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Phytotoxic activity in *Flourensia campestris* and isolation of (−)-hamanasic acid A as its active principle compound

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ABSTRACT

An aqueous extract from *Flourensia campestris* (Asteraceae) dry aerial parts showed strong inhibition on the germination and growth of *Lactuca sativa*. Based on bio-guided chromatographic fractionation of aq. extracts from dry and fresh leaves and spectroscopic means, (-)-hamanasic acid A (7-carboxy-8-hydroxy-1(2), 12(13)-dien-bisabolene (**1**)) was isolated as the most inhibitory active principle on germination (EC_{50} = 2.9 mM) and on root (ECr_{50} = 1.5 mM)/shoot (ECs_{50} = 2.0 mM) growth. As measured by GC, and correlated with a simple designed 2D-TLC, compound **1** was distributed throughout the plant, with a remarkably high concentration (1.6%) in the leaves and the inflorescences. At least a quarter of the amount of **1** was found in aqueous extracts suggesting that leaching would be a key route for its release into the environment. By contrast, leaf essential oils (HD) between 0.5 and 1.5 μl ml^{-1} did not show herbicidal effects and **1** was not found in them (TLC) nor among volatiles (HS-SPME). Volatile compositions were assessed by GC-FID and GC–MS and led to the identification of 23 compounds (4 monoterpenes and 19 sesquiterpenes) with a wide seasonal (spring–summer%) variation, represented principally by bicyclogermacrene (37–6%), spathulenol (4–32%), globulol (20–0%), beta-caryophyllene (15–6%), caryophyllene oxide (1–13%) and bicycloelemene (10–1%), respectively. The high amount of **1** in *F. campestris* together with its feasibility of being extracted with water suggest that (−)-hamanasic acid A is an allelochemical in this species. Species-specific studies must be carried out to evaluate the potential of **1** as a natural herbicidal compound.

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1. Introduction

The genus *Flourensia* (Asteraceae) belongs to the subtribe Ecliptinae, tribe Heliantheae (Robinson, 1981), and comprises about 32 species of resinous shrubs that grow from the southern United States south to Argentina and Chile. *Flourensia campestris* is an endemic species of the arid central region of Argentina (Córdoba, Santiago del Estero and Catamarca provinces (Dillon, 1984; Sáenz, 2000)). It is commonly known as "chilca" and frequently used as an aromatic, a tintorial and – especially their roots – as firewood (Paz, 1928). It grows at high density, frequently forming almost pure populations known as "chilcales" (Luti et al., 1979). The production of secondary metabolites (allelochemicals) may play an important role (allelopathy) in the adaptation and colonization of this species in its natural environment, which is characterized by abiotic stresses (e.g., poor soils, droughts, high temperatures, elevated UV-B) (Aphalo et al., 1999; Ballaré et al., 1999; Weston, 2005).

Resins, essential oils and vegetal extracts of *Flourensia* spp. species have been reported as having insect antifeedant (Diaz-Napal et al., 2009; Faini et al., 1997; García et al., 2007), phytotoxic (Mata et al., 2003; Palacios et al., 2007), antifungal, antialgal and antitermite properties (Tellez et al., 2001). Most of the metabolites isolated from this genus have been sesquiterpenes, prenylflavonoids and benzofuran derivatives (Uriburu et al., 2007). In *F. campestris*, benzofuran and flavonoid derivatives have also been documented (Dillon and Mabry, 1977; Fukai and Nomura, 1990; McCormick et al., 1986; Uriburu et al., 2004). Allelochemicals can be released into the environment by a variety of mechanisms: volatilization from leaves, exudation from roots, and leaching from leaves and plant litter by precipitation (Vyvyan, 2002). These compounds, that suppress or eliminate competing plant species near the source plant, have received special attention for their ecological

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implications and also due to their agricultural potential as selective natural herbicides (Benner, 1996; Duke et al., 2000). As part of our studies on the ecophysiological adaptations of native plants of Argentina (Izaguirre et al., 2003; Robson et al., 2005; Silva et al., 2008 pers. communication; Zaller et al., 2004) a focus on the elucidation of phytotoxic effects from *F. campestris* is being pursued. In order to mimic allelopathic phenomena, mild aqueous extracts from leaves of *F. campestris* were used to assess the mean effective concentrations that inhibit germination (EC_{50}), root (ECr_{50}) and shoot (ECs_{50}) growth of *L. sativa*, followed by bio-guided fractionation and spectroscopic identification of the corresponding bioactive compounds. Also bioassayed were the essential oils and their volatile compounds were identified from leaves of *F. campestris*. The amounts of the isolated phytotoxic compound were measured in aq. extracts and different plant organs; its putative role as allelochemical was also addressed.

2. Results and discussion

2.1. Identification and elucidation of the phytocompound

In order to identify the responsible compounds for the phytotoxic effects, dry leaf aq. extracts were partitioned with EtOAc (10:2). Based on bio-guided chromatographic fractionation of the EtOAc extracts and by spectroscopic means and polarimetry, (−)-hamanasic acid A, (7-carboxy-8-hydroxy-1(2), 12(13)-dien-bisabolene (**1**), Fig. 1a) was isolated and identified as the main active principle exerting a herbicidal effect on germination and on root/shoot growth of *L. sativa*. Compound **1** was also isolated from the aq. extracts of fresh leaves following the same protocols as those for dry leaves. The compound was obtained as a colorless syrup and the ^1H, ^{13}C, ^1H-^1H COSY NMR spectroscopic data (in $CDCl_3$) were consistent with that for (+)-hamanasic acid A (**2**) previously described by Hashidoko et al. (1991) from *Rosa rugosa* leaves (Rosaceae). The HMBC cross-peaks between H-10/C-4, C-9 and C-8 (Fig. 1b), H-12/C-14, C-11, C-15 and C-9, and the correlation H-2/C-6, C-3, C-4, C-1 and C-7 were in accordance with fragments C, A and B, respectively, described for **2** by ^1H, ^{13}C and ^1H-^1H COSY NMR spectroscopic analysis (Hashidoko et al., 1991). Compound **1** showed an $[\alpha]_D$ − 63.0 (levorotatory) instead of $[\alpha]_D$ + 68 (dextrorotatory) as was reported for **2.** Uriburu (2002) described **1** in a closely related species, *Flourensia fiebrigii*, but with a significant difference in the rotational value ($[\alpha]_D$ − 94.4). The current work is the first report of phytotoxic activity of (−)-hamanasic acid A (**1**) and its presence in *F. campestris*.

