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Volatiles and Nonvolatiles in *Flourensia campestris* Griseb. (Asteraceae), How Much Do Capitate Glandular Trichomes Matter?

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The distribution and ultrastructure of capitate glandular trichomes (GTs) in *Flourensia* species (Asteraceae) have been recently elucidated, but their metabolic activity and potential biological function remain unexplored. Selective nonvolatile metabolites from isolated GTs were strikingly similar to those found on leaf surfaces. The phytotoxic allelochemical sesquiterpene (–)-hamanasic acid A ((–)-HAA) was the major constituent (*ca.* 40%) in GTs. Although GTs are quaternary ammonium compounds (QACs)-accumulating species, glycine betaine was not found in GTs; it was only present in the leaf mesophyll. Two (–)-HAA accompanying surface secreted products: compounds 4-hydroxyacetophenone (piceol; **1**) and 2-hydroxy-5-methoxyacetophenone (**2**), which were isolated and fully characterized (GC/MS, NMR), were present in the volatiles found in GTs. The essential oils of fresh leaves revealed *ca.* 33% monoterpenes, 26% hydrocarbon- and 30% oxygenated sesquiterpenes, most of them related to cadinene and bisabolene derivatives. Present results suggest a main role of GTs in determining the volatile and nonvolatile composition of *F. campestris* leaves. Based on the known activities of the compounds identified, it can be suggested that GTs in *F. campestris* would play key ecological functions in plant-pathogen and plant-plant interactions. In addition, the strikingly high contribution of compounds derived from cadinene and bisabolene pathways, highlights the potential of this species as a source of high-valued bioproducts.

Keywords: (–)-hamanasic acid A, glycine betaine, 4-hydroxyacetophenone, 2-hydroxy-5-methoxyacetophenone, essential oils.

Introduction

Flourensia campestris Griseb. (Asteraceae, subfamily Asteroideae, tribe Heliantheae) is an endemic resinous species from the center of Argentina which produces a high complex mixture of secondary metabolites. Some of these metabolites have been chemically and/or biologically characterized from different plant aerial organs, including the phytotoxic sesquiterpene (–)-hamanasic acid A ((–)-HAA; 8-hydroxybisabola-1,12-diene-7-carboxylic acid),[1] volatile components,[1] quaternary ammonium compounds (QACs) -glycine betaine-,[2] and benzofuran and flavonoid derivatives.[3][4] However, no previous studies have reported the exact localization of metabolites in *Flourensia*. Knowledge of the occurrence of any given metabolite is fundamental not only to understand the possible functional roles that metabolites may play in the species, but also to find applications in underpinning biosynthetic pathways, prospective bioengineering, or targeted harvesting for industrial uses.

Glandular trichomes have been signaled as major production, storage and secretion sites in secondary metabolites.[5 – 7] In the case of *Flourensia*, recent works by *Silva et al.*[1][8] provide a detailed description of the ultrastructure and distribution of capitate glandular trichomes (GTs) and secretory ducts in *F. campestris* and *F. oolepis*, and the distribution of (–)-HAA in all aerial organs. Although the evidence gathered so far strongly suggests the involvement of GTs as the

source of (–)-HAA, its occurrence in isolated GTs has not been assessed. Thus, in the present study we investigated the presence of the nonvolatile (–)-HAA in GTs of *F. campestris*. In addition, two (–)-HAA accompanying surface secreted products, found together with (–)-HAA during regular extractions, were properly isolated and chemically characterized, and their presence was also investigated in GTs.

GTs, typically embedded in the epidermis, are exposed to the same array of abiotic stresses as the whole leaf, and hence it could be expected that their functionality would be protected by mechanisms similar to those found in the leaf mesophyll. In this sense, the role of compatible solutes in abiotic stress tolerance has been extensively studied.[9][10] In the case of *Flourensia*, we have previously demonstrated that all species which were investigated are QAC accumulators, specifically glycine betaine (*F. campestris*, *F. oolepis*, *F. hirta*, *F. niederleinii*, *F. riparia*, *F. fiebrigii*, *F. macroligulata*, and *F. heterolepis*).[2] In *F. campestris*, we have also shown that glycine betaine content and distribution within plants are regulated by UV-B. We hypothesized that glycine betaine could also be operative as a protection mechanism in GTs; therefore, we investigated its occurrence in the nonvolatile metabolome of GTs.

Flourensia species are rich in essential oils.[11][12] In *F. campestris*, *Silva et al.*[1] partially described the composition of leaf essential oils, all volatile compounds were obtained through headspace solid-phase micro-extraction (HS-SPME) and GC/MS analysis. *Silva et al.*[8] also reported that some of these volatiles were present in the resins collected from secretory ducts, posing the question of the extent to which GTs or other secreting structures may be responsible for volatile production. In the present article, we performed a complete characterization of essential oils from whole leaves obtained through hydrodistillation, and investigated which of those compounds were also part of the volatile metabolome of GTs in *F. campestris*.

A large variety of methods and techniques have been devised to detach and isolate GTs in other plant species.[5][7] However, none were intended for isolating GTs of heavily resinous species. The work by *Keene* and *Wagner*,[13] with tobacco leaves, is one of the few available records in which the applied isolation techniques resulted in a complex mixture of head trichomes and sticky exudates from leaf epidermis. As many *Flourensia* species, the leaves of *F. campestris* are clearly distinguished from the surrounding vegetation by a characteristic glow derived from the dense layer of sticky materials present on their surfaces.[8] These resinous secreted products make isolation of GTs a much more difficult task,[14] turning necessary the development of a novel and efficient technique that allows both, detachment and isolation of preserved GTs.

