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Phytotoxic Activity of *Salvia x jamensis*

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A study has been carried out on the surface exudate of *Salvia x jamensis*, which showed a significant phytotoxic activity against *Papaver rhoeas* L. and *Avena sativa* L.. Bioguided separation of the exudate yielded active fractions from which 3β-hydroxy-isopimaric acid (**1**), hautriwaic acid (**2**), betulinic acid (**3**), 7,8β-dihydrosalviacoccin (**4**), isopimaric acid (**5**), 14α-hydroxy-isopimaric acid (**7**), 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide (**8**), cirsiliol (5,3',4'-trihydroxy-6,7-dimethoxyflavone, **9**) and two new neoclerodane diterpenes (**6** and **10**) were isolated. The structures of **6** and **10** were identified as 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide and 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide respectively on the basis of spectroscopic data analysis. All compounds, but **7**, **8** and **10**, were active in inhibiting the germination of the tested species.

Keywords: *Salvia*, diterpenes, triterpenes, flavonoids, phytotoxic activity.

Phytotoxic chemicals originated by aerial parts of the plants are known to be released into the environment by different ways as foliar leaching, volatilization or residue decomposition [1,2]. Many of these compounds are found in the complex secretion product originated in the epidermal secretory structures [3]. The surface exudate of *Salvia x jamensis* J. Compton (Lamiaceae), whose platelet antiaggregating activity we described previously [4], showed a good antigerminative activity against *Papaver rhoeas* L. and *Avena sativa* L. (Final germination [5] 0.4±0.7% against *Papaver* and 0.0 % against *Avena* at 5 mg/L).

Isolation and identification of the compounds. Bioguided separation of the exudate with column chromatography yielded active fractions (Table 1). From fractions III and IV, 3β-hydroxy-isopimaric acid (**1**), hautriwaic acid (**2**), betulinic acid (**3**), 7,8β-dihydrosalviacoccin (**4**), isopimaric acid (**5**), 14α-hydroxy-isopimaric acid (**7**), 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide (**8**), cirsiliol (5,3',4'-trihydroxy-6,7-dimethoxyflavone, **9**) and two new neoclerodane diterpenes (**6** and **10**) were isolated.

Table 1: Effect of the various fractions of *Salvia x jamensis* exudate on the Final germination (maximum average percentage of seeds that germinated during the experiment) and early growth of *Papaver rhoeas* L. and *Avena sativa* L. at various concentrations. Data are expressed as mean (± S.D.) of three triplicates. Significant differences among the means were evaluated using the Tukey's honest significant difference test. Values with the same letters in a column are not significantly different at the 0.05 probability level. The letters are given following increasing values.

Fraction groups of *S. x jamensis*	*Papaver rhoeas* L.			*Avena sativa* L.		
	2 mg/L	5 mg/L	10 mg/L	2 mg/L	5 mg/L	10 mg/L
I	26.7±2.1^{c}	6.3±0.6^{b}	6.0±1.7^{b}	50.3±2.5^{c}	26.3±2.5^{c}	20.0±2.0^{c}
II	22.7±3.1^{c}	8.7±2.3^{b}	6.7±1.2^{b}	31.7±2.5^{b}	13.7±2.5^{b}	9.7±2.1^{b}
III	6.7±2.5^{a}	0.0^{a}	0.0^{a}	5.0±2.0^{a}	0.0^{a}	0.0^{a}
IV	15.0±3.6^{b}	0.0^{a}	0.0^{a}	6.0±1.7^{a}	0.0^{a}	0.0^{a}

Table 2: NMR spectral data for compound **6**: δ values, $CDCl_3$, ^{13}C NMR at 125 MHz, ^1H NMR at 500 MHz.

C	^{13}C	^1H (*J* in Hz)	HMBC correlations of the C
1	26.6	1.61 ddd (5.3, 12.0, 12.4), 1.80 m	-
2	23.3	2.52, 2.57 both m	-
3	135.6	6.78 m	1.80, 2.52, 2.57
4	139.5	-	4.47
5	49.3	-	1.80, 1.84, 2.07, 4.47, 6.78
6	26.2	1.84, 2.10 both m	1.97, 3.88, 4.47
7	19.4	1.97, 2.07 both m	-
8	42.9	3.53 dd (4.2, 12.0)	2.68
9	40.8	-	0.91, 2.68, 3.53
10	77.3	-	0.91, 1.80, 2.52, 2.57, 2.68
11	40.3	1.67, 2.68 both m	0.91, 3.53
12	71.9	5.38 dd (6.2, 11.2)	1.67
13	125.4	-	1.67, 5.38, 6.48, 7.47
14	109.7	6.48 br s	5.38
15	144.8	7.47 br s	7.52
16	140.7	7.52 br s	5.38, 6.48
17	176.0	-	3.53
18	168.9	-	4.47
19	73.7	4.47 d (6.8), 3.88 dd (2.0, 6.8)	-
20	21.0	0.91 s	1.67, 2.68, 3.53

Compounds **1**, **3**, **4**, **5**, **7**, **8** were previously isolated from this plant [4] and compounds **2** and **9** were identified by comparison of their physical and spectroscopic data with those published in the literature [6,7].

IR absorption bands at 3495 (-OH groups), 1755, 1725 (two lactone rings), and 3121, 1502, 868 (β-substituted furan ring) cm^{-1} and 20 carbon resonances in the ^{13}C NMR suggested for **6** an oxygenated clerodane diterpenoid structure. By inspection of ESI-MS, HR-MS, COSY, HSQC and HMBC spectra it was possible to assign all the protons and carbons (Table 2) as belonging to the planar structure **6** (Figure 1). ROESY correlation H-8/H-12 showed that these protons are on the same side. The lack of correlation between H8 and CH_3-20 suggested that they are on opposite sides. ROESY correlations CH_3-20/CH_2-19 showed that these groups are on the same side. ROESY correlations of H-1 α axial (δ_H = 1.61) with CH_3-20 and H-19 *endo* could be explained by a 5α,10β *trans*-fused structure and thus by a β orientation of the 10-OH. From these results, **6** is 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide. A stereoisomer of **6** was isolated from *Salvia haenkei* [8].