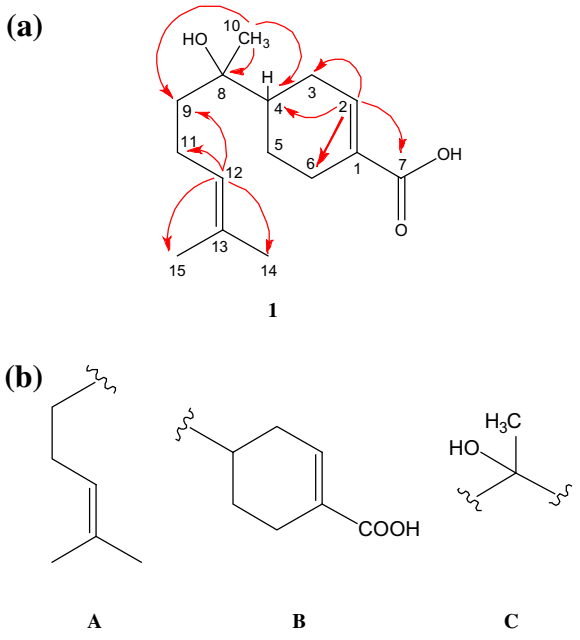

Fig. 1. (a) Compound **1**, most relevant HMBC correlations (red arrows); b) Fragments A, B and C as previously described by Hashidoko et al. (1991) for (+)-hamanasic acid A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

There are differences in the occurrence between compounds **1** and **2**. Hashidoko et al. (1991) reported that **2** is present in glandular secretions of *R. rugosa* leaves, being in its ester form ((+)-bisaborosaol A, methoxycarbonyl group at C-7) and is the other major bisabolene constituent found in the exudates. In contrast, the ester of **1** in *F. campestris* leaves was not detected when the same methodology was carried out (i.e., TLCs) as described by Hashidoko et al. (1991). Moreover, compound **1** remains the main form present in the aq. extracts of both fresh and dry leaves, as well as in whole tissues extracted with solvents (Table 1).

Despite that very little is known about sesquiterpenoid modes of action and specific molecular targets of most phytotoxins, if biological actions of secondary metabolites are mediated by coupling to specific sites (Duke and Oliva, 2004; Mata et al., 2003; Weston and Inderjit, 2007) then it could be speculated that compound **2**, because of its different spatial conformation, may not have any phytotoxic activity. The importance of stereochemistry in determining the phytotoxic activity of a compound has been previously demonstrated. Reynolds (1987) observed that the monoterpenoid (+)-carvone caused a 50% reduction in lettuce seed germination at 0.052 mM, while 0.38 mM was necessary for its isomer (−)-carvone. In the case of hamanasic acid A, in contrast, the isomeric forms (**1** and **2**) are produced by different species (*F. campestris* and *R. rugosa*, respectively). The evaluation of the phytotoxicity of **2** is necessary in order to elucidate to what extent differences in configuration are involved in their biological activities.

Arising from the cytosolic mevalonate pathway (Adam and Zapp, 1998; Aharoni et al., 2006), compound **1** belongs to the (−)-α-bisabolol type, containing an oxygenated functionality at C-8. Another characteristic feature of this cyclic sesquiterpene is oxygenation of the pendant allylic carbon at C-7. Similarly oxygenated bisabolanes have been isolated from pine and fungal sources (Hamasaki et al., 1975) and examples of C-7, C-8-dioxygenated bisabolanes have been isolated from the Compositae (Jaensch et al., 1989; Zdero and Bohlmann, 1989).

The fact that **1** could be isolated from both aq. extracts of dry and fresh leaves of *F. campestris* confirms that the bioactive compound is not a result of decomposition and/or enzymatic degradation during drying. It also suggests a putative biogenetically determined role that is operating during and after the life cycle of the leaves. The concentration of **1** found in dry leaf tissue by GC analysis was very high (16 mg g^{-1} (68 μmol g^{-1}, Table 1))

Table 1
Concentrations of (−)-hamanasic acid A (**1**) in *F. campestris*.

EtOAc extracts	(−)-hamanasic acid A (**1**)	
	(mg g^{-1} dry wt)	(mM)
Dry tissue		
Leaves	16.0 ± 0.7	NA
Inflorescences	17.0 ± 0.6	NA
Roots	0.58 ± 0.01	NA
3% aq. extracts		
Fresh leaves	7.8 ± 0.4	1.0 ± 0.1
Dry leaves	4.9 ± 0.3*	0.64 ± 0.06*

Means ± s.e. from two independent experiments with three assays for each determination ($n = 6$) are shown. NA, not applicable. Asterisks represent significant differences between dry and fresh leaves ($p < 0.05$).

accounting for 1.6% of total leaf biomass. A similar content (1%) was reported for the known phytotoxic compound L-DOPA described in *Mucuna pruriens* (Fujii and Hiradate, 2005). These concentrations are exceptionally high when compared to those generally found for a vast number of secondary metabolites with phytotoxic activity from different species, whose concentrations are in the order of μg or ng per g of whole tissue (Hirai et al., 2000; Kato-Noguchi, 2003a,b).

The presence of (−)-hamanasic acid A (**1**) was also investigated in dry tissue of inflorescences (capitula) and roots of *F. campestris* (Table 1). Results show that it is present in all the organs, with strikingly similar high concentrations in leaves and capitula, and with much lower values in roots. This pattern coincides with the distribution of other secondary metabolites produced by glandular trichomes in *Artemisia annua* (Duke et al., 1994; Ferreira et al., 1995). Results from histological studies indicate that nearly all flower structures in the capitulum of *F. campestris* present a high density of glandular trichomes (Delbón et al., 2007; Tourn, 2010 pers. communication), which could explain the high values of **1** measured in inflorescences as discussed below. The fact that **1** is present throughout the plant would indicate that it is constitutively produced in *F. campestris* as a major defence weapon with probably different targets and modes of action.