In order to assess their metabolic productivity and discriminate those compounds exclusively produced by GTs from those found elsewhere in the leaves, we used different extraction methods and compound identification techniques on isolated GTs, whole leaves, surface secreted products and peeled leaves. The possible functional roles of GTs in *Flourensia* are discussed on the basis of the array of metabolites detected in these secretory structures. The potential of the species as a source of valuable bioproducts is also addressed.

Results and Discussion

Isolation of GTs

The technique developed for the isolation of GTs of this resinous species (*Figure 1,A*) rendered optimal results in terms of simplicity, cellular integrity, and low contamination. *Figure 1,B*, and *C* shows the typical distribution of GTs on fresh tissue, found together with filiform shaped trichomes (PhTs). The latter have been previously described as non glandular trichomes for this species and *F. oolepis*,[15] although they are strikingly similar to the uniseriate linear glandular trichomes found in *Helianthus annus* and other genera of the Heliantheae.[16][17] The adaxial surface of *F. campestris* leaf adhered to the adhesive tape showed dislodged trichomes immersed in abundant surface secreted products (not shown). This novel method removed *ca.* 75% of GTs present on the leaf surface, without damaging the epidermis or altering the structure and integrity of GTs as inspected by light microscopy. *Figure 1, D* shows the cellular structure of the GTs at light microscopy. As described in detail by *Silva et al.*,[8] these GTs are biseriate with 6 – 8 head celled, a two celled short-stalk (with cutinized lateral walls) and three basal cells.

Figure 2 illustrates different steps during the isolation process: (*A* and *B*) the starting material detached from the tapes, usually containing the different types of trichomes and variable proportions of sticky resin plaques, and small debris; (*C* and *D*) the final purified, highly preserved fraction of GTs.

The integrity of GT form and structure was monitored by light microscopy at high magnifications (400 – 1000×), specifically observing the presence of complete GTs (*i.e.*, preserved head cells not separated from stalk cells). Hemocytometer counting of the purified GT fraction showed that as much as

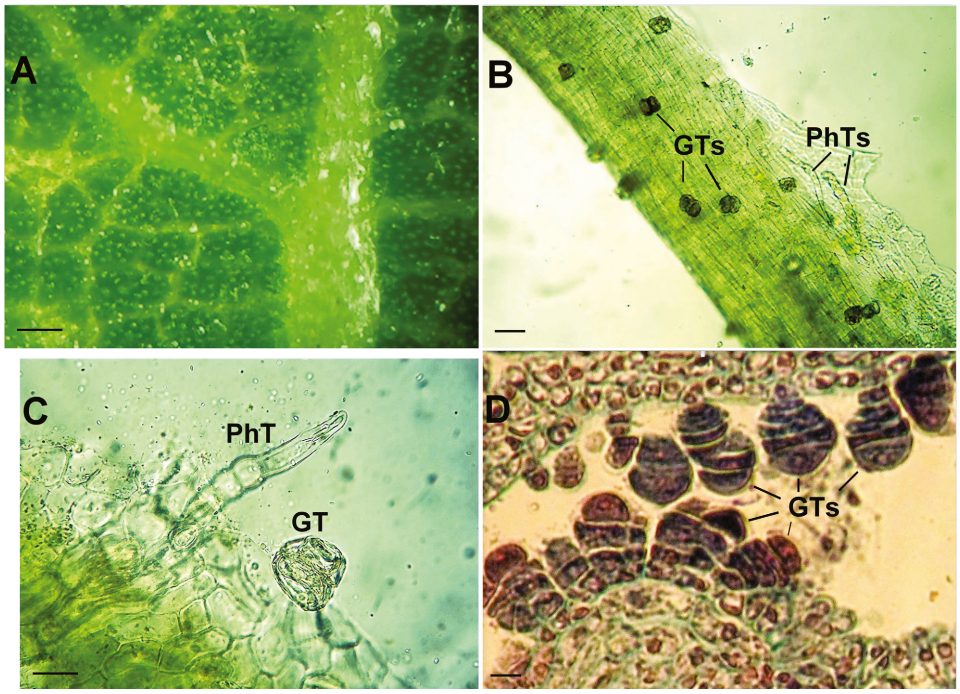


Figure 1. Light microscopy photographs of aerial organs of *Flourensia campestris*. *A*) Characteristic gloss of fresh leaves due to high loads of resin deposited on the epidermis. *B*), *C*) Rinsed fresh leaves showing the predominant location of capitates glandular trichomes (GTs) on leaf veins usually associated with filiform trichomes (PhTs). *D*) GTs on a longitudinal section of a fixed and stained (safranin combined with fast green) young leaf and corolla of tubular flower. Scale bars: *A* = 500 μm, *B* = 60 μm, *C* = 20 μm, *D* = 10 μm.

$43 \pm 5 \times 10^3$ GTs g^{-1} FW could be regularly obtained ($n = 3$). The yield was estimated to be 10 – 15%, based on the total epidermal leaf surface detached with the tapes and the average density of GTs (*ca.* 120 GTs mm^{-2}) present on the adaxial leaf side.[8]

Direct comparisons with other methods used for isolating trichomes cannot be made due to differences in starting material, trichome density and the way in which results are reported.

As already proposed,[18] GTs may be considered as exceptionally advantageous experimental organs for the elucidation of secondary metabolites and their complete biosynthetic pathways to explore the protective mechanisms against abiotic and biotic stresses, and for genomic sequencing.