IR absorption bands at 3560, 3520 (-OH groups), 1755, 1720 (two lactone rings), and 3130, 1500, 875 (β-substituted furan ring) cm^{-1} and 20 carbon

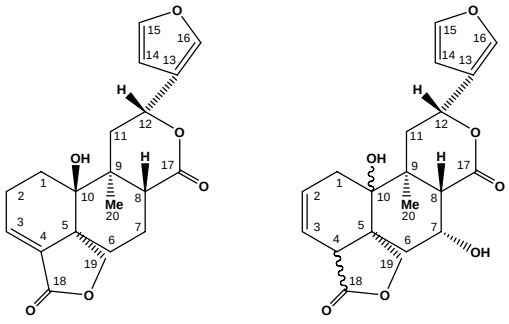


Figure 1: Structure of compound **6** and of compound **10**.

Table 3: NMR spectral data for compound **10**: δ values, $CDCl_3$, ^{13}C NMR at 125 MHz, ^1H NMR at 500 MHz.

C	^{13}C	^1H (*J* in Hz)	HMBC correlations of the C
1	30.1	2.22 m, 2.63 nd (7.9)	6.00
2	126.3	6.00 m	2.22
3	123.2	5.98 m	2.22
4	51.8	2.88 br s	5.12, 6.00
5	46.4	-	2.19, 5.12
6	35.0	1.95 dd (13.0, 3.0), 2.19 m	4.15, 5.12
7	65.7	4.56 br s ($W_{1/2}$ = 8)	2.19, 3.37
8	45.1	3.37 br s	1.28, 1.57, 2.19, 2.76
9	41.0	-	1.28, 1.57, 2.22, 2.76, 3.37
10	74.7	-	1.28, 2.22, 2.76, 3.37
11	40.9	1.57 dd (11.6, 13.5), 2.76 dd (5.5, 13.5)	1.28, 3.37, 5.32
12	72.2	5.32 dd (5.5, 11.6)	1.57, 2.76
13	124.1	-	1.57, 5.32, 6.45, 7.44
14	108.9	6.45 br s	5.32, 7.48
15	144.2	7.44 br s	6.45, 7.48
16	140.1	7.48 br s	5.32, 6.45, 7.44
17	176.8	-	3.37
18	175.1	-	5.12
19	73.7	4.15 d (8.6), 5.12 d (8.6)	2.19
20	24.8	1.28 s	1.57, 2.76, 3.37

resonances in the ^{13}C NMR suggested an oxygenated clerodane diterpenoid structure for **10**. By inspection of ESI-MS MS, HR-MS, COSY, HSQC and HMBC spectra it was possible to assign all the protons and carbons (Table 3) and to get the planar structure for **10**. ROESY correlation H-8/H-12 showed that these protons are on the same side. The lack of correlation between H8 and CH_3-20 suggested that they are on opposite sides. The axial orientation of the hydroxyl group at C-7 was indicated by the small *J* of the H-7 (br s). The strong deshielding effect on the *exo* H-19 (δ_H = 5.12) as compared to the *endo* H-19 (δ_H = 4.15), the chemical shift of CH_3-20 (δ_H = 1.28), which is similar to that of **8** [4] and ROESY correlations CH_3-20/CH_2-19 showed that these last groups and the 7-OH are on the same α side. The relative stereochemistry of C-4 and C-10 could not

Table 4: *Papaver rhoeas* L. germination indices after exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations. Percent germination data were arcsin-square root transformed for analysis to meet the requirements of the test. Retransformed data are presented in the results. Significant differences among the means were evaluated by one-way ANOVA, using the Tukey's honest significant difference test. Values with the same letters in a column are not significantly different at the 0.05 probability level. The letters are given following increasing values.