A high concentration of **1** was also found in the 3% aq. leaf extracts (Table 1) through GC analysis, with a higher yield in fresh (as compared to dry) tissue, equivalent to 1 mM and 0.64 mM, respectively. This was correlated with the levels of **1** in both tissues, as also indicated by 2D-TLC. Although the total mass of EtOAc soluble compounds obtained from aq. extracts of fresh leaves showed a loss of ca. 50% by drying (see Section 4.4.2), ca. 63% of **1** was still present in the aq. extracts from dry tissue, suggesting its presence is highly preserved during leaf desiccation. These results show that ca. 30% of the amounts present in the leaves of *F. campestris* could be recovered in the aqueous extract, suggesting that leaching would be a key route for release of **1** into the environment. In fact, since the early report of Lee and Monsi (1963), who documented how rain or dew washing the leaves of red pine (*Pinus densiflora*) was harmful to crops growing under pines, there has been an increased interest in quantifying plant leachates under relevant field situations (Chou, 1999; Kohli et al., 2001; Macías et al., 1997). Leaching of phytotoxic compounds may be particularly important in arid and semiarid regions wherein germination is cued by temperature and seasonal precipitation. In the case of *F. campestris*, the washing of **1** from foliage during rain episodes could exert an important inhibitory effect on seed bank germination, thus reducing potential competition in these resource-limited environments.

Compound **1** is distributed throughout the plant (Table 1), with very low concentrations in roots and stems (2D-TLC, data not shown), and high concentrations in leaves and capitula. Short dips of whole fresh *F. campestris* leaves and capitula in EtOAc or in water indicated a high concentration of **1**, accounting for ca. 20–30% (2D-TLC, data not shown) of the total surface-accumulated secondary metabolites leached. These organs, with higher levels of **1** (as measured by GC, Table 1) also exhibit higher density of glandular trichomes (Delbón et al., 2007; Tourn et al., 2010 pers. communication). In the same way, the isomer of **1** (compound **2**) was found in a high concentration in an isolated fraction of glandular trichomes from *R. rugosa* leaves (Hashidoko and Urashima, 1995). In spite of the growing consensus that secondary compounds that accumulate outside the cuticle on plant surfaces (terpenoids, phenylpropanoids, etc.) are almost exclusively produced by glandular secreting trichomes (Urzúa, 2004; Wagner et al., 2004), this fact remains to be established, especially since compound **1** was also found in glandless organs (Tourn et al., 2010 pers. communication).

Bisabolanoid derivatives have been shown to exhibit a wide range of bioactivities with antifeedant (Matsui et al., 2010), anti-inflammatory, bactericidal and antimicotic properties (Harbourne et al., 1999) and with antifungal, antibacterial and cytotoxic activities (Mikhova et al., 2004). Phytotoxic activity for this class of compounds was observed by Macías et al. (1998) in an extract from *Helianthus annuus* over monocotyledon (*Allium cepa*) and dicotyledon species (*Lactuca sativa* and *Lepidium sativum*). Alarcón et al. (2007) also reported phytotoxic effects of two bisabolene-type sesquiterpenes from *Lagascea mollis* (Asteraceae) on the weed *Sorghum halepense*. According to these results and the general distribution of (−)-hamanasic acid A found in *F. campestris*, it may be speculated that **1** could exert allelopathic effects on neighboring vegetation through leaf leaching, and serve as a feeding deterrent, antifungal or bactericidal compound protecting different plant organs at different developmental stages. The ecological significance of compound **1** in nature remains to be elucidated.

2.2. Biological activity

The aq. extracts of dry leaves from *F. campestris* showed a strong inhibitory effect on germination and on root/shoot growth of *L. sativa*. The mean effective concentrations of aq. extracts that inhibited germination (ECg_{50}), root (ECr_{50}) and shoot (ECs_{50}) growth were 3.9, 1.5 and 2.0%, respectively (Fig. 2, top). The EtOAc fraction from aq. extracts showed EC_{50} of 1.7, 0.8 and 1.0 mg ml^{-1} in the bioassays, respectively (Fig. 2, bottom). The dose response curve for isolated (−)-hamanasic acid A showed EC_{50} of 2.9, 1.5 and 2.0 mM, respectively (Fig. 3).

Specific phytotoxic activity of secondary metabolites using *L. sativa* seeds in bioassays have been reported within a wide range of concentrations, from nM to mM ((nM) Macías et al., 1998; (μM) Goto et al., 2001; Kato-Noguchi, 2003a; (mM) Hirai et al., 2000; Sánchez-Moreiras et al., 2008). In our bioassays with the same species it was found that the specific activity (ECg_{50}) of the isolated **1** corresponds to the mM range (below 3 mM, Fig. 3). The ECg_{50} on *L. sativa* seeds for 2,4-D, a synthetic herbicide used world wide exclusively for broadleaf weeds, also falls within the mM range, specifically 0.86 mM (Palacios et al., 2010). Nevertheless, some researchers have argued that if a compound is substantially active *in vitro* only at mM concentrations, it is unlikely to act as a phytotoxin in nature (e.g., Duke and Dayan, 2006). While there may be some controversy regarding the level of concentration and activity in natural environments, it is important to note that due to the high concentration of **1** in the leaf tissue (ca. 1.6%) and its high feasibility of being extracted with water, could indeed be a potent allelochemical in *F. campestris*, being in agreement with similar findings reported for other species (Hirai et al., 2000; Kato-Noguchi, 2003a).

The three response parameters selected to assess potential allelopathic activity (seed germination, root and shoot growth) exhibited a general response pattern throughout bioassays. Root growth was the most sensitive variable, followed by shoot growth and seed germination. In fact, drastic inhibition of root growth (by 70%) and shoot growth (by 40–50%) was achieved in all bioassayed media (aq. and EtOAc fractions and pure **1**) at concentrations that had no inhibitory effect on seed germination (Figs. 2 and 3). In the EtOAc fraction of the aq. extracts and with isolated **1**, root growth was almost nil (<10%) or completely arrested at ca. 2.6 mM of **1**, while slightly higher concentrations were needed to attain the same inhibitory effects when shoot growth was considered (ca. 4 mM and 3 mM, respectively). However, it should be noted that only the EtOAc fraction, at a concentration below 1 mM of **1**, showed a small, but consistent, promotion of root and shoot growth (ca. 10%, Fig. 2 bottom)