(–)-HAA in GTs

Our present results are the first to report that GTs are indeed the site of storage of (–)-HAA, as revealed by the extracts obtained from isolated and purified fractions of leaf GTs analyzed by TLC and 2D-TLC (*Figure 3*). Since surface secreted resins bear a high concentration of (–)-HAA, GTs had to be freed from any residual (–)-HAA that could remain adhered to their surface prior to final extraction. Therefore, some fractions of isolated GTs were subjected to subsequent rinses with sodium dodecyl sulfate 1%. A high concentration of (–)-HAA (most prominent band, $R_f \approx 0.45$) was found in the starting material, and the supernatant and first wash from the isolated GTs (*Figure 3,A*, lanes 1 – 3). At this step, when partly washed isolated GTs were lysed, a high concentration of (–)-HAA could be observed (*Figure 3,A*, lane 4). Finally, (–)-HAA was practically absent in further washes of the intact isolated GTs (*Figure 3,A*, lane 5) and was found to be stored in totally washed GTs as revealed after their lyses and extraction (*Figure 3,A*, lane 6, arrow). The 2D-TLC depicted in *Figure 3,B* corroborates this result: (–)-HAA was absent in the last wash of isolated GTs (left run, oblique arrow) compared to a high concentration of (–)-HAA found in the interior of isolated, lysed GTs (right run, oblique arrow). The lyses and extraction of uncontaminated GTs revealed that (–)-HAA accounted for as much as *ca.* 40% of *F. campestris* GTs nonvolatile metabolome as judged by its TLC density at UV or with I_2 stain.[8] GC/MS analysis performed with the polar phase of the hexane partition from isolated lysed GTs and obtained using standard (–)-HAA under the same conditions yielded identical retention indexes (*LRI* = 2168 ± 1) and mass

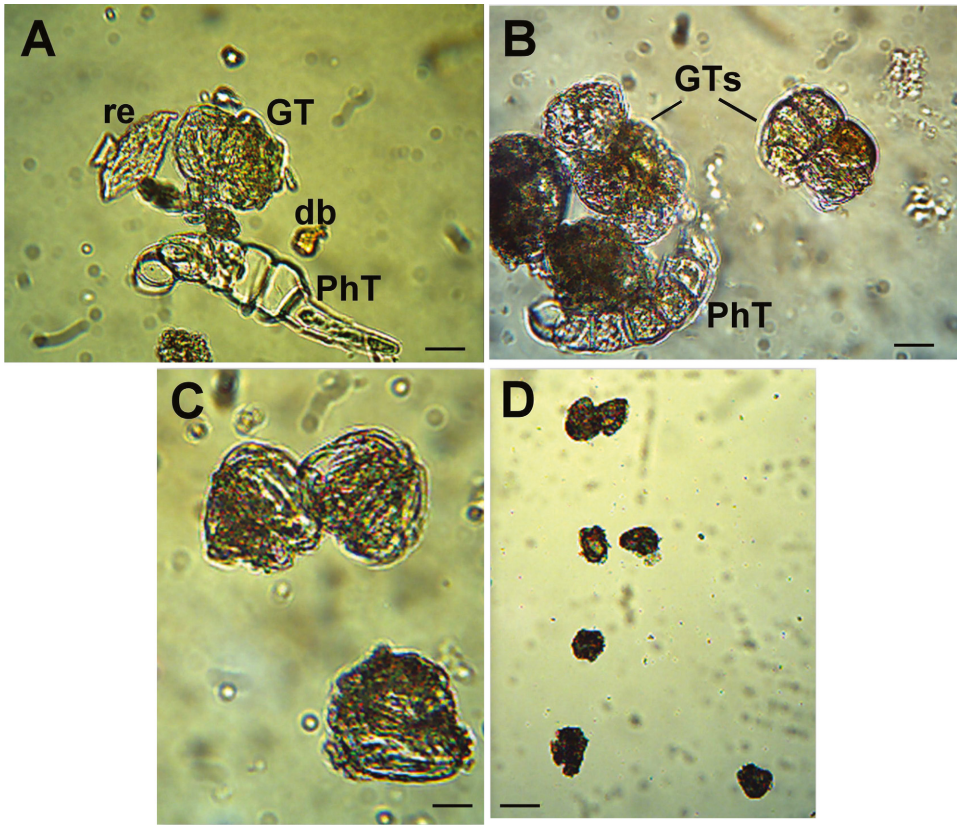


Figure 2. Isolation of GTs. Light microscopy inspections obtained from the isolation steps of GTs (average size: 30 μm). *A*) Starting material showing trichomes (GTs and PhTs), small plaques of resins (re) and debris (db). *B*) Further isolation steps showing only both types of trichomes. *C*), *D*) Final purified fraction showing only GTs. Scale bars: *A* and *B* = 10 μm; *C* = 7 μm; *D* = 40 μm.

spectra (*see General, Experimental Section*), confirming the presence of (–)-HAA in GTs. Quantitative analysis showed that (–)-HAA was at least ten times more concentrated in extracts from isolated GTs compared to whole leaves (190 ± 2 *vs.* 17.9 ± 0.1 mg (–)-HAA g^{-1} DW, respectively, $n = 3$).

Figure 3,A also shows that the profile of secondary metabolites found in the selected nonvolatile metabolome of isolated GTs (lane 6) is strikingly similar to that obtained from the starting material (lane 1), which included both, products deposited on the leaf surface and GTs. These results strongly suggest that GTs are the main source of the compounds secreted on the leaf surface, in agreement with similar reports for other species.[7][19] The confirmation of the high concentration of (–)-HAA found inside isolated GTs would also indicate that (–)-HAA is stored to some extent as a final product, thus ready to be secreted. Scanning electron microscopy observations and ultrastructural studies of GTs in *F. campestris*[8] revealed that compounds are released through cracks or pores once trichomes have reached maturity. This physical mechanism of secretion would need high stores of chemically and biologically active metabolites readily available, as seems to be the case of (–)-HAA. Studies of the developmental gene expression during trichome development in anther appendages of disc florets in *Helianthus annus*[20] would support this notion. The authors have shown that sesquiterpene synthases are exclusively up regulated during the biosynthetically active stages (pre-secretory) of the trichomes and not expressed during the post-secretory stage. In relation to the synthesis of (–)-HAA, an increasing number of studies have shown that terpene biosynthesis takes place within GTs.[5 – 7][19 – 21] The analysis of the volatiles identified in GTs of *F. campestris* (see below) revealed the presence of known precursors of the bisabolene-type terpene biosynthetic pathways studied in other species, suggesting that, at least in part, the synthesis of the bisabolene (–)-HAA would take place within GTs. The fact that (–)-HAA was not found in the internal secretory ducts of leaves[8] -looking for its possibility of being translocated from the interior to the leaf surface- supports the contention that (–)-HAA is not only stored, but also synthesized by GTs. An efficient transport or insufficient analytical sensitivity may also account for the observed distribution of compounds and their eventual absence in TLC and GC/MS analyses.

