (% different from control)						Root length (% different 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		−3	−5	−15	−20	6		−22	−7	11	−13	8	1	−3	2	4	9	
4	−26	0	−8	−2	2	−11	−6	17	17	−17	−3	8	−11	5	0	−8	5	8
6		−2	9	14	−6	−6		27	5	−5	−10	12		10	5	6	5	−4
11		−2	−7	−20	22	7		27	−7	5	3	−7		−2	−10	−1	−8	−20
14		−6	−11	−5	6	−9		21	−14	−3	−1	−1		6	−7	−1	1	15
15	−23	−14	15	−9	6	12	−11	−2	27	−1	23	−2	−9	2	8	−1	14	−3
16		0	−7	18	−2	8		−1	9	20	12	22		−5	−2	1	−5	−2
Allium cepa L.																		
1	−31	−2	−58	−10	−25	−40	−54	−42	−26	−42	−20	−28	40[b]	−2	26	−19	−8	2
2		−17	−4	−35	−31	−50		−7	−28	−19	−42	−4		6	2	−5	−13	27
4	−54	15	−6	−17	−69	−27	−31	3	−14	−35	−26	−25	−37	12	16	−18	−41[b]	10
6		−2	−8	−13	−15	−6		1	6	11	−7	−16		7	16	6	33[b]	11
7	−15	−2	8	4	−12	8	11	−23	15	−18	−19	4	11	−3	3	−8	−12	−3
14		−29	4	−8	8	10		6	1	−37	−21	2		17	6	−5	6	3
15	−10	−15	−10	−13	−15	4	−26	19	9	−16	−15	17	10	8	21	−1	−5	26
16	13	19	29	−6	12	2	−38	−13	19	8	−11	−3	−29	5	18	11	−4	8

Values presented as percentage differences rom the control (e.g. 16% means 116% compared with the control).Values are significative different from the control with $P > 0.05$ for the Welch's test.[a]$P < 0.01$.[b]$0.01 < P < 0.05$.

has been described. The loliolides are also inhibitory. Both, loliolide and isololiolide inhibit the germination of tomato (*L. esculentum*; **21**: −16%, 10^{-5} M, −19%, 10^{-9} M, −19%, 10^{-8} M; **22**: −27%, 10^{-5} M, −28%, 10^{-6} M) and stimulate the germination of barley (*H. vulgare*; **21**: 29%, 10^{-5} M, 39%, 10^{-7} M; **22**: 20%, 10^{-5} M, 23%, 10^{-8} M); no other significative values are observed for the rest of parameters and species, excepting isolated cases. Otherwise, neither the diterpene **20**, nor the cyclitol **23** present any relevant activities.

2.2. Germination effects

There is a differential sensitiveness among tested species. The two varieties of lettuce show few effects with alternate slight positive and negative values, while tomato shows a general trend to stimulation with upper and more homogeneous values. Among the two monocotyledons tested there are also different behaviours between active compounds, with onion being more sensitive to inhibition than barley. These differences have been observed before for other kinds of compounds (Macías, Simonet & Galindo, 1995; Macías, Simonet, Esteban & Galindo, 1996; Macías, Simonet & Galindo, 1997; Macías et al., 1998) and should be an expression of the differences in the metabolism and active sites between species.

2.3. Isoflavonoids

Among tested species the most important effects are shown on onion, strongly inhibited by compounds **1** (−31%, 10^{-4} M; −58%, 10^{-6} M; −40%, 10^{-9} M), **2** (−17%, 10^{-5} M; −35%, 10^{-7} M; −50%, 10^{-9} M) and **4** (−54%, 10^{-4} M; −17%, 10^{-7} M; −69%, 10^{-8} M).

Tomato was also affected strongly. In this case, germination is homogeneously stimulated by compounds **1** (25%, 10^{-5} M; 23%, 10^{-6} M), **2** (19%, 10^{-5} M; 26%, 10^{-7} M, 26%, 10^{-8} M) and **4** (21%, 10^{-6} M; 36%, 10^{-7} M; 21%, 10^{-8} M), in all the range of concentrations and with similar levels of activity.

The effects in the other species are of less importance with different profiles of activity, indeed among the two varieties of lettuce. Thus, while the Nigra variety is only slightly stimulated by **2** (maximum value: 22%, 10^{-7} M), the Roman variety is inhibited with little and dispersed values by **2** and **4**. Barley shows no significant activities for any of the compounds. Comparison between structures and activities of compounds **1** and **4** shows no relevant differences in the open structure of isoflavone and the ring closed pterocarpan.