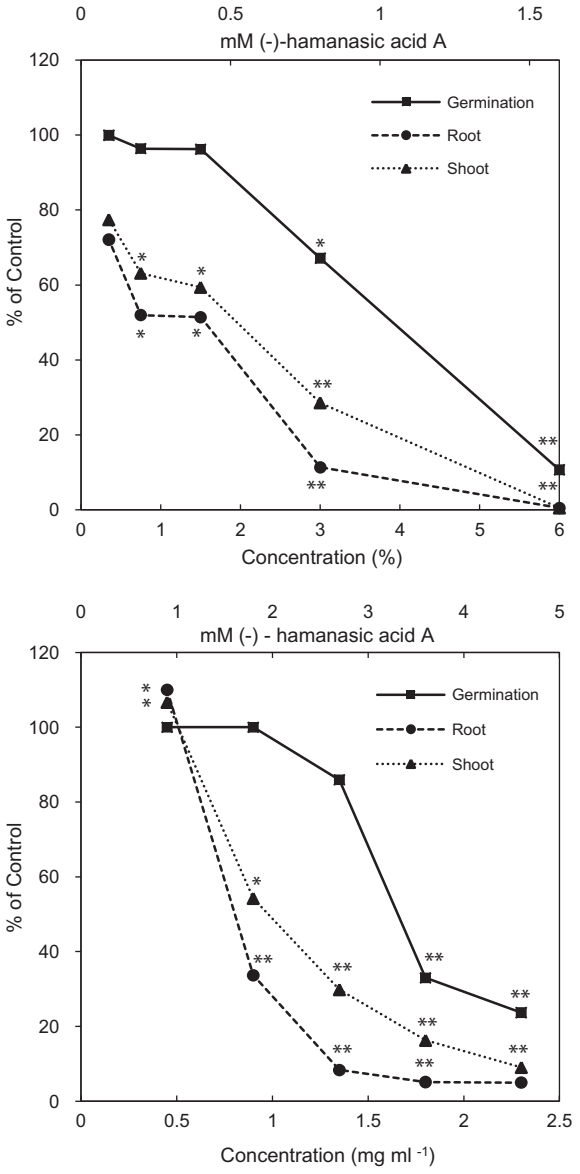

Fig. 2. Effects of aq. dry leaf extracts (top) and their EtOAc extracts (bottom) from *F. campestris* on germination and root and shoot growth of *L. sativa*. The concentrations of (−)-hamanasic acid A (**1**) (mM) as measured by GC in the extracts are also shown. Points represent the mean of three independent bioassays. (*), (**); significantly different from the control $p < 0.05$ and $p < 0.01$, respectively.

albeit abnormal morphology was also observed in this case (i.e., slender, glassy, no root hairs). Promotion of growth has been also documented before for the lower-range concentrations of different plant extracts or specific compounds (Belz and Cedergreen, 2010; Calabrese and Baldwin, 2001; Goto et al., 2001; Hirai et al., 2000). Total seed germination was significantly reduced at concentrations higher than 2.6 mM of **1** for the EtOAc fraction of the aq. extract and the isolated **1** (cf. Figs. 2-bottom and 3), and maximum inhibition of seed germination was 75 and 98%, respectively, attained at the higher concentrations tested. The similarity between the specific phytotoxic activities (EC_{50}) and response patterns between the EtOAc extracts from leaf aq. extracts and those of isolated **1** (Fig. 3) strongly suggest that (−)-hamanasic acid A (**1**) is the main compound responsible for the inhibitory effects reported. This is in line with the results obtained during the bio-guided fractionation where no other phytotoxic compound was detected.

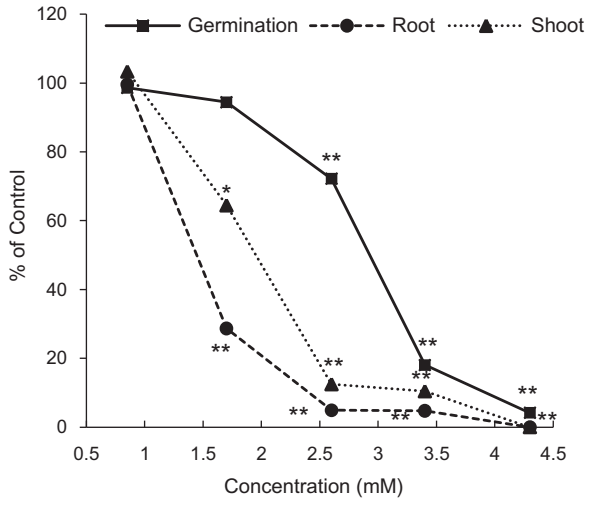

Fig. 3. Effects of (−)-hamanasic acid A (**1**) on germination and root and shoot growth of *L. sativa*. Points represent the mean of three independent bioassays. (*), (**); significantly different from the control $p < 0.05$ and $p < 0.01$, respectively.

Although the trend in the response of each parameter was similar among the tested solutions, the specific phytotoxic activity detected in the bioassays performed with the aq. extract was 3-fold higher than that observed in the EtOAc extracts and with the isolated **1** (cf. Figs. 2 and 3). Highly significant reductions in growth of roots and shoots were attained at a concentration of 3% in aq. leaf extracts, and germination was completely arrested at ca. 6% (Fig. 2 top). The calculated concentrations of **1** at these percentages in the aq. extracts were as low as ca. 0.8 mM and 1.6 mM, respectively. The difference in bioactivity observed could not be explained on the basis of **1** concentration (alone). Recovery of **1** through EtOAc extraction from the aq. phase was very efficient (more than 95%); hence the higher phytotoxicity of the aq. extracts could be explained by the presence of another phytotoxin which has not been extracted with EtOAc (the phase used for bioassay-guided fractionation) and/or the action of natural detergents that would raise the solubility of **1**, and thus its bioactivity. In this regard, Kato-Noguchi (2001) found that the water soluble fraction of *Melissa officinalis* showed the greatest allelopathic potential compared to other extraction media. The low solubility of **1** in water (ca. 0.1 g L^{-1}) may favor its persistence within the soil layer in which most seed germination and growth occurs, as is the case of several soil-applied herbicides and allelochemicals which are highly lipophilic (e.g., sorgolene). Nevertheless, the specific bioactivity of **1** in different buffered solutions or emulsifiers, and in soil solutions, aimed at exploring its potential use as a natural herbicide, deserves further investigation. In the same vein, determination of other (more polar) compounds present in the aq. extract, with known or potential allelopathic effects remains to be explored.