Index	Compound	compound concentration (mg/L)			
		2	5	10	20
Total germination (G_T)	**1**	15.7 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	13.3 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	11.3 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	20.7 ^{a}	12.0 ^{b}	6.3 ^{b}	0.0 ^{a}
	5	87.0 ^{c}	84.7 ^{c}	31.3 ^{c}	11.3 ^{b}
	6	20.3 ^{a}	11.3 ^{b}	6.0 ^{b}	0.0 ^{a}
	7	74.7 ^{c}	78.0 ^{c}	78.4 ^{d}	78.4 ^{c}
	8	78.0 ^{c}	80.0 ^{c}	71.7 ^{d}	79.7 ^{c}
	9	44.3 ^{b}	15.6 ^{b}	5.5 ^{b}	0.0 ^{a}
	10	81.7 ^{c}	82.3 ^{c}	82.3 ^{d}	83.0 ^{c}
	control	83.0 ^{c}	83.0 ^{c}	83.0 ^{d}	83.0 ^{c}
Speed of germination (S)	**1**	2.39 ± 0.22 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	1.80 ± 0.23 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	1.50 ± 0.23 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	2.98 ± 0.13 ^{a}	1.60 ± 0.14 ^{b}	0.87 ± 0.12 ^{a}	0.0 ^{a}
	5	13.66 ± 0.12 ^{e}	13.24 ± 0.11 ^{c}	4.29 ± 0.34 ^{b}	1.45 ± 0.14 ^{b}
	6	2.97 ± 0.07 ^{a}	1.46 ± 0.23 ^{b}	0.77 ± 0.04 ^{a}	0.0 ^{a}
	7	11.68 ± 0.07 ^{c}	12.62 ± 0.49 ^{c}	12.18 ± 0.46 ^{c}	12.26 ± 0.58 ^{c}
	8	12.56 ± 0.24 ^{d}	12.92 ± 0.14 ^{c}	11.81 ± 0.31 ^{c}	12.75 ± 0.44 ^{c}
	9	6.24 ± 0.33 ^{b}	2.06 ± 0.31 ^{b}	0.66 ± 0.29 ^{a}	0.0 ^{a}
	10	12.72 ± 0.07 ^{d}	12.83 ± 0.16 ^{c}	12.87 ± 0.14 ^{d}	12.86 ± 0.17 ^{c}
	control	12.95 ± 0.24 ^{d}	12.95 ± 0.24 ^{c}	12.95 ± 0.24 ^{d}	12.95 ± 0.24 ^{c}
Speed of accumulated germination (AS)	**1**	8.2 ± 1.0 ^{b}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	5.27 ± 0.76 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	4.21 ± 0.81 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	9.65 ± 0.59 ^{b}	4.51 ± 0.43 ^{b}	2.70 ± 0.32 ^{a}	0.0 ^{a}
	5	47.38 ± 0.18 ^{e}	45.85 ± 0.39 ^{c}	12.7 ± 1.3 ^{b}	3.85 ± 0.29 ^{b}
	6	9.65 ± 0.66 ^{b}	3.91 ± 0.86 ^{b}	2.04 ± 0.29 ^{a}	0.0 ^{a}
	7	40.6 ± 1.6 ^{d}	44.9 ± 2.6 ^{c}	42.2 ± 1.4 ^{c}	42.5 ± 2.9 ^{c}
	8	45.1 ± 1.1 ^{e}	46.43 ± 0.59 ^{c}	43.4 ± 1.8 ^{c}	45.1 ± 1.1 ^{c}
	9	19.4 ± 1.3 ^{c}	5.91 ± 0.88 ^{b}	1.40 ± 0.60 ^{a}	0.0 ^{a}
	10	43.85 ± 0.25 ^{e}	44.24 ± 0.20 ^{c}	44.56 ± 0.42 ^{c}	44.07 ± 0.49 ^{c}
	control	44.7 ± 1.1 ^{e}	44.7 ± 1.1 ^{c}	44.7 ± 1.1 ^{c}	44.7 ± 1.1 ^{c}
Coefficient of the rate of germination (CRG)	**1**	12.28 ± 0.15 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	11.77 ± 0.10 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	11.72 ± 0.13 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	12.03 ± 0.09 ^{a}	11.78 ± 0.04 ^{c}	11.79 ± 0.28 ^{c}	0.0 ^{a}
	5	12.52 ± 0.03 ^{a}	12.48 ± 0.04 ^{d}	11.85 ± 0.13 ^{c}	11.50 ± 0.16 ^{b}
	6	12.10 ± 0.26 ^{a}	11.52 ± 0.07 ^{b}	11.49 ± 0.23 ^{c}	0.0 ^{a}
	7	12.46 ± 0.10 ^{a}	12.63 ± 0.13 ^{d}	12.42 ± 0.07 ^{d}	12.48 ± 0.10 ^{c}
	8	12.56 ± 0.07 ^{a}	12.59 ± 0.04 ^{d}	12.62 ± 0.10 ^{d}	12.59 ± 0.04 ^{c}
	9	11.96 ± 0.09 ^{a}	11.56 ± 0.10 ^{b}	10.98 ± 0.11 ^{b}	0.0 ^{a}
	10	12.47 ± 0.04 ^{a}	12.48 ± 0.02 ^{d}	12.49 ± 0.01 ^{d}	12.46 ± 0.03 ^{c}
	control	12.49 ± 0.07 ^{a}	12.49 ± 0.07 ^{d}	12.49 ± 0.07 ^{d}	12.49 ± 0.07 ^{c}

be determined. From these results **10** is 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide. For both the molecules the whole structure was confirmed by tandem ESI-ion trap mass spectrometry in HPLC-MS and MS^2 experiments conducted as described below. The HR mass spectrometry analysis was performed in DIA (direct infusion analysis) in the negative ion mode to assess the elemental composition.

Dose–response studies. The activity of the isolated compounds on the germination of *Papaver rhoeas* L. and *Avena sativa* L. is described by the data reported in Table 4 and 5. In respect of both species, germination was measured to be significantly low at all the concentrations of **1**, **2**, **3**, **4**, **5**, **6**, and **9** and the decrease exhibited a strong reciprocal correlation with the increasing concentration. At the highest compound concentration (20 mg/L), germination capacity, calculated by means of the Total germination index (G_T) of *Papaver* seeds [9,10] (Table 4), was significantly lower than control after exposure to **1**, **2**, **3**, **4**, **6** and **9**. **5** resulted less active, while **7**, **8** and **10** were not active. At the lowest tested concentration (2 mg/L), G_T values of *Papaver* (Table 4) showed the lowest delay in inhibiting

Table 5: *Avena sativa* L. germination indices after exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations. Percent germination data were arcsin-square root transformed for analysis to meet the requirements of the test. Retransformed data are presented in the results. Significant differences among the means were evaluated by one-way ANOVA, using the Tukey's honest significant difference test. Values with the same letters in a column are not significantly different at the 0.05 probability level. The letters are given following increasing values.