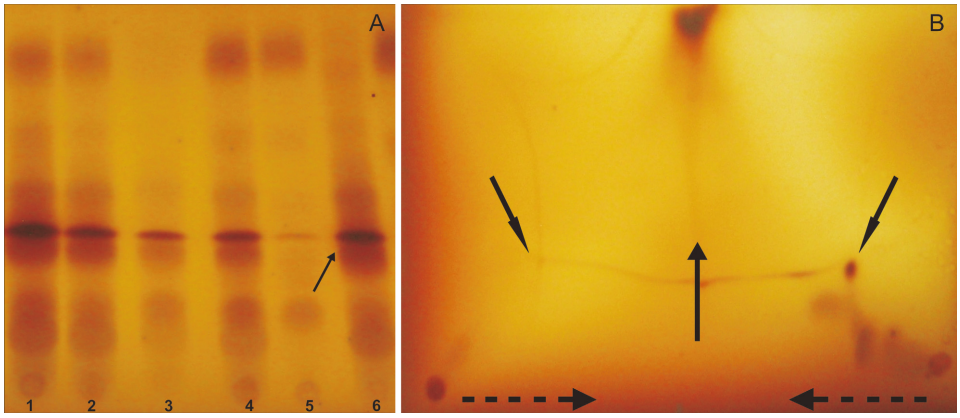


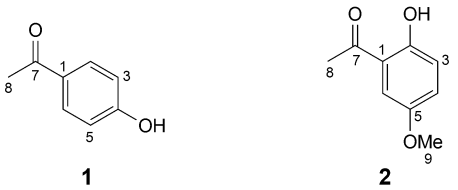
Figure 3. Occurrence of (–)-HAA in isolated GTs. *A*) TLC with concentration zone using chloroform/ethyl acetate/methanol (5:1:0.5) as solvent and stained with I_2 vapors. Most stained bands at $R_f \approx 0.45$ correspond to (–)-HAA. Samples extracted with ethyl acetate (10 mg ml^{-1}): extract of starting material (lane 1), supernatant from isolated GTs fractions (lane 2), first wash of isolated GTs with SDS 1% (lane 3), remaining isolated GTs extract (lane 4), second and last wash of isolated GTs (lane 5), final isolated GTs extract showing the enriched fraction at (–)-HAA (lane 6, arrow). *B*) 2D-TLC showing (–)-HAA stores (right oblique arrow) in the final isolated GT extracts (right run) against previous SDS wash of isolated GTs (left run) where no (–)-HAA could be found (left oblique arrow). The horizontal dashed arrows and the vertical arrow represent the two dimensional runs of the 2D-TLC.

The high amounts of (–)-HAA in GTs may serve different functional roles in *F. campestris*. Based on the fact that phytotoxic (–)-HAA is easily extracted with water,[1] and on our observations that after a heavy rainfall (–)-HAA concentration in leaves falls abruptly as a result of deposits being washed out from the leaf surface (data not shown), this molecule could be signaled as an important allelochemical. Preliminary results from our group have also shown that (–)-HAA can be very effective in reducing growth and biofilm in pathogenic bacteria,[22] suggesting a significant protective role of the surface deposited (–)-HAA that would act as a barrier against microbial attack and/or proliferation.

Chromatographic fractionation from the original protocol of (–)-HAA isolation,[1] employing dry or fresh leaf aqueous extracts partitioned with ethyl acetate and followed by GC/MS analysis and ^1H, ^{13}C, $^1H,^1H$-COSY NMR spectroscopic data yielded 4-hydroxyacetophenone (piceol; **1**) and 2-hydroxy-5-methoxyacetophenone (**2**) (*Figure 4*).

Compounds **1** and **2** were also found in leaf rinses (data not shown), constituting the first record of these metabolites as components of leaf surface secreted products in *F. campestris*. Ethyl acetate extracts of purified GTs could not reveal the presence of **1** and **2**, possibly due to their low concentration (similar to that of volatiles, *ca.* 1/100 of (–)-HAA). However, GC/MS of hexane partition from isolated GTs allowed identifying two minor peaks with *LRI* and MS spectra that corresponded to those of pure **1** and **2**, thus confirming their presence in GTs (*Figure S1*).

The isolation of **1** and **2** as natural products in *F. campestris* has chemotaxonomic implications. The



1 **2**

Figure 4. Chemical structures of isolated acetophenones. 4-Hydroxyacetophenone (**1**) and 2-hydroxy-5-methoxyacetophenone (**2**) isolated from *Flourensia campestris* leaves and found in GT stores.

4-hydroxyacetophenone derivatives have been proposed as precursors of benzofurans, widely distributed in *Flourensia*, including *F. campestris*, and other Asteraceae.[3][4][23] Furthermore, the notion of a chemotaxonomic link is strengthened by results found in *Flourensia* species that belong to the same South-American linage as that in *F. campestris*,[24] such as the presence of 4-hydroxyacetophenone in leaves of *F. riparia*.[3] Taking into account the antimicrobial properties of acetophenone derivatives,[25] the occurrence of these precursors in GTs strongly suggests their functional roles against biotic stress. Present results would also indicate that, at least in part, the synthesis of benzofurans and acetophenone derivatives in *F. campestris* leaves may take place in GTs.