It is worthy to note that root morphology was visibly altered in the germinated seeds under all bioassayed conditions compared to the controls. Spindly roots and clubbing, were typically observed in the majority of germinated seeds growing in aq. extracts at concentrations as low as 0.35%, and very sparse or no root hairs was a prominent feature. At concentration of 3% or higher, root tip browning and/or necrosis was visible. The same abnormalities in root growth were also observed in seeds incubated in the EtOAc extracts at all concentrations, although root tip browning was the most conspicuous damage. When incubated with **1** all germinated seeds showed root tip browning, and necrosis was evident at the higher concentrations. These or similar abnormalities in root growth and development have been observed when seeds are allowed to germinate or seedlings to grow in the presence of certain other

allelochemicals (Gniazdowska and Bogatek, 2005; Lotina-Hennsen et al., 2006). The type of morphological changes experienced by germinated seeds may also yield important information regarding the possible targets and modes of action of a specific compound (as in isolated **1**) or the mixture (as in the aq. extracts).

At present, only one report has been found in which the phytotoxic activity of *F. campestris* was investigated. Palacios et al. (2010) showed that leaf ethanolic extracts from *F. campestris* at 10 mg ml^{-1} had no significant effect on seed germination (*Avena sativa* and *Raphanus sativus*). In contrast, strong inhibitions of seed germination and of root/shoot growth of *L. sativa* were found when incubated with the ethanolic extracts from dry leaves of *F. campestris* at 10 and 5 mg ml^{-1}, obtained following the methodology described by Palacios et al. (2010). Seed germination was reduced by 93 and 66% at 10 and 5 mg ml^{-1}, respectively. The lower concentration drastically inhibited root and shoot lengths by 96 and 85%, respectively, whereas no growth was detected at 10 mg ml^{-1} in germinated seeds. This was correlated with a high concentration of **1** in the ethanolic extracts as assessed by 2D-TLC. These striking differences between our results and those of Palacios et al. (2010) may be due to species-specific effects of **1**. New assays to corroborate the phytotoxic effects of **1** on weed and crop species, aimed at exploring its potential as a natural herbicidal compound and its putative participation as allelochemical agent, are currently underway.

Based on the strict correlation found between concentration of **1** measured by GC and that obtained with the novel 2D-TLC technique devised during this study, the 2D-TLC can be proposed as a fast and simple screening technique for the identification and semi-quantification of **1** in plant extracts (Fig. 4). This technique could be particularly valuable in the search of new sources of **1** regarding its potential application as herbicidal agent.

2.3. Essential oils

Essential oil fractions have proved to be the source of many allelochemicals with phytotoxic activity in a variety of plant species (Dayan and Duke, 2006; Mancini et al., 2009; Weidenhamer et al., 1993). The essential oils (HD) of *F. campestris* dry leaves harvested during the spring and summer seasons, did not show effects on germination nor on root/shoot growth of *L. sativa* through bioassay at conventional doses (0.5, 1.0 and 1.5 µl ml^{-1}). As established by TLC, compound **1** was not present in this fraction, remaining at high concentration in the aq. phase of the HD apparatus. TLC also indicated the higher polarity of pure **1** as compared to the compounds present in the essential oils. Volatile compounds constitute a fraction typically associated with essential oils but obtained through another methodology. Because the emitted volatile fraction plays a fundamental role in a plant's life, headspace solid phase microextraction (HS-SPME) was used to obtain a rapid fingerprint of leaf volatiles. Different studies demonstrate that HD and HS-SPME could be used as complimentary extraction techniques in order to obtain the complete characterization of plant volatiles (El Amine Dib et al., 2010; Nezhadali and Zarrabi-Shirvan, 2010).

The current work is the first to report the composition of dry leaf volatiles (HS-SPME) in *F. campestris*. Through GC and GC–MS we were able to identify 23 compounds, 4 monoterpenes and 19 sesquiterpenes (Table 2), were identified which accounted for ca. 95% of the total volatiles measured. As documented for other species (Mancini et al., 2009), a wide seasonal variation in its composition was observed. The monoterpene fraction accounted for up to (spring–summer%) 0.7–13.1% while the sesquiterpene fraction represented 96.0–79.9%, reflecting a shift towards the sesquiterpene production during the spring season. In summer, the monoterpenoid fraction was characterized by similar quantities of monoterpenoid hydrocarbons and oxygenated monoterpenoids (5.8% and 7.2%, respectively). The sesquiterpene fraction during the spring showed a higher quantity of sesquiterpene hydrocarbons (71.0%), mainly composed of bicyclogermacrene (36.7%), beta-caryophyllene (15.3%) and bicycloelemene (10.2%), while oxygenated sesquiterpenes accounted for 25.0%, representatives of which are globulol (19.6%) and spathulenol (4.1%). In contrast, during summer the pattern was inverse, sesquiterpene hydrocarbons accounted for 25.0%, mainly represented by beta-caryophyllene (6.0%) and bicyclogermacrene (5.6%), while oxygenated sesquiterpenes constituted the larger fraction (54.9%) with spathulenol (32.2%) and caryophyllene oxide (13.4%) as major compounds.

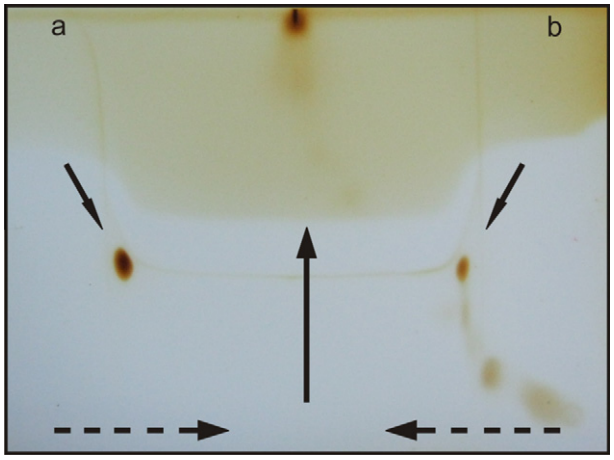

Fig. 4. 2D-TLC showing the identification and semi-quantification of (−)-hamanasic acid A (**1**) (a) in EtOAc extract from leaf aq. extracts of *F. campestris* (b) stained with I_2 vapors. Long broken and whole arrows indicate the first and second dimensional runs, respectively. Compound **1** in both runs is indicated by short arrows.