Index	Compound	compound concentration (mg/L)			
		2	5	10	20
Total germination (G_T)	**1**	27.6 ^{b}	12.6 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	21.3 ^{b}	11.3 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	11.3 ^{a}	11.3 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	32.3 ^{b}	10.6 ^{a}	3.3 ^{b}	0.0 ^{a}
	5	73.4 ^{c}	73.0 ^{b}	28.7 ^{c}	9.7 ^{b}
	6	13.2 ^{a}	13.0 ^{a}	4.9 ^{b}	0.0 ^{a}
	7	72.7 ^{c}	79.7 ^{b}	74.4 ^{d}	74.8 ^{d}
	8	73.7 ^{c}	73.0 ^{b}	74.7 ^{d}	72.4 ^{d}
	9	78.0 ^{c}	77.0 ^{b}	70.0 ^{d}	52.3 ^{c}
	10	79.3 ^{c}	78.0 ^{b}	79.4 ^{d}	82.4 ^{e}
	control	83.0 ^{c}	83.0 ^{b}	83.0 ^{d}	83.0 ^{e}
Speed of germination (S)	**1**	4.52 ± 0.53 ^{a}	1.85 ± 0.15 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	3.36 ± 0.40 ^{a}	1.64 ± 0.24 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	1.79 ± 0.19 ^{a}	1.71 ± 0.05 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	4.94 ± 0.47 ^{a}	1.67 ± 0.33 ^{a}	0.50 ± 0.08 ^{a}	0.0 ^{a}
	5	21.2 ± 1.3 ^{b}	23.79 ± 0.48 ^{c}	4.65 ± 0.27 ^{b}	1.58 ± 0.15 ^{b}
	6	2.14 ± 0.44 ^{a}	1.90 ± 0.14 ^{a}	0.77 ± 0.26 ^{a}	0.0 ^{a}
	7	24.4 ± 1.0 ^{c}	24.4 ± 1.2 ^{c}	23.4 ± 1.3 ^{d}	23.0 ± 1.0 ^{d}
	8	24.97 ± 0.95 ^{c}	24.3 ± 2.5 ^{c}	23.09 ± 0.51 ^{d}	23.78 ± 0.56 ^{d}
	9	19.25 ± 0.53 ^{b}	18.82 ± 0.71 ^{a}	15.42 ± 0.87 ^{c}	8.72 ± 0.50 ^{c}
	10	24.40 ± 0.40 ^{c}	24.09 ± 0.24 ^{c}	24.17 ± 0.51 ^{d}	24.61 ± 0.35 ^{e}
	control	24.72 ± 0.36 ^{c}	24.72 ± 0.36 ^{c}	24.72 ± 0.36 ^{d}	24.72 ± 0.36 ^{e}
Speed of Accumulated Germination (AS)	**1**	16.2 ± 2.4 ^{a}	6.07 ± 0.47 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	11.8 ± 1.9 ^{a}	5.3 ± 1.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	6.21 ± 0.80 ^{a}	5.67 ± 0.32 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	16.5 ± 1.7 ^{a}	5.7 ± 1.2 ^{a}	1.68 ± 0.32 ^{a}	0.0 ^{a}
	5	80.6 ± 6.5 ^{c}	91.9 ± 1.9 ^{c}	16.3 ± 1.1 ^{b}	5.52 ± 0.86 ^{a}
	6	7.7 ± 1.8 ^{a}	6.01 ± 0.67 ^{a}	2.57 ± 0.85 ^{a}	0.0 ^{a}
	7	95.1 ± 3.6 ^{d}	91.9 ± 5.6 ^{c}	89.7 ± 5.7 ^{d}	87.1 ± 5.3 ^{c}
	8	98.0 ± 3.4 ^{d}	94 ± 10 ^{c}	87.6 ± 3.1 ^{d}	91.4 ± 3.6 ^{c}
	9	70.9 ± 2.3 ^{b}	70.2 ± 3.0 ^{b}	55.8 ± 3.7 ^{c}	30.2 ± 2.0 ^{b}
	10	95.1 ± 1.7 ^{d}	93.2 ± 1.2 ^{c}	93.6 ± 1.8 ^{d}	95.0 ± 1.5 ^{c}
	control	94.4 ± 1.9 ^{d}	94.4 ± 1.9 ^{c}	94.4 ± 1.9 ^{d}	94.4 ± 1.9 ^{c}
Coefficient of the rate of germination (CRG)	**1**	12.57 ± 0.11 ^{a}	12.07 ± 0.34 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	12.38 ± 0.17 ^{a}	12.04 ± 0.25 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	12.51 ± 0.32 ^{a}	12.37 ± 0.12 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	12.32 ± 0.12 ^{a}	12.47 ± 0.02 ^{a}	12.10 ± 0.56 ^{b}	0.0 ^{a}
	5	14.66 ± 0.19 ^{b}	15.05 ± 0.02 ^{c}	12.55 ± 0.27 ^{b}	12.58 ± 0.40 ^{b}
	6	12.47 ± 0.63 ^{a}	12.15 ± 0.42 ^{a}	12.37 ± 0.39 ^{b}	0.0 ^{a}
	7	15.20 ± 0.11 ^{b}	14.95 ± 0.21 ^{c}	14.96 ± 0.19 ^{d}	14.85 ± 0.31 ^{c}
	8	15.16 ± 0.05 ^{b}	15.13 ± 0.21 ^{c}	14.92 ± 0.01 ^{d}	15.06 ± 0.22 ^{c}
	9	14.13 ± 0.12 ^{b}	14.07 ± 0.15 ^{b}	13.70 ± 0.22 ^{c}	12.76 ± 0.09 ^{b}
	10	14.89 ± 0.06 ^{b}	15.01 ± 0.02 ^{c}	14.91 ± 0.07 ^{d}	14.84 ± 0.03 ^{c}
	control	14.94 ± 0.10 ^{b}	14.94 ± 0.10 ^{c}	14.94 ± 0.10 ^{d}	14.94 ± 0.10 ^{c}

germination for **1**, **2**, **3**, **4** and **6**.G_T values of *Avena* seeds (Table 5) gave similar results, with the exception of that related to **9**. As G_T index doesn't consider slackening in germination [9], germination progress was evaluated by means of the three indices used by Chiapusio [9] and Allaie [10], i.e. Speed of germination (S), Speed of accumulated germination (AS) and Coefficient of the rate of germination (CRG). For both *Papaver rhoeas* and *Avena sativa*, all calculated indices were significantly influenced by the different concentrations of **1**, **2**, **3**, **4**, **5**, **6** and **9** (Tables 4 and 5). At all the tested concentrations against *Papaver* and *Avena*, **1**, **2**, **3**, **4**, and **6**, gave S and AS values significantly lower than those obtained for the other compounds, as found for G_T index. Moreover, as well as for G_T values, S values of **1**, **2**, **3**, **4**, and **6** against *Papaver* showed the lowest delay in inhibiting germination at the lowest concentration (2 mg/L), while AS indicated that only **2** and **3** showed the lowest delay. As S considers the number of germinated seeds between two exposure times and AS considers the cumulative number of germinated seeds at each exposure time [9], S index appears to be the most sensitive [9,10]. This difference was not noted with *Avena* results (Table 5).