The other isoflavonoids tested (**6**, **7** and **11**) show no relevant activities on any of the species, except some isolated values for **7** and **11** in lettuce cv. Roman. Similar behaviours are found for active isoflavonoids in each tested species. Thus, isoflavones (**1**, **2**), pterocarpans (**4**, **6**) and coumestans (**7**, **11**) seem to have similar modes of action when active.

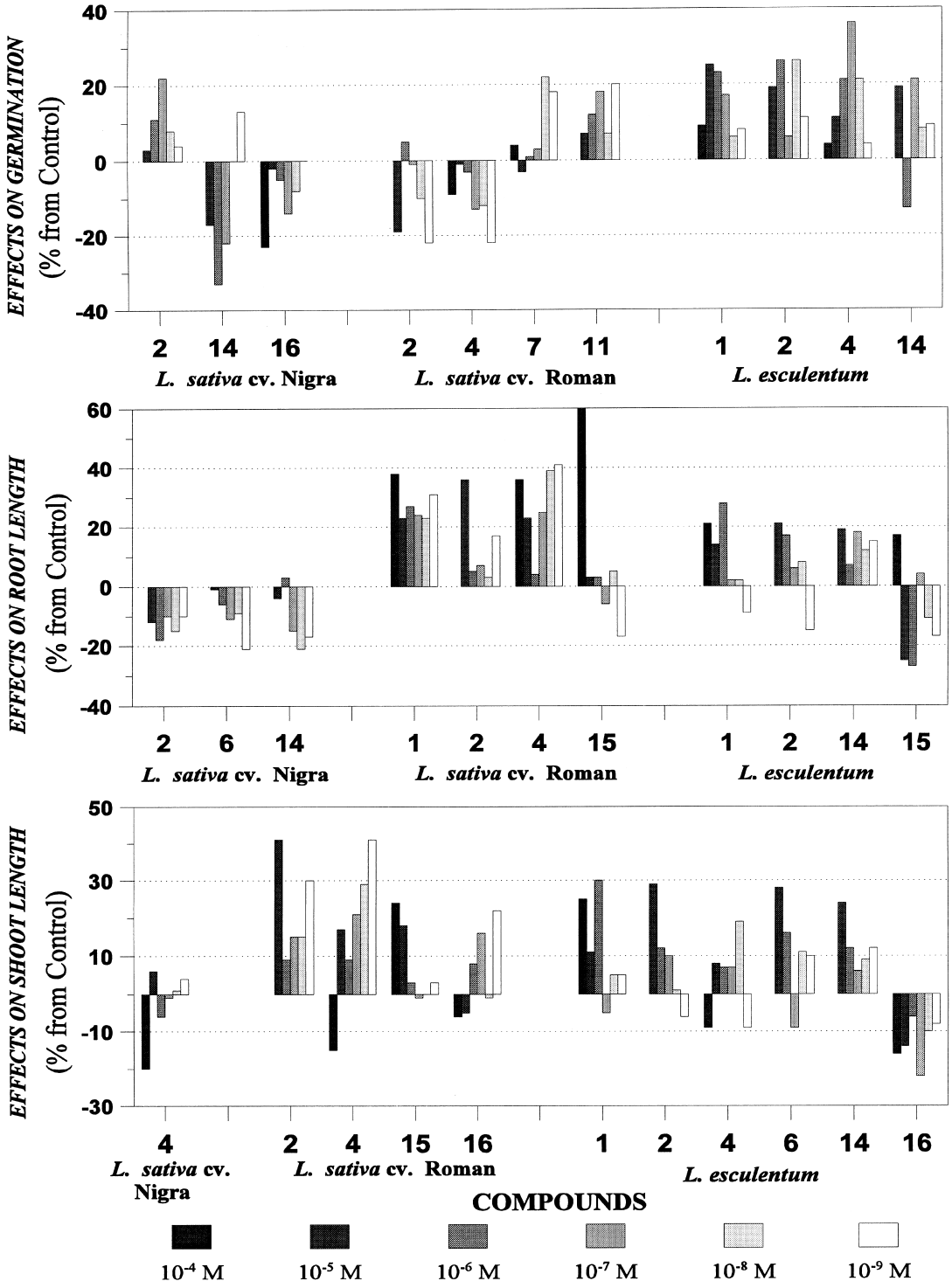


Fig. 4. Effects of active compounds on germination and growth of dicotyledons.