Table 2
Composition of leaf volatiles from *F. campestris* harvested in two different seasons.

Compound	Spring (%)	Summer (%)
α-Terpinolene	0.0	3.8
Isoterpinolene	0.0	3.4
α-Pinene	0.7	5.3
β-Pinene	0.0	0.5
Bicycloelemene	10.2	0.7
α-Copaene	0.7	0.0
β-Caryophyllene	15.3	6.0
Allo-Aromadendrene	0.7	0.0
α-Humulene	0.9	0.6
β-Selinene	0.0	0.6
Aromadendrene	1.3	0.0
Germacrene	0.0	3.6
Bicyclogermacrene	36.7	5.6
α-Amorphene	1.2	2.0
δ-Cadinene	1.9	1.6
α-Agarofuran	0.0	1.6
Spathulenol	4.1	32.2
Caryophyllene oxide	1.3	13.4
β-Maalinene	0.0	1.3
Bicyclo (4,4,0)	2.1	3.2
β-Eudesmol	0.0	4.3
α-Cadinol	0.0	3.3
Globulol	19.6	0.0
Monoterpene hydrocarbons	0.7	5.9
Oxygenated monoterpenes	0.0	7.2
Total monoterpenes	0.7	13.1
Sesquiterpene hydrocarbons	71.0	25.0
Oxygenated sesquiterpenes	25.0	54.9
Total sesquiterpenes	96.0	79.9

Compounds are listed in order of their elution. All compounds were identified by GC (retention index identical to bibliography) and MS (identification based on comparison of mass spectra).

These interesting findings confirm that the chemical composition of essential oils depends strictly on the collection period. In *F. campestris*, vigorous vegetative growth and resprouting from perennating structures take place during spring, while flowering and seed production is concentrated in summer. Thus, phenological stage and prevailing environmental conditions may be regulating essential oil composition, triggering production of specific compounds. In fact, the current results show that 13 compounds were exclusively produced either in spring (four sesquiterpenoids) or summer (three monoterpenoids and six sesquiterpenoids) (Table 2).

The degree of oxygenation in terpenoids has been positively correlated with increased biological activity. For instance, high percentage of oxygenated monoterpenes has been linked to potent phytotoxic activity (Rolim de Almeida et al., 2010; Vokou et al., 2003). Hence, the lack of inhibitory effects of the essential oils from *F. campestris* may be at least partially explained by the low contribution of oxygenated monoterpenes. On the other hand, Verdeguer et al. (2009) have shown that in bioassays with *Eucalyptus camaldulensis* essential oil, rich in the oxygenated sesquiterpene spathulenol, seed germination and seedling growth of *Amaranthus hybridus* and *Portulaca oleracea* were completely inhibited. As mentioned above, although spathulenol was a major component of *F. campestris* volatiles in summer, bioassays did not show inhibitory effects. Bioassays using higher doses of the isolated essential oil, as well as seeds of other plant species should be addressed in order to further unravel its potential phytotoxic effects.

3. Conclusions

In the present work, underlying reasons for phytotoxic effects of *F. campestris* were investigated. It was demonstrated that this endemic species is able to produce elevated amounts of a phytotoxic compound identified as (−)-hamanasic acid A (**1**). In bioassays using *L. sativa* as a test system, this compound drastically inhibited seed germination (by 90%), and completely arrested root and shoot growth at the higher concentrations tested (ca. 4 mM). This compound accumulates on plant surfaces, being easily leached out by water in fresh and dry tissues. These facts suggest both, its putative allelochemical role and its potential as a natural herbicidal agent. Based on the concentration and generalized distribution of (−)-hamanasic acid A (**1**) in roots, leaves and inflorescences, it can be proposed that it is constitutively produced exerting diverse biological activity, as has been documented for this kind of bisabolanoids. The compounds present in leaf essential oils did not show phytotoxic effects, at least at the bioassayed concentrations (0.5–1.5 μl ml^{-1}). Nevertheless, some of the volatiles identified in *F. campestris* in high concentration (e.g., spathulenol) have been described as phytocompounds on other species suggesting they might also be involved in the allelopathic action of *F. campestris*.

Based on our results, it is plausible that (−)-hamanasic acid A (**1**), and probably other compounds described here, may be key components of the adaptive strategies displayed by *F. campestris* in its natural environment.

4. Experimental section

4.1. General

Solvents were used from commercial sources without purification. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), DEPT, COSY, HMBC and HSQC spectra were recorded at room temperature with Bruker AC 400 spectrometers. The spectra were recorded in $CDCl_3$ and the solvent signals (7.26 ppm for ^{1}H-RMN and 77.16 ppm for ^{13}C-RMN) were used as reference (Gottlieb et al., 1997). Coupling constants, *J*, are reported in hertz (Hz). Optical rotation was measured on a Jasco P-1010 polarimeter for solutions in acetone (ca. 0.5%) at 25 °C. GC–MS for **1** identification was obtained on a GC-17A Shimadzu and QP-5000 MS Shimadzu. The GC column was HP 5% phenylmethylsilicone (Alltech) (30 m × 0.32 mm i.d.). The linear temperature program was from 150–300 °C, at the rate of 10 °C min^{-1}, and the carrier gas was He (1 ml min^{-1}). Volatile compounds data were obtained on a Shimadzu GC-RIA-FID with a CBP-1 column (30 m × 0.25 mm i.d.) and on a GC-HP 5890 fitted with a fused-silica DB-5 column (30 m × 0.25 mm i.d.) coupled to a MS-HP 5971A mass selective detector at 70 eV. Temperatures for GC and GC–MS analysis were programed from 60 to 260 °C at 3 °C min^{-1}, injector and detector temperatures at 280 °C and the carrier gas used was He (1 ml min^{-1}).