Glycine Betaine

Studies from various authors reported the presence of organic acids, polysaccharides, proteins, terpenes, and phenyl compounds in GTs of several species[14][21][26]

that have been suggested to be involved in abiotic protection. Recent work from *Balcke et al.*[6] in tomato, a non QAC-accumulating species, found that glandular trichomes cope with oxidative stress by producing high levels of polyunsaturated fatty acids, oxylipins, and glutathione.

Taking into consideration that the effective protection of QACs against several abiotic stresses has been extensively documented (for a review see *Chen* and *Murata*[9]), and that *F. campestris* is a glycine betaine accumulating species,[2] we hypothesized that glycine betaine could play an important role functioning as an osmoprotectant and/or inducing ROS-scavenging enzymes in GTs.

Contrary to our expectations, glycine betaine was not found in GT nonvolatile metabolome, or on the surface of the leaves, but only in samples containing leaf mesophyll (*Figure S2*). Moreover, the concentration in whole leaves was practically the same as that found in peeled leaves. More studies are being carried out to determine how these highly exposed secretory cells are protected against abiotic stress in otherwise QAC-accumulating species.

Volatiles

Hydrodistillation extractions gave pale yellow oils with yields (mean of duplicates) of 0.78% (dry leaves, spring 2007), 0.24% (fresh leaves, summer 2014), and 0.88% (dry leaves, summer 2014). *Table 1* shows results of the entire volatile composition of whole leaves and different leaf fractions obtained through hydrodistillation and/or solvent extraction. From a total of 23 compounds originally identified through HS-SPME,[1] hydrodistillation of the same plant material (dry leaves, spring 2007) allowed identifying 30 compounds. As compared to the essential oils from dry leaves 2014 (summer), dry leaves of 2007 showed a lower content in monoterpenes and hydrocarbon sesquiterpenes, and a higher content of oxygenated sesquiterpenes (*Table 1*). These differences may be attributed to the known seasonal intra-specific variation between specimens and inter-annual composition of plant volatiles. Despite the oils of *F. campestris* dry leaves have shown to be quite stable (data not shown), a possible effect resulting from long storage of 2007 samples should be taken into account. When the same plant material was used (summer 2014), only minor differences in the essential oil composition were observed between dry and fresh tissues (*Table 1*). In all cases, volatiles from essential oils had *LRI*s lower than 1714. From the identified compounds, sesquiterpenes predominated over monoterpenes (*ca.* 60% *vs.* 33%, as percentage of total peak area) in the oils of fresh tissue from where GTs were isolated, most of which were related to cadinene and bisabolene derivatives (*Table 1*).

When the same batch of fresh leaves (summer 2014) was extracted with solvents, the recovery of volatiles was significantly lower as compared to hydrodistillation, and only volatiles from the sesquiterpene fraction could be identified (*Table 1* and *Figure S3*). Since monoterpenes could not be identified with the solvent extraction used, the presence of these compounds in GT metabolome cannot be ruled out, and would deserve further investigation. Between 20% and 40% of the compounds found in solvent extracted samples exhibited *LRI*s higher than 1714 (*Figure S1*). For this reason, these metabolites were, strictly speaking, not considered as volatiles, and therefore their identification was not addressed in the present study.

The analysis of the volatile metabolome of GTs through solvent extraction and GC/MS analysis allowed identifying 13 (out of 15) compounds (*Table 1*): 4-hydroxyacetophenone (**1**), 2-hydroxy-5-methoxyacetophenone (**2**),1-methyl-1,3-cyclohexadiene, *n*-tridecanol, and the sesquiterpenes germacrene A, nerolidol, carotol, ledol, τ-cadinol and/or τ-muurolol, α-cadinol, *epi*-β-bisabolol, β-bisabolol and (*Z*)-nerolidyl acetate. Most of these volatiles were also detected in the rinses of leaf surfaces (adaxial and abaxial) and in whole leaf extracts. The adaxial leaf side showed two compounds, ethyl butanoate and 2-buthoxyethanol, at least ten times more concentrated than those in the abaxial sides, and also more concentrated than those in whole leaves (*Table 1*). These compounds were absent in GTs and in the essential oils studied, and hence corresponded to compounds that were lost during hydrodistillation. In the adaxial side, *epi*-β-bisabolol and β-bisabolol (14.2% and 12.8% of total peak area, respectively) could be individually identified, while in the abaxial side, 33.7% of total peak area was attributed solely to β-bisabolol. Except for these differences, both leaf sides showed similar qualitative and quantitative compositions (*Table 1*). However, we cannot discard that such differences may account for a distinctive metabolic composition of the GTs present in the adaxial and abaxial epidermis. It should be noted that quantification was based on peak response values, thus limiting comparison across different classes of compounds. Regarding the question of whether GTs in *F. campestris* may be the site of volatile synthesis, our present results and those from other studies strongly support this notion. In GTs, most of the identified volatiles from the oxygenated sesquiterpenes present in