2.4. Other phenolics

Compound **14** is the only flavonoid tested and its behaviour is different than those of isoflavonoids: both tomato and onion are stimulated, except for weak negative values at the higher concentrations in onion (−29%, 10^{-5} M), while lettuce is slightly inhibited in the Nigra variety and non-active with the other. Barley is again not affected.

Compound **15** does not show any relevant activities, excepting some slight negative values at higher concentrations for lettuce cv. Roman (−14%, 10^{-4} M; −22%, 10^{-5} M) and barley (−23%, 10^{-4} M). There are no previous reports of allelopathic activities for lignans.

The coumarin **16** shows only moderate phytotoxic properties at 10^{-4} M (*L. sativa* cv. Nigra: −23%, germination; −13% root; −11%, shoot; *L. sativa* var. Roman: −14%, germination; *L. esculentum*: −16%, shoot; *A. cepa*: −38%, root; −29%, shoot) which is in good agreement with previous studies (Macías, Galindo, Massanet, Rodríguez-Luis & Zubía, 1993), where coumarins showed phytotoxic activities only at concentrations of 10^{-4} M or higher.

2.5. Growth effects

In the same way shown for germination, there are different behaviours depending on the species. With regard to the two varieties of lettuce, the cv. Nigra is less sensitive than the cv. Roman, both for shoot and root parameters, the latter being stimulated, while the former, when affected, is slightly inhibited. Tomato is also stimulated.