GC-FID (GC-hydrogen flame ionization detector, Agilent GC 6890) equipped with autosampler and injector was used for quantification of **1**. The column was ZB-5HT (15 m × 0.32 mm i.d.). The injector temperature was set to 200 °C with an injection volume of 4 μl, split ratio of 10 and N_2 flow of 2 ml min^{-1}. The oven temperature program began at 60 °C with a ramp rate of 3 °C min^{-1}. The final temperature was 290 °C (200–245 °C, 40 °C min^{-1}; 245–255 °C, 1 °C min^{-1}; 255–290 °C, 50 °C min^{-1}) which was held for 5 min making a total run time of 18 min per sample. FT-IR spectra were obtained through a FTIR-8501 Shimadzu. Silica gel 60 (Merck) was used for TLC (aluminum sheets, F_{254}) and for column chromatography CC (70–230 mesh). UV spectra were recorded on a Metrolab 2500.

4.2. Plant material

F. campestris Griseb was collected (unless specified) from October 2007 to November 2009, in natural areas corresponding to the Punilla Valley, Córdoba province, Argentina. A voucher specimen (BAA 26.498) is deposited at the Herbario "Gaspar Xuárez" of the Facultad de Agronomía, Universidad de Buenos Aires, Argentina.

4.3. Extraction and isolation

4.3.1. Bioactive compound

The air dried aerial parts of *F. campestris* (120 g) were extracted with H_2O (2 L) for 24 h at 22 °C and partitioned with EtOAc (10:2) as described below (Section 4.4.2). The organic phase was dried at 30 °C under a N_2 flow, giving a green syrup. The resulting EtOAc extracts (ca.: 1 g) were next fractionated by silica gel CC (1.5 cm i.d., 47 cm; 50 g silica) eluted at a flow rate of 5 ml min^{-1} using $CHCl_3$–EtOAc–MeOH (5:1:0.25; Solvent 1). The collected 1 min fractions were bioassayed as described below (Section 4.5). Fractions were subjected to TLC using the same solvent and detected under UV_{254} and UV_{365} illumination and with I_2 vapors. Bioactive fractions (F_1 (Fr.34–48), ca.100 mg) were pooled and dried and passed through the same CC system using toluene–$CHCl_3$–EtOAc–MeOH (1:6:0.5:0.25). Bioactive fractions (F_2 (Fr.80–100), ca. 50 mg) were next fractionated by a 5 ml CC (0,5 cm i.d., 25 cm; 5 g silica) eluted at a flow rate of 1 ml min^{-1} using toluene–$CHCl_3$–EtOAc–MeOH–HOAc (1:6:0.5:0.15:0.15; Solvent 2) to afford a main isolated fraction F_3 (Fr.7–15). Prior to bioassay, and in order to avoid any side-effects over seed germination, AcOH from F_3 was removed by washing the sample with $CHCl_3$ in a 1 ml CC and eluting with EtOAc–MeOH (50:50). F_3 yielded ca. 25 mg (0.02% in dry wt basis) of a colorless syrup with an isolated bioactive compound (*Rf* = 0.41, Solvent 1) characterized as a levorotatory isomeric form ((−)-hamanasic acid A (**1**)) of (+)-hamanasic acid A (**2**) by analyses of ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HMBC and HSQC spectra in $CDCl_3$, and by GC–MS, FT-IR, polarimetry and by comparison to previously reported data (Hashidoko et al., 1991).

Equivalent dry wt mass of fresh aerial parts (400 g, considering that fresh plant material lost 70% of its weight by drying) was extracted and processed in the same way. Each step of the fractionation gave similar yields and results as compared to dry aerial parts and the isolated bioactive compound was also identified as **1** by spectral means and polarimetry.

4.3.2. Volatile compounds

The volatiles emitted from *F. campestris* air dried leaves were investigated through HS-SPME using 100 μm fibers coated with PDMS. Plant material was harvested in December 2005 (spring season) and February 2006 (summer season) in the same natural areas as described above (Section 4.2). Briefly, chopped leaf samples were kept at 80 °C, equilibrated and extracted during 20 min. For each sample, at least three replicates of extractions were performed. Headspace samples were analyzed by GC-FID and GC–MS (Section 4.1). Identification of the components was based (i) on the comparison of their GC retention indices (König et al., 2001) and (ii) on computer matching with commercial mass spectral libraries (NIST, 2005).

4.4. Extract preparation and essential oil isolation

4.4.1. Aq. extracts

Air dried whole leaves of *F. campestris* were extracted at 3% or 6% (w/v) in distilled H_2O (3 or 6 g of plant material in 100 ml) for 24 h at 22 °C. Fresh leaves were extracted in the same way on a dry wt basis, considering 70% water content. The average (mean ± s.e., $N = 5$) pH, conductivity (μS cm^{-1}) and DTS (ppm) of 3% aq. extracts of dry and fresh leaves were 5.5 ± 0.2 and 5.6 ± 0.2, 2636 ± 180 and 2073 ± 163 μS cm^{-1} and 1319 ± 114 and 1038 ± 97 ppm, respectively.

4.4.2. EtOAc extract from aq. extracts

Aq. extracts were partitioned with EtOAc (10:2) and organic phases obtained after centrifugation (10 min, 3000 g) corresponded to the EtOAc extracts used both, in bioassays, and for calculating the concentration of **1** by GC in the aq. extracts (see Section 4.6.1). Obtained yields ($N = 5$) were 7.4 ± 0.3 mg g^{-1} (ca. 0.7% w/w) and 15.2 ± 0.5 mg g^{-1} (ca. 1.5% w/w) of dry and fresh leaves, respectively.

4.4.3. EtOAc extract from dry tissue

Powdered air dried leaves, roots and inflorescences (50 mg) were extracted twice using EtOAc (1.5 and 0.5 ml), vortexed for 2 min and centrifuged (10 min, 300 g). The EtOAc phases were pooled, dried at 30 °C under N_2 flow giving a green syrup which was weighed. Extracts ($N = 3$) expressed as percentage wt of air dried material yielded 11.4 ± 1.0, 5.5 ± 0.7 and 8.1 ± 0.9%, respectively.

4.4.4. Ethanolic extract from dry tissue

Air dried leaves (10 g) were extracted by 48 h maceration with 100 ml EtOH as was described by Palacios et al. (2010). Yields of each viscous extract ($N = 3$), obtained after solvent removal, and expressed as percentage wt of air dried plant material (17.5 ± 1.3%) were similar to those reported by Palacios et al. (2010) (11.8%).