Table 6: LC_{50} and LC_{90} for *Papaver* and *Avena* germination inhibition after exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations, calculated by linear interpolation between two adjacent values. Data are means (± S.D.) of three replicates. LC_{50} and LC_{90} are expressed as 95% confidence interval. (NC= not calculated)

Compound	*Papaver rhoeas* L.						*Avena sativa* L.					
	LC_{50} (mg/L)			LC_{90} (mg/L)			LC_{50} (mg/L)			LC_{90} (mg/L)		
1	1.23	±	0.15	3.41	±	0.47	1.50	±	0.29	6.7	±	1.3
2	1.19	±	0.15	3.13	±	0.73	1.35	±	0.21	6.3	±	1.4
3	1.16	±	0.15	2.80	±	0.87	1.16	±	0.18	5.4	±	4.4
4	1.33	±	0.14	8.3	±	2.9	1.64	±	0.31	6.6	±	3.2
5	8.96	±	0.53		NC		8.50	±	0.41		NC	
6	1.32	±	0.17	7.8	±	3.0	1.19	±	0.24	7.5	±	3.4
7		NC			NC			NC			NC	
8		NC			NC			NC			NC	
9	2.30	±	0.76	8.7	±	3.8		NC			NC	
10		NC			NC			NC			NC	

The values of CRG of all compounds were similar to those obtained for the other considered germination indices against both *Papaver* and *Avena* (Tables 4 and 5) at the highest compound concentration used (20 mg/L); the differences from the other germination indices, observed at the lower tested concentrations, are probably ascribable to the lower sensitivity of this index in respect to the other indices [9].

From the dose-response studies, LC_{50} and LC_{90} [11] were determined for both the species (Table 6) and proved to be consistent with the results obtained previously: the lowest values of LC_{50} and LC_{90} against *Papaver* and *Avena* were yielded by **1**, **2**, **3**, **4** and 6, whereas LC_{50} and, consequently LC_{90}, for **7**, **8** and **10** could not be calculated. It can be highlighted that LC_{90} values registered for *Avena* were doubled with respect to those found for *Papaver*. Results for **5** and **9** were consistent with those obtained for the other considered parameters.

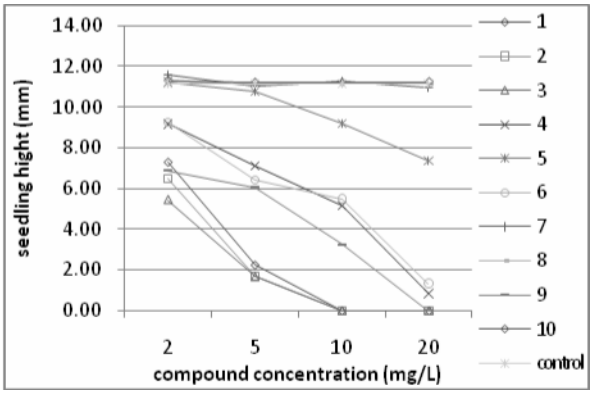
Growth experiments. In addition to germination, even the subsequent growth of the seedlings of the both species was reduced at the various concentrations of several *Salvia x jamensis* metabolites (Figure 2). **1**, **2**, **3**, **4** and **6** significantly reduced the seedling height, the cotyledon length, the root length and the chlorophyll content of *Papaver rhoeas* L. and *Avena sativa* L. at the highest tested concentration (20 mg/L); at the same concentration **9** was active only against *Papaver*, while **5** was less active than the other compounds, but effective against the both species. **7**, **8** and **10** were inactive. The evaluation of seedling fresh weight and dry weight of both *Papaver* and *Avena* seedlings at the highest concentration (20 mg/L) gave similar results (Figure 3). **1**, **2**, **3**, **4**, **6** and **9** significantly reduced the fresh weight of *Papaver* seedlings; **5** showed the same activity of **1**, **2**, **3**, **4**, **6** and **9** was less active; **7**, **8** and **10** were inactive on *Avena* seedlings.

On the whole, the tested compounds differed greatly in their biological activity. **7**, **8** and **10** were essentially inactive, whereas **1**, **2**, **3**, **4** and **6** were phytotoxic against both the tested species, whereas **9** was active only against *Papaver*. **5** had significant inhibitory activity on germination of both *Papaver* and *Avena*, but its effect on the subsequent growth of these species was significantly lower than those of the other active compounds.

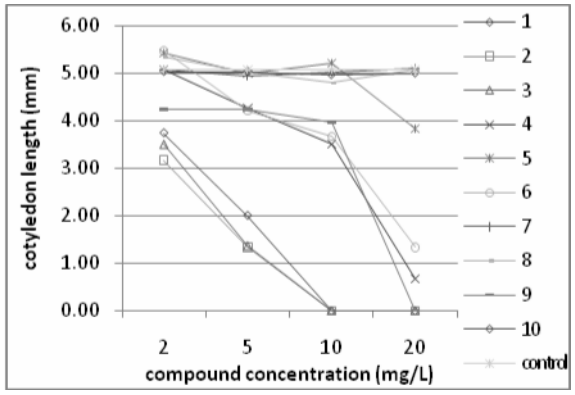
The phytotoxic activity of some of the considered diterpenoidic as well as flavonoidic compounds is consistent with literature where phytotoxicity has been described for these types of substances, and particularly for clerodane and ent-labdane diterpenes [12,13].

To our knowledge this is the first report on phytotoxic activity of isopimarane derivatives. However, it is clear from the present study that structure-activity relationships need to be deeply investigated, as clerodane and isopimarane structures resulted present in both active and inactive compounds.