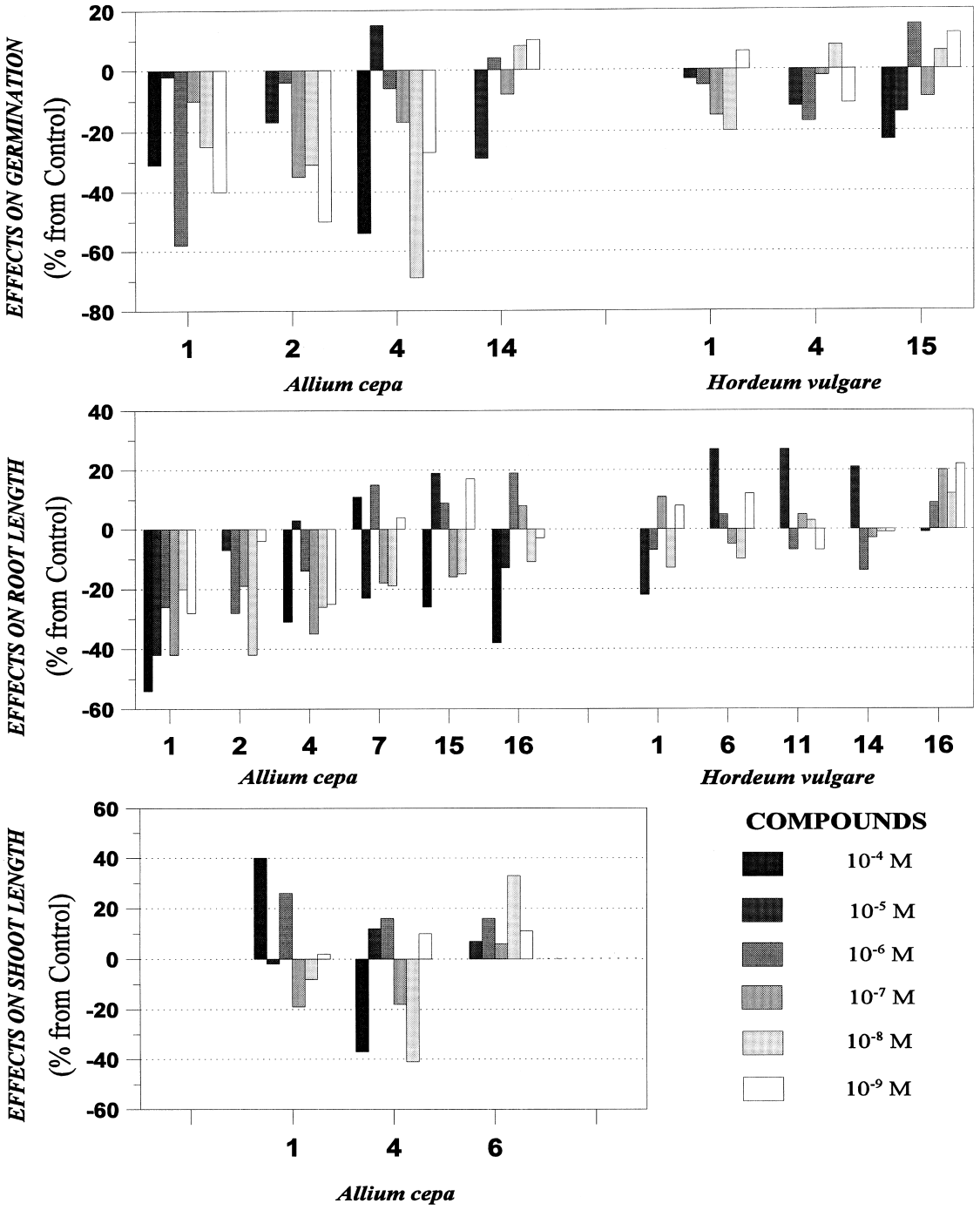


Fig. 5. Effects of active compounds on germination and growth of monocotyledons.

With regard to monocotyledons, the sensitiveness of onion is again greater than that of barley, the behaviours also being different. Root is generally inhibited in onion, while barley is weakly stimulated, when affected. The shoot length parameter is only affected in onion by a few compounds and shows no general trends.

2.6. Isoflavonoids

Onion roots are again the most deeply inhibited by the isoflavonoids **1** (−42% at 10^{-5} M and 10^{-7} M), **2** (−28%, 10^{-6} M; −42%, 10^{-8} M) and **4** (−31%, 10^{-4} M; −35%, 10^{-7} M; −25%, 10^{-9} M), lettuce cv. Nigra roots being the other species inhibited, but to a much lower extent (all values falling below −20%).

Radicles and hypocotyls of lettuce cv. Roman are strongly stimulated at almost all concentrations by compounds **1**, **2** and **4**, with an average value of 30% for radicles and 25% for hypocotyls. Tomato is also stimulated by compounds **1**, **2** and **6**, but with lower values than lettuce. Barley is again not significantly affected.

Compounds **6**, **7** and **11** show no relevant activities. Only shoots of tomato and onion are stimulated by **6**, the effect being deeper in the former. Roots of barley are weakly stimulated at the upper concentration (27%, 10^{-5} M) by **6** and **11**.

There is an important difference between medicarpin (**4**) and 3-hydroxymedicarpin (**6**), the former being active in more cases. Since the only structural difference between them is the presence of an hydroxyl moiety at C-3, such difference could be related to the different behaviour shown by them.

2.7. Other phenolics

Compound **14** shows only weak effects on the growth of one of the three dicotyledons, stimulating both parameters of tomato. With respect to monocotyledons, only the roots of barley are weakly stimulated at 10^{-5} M. No other significant effects are observed.

The root length of lettuce cv. Roman is strongly enhanced at the upper concentration (60%, 10^{-4} M) of compound **15**, while shoot length is only weakly affected. Excepting a slight inhibiting activity on the roots of tomato, no other important effects are observed.

2.8. Conclusions

Attending to the three objectives presented at the beginning of this study we can conclude the following:

(1) With regard to its possible use as green manure, it has been reported for other species of sweetclover and trefoil that its high content in formononetin (precursor of the active compound equol) and coumestrol is harmful to herbivores due to their estrogenic and oestrogenic effects (Batterham et al., 1971; Cox & Branden, 1974; Shutt, 1976). Keeping in mind the extraction procedure followed in this work, the levels of formononetin (**1**) and coumestrol (**7**) obtained (4×10^{-4}% from the fresh plant weight for **1** and 8×10^{-5} % for **7**) and the difference between this methodology and the extraction that animals carry out during digestion, we can propose that the isolated amounts are within the non-toxic levels necessary for use as a forage resource (toxic levels are among 25–300 μg/ml in blood) (Smith, 1982; Vanetten, 1972), though it is necessary to accomplish the corresponding bioassays with fresh plants and animals.

(2) The results of the analysis of the phenolic fractions of *M. messanensis* have shown that the unique simple coumarin isolated is 3-hydroxycoumarin (**16**), which cannot be a natural precursor of dicoumarol, responsible of the haemorrhagic effects on herbivores (Casper, Alstad, Monson & Johnson, 1982; Blackley, 1985), because of the biogenetic pathway of this compound. No other coumarins, dicoumarol or dicoumarol precursors have been detected.

(3) The presence of isoflavonoids in *M. messanensis* confirms the ability to generate phytoalexins related with medicarpin for self-defence and, thus, its usefulness as forage resource resistant to fungus attack when stored, as previously described for other species of this genus (Ingham, 1977; Ingham, 1976).