Table 1. GC/MS analysis of volatiles from *Flourensia campestris*

No.[a]	Compound	*LRI*[b]	Composition (% peak area)[c]							Ident.[d]
			Essential oils (hydrodistillation)			Hexane partition extracts				
			Dry leaves		Fresh leaves	Fresh leaves summer 2014				
			Spring 2007	Summer 2014	Summer 2014	Adaxial rinse	Abaxial rinse	Whole leaf	Glandular trichomes	
1	1-Methyl-1,3-cyclohexadiene	769 ± 3	0.2	–	0.4	–	–	–	8.0	1, 3
2	Ethyl butyrate	805 ± 3	–	–	–	16.6	1.0	11.8	–	1, 2, 3
3	Santolinatriene	903 ± 2	5.3	12.6	8.8	–	–	–	–	1, 2, 3
4	2-Butoxyethanol	912 ± 3	–	–	–	14.2	1.4	5.9	–	2, 3
5	α-Pinene	937 ± 1	7.2	6.0	5.8	–	–	–	–	1, 2, 3
6	β-Pinene	980 ± 1	1.1	1.0	0.8	–	–	–	–	2, 3
7	β-Myrcene	989 ± 1	0.8	4.5	3.7	–	–	–	–	1, 2, 3
8	N.I.	997	–	–	–	–	0.8	–	0.4	–
9	N.I.	1028	0.4	–	0.2	–	–	–	–	–
10	β-*cis*-Ocimene	1040	–	19.6	12.5	–	–	–	–	2, 3
11	β-*trans*-Ocimene	1048	–	2.2	1.6	–	–	–	–	1, 2, 3
12	N.I.	1129	–	0.4	0.3	–	–	–	–	–
13	N.I.	1158 ± 1	–	0.2	0.6	–	–	–	–	–
14	N.I.	1274 ± 2	1.0	0.3	0.7	–	–	–	–	–
15	N.I.	1331	–	0.4	0.4	–	–	–	–	–
16	α-Copaene	1373	0.5	–	0.2	–	–	–	–	1, 2, 3
17	N.I.	1385 ± 1	–	0.2	–	–	2.4	–	0.6	-
18	N.I.	1394	–	–	–	2.6	–	1.5	–	-
19	(*E*)-β-Caryophyllene	1420 ± 3	3.0	4.7	4.1	–	–	–	–	1, 2, 3
20	Aromadendrene	1441 ± 4	1.8	0.6	1.1	–	–	–	–	1, 2, 3
21	α-Humulene	1452	–	0.5	–	–	–	–	–	1, 2, 3
22	N.I.	1456	0.6	0.5	–	–	–	–	–	–
23	N.I.	1465	–	0.4	0.5	–	–	–	–	–
24	N.I.	1470	–	–	0.6	–	–	–	–	–
25	4-Hydroxyacetophenone	1472	–	–	–	–	–	–	4.2	3, 4
26	β-Acoradiene	1473	1.0	–	–	–	–	–	–	1, 2, 3
27	α-Amorphene	1481 ± 2	0.3	0.4	0.6	–	–	–	–	1, 2, 3
28	δ-Selinene	1492 ± 2	1.4	8.0	5.5	–	–	–	–	1, 2, 3
29	2-Hydroxy-5-methoxyacetophenone	1502	–	–	–	–	–	–	3.0	3, 4
30	β-Bisabolene and/or (*Z*)-α-Bisabolene	1503 ± 2	0.7	0.9	1.7	–	–	0.2	–	1, 2, 3
31	Germacrene A	1509 ± 2	0.3	14.6	10.0	–	–	–	0.2	1, 2, 3
32	(*Z*)-γ-Bisabolene	1515	1.9	–	–	–	–	–	–	1, 2, 3

Table. (cont.)

No.[a]	Compound	*LRI*[b]	Composition (% peak area)[c]							Ident.[d]
			Essential oils (hydrodistillation)			Hexane partition extracts				
			Dry leaves		Fresh leaves	Fresh leaves summer 2014				
			Spring 2007	Summer 2014	Summer 2014	Adaxial rinse	Abaxial rinse	Whole leaf	Glandular trichomes	
33	N.I.	1518	0.6	0.6	–	–	–	–	–	–
34	δ-Cadinene	1524 ± 1	0.5	1.1	1.1	–	–	–	–	1, 2, 3
35	(*E*)-γ-Bisabolene	1529	–	–	1.8	–	–	–	–	1, 2, 3
36	N.I.	1534	–	–	0.5	–	–	–	–	–
37	Nerolidol	1560 ± 2	10.3	1.4	0.8	4.4	5.0	3.0	1.4	1, 2, 3
38	*n*-Tridecanol	1574 ± 2	0.6	–	1.0	–	–	–	2.0	1, 2, 3
39	Caryophyllene oxide	1582	–	0.2	0.8	–	–	–	–	1, 2, 3
40	Epiglobulol	1588 ± 1	22.3	4.4	0.6	–	–	–	–	1, 2, 3
41	Viridiflor	1592	5.1	–	–	–	–	–	–	1, 2, 3
42	Carotol	1594	2.3	1.5	0.5	–	–	25.2	6.0	1, 2, 3
43	Ledol	1601 ± 2	1.2	1.4	8.8	–	–	3.8	1.0	1, 2, 3
44	N.I.	1606	0.3	–	–	–	–	–	–	–
45	N.I.	1613	1.8	–	3.2	–	–	–	–	–
46	Bisaboladien-4-ol	1619 ± 1	1.2	0.4	2.0	–	–	–	–	1, 2, 3
47	γ-Eudesmol	1632	5.9	–	0.6	–	–	–	–	1, 2, 3
48	τ-Cadinol and/or τ-Muurolol	1638 ± 2	0.7	2.9	–	19.9	27.3	3.0	13.0	1, 2, 3
49	α-Cadinol	1653 ± 3	8.8	4.6	3.7	11.1	21.5	9.2	25.2	1, 2, 3
50	7-*epi*-α-Eudesmol	1661	2.8	–	–	–	–	–	–	1, 2, 3
51	N.I.	1664 ± 1	3.9	1.4	0.4	–	–	–	–	–
52	Bisabol-11-ol	1668 ± 1	2.2	0.8	0.2	–	–	3.8	–	1, 2, 3
53	*epi*-β-Bisabolol	1671 ± 1	–	0.2	6.6	15.7	–	6.4	19.0	1, 2, 3
54	β-Bisabolol	1673	0.9	–	–	14.2	40.4	6.2	9.0	1, 2, 3
55	(*Z*)-Nerolidyl acetate	1679 ± 2	0.8	0.9	–	1.3	–	14.9	7.0	1, 2, 3
56	α-Bisabolol	1688 ± 1	0.3	0.2	6.2	–	–	1.0	–	1, 2, 3
57	N.I.	1702	–	–	1.1	–	–	–	–	–
58	N.I.	1714	–	–	–	–	–	4.1	–	–
Number of identified compounds		30	26	28	8	6	13	13		
Grouped components [%]										
Monoterpene hydrocarbons		14.4	45.9	33.2	–	–	–	–		
Oxygenated monoterpenes		–	–	–	–	–	–	–		
Total monoterpenes		14.4	45.9	33.2	–	–	–	–		
Sesquiterpene hydrocarbons		11.4	30.8	26.1	–	–	0.2	0.2		
Oxygenatedsesquiterpenes		64.8	18.9	30.8	66.6	94.2	76.5	81.6		