Triterpenes are usually considered to be not highly phytotoxic, but are thought to be co-solubilizant agents of other bioactive compounds [13]. Nevertheless, the phytotoxic activity of betulinic acid against other dicotyledon and monocotyledon species was previously described [14-16]. The presence of



(a)



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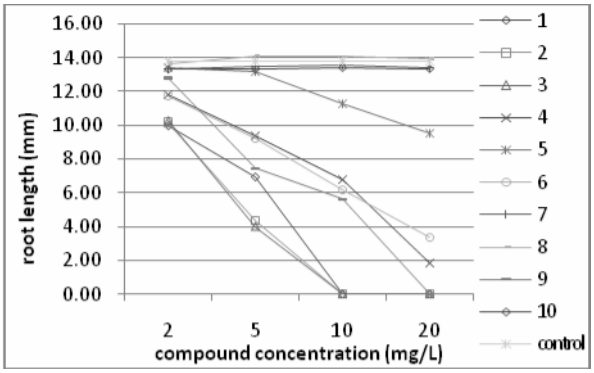
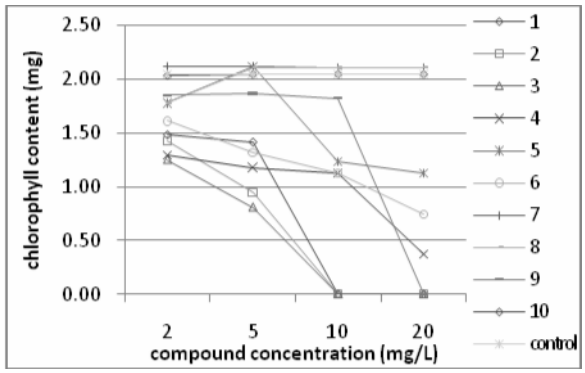
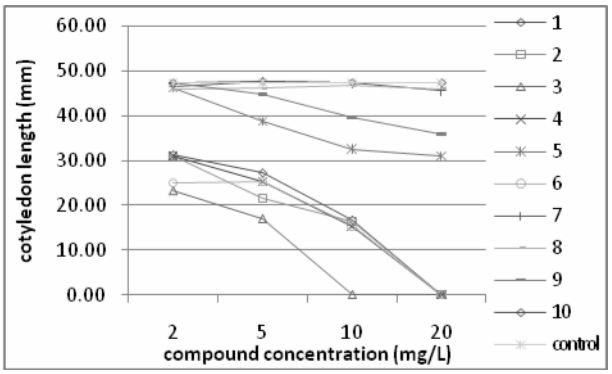


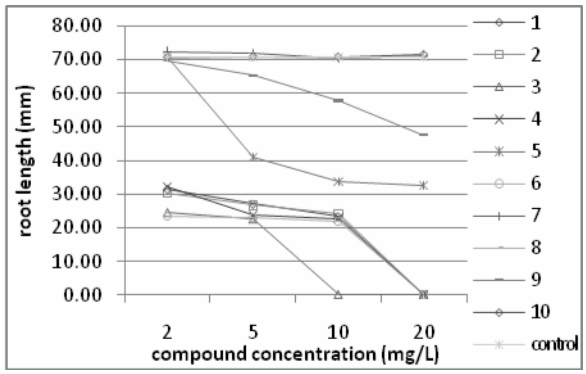
(c)



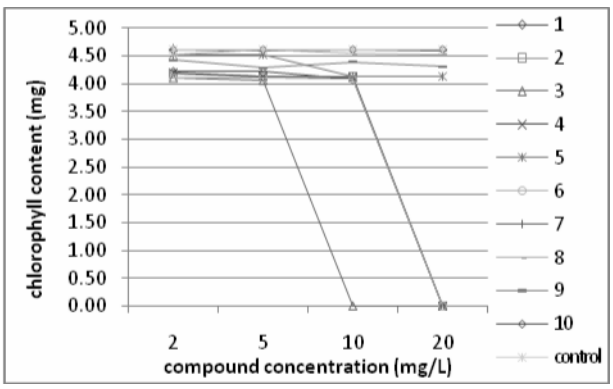
(d)



(e)



(f)



(g)

Figure 2: *Papaver rhoeas* L. and *Avena sativa* L. seedlin growth parameters after ten days exposure to *Salvia x jamensi* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwai acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, 4 isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod 3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod 3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16 epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19- diolide, **10**) at various concentrations. *Papaver rhoeas* L.: (a seedling height; (b) cotyledon length; (c) root length; (d chlorophyll content, fresh weight. *Avena sativa* L.: (e) cotyledo length; (f) root length; (g) chlorophyll content, fresh weigh Standard deviation bars are omitted for clarity.