(4) With regard to its phytotoxic effects, excepting the simple phenolic acids **17**, **18** and **19**, only phenolics **1**, **2**, **4**, **14** and **16** show moderated activities. In previous papers (Macías, Simonet & Esteban, 1994; Macías, Simonet & Galindo, 1995; Macías, Simonet, Esteban & Galindo, 1996; Macías, Simonet & Galindo, 1997; Macías, Simonet & Galindo, 1998) we have described the phytotoxic activity of several triterpenes and steroids. They are also the major constituents of the plant. So, both due to the total amounts of them obtained and the levels of activity shown, they can be proposed as the compounds responsible for the observed allelopathic effects. In this way, though the role of flavonoids in the plant ought to be other than a defensive role against other plants and should be related to self-defence agents towards pathogens their phytotoxicity should contribute to the macroscopic observed allelopathic activity of *M. messanensis*. Only one point remains unclear: two saponins have been isolated and elucidated (**24**, **25**), but evidence of other saponins has also been obtained. A study aiming to determine the saponin content of sweetclover and its phytotoxicity is needed to complete the picture of the ecological chemistry of this plant and to answer the questions proposed in previous work (Macías, Simonet, Esteban & Galindo, 1996).

3. Experimental

3.1. Plant material

Melilotus messanensis (L.): All was collected on 11 April 1991 in Trebujena, Cádiz, Spain (a voucher specimen is deposited at the University of Seville Herbarium, Spain, SEV-7992) when the plant was at the beginning of the flowering.

3.2. Extraction and isolation

Fresh plants (2.5 kg) were soaked in MeOH for 24 h at 25° in the dark. The MeOH extract (33 g) was dissolved with 0.8 l of H_2O and extracted (4×) with 0.8 l of EtOAc. The combined extracts were dried over Na_2SO_4 and evapd *in vacuo* to yield 4.7 g of MA extract. The aq. fr. was re-extracted (10×) with 0.8 l of *n*-ButOH and the combined extracts were evapd *in vacuo* to yield 7 g of MB extract.

The MA extract was sepd by CC on silica gel using CH_2Cl_2–Me_2CO mixts of increasing polarity yielding 7 frs after comparison by TLC. Frs C and E were sepd using gel-filtration chromatography with Sephadex LH-20 as stationary phase and *iso*-PrOH as eluent. The phenolic fr from C was chromatographed using HPLC (Hibar Si60 column) with $CHCl_3$–EtOAc (19:1) as eluent and UV 254 nm as detector, yielding **4** (14 mg), **8** (2 mg) and **12** (1 mg). The non-phenolic fr. of E was sepd by silica gel CC using *n*-hexane–EtOAc (4:1) and chromatographed again using HPLC (Hibar Si60 column) with *n*-hexane–EtOAc (3:2) as eluent and a refraction index detector, yielding **20** (3 mg), **21** (2 mg) and **22** (0.5 mg). The phenolic fr. of E was chromatographed using HPLC (RP-18 column) with MeOH–H_2O (7:3) as eluent and a refraction index detector and chromatographed again using HPLC (Hibar Si60 column), *n*-hexane–Me_2CO (3:1) and a refraction index detector, yielding **1** (10 mg), **2** (2 mg), **3** (1 mg), **5** (0.5 mg), **6** (1 mg), **7** (2 mg), **9** (0.5 mg), **10** (0.5 mg), **11** (0.5 mg), **13** (1 mg), **14** (1 mg), **15** (2 mg), **17** (1 mg), **18** (4 mg) and **19** (4 mg). Fr. G was sepd using silica gel CC and CH_2Cl_2–MeOH (19:1) as eluent yielding **24** (5 mg).

The MB extract was sepd by CC on silica gel using $CHCl_3$–MeOH–H_2O mixts of increasing polarity, yielding 47 × 50 frs which were reduced to 8 frs after comparison by TLC. Frs C and D were sepd using gel-filtration CC, using Sephadex LH-20 and MeOH–H_2O (1:1) as eluent. Intermediate frs were chromatographed using HPLC (RP-18 column, MeOH–H_2O (3:2) as eluent), yielding **16** (2 mg), **23** (100 mg) and **25** (2 mg).

3.3. Identification

Known compounds were identified by comparison of their physical and spectroscopic data (m.p., [α], IR, MS, ^{1}H NMR and ^{13}C NMR) with those previously reported in the literature. Structures of new compounds were established based on the analysis of their spectroscopic data.