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Table. (cont.)

No.[a]	Compound	*LRI*[b]	Composition (% peak area)[c]							Ident.[d]
			Essential oils (hydrodistillation)			Hexane partition extracts				
			Dry leaves		Fresh leaves	Fresh leaves summer 2014				
			Spring 2007	Summer 2014	Summer 2014	Adaxial rinse	Abaxial rinse	Whole leaf	Glandular trichomes	
	Total sesquiterpenes		76.2	49.9	56.9	66.6	94.2	76.7	81.8	
	Others		0.8	0.0	1.4	30.8	2.4	17.7	17.2	
	Total volatiles ($LRI \leq 1714$) identified		91.4	95.6	91.5	97.4	96.8	94.4	99.0	
	Not identified (N.I.)		8.6	4.4	8.5	2.6	3.2	5.6	1.0	

N.I., not identified. [a] Order of elution on apolar column (*DB-5*). [b] Means ($n = 2$) and means ± SD ($n \geq 3$) of Linear Retention Indices from identified components in the different samples with similar retention indexes and mass spectra. [c] Percentages as means of technical replicates with differences within 5% of the means. [d] Identification of compounds with: 1-*LRI* datasets from literature, 2-*LRI* (NIST), 3-MS (NIST), 4-*LRI*, and MS of Standards.

the essential oils corresponded to cadinene and bisabolene derivatives. Such compounds have been found to share farnesyl, nerolidyl, and bisabolyl cations as common precursors, and to be substrates, by-products or final products of cadinene and/or bisabolene synthases in other plant species.[27][28] Moreover, the fact that GTs contain particularly large stores of another bisabolene derivative, the nonvolatile (–)-HAA, provides additional evidence that synthesis takes place within GTs through metabolic routes that could be similar to those elucidated for other species.[21] However, further studies involving transcriptome analysis and identification of corresponding encoding genes for terpene synthases[28] are in order. With respect to the biological activities of the volatiles found in GTs, cadinene derivatives,[29] nerolidol,[30] and ledol[31] are related to antifungal activities. Particularly, τ-cadinol and α-cadinol were reported as two of the three most active antifungal compounds found in the leaf essential oil of *Neolitsea parvigemma*.[32] *Jasicka-Misiak et al.*[33] described a strong fungicidal effect of carotol, the carotane derivative found in GTs, against *Alternaria alternate*. In turn, bisabolene derivatives could potentiate plant antimicrobial response through enhancing pathogen membrane permeability, as has been shown for the precursor of sesquiterpene β-bisabolol, β-bisabolene, which restores the effectiveness of ampicillin against resistant *Staphylococcus aureus*.[34] Thus, volatile metabolome of GTs in *F. campestris* would be involved in defense mechanisms against biotic stress, specifically storing, secreting and probably synthesizing bioactive sesquiterpenes as defense weapons against microbial attack.

In addition to the important ecophysiological role that volatiles could play in *F. campestris*, GT metabolome may be considered as a source of bioactive compounds with a diverse range of uses in different industries. In this sense, bisabolene and bisabolol isomers have been found to exhibit anti-inflammatory and cytotoxic effects, with strong potential for medical and pharmaceutical applications.[35] Bisabolol and nerolidol may increase dermal absorption of other substances, being useful vehicles for other drugs;[36] they can also enhance bacterial permeability and susceptibility to clinically important antibiotic compounds.[37] Moreover, based on the hypothesis that a highly expressed bisabolene synthase is operative in leaves and GTs, *F. campestris* could have a potential application for candidate biofuels. *Peralta-Yahya et al.*[38] recently proposed bisabolane as a drop in biosynthetic alternative to D2 diesel, and to engineer microbial platforms for the production of its immediate precursor, bisabolene.

Conclusions

In *F. campestris*, although the ultrastructure of GTs has been studied in detail, their phytochemical composition and biological function were still unknown. A new method was devised that allowed an efficient isolation and purification of GTs from these resinous leaves.

Most of the compounds present in the nonvolatile metabolome of GTs in *F. campestris* were found deposited on the surface of the leaves. The phytotoxic bisabolene (–)-HAA was the major constituent of the nonvolatile fraction (*ca.* 40%), highlighting the role of GTs in allelopathic interactions. Despite being QAC-accumulating species, glycine betaine was not found in the nonvolatile metabolome of GTs, and therefore other alternative mechanisms would be operative in protection of GT functionality.