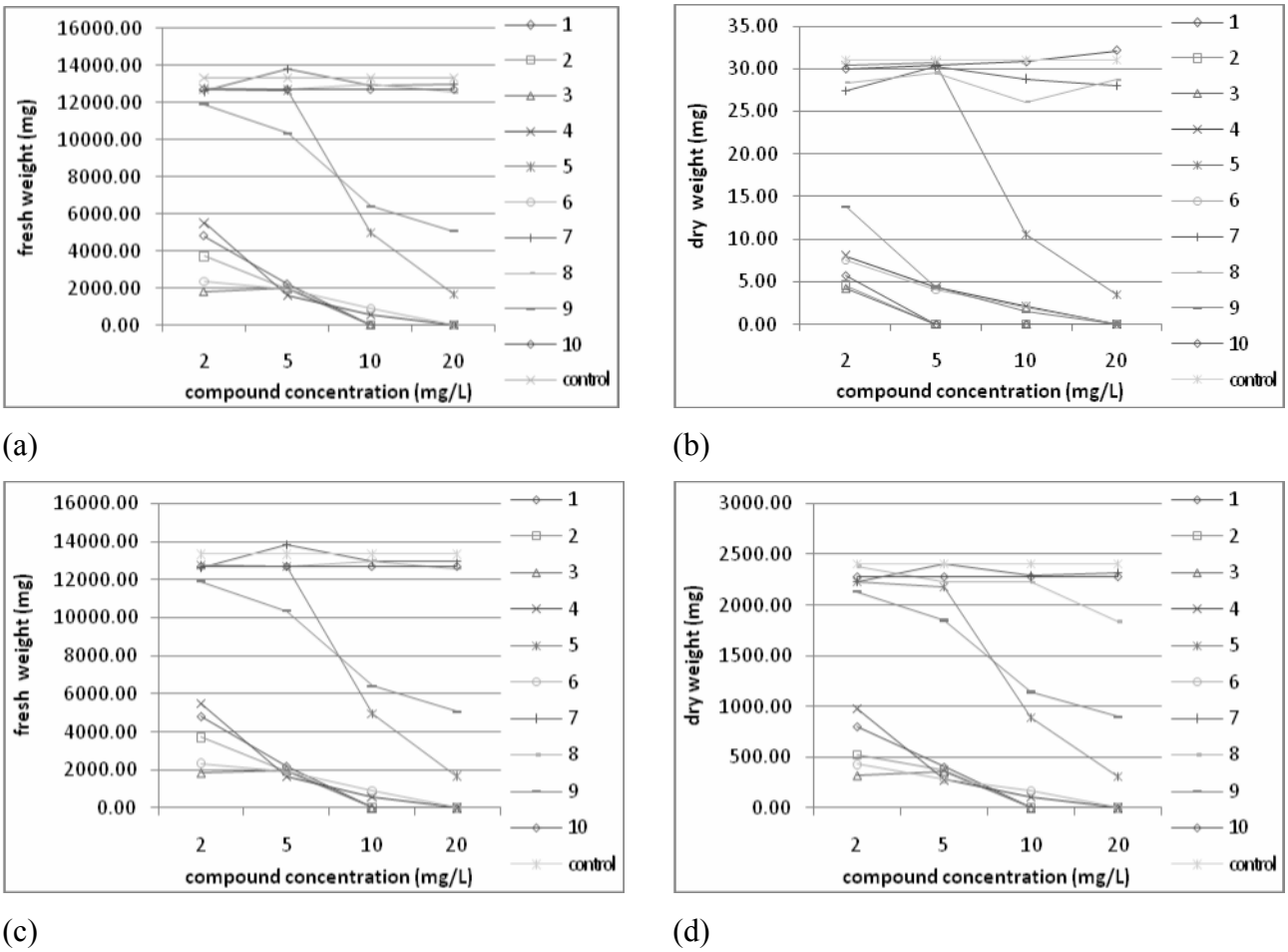


Figure 3: *Papaver rhoeas* L. and *Avena sativa* L. seedling fresh and dry weight after ten days exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations. *Papaver rhoeas* L.: (a) seedling fresh weight; (b) seedling dry weight. *Avena sativa* L.: (c) seedling fresh weight; (d) seedling dry weight. Standard deviation bars are omitted for clarity.

ursolic acid in the exudate of *S. jamensis* [4] may be useful for the allelopathic activity of these active liphophilic compounds since it was suggested that ursolic acid and other natural detergents, which are released from a source plant, enhance the solubilization of allelopathic lipids via micellization [17].

These laboratory bioassays will be followed by greenhouse studies to verify whether these effects take place also in the natural environment [12].

Experimental

General Experimental Procedures: Melting points are uncorrected and were measured on a Tottoli melting point apparatus (Büchi). Silica gel 60 (Merck 230-400 mesh) were used for column chromatography; aluminum sheets of silica gel 60 F_{254} (Merck 0.2 mm thick) with $CHCl_3$/MeOH/HCOOH (10:0.5:0.1) as an eluent were used for TLC and the spots were detected by spraying 50% H_2SO_4, followed by heating. HPLC-MS and MS^2 experiments were performed on an 1100 MSD HPLC-MS iontrap system (Agilent Technologies, Palo Alto, CA USA) equipped with a G1313A autosampler and diode array detector coupled with an electrospray ion source. The HPLC eluents were A: water with 0.1% formic acid and B: methanol with 0.05% formic acid. Starting from a A:B composition of 80:20 the linear gradient reached the 100% of B concentration in 30 min at a flow rate of 0.2 mL/min. The column employed was a Zorbax C18 150x2.1 mm ID, 3.5 μm particle size. Semi-preparative HPLC was carried out using a Waters W600 pump (Waters Corporation, Milford, USA) equipped with a Rheodyne Delta 600 Injector (with a 100 μL loop) and a Waters 2414 Refractive Index detector. The semi-preparative reversed-phase chromatography was performed at room temperature on a chemically

bonded stationary phase, 10μm μBondapack C18 column (7.8x300 mm ID) (Waters). The elution mixture (helium-degassed) was composed of CH_3OH/H_2O 40:60. The flow rate was 2.0 mL/min. The preparative HPLC was conducted on a Shimadzu LC8A system using the same eluents employed in the HPLC-MS analysis. The linear gradient from 30% to 90% of B in 40 minutes was performed at 15 ml/min on a 250x21.2 mm ID column (Phenomenex Luna C18). Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were obtained by a HP 8453 diode array spectrophotometer (Hewlett Packard, USA). IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer. NMR experiments were performed on a Bruker DRX-500 spectrometer. The standard pulse sequence and phase cycling were used for all 2D experiments: DQF-COSY, HSQC, HMBC, and ROESY spectra. The ROESY spectra were acquired with t_{mix}=400 ms. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software. ESI-MS measures were carried out in the negative ion mode within a mass range containing the expected m/z signals. The acquisition parameters were set time by time in DIA (direct infusion analysis) to obtain the optimal signal to noise ratio for each molecule. The HR mass spectrometry analyses were performed in DIA (direct infusion analysis) in the negative ion mode on a QSTAR XL system (Applied Biosystems, Toronto Canada) to assess the elemental composition. The calculations were performed using a utility integrated in the dedicated software and the calculated elemental formulas were unambiguous for both the compounds.

Plant Material: Aerial parts of *S. x jamensis* J. Compton were obtained from Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga. Italy). The species has been identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).

Commercial seeds of *Papaver rhoeas* L. (La Semeria-www.Lasemeria.it- Italy) and *Avena sativa* L. (Il Monastero-Sementi-Italy), chosen as model species of dicotyledonous and monocotyledonous plants [10], were used. Damaged and undersized seeds were discarded; the seeds to be assayed were selected based on the uniformity of size. Germination of *Papaver* and *Avena* was tested before use and it was about 83% and 80% respectively.