3.4. Vestitone (3)

^{1}H NMR (399.952 MHz, $CDCl_3$): δ 3.74 (3H, *s*, -OCH_3), δ 3.89 (1H, *dd*, $J_{2a,3}=3$ Hz, $J_{2b,3}=5$ Hz, H-3), δ 4.79 (1H, *dd*, $J_{2a,2b}=12$ Hz, $J_{2b,3}=5$ Hz, H-2b), δ 4.94 (1H, *dd*, $J_{2a,2b}=12$ Hz, $J_{2a,3}=3$ Hz, H-2a), δ 6.41 (1H, *d*, $J_{6,8}=2$ Hz, H-8), δ 6.46 (1H, *dd*, $J_{5',6'}=8$ Hz, $J_{3',5'}=3$ Hz, H-5′), δ 6.50 (1H, *dd*, $J_{5,6}=9$ Hz, $J_{6,8}=3$ Hz, H-6), δ 6.53 (1H, *d*, $J_{3',5'}=3$ Hz, H-3′), δ 7.40 (1H, *d*, $J_{5',6'}=9$ Hz, H-6′), δ 7.83 (1H, *d*, $J_{5,6}=9$ Hz, H-5).

3.5. 3-Hydroxymedicarpin (6)

$[\alpha]_D^{25}$ −209° ($CHCl_3$, *c* 0.07); ^{1}H NMR (399.952 MHz, $CDCl_3$): δ 3.74 (3H, *s*, -OCH_3), δ 3.91 (1H, *d*, $J_{2a,2b}=11$ Hz, H-2a), δ 4.10 (1H, *d*, $J_{2a,2b}=11$ Hz, H-2b), δ 5.20 (1H, *s*, H-4), δ 6.28 (1H, *d*, $J_{6,8}=2$ Hz, H-8), δ 6.36 (1H, *d*, $J_{3',5'}=2$ Hz, H-3′), δ 6.49 (1H, *dd*, $J_{5,6}=8$ Hz, $J_{6,8}=2$ Hz, H-6), δ 6.51 (1H, *dd*, $J_{5',6'}=9$ Hz, $J_{3',5'}=2$ Hz, H-5′), δ 7.42 (1H, *d*, $J_{5,6}=8$ Hz, H-5); ^{13}C NMR(100,577 MHz, $CDCl_3$): δ 56.0 (−OCH_3), δ 70.9 (C-2), δ 77.2 (C-3), δ 86.2 (C-4), δ 97.6 (C-3′), δ 104.1 (C-8), δ 108.2 (C-6), δ 111.1 (C-5′), δ 113.1 (C-10), δ 122.5 (C-1′), δ 125.1 (C-6′), δ 133.2 (C-5), δ 157.4 (C-9), δ 160.1 (C-7), δ 162.2 (C-2′), δ 163.7 (C-4′).

3.6. 4′-O-Methylcoumestrol (8)

^{1}H NMR (399.952 MHz, $CDCl_3$): δ 3.88 (3H, *s*, -OCH_3), δ 6.89 (1H, *dd*, $J_{5,6}=9$ Hz, $J_{6,8}=2$ Hz, H-6), δ 6.91 (1H, *d*, $J_{6,8}=2$ Hz, H-8), δ 7.01 (1H, *dd*, $J_{5',6'}=8$ Hz, $J_{5',3'}=2$ Hz, H-5′), δ 7.15 (1H, *d*, $J_{3',5'}=2$ Hz, H-3′), δ 7.80 (1H, *d*, $J_{5,6}=9$ Hz, H-5), δ 7.91 (1H, *d*, $J_{5',6'}=8$ Hz, H-6′).

3.7. 7-Hydroxy-4′,5′-dimethoxycoumestan (9)

^{1}H NMR (399.952 MHz, $CDCl_3$): δ 3.98 (3H, *s*, -OCH_3), δ 4.00 (3H, *s*, -OCH_3), δ 6.91 (1H, *dd*, $J_{5,6}=9$ Hz, $J_{6,8}=2$ Hz, H-6), δ 6.96 (1H, *d*, $J_{6,8}=2$ Hz, H-8), δ 7.19 (1H, *s*, H-3′), δ 7.52 (1H, *s*, H-6′), δ 7.85 (1H, *d*, $J_{5,6}=9$ Hz, H-5).