4.4.5. Short dips

Fresh leaves and capitula (3.5 g) were washed with gentle agitation in of cold EtOAc or distilled H_2O (15 ml) during 20 s as was already described (Urzúa, 2004; Wagner et al., 2004). Samples were then subjected to 2D-TLC (Section 4.6.2).

4.4.6. Essential oils

Air dried leaves (100 g) from plant material harvested in spring and summer (see Section 4.3.2) were cut into small pieces and then submitted to hydro-distillation for 3 h (Clevenger type apparatus, with a separated extraction chamber). The resulting essential oils (pale yellow) were dried over anhydrous-sodium sulfate, both yielding ca. 1.1% (v/w), on a dry wt basis.

4.5. Bioassays

The bioactivity of aq. and EtOAc extracts and essential oils was evaluated on seeds of lettuce (*Lactuca sativa*, Grand Rapids). Fifty seeds of lettuce were placed in a 9.5 cm Petri dish lined with one sheet of filter paper previously moistened with of each test solution (3 ml), and allowed to germinate in a growth chamber in the darkness at 22 ± 1 °C. Controls received of distilled H_2O (3 ml). In the case of solvents used, these were evaporated at 40 °C before adding H_2O. Each treatment and whole bioassays were replicated three times.

Aq. extracts were bioassayed at 6% (w/v) as well as serial dilutions with H_2O at 4.5, 3.0, 1.5 and 0.75%. EtOAc extracts from aq. extracts were bioassayed between 0.45 and 2.3 mg ml^{-1} (final concentrations on a dry wt basis). Because quantities of isolated fractions from LC and pure compounds were limited, these were bioassayed using 25 seeds of lettuce ($N = 3$) placed in a 5.0 cm Petri dish lined with one sheet of filter paper previously moistened with of each test solution (1.5 ml).

Essential oils were diluted at 0.5, 1.0 and 1.5 μl ml^{-1} with EtOAc. Three ml of each dilution was placed in a Petri dish and evaporated, followed by addition of 3 ml 0.1% (v/v) aq. Tween 20. Bioassays performed with this detergent alone showed no appreciable differences compared to controls with water.

Seed germination process was assessed at 24 h interval during 3 days. A seed was considered germinated when root protrusion was evident (ca. 1 mm). After this period, lengths of roots and shoots (hypocotyls) of 50% randomly chosen lettuce seedlings per Petri dish were determined. Controls showed (mean ± s.e.) 93.7 ± 0.5% of germination and 2.1 ± 0.1 cm of root and 1.1 ± 0.1 cm of shoot growth. Germination and growth responses, expressed as percentage of the controls were plotted against treatments concentrations. Effective concentrations capable of inhibiting 50% of germination, root growth or shoot growth were calculated as ECg_{50}, ECr_{50} and ECs_{50}, respectively. The results were analyzed by ANOVA ($p < 0.05$).

4.6. Quantification of (−)-hamanasic acid A (**1**)

4.6.1. Gc-fid

EtOAc extracts (Sections 4.4.2 and 4.4.3) were re-suspended in toluene (1 ml) plus BSTFA (950 + 50 μl, respectively) prior to analysis. The (−)-hamanasic acid A (**1**) concentration was assessed by GC-FID using a calibration standard curve. Six points standard curve of **1** (0.005–1.0 mg ml^{-1}) diluted in toluene-BSTFA was used for quantification by interpolating the peak height on the chromatograms of GC (retention time: 5.8 min) to a standard curve constructed by the peak height of pure **1** isolated from leaves as described earlier. The standard curve was made by triplicate showing a linear response along the interval studied ($r = 0.997$).

4.6.2. 2D-TLC

For a rapid identification and semi-quantification of compound **1**, a novel 2D-TLC methodology was developed. For this screening technique, two samples (compound **1** against a plant extract, or a plant extract against another plant extract) are run from the opposite edges at the bottom of a chromatogram (10 × 6.5 cm, silica gel aluminum sheets). Solvent 1 plus 0.25 HOAc (*Rf* of **1** = 0.5) is used

in the first run, in which one sample at a time is allowed to run until the solvent front reach the center. Then, the confronted runs are allowed to migrate in the second dimension using solvent 2. Compound **1** is visualized in the second dimension at *Rf* = 0.35, as a single blue spot through UV_{254} light, and stained strongly with I_2 vapors (see Fig. 4). The semi-quantitative results obtained with this simple methodology were in agreement with the accurate concentration of **1** obtained by GC standard curve in all the plant extracts assayed.

4.7. Compound 1: 7-carboxy-8-hydroxy-1(2),12(13)-dien-bisabolene

Colorless syrup; $[\alpha]_D^{25}$ −63.0 (*c* 0.70, acetone); UV (EtOH) λ_{max} nm (log ε): 235 (3.42); (^{1}H-RMN ($CDCl_3$), δ (ppm): 1.14 (s; 3H; 10); 1.27 (dddd; *J* = 12.3 Hz; *J* = 12.3 Hz; *J* = 12.5 Hz; *J* = 5.3 Hz; 1H; 5b); 1.50–1.57 (m, 2H; 9); 1.62 (s; 3H; 14); 1.62 (dark signal; 1H; 4); 1.68 (s; 3H; 15); 2.04–2.07 (m; 4H; 11, 3a, 5a); 2.17 (m; 1H; 6b); 2.30 (broad d; *J* = 18.8 Hz; 1H; 3b); 2.53 (broad d; *J* = 17.6 Hz; 1H; 6a); 5.12 (broad t; *J* = 7.0 Hz, 1H, 12); 7.10 (broad t; *J* = 2.5 Hz; 1H; 2). ^{13}C-RMN ($CDCl_3$), δ (ppm): 18.0 (15); 22.4 (1); 22.9 (5); 23.7 (10); 25.2 (6); 26.0 (14); 27.9 (3); 40.2 (9); 42.6 (4); 74.4 (8); 124.6 (12); 130.2 (1); 132.1 (13); 142.2 (2); 172.6 (7). GC–MS (EI-70 eV), *m*/*z* (%): 234 (7) [M^+-H_2O], 219 (2), 191 (6), 164 (3), 123 (9), 109 (81), 93 (11), 82 (33), 81 (17), 79 (33), 69 (100), 55 (24).Compound **1** was established to be a levorotatory isomer of **2** (7-carboxy-8-hydroxy-1(2),12(13)-dien-bisabolene, (Hashidoko et al., 1991).

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