Extraction and isolation of the active components: Extraction of leaf surface constituents was performed as previously described [4]. The exudate (18 g) was chromatographed in portions of 1.5 g on Sephadex LH-20 column (60x3 cm) using $CHCl_3$/MeOH (7:3) as an eluent to give, in order of elution, four fraction groups: fraction group I (3g) with waxy compounds (from 0 to 170 mL), fraction group II (3g) with very crude ursolic acid (from 170 mL to 220 mL), fraction group III (12 g) with the mixture of **1-8** and **10** (from 220 mL to 290 mL) and fraction group IV (0.2 g) (from 295 mL to 365 mL) with **9**. Fraction group III was chromatographed in portions of 4 g on a silica gel column (40 x 4 cm), eluting with mixtures of *n*-hexane/$CHCl_3$ [50:50 (4.1 L), 40:50 (2.1 L), 33:67 (1.0 L), 25:75 (1.0 L), 10:90 (11.0 L], then with $CHCl_3$ (8.3 L), and then with $CHCl_3$/MeOH [95:5 (3.4 L)]. Elution with *n*-hexane/$CHCl_3$ (40:50) (from 0.2 L to 2.1 L) and with *n*-hexane/$CHCl_3$ (33:67) and 25:75 afforded fractions with **5** (crystallized from MeOH: 2.05 g). Elution with *n*-hexane/$CHCl_3$ (10:90) afforded at first fractions with **3** (from 1.0 to 1.4 L; crystallized from EtOH: 0.2 g), followed by fractions with ursolic acid (from 1.6 to 2.7 L; crystallized from EtOH: 1.4 g), fractions with **4** (from 2.7 to 3.0 L; crystallized from $CHCl_3$/MeOH: 0.14 g), a mixture of **6** and **10** (from 3.1 to 3.8 L), and finally fractions with **8** (from 3.8 to 11.0 L; crystallized from $CHCl_3$/MeOH: 0.58 g). Elution with $CHCl_3$ afforded fractions with **7** (from 3.5 to 6.0 L; crystallized from MeOH/H_2O: 0.25 g). Elution with $CHCl_3$/MeOH 95:5 afforded at first fractions with very crude **2** (from 0.1 to 0.9 L; purified with semi-preparative RP-HPLC (20 mg), then fractions with **1** (from 1.1 to 2.0 L**,** crystallized from MeOH/H_2O: 50 mg). The mixture of **6** and **10** was purified by preparative RP-HPLC yielding 13 mg of **6** and 17 mg of **10**. Fraction group IV (0.2 g) was crystallized from $CHCl_3$/MeOH yielding 36 mg of **9**.

Germination and growth test: *Dose–response studies* The seeds were surface sterilized in 2% sodium hypochlorite under vacuum for 20 min, rinsed 3 times in sterile distilled water, and dipped for 24 h in sterile distilled water for imbibition prior to the germination trial. The seeds were then equidistantly placed in 9 cm and 15 cm diameter Petri dishes respectively for *Papaver* and *Avena* (50 seeds per Petri dish, 3 replicates per treatment) lined with three layers no. 1 Whatman sterilized filter paper. Standard solutions of 2, 5, 10 and 20 μg/mL of each compound were obtained by dissolving each weighed compound in DMSO and diluting with the

appropriated quantity of sterile distilled water. A similar treatment with distilled water, containing the same DMSO concentration, served as control. The filter paper was treated with the above solutions, and the Petri dishes were sealed with parafilm and placed in a growth chamber at 25±2°C under 16h/8h light/dark photoperiod and light intensity of 65 µmol m^{-2} s^{-1}. Emergence of the radicle (≥1 mm) was used as an index of germination and it was recorded daily in replicate. Germination counts were conducted for a period of 10 days. Seed germinability was assessed by the total germination of the seeds at the end of the test [10]; germination progress or germination rate was evaluated by the calculation of other indices, i.e. speed of germination, speed of accumulated germination and coefficient of the rate of germination [9,10]. The lethal concentrations needed to reduce germinability by 50% (LC_{50}) and by 90% (LC_{90}) were calculated by linear regression between the two adjacent values respectively comprehending the 50% and the 10% response of the final cumulative percentage of germination at the end of tests [5] for each compound [11].

Growth experiments: In another set of experiments, the effects of the same solutions of *Salvia x jamensis* compounds plus control solution were studied on the early growth of *Papaver rhoeas* L. and *Avena sativa* L., using the same assay procedure. After 10 days, seedling growth, in terms of seedlings height, cotyledons length and roots length were recorded with callipers. Fresh weights and dry weights of the seedlings were also measured.

Chlorophyll content: Chlorophyll was extracted from seedlings using dimethyl sulphoxide following the method of Lichtenthaler [18] and estimated using the equation of Arnon [19]. Chlorophyll content was expressed on the basis of the fresh weight of the tissue.

Statistical analysis: All the activities were studied in triplicate. Significant differences were statistically evaluated using analysis of variance (one-way ANOVA) and the Tukey test on untransformed data. For analysis of variance, percent germination data (Total germination) were arcsine-square root transformed to meet the requirements of the test [10]. For all tests, statistical significance was set at $P<0.05$. SYSTAT (Version 8.0 – SPSS Inc., 1998) software was used for statistical analysis of the data.

Compound 6

MP: >260ºC (dec.).
$[\alpha]_D^{25}$: -53.1 (*c* 0.1, CH_3OH).
IR (KBr): 3495, 3121, 1755, 1725, 1502, 868 cm^{-1}.
UV/Vis λ_{max} (CH_3OH) nm (log ε): 207 (4.04), 239 (3.09).
^1H NMR and ^{13}C NMR: Table 2.
HR-MS: *m/z* 357.1352 $[M-H]^-$ (3.88 ppm error).

Compound 10

MP: >230ºC (dec.).
$[\alpha]_D^{25}$: -125.0 (*c* 0.1, $CHCl_3$).
IR (KBr): 3560, 3520, 3130, 1755, 1720, 1500, 875 cm^{-1}.
UV/Vis λ_{max} (CH_3OH) nm (log ε): 207 (3.72).
^1H NMR and ^{13}C NMR: Table 3.
HR-MS: *m/z* 373.1303 $[M-H]^-$ (4.21 ppm error).

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