3.8. Melimessanol A (**10**)

$C_{16}H_{10}O_6$, white amorphous solid; UV λ_{max}^{MeOH} nm: 230, 314, 353, 366sh; EIMS (70 eV) *m/z* (rel. int.): 298 $[M]^+$ (100), 283 $[M–CH_3]^+$ (71), 255 (28), 227 (8); ^1H NMR (399.952 MHz, $CDCl_3$): δ 4.00 (3H, *s*, -OCH_3), δ 6.94 (1H, *dd*, $J_{5',6'}=8$ Hz, $J_{3',5'}=2$ Hz, H-5′), δ 6.99 (1H, *s*, H-8), δ 7.13 (1H, *d*, $J_{3',5'}=2$ Hz, H-3′), δ 7.45 (1H, *s*, H-5), δ 7.92 (1H, *d*, $J_{5',6'}=8$ Hz, H-6′).

3.9. 7,2′-Dihydroxy-4′-methoxy-3-arylcoumarin (**11**)

^1H NMR (399.952 MHz, $CDCl_3$): δ 3.80 (3H, *s*, -OCH_3), δ 6.55 (1H, *dd*, $J_{5',6'}=8$ Hz, $J_{5',3'}=3$ Hz, H-5′), δ 6.57 (1H, *d*, $J_{3',5'}=3$ Hz, H-3′), δ 6.84 (1H, *dd*, $J_{5,6}=9$ Hz, $J_{6,8}=2$ Hz, H-6), δ 6.84 (1H, *d*, $J_{6,8}=2$ Hz, H-8), δ 7.17 (1H, *d*, $J_{5',6'}=8$ Hz, H-6′), δ 7.41 (1H, *d*, $J_{5,6}=9$ Hz, H-5), δ 7.79 (1H, *s*, H-4).

3.10. Melimessanol B (**12**)

$C_{18}H_{16}O_6$, white amorphous solid; UV λ_{max}^{MeOH} nm: 283, 319; EIMS (70 eV) *m/z* (rel. int.): 328 $[M]^+$ (100), 313 $[M–CH_3]^+$ (23), 297 (9), 282 (8), 267 (14); ^1H NMR (399.952 MHz, $CDCl_3$): δ 3.78 (3H, *s*, -OCH_3), δ 3.82 (3H, *s*, -OCH_3), δ 3.86 (3H, *s*, -OCH_3), δ 6.50 (1H, *dd*, H-5′), δ 6.51 (1H, *d*, H-3′), δ 6.95 (1H, *dd*, $J_{5,6}=9$ Hz, $J_{6,8}=2$ Hz, H-6), δ 7.03 (1H, *d*, $J_{6,8}=2$ Hz, H-8), δ 7.40 (1H, *d*, $J_{5'.6'}=9$ Hz, H-6′), δ 7.85 (1H, *d*, $J_{5,6}=9$ Hz, H-5).

3.11. Melimessanol B acetate (**12a**)

$C_{20}H_{18}O_7$, white amorphous solid; EIMS (70 eV) *m/z* (rel. int.): 370 $[M]^+$ (32), 328 $[M–CH_2CO]^+$ (61), 313 $[M–CH_2CO–CH_3]^+$ (19), 297 (5), 282 (4), 267 (8); ^1H NMR (399.952 MHz, $CDCl_3$): δ 2.33 (3H, *s*, -OAc), δ 3.80 (3H, *s*, -OCH_3), δ 3.82 (3H, *s*, -OCH_3), δ 3.87 (3H, *s*, -OCH_3), δ 6.77 (1H, *d*, $J_{3',5'}=2$ Hz, H-3′), δ 6.83 (1H, *dd*, $J_{5',6'}=8$ Hz, $J_{3',5'}=2$ Hz, H-5′), δ 6.97 (1H, *dd*, $J_{5,6}=9$ Hz, $J_{6,8}=2$ Hz, H-6), δ 7.04 (1H, *d*, $J_{6,8}=2$ Hz, H-8), δ 7.46 (1H, *d*, $J_{5'.6'}=8$ Hz, H-6′), δ 7.88 (1H, *d*, $J_{5,6}=9$ Hz, H-5).