Regarding the volatile metabolome of GTs, a complete characterization of the essential oils was obtained through hydrodistillation of whole leaves. Volatiles found in fresh leaves, from where GTs were isolated, yielded *ca.* 33% monoterpenes, 26% hydrocarbon- and 30% oxygenated sesquiterpenes, most of them related to cadinene and bisabolene derivatives. In all samples analyzed (dry or fresh tissues), volatiles from essential oils had *LRI*s lower than 1714. Monoterpenes could not be elucidated when solvent extraction methods were used, thus their presence in GTs remains to be investigated. In the volatile metabolome of GTs, 13 compounds could be identified, mostly corresponding to cadinene and bisabolene derivatives, largely recognized by their antifungal and antimicrobial activities. Two volatiles newly described (**1** and **2**) were found in GT stores and also deposited on the surface of the leaves. These compounds known as precursors of antimicrobial acetophenone derivatives and of benzofurans are widely distributed in *Flourensia* and, specifically, in *F. campestris*. The composition of the volatile metabolome of GTs suggests that these structures would be involved in defense mechanisms against biotic stress, specifically storing, secreting and synthesizing bioactive compounds as a defense weapon against microbial attack.

The remarkable stores of nonvolatile and volatile compounds with a bisabolene backbone found in GTs suggest the existence of specific enzymes with high catalytic yield. If this is the case, the potential application of this apparently high efficient bisabolene terpene synthase(s) in biofuel production remains to be explored.

Altogether, the present article shows strong evidences about the significant contribution of GTs to *F. campestris* metabolome, with the capacity to produce large quantities of secondary metabolites which would play key functional ecological roles in plant-pathogen and plant-plant interactions, also highlighting the potential of this native species as a source of valuable bioactive compounds.

Experimental Section

General

Analytical-grade solvents were used from commercial sources. Ethyl acetate or ethanol extracts from plant tissues or from trichome isolation fractions, and volatiles from hexane partitions and hydrodistillation were analyzed by GC/MS on a *PerkinElmer Clarus 600*, and Turbo Mass 5.4.2 for data acquisition; in *m/z* (%). The GC column was *DB-5* (60 m, 0.25 mm i.d., 0.25 μm particle size; *PerkinElmer*), and the carrier gas used was He (49.6 psi). Running program: the initial temperature of 60 °C was gradually increased after 2 min to 300 °C by a ramp of 5 °C min^{-1} and held for 5 min, injector at 300 °C. The injector was used in split mode (20 ml min^{-1}) with the inlet temperature set to 300 °C. Samples were diluted to 1:20 with ethyl acetate or ethanol, and to 1:100 with hexane (volatiles). Silica gel *60* (*Merck*) was used for regular TLC, 2D-TLC and for TLC with concentration zone (aluminum sheets, F_{254}) and for CC (70 – 230 mesh).

Identification and Quantification of Compounds

GC Linear Retention Indices (*LRI*s) of compounds were determined according to the retention times of a series of *n*-alkanes (C_7 – C_{23}) with linear interpolation, using the equation of *Van den Dool* and *Kratz*[39] based on the equation developed by *Kovátz*.[40] Identification of the components from duplicate runs was based on: *a*) comparison of their GC *LRI*s with *i*) those of authentic compounds, *ii*) the widely used datasets of *Adams*[41] and *Babushok et al.*[42] (50% *LRI*s range), using data restricted to *DB-5* column, 20 – 60 m × 0.25 mm i.d., 0.25 μm film thickness and similar temperature ramp conditions. As suggested by *D'Acampora Zellner et al.*,[43] only those calculated *LRI* values that fall within a determined range (*i.e.*, ± 5 units for methyl silicone stationary phases) were considered acceptable. *b*) Comparison of their MS through computer matching with commercial mass spectral libraries[44] and mass spectra of our personal library. Pure (–)-HAA under same conditions showed a *LRI*: 2168 ± 1 (mean ± SE, $n = 3$), and *m/z* (%): 234 (12, $[M - H_2O]^+$), 219 (2), 191 (6), 164 (3), 149 (14), 123 (9), 109 (100), 105 (9), 79 (22), 69 (65), 55 (20), 43 (35), 39 (6).

GC/MS for identification of **1** and **2** were obtained on a *GC-17A Shimadzu* and *QP-5000 MS Shimadzu*. The GC column was HP 5% phenyl methyl silicone (*Alltech*) (30 m × 0.32 mm i.d.). The linear temperature program was from 150 to 300 °C, at a rate of 10 °C min^{-1}, and the carrier gas was He (1 ml min^{-1}). ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), DEPT, COSY, HMBC, and HSQC spectra for **1** and **2** were recorded at room temperature with *Bruker AC 400* spectrometer. The spectra were recorded in $CDCl_3$ and the solvent signals (7.26 ppm for ^{1}H-NMR and 77.2 ppm for ^{13}C-NMR) were used as a reference.[45] Coupling constants, *J*, are reported in Hz.

Relative amounts of individual components were calculated based on their GC peak areas on the capillary columns, without FID response correction factor. Nevertheless, the limitations of the quantification method used, particularly when comparing across different classes of compounds, are acknowledged in the discussion of results.

Plant Material

Plant material was collected in a natural area corresponding to the Punilla Valley, Córdoba Province, Argentina, in a typical shrub community (total plant cover 70 – 90%) dominated by the evergreen shrub *Flourensia campestris*. Fresh leaves from 15 randomly selected specimens of *F. campestris* were collected in spring 2007 and in early summer (January) of 2014. Plant material was used immediately after harvest, or air-dried and stored at *ca.* 20 °C, and was determined by Drs. *J. J. Cantero* and *G. M. Tourn*. A voucher specimen (BAA 26.498) was deposited with the Herbarium Gaspar Xuárez, School of Agronomy, Universidad de Buenos Aires, Argentina.

Isolation of Capitate Glandular Trichomes (GTs)

The first steps of the isolation method were similar to those described by *Siebert*.[19] Clear adhesive tape (*Stiko*, 48 mm wide, commercial use) was used to remove leaf trichomes from fresh leaves. The adhesive tape itself was analyzed through TLC and GC/MS in order