3.12. Melimessanol C (**13**)

$C_{17}H_{14}O_5$, white amorphous solid; UV λ_{max}^{MeOH} nm: 241, 344; EIMS (70 eV) *m/z* (rel. int.): 298 $[M]^+$ (1), 279 (26), 167 (37), 149 (100); ^1H NMR (399.952 MHz, CD_2Cl_2): δ 3.82 (3H, *s*, -OCH_3), δ 3.83 (3H, *s*, -OCH_3), δ 6.66 (1H, *dd*, $J_{5',6'}=8$ Hz, $J_{3',5'}=2$ Hz, H-5′), δ 6.72 (1H, *d*, $J_{3',5'}=2$ Hz, H-3′), δ 6.95 (1H, *dd*, $J_{4,5}=8$ Hz, $J_{5,7}=2$ Hz, H-5), δ 7.06 (1H, *d*, $J_{5,7}=2$ Hz, H-7), δ 7.44 (1H, *d*, $J_{5',6'}=8$ Hz, H-6′), δ 8.02 (1H, *d*, $J_{4,5}=8$ Hz, H-4), δ 10.01 (1H, *s*, C*H*O).

3.13. Melimessanol C TBDMS derivative (**13t**)

^1H NMR (399.952 MHz, $CDCl_3$): δ 3.82 (3H, *s*, -OCH_3), δ 3.83 (3H, *s*, -OCH_3), δ 6.52 (1H, *d*, $J_{3',5'}=2$ Hz, H-3′), δ 6.57 (1H, *dd*, $J_{5',6'}=8$ Hz, $J_{3',5'}=2$ Hz, H-5′), δ 6.96 (1H, *dd*, $J_{4,5}=9$ Hz, $J_{5,7}=2$ Hz, H-5), δ 7.04 (1H, *d*, $J_{5,7'}=2$ Hz, H-7), δ 7.45 (1H, *d*, $J_{5',6'}=8$ Hz, H-6′), δ 8.08 (1H, *d*, $J_{4,5}=9$ Hz, H-4), δ 10.01 (1H, *s*, C*H*O); ^{13}C NMR (100.577 MHz, $CDCl_3$): δ 55.8 (-O*C*H_3), δ 95.9 (C-3′), δ 99.6 (C-7), δ 107.8 (C-5′), δ 112.9 (C-5), δ 122.5 (C-4), δ 132.8 (C-6′), δ 187.9 (*C*HO).

3.14. 3-Hydroxycoumarin (**16**)

$C_9H_6O_3$, red crystals; EIMS (70 eV) *m/z* (rel. int.): 162 $[M]^+$ (43), 161 (47), 147 (16), 143 (22), 132 (29), 115 (50), 104 (33), 44 (87); ^1H NMR (399.952 MHz, M_eOD-du): δ 7.14 (1H, *s*, H-4), δ 7.18 (1H, *dd*, $J_{5,6}=J_{6,7}=8$ Hz, H-6), δ 7.28 (1H, *d*, $J_{7,8}=8$ Hz, H-8), δ 7.33 (1H, *dd*, $J_{6,7}=J_{7,8}=8$ Hz, H-7), δ 7.49 (1H, *d*, $J_{5,6}=8$ Hz, H-5); ^{13}C NMR (100.577 MHz, $CDCl_3$): δ 114.7 (C-8), δ 116.1 (C-5), δ 122.7 (C-10), δ 124.0 (C-6), δ 127.2 (C-7), δ 127.9 (C-4), δ 134.7 (C-3), δ 147.2 (C-9), δ 160.9 (C-2).

3.15. Bioassays

Seeds of *Lactuca sativa* L. cv. Roman and cv. Nigra, *Lycopersicum esculentum* L. cv. Tres Cantos, *Allium cepa* L. cv. Valenciana and *Hordeum vulgare* L. cv. Wellam were obtained from FITÓ, S. L. (Barcelona, Spain). All undersized or damaged seeds were discarded and the assay seeds were selected for uniformity. Germination and growth bioassays were as follows: *L. sativa*, *L. esculentum* and *A. cepa*, 25 seeds per dish, 5 ml test soln, 5 days dark, 25° and four replicates of each concentration; *H. vulgare*, 10 seeds per dish, 5 ml test soln., 5 days dark, 25° and ten replicates of each concentration (Macías, Castellano & Molinillo, 1998).

Test solns (10^{-4} or 10^{-5} M) were prepd using H_2O–MES (2-[*N*-morpholino]ethanesulfonic acid, 10 mM), and the rest 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 vapour pressure osmometer (WESCOR 5500) and ranged between 30 and 38 mOsmolar.

Data are presented as percentage differences from control in graphics and tables (Figs. 4 and 5 and Tables 2 and 3). Thus, zero represents the control; positive values represent stimulation of the studied parameter, and negative values represent inhibition.

3.16. Statistical treatment

Germination, root and shoot length values were tested by the Welch test; differences between experimentals and controls were significant ($P = 0.01$) (Tables 2 and 3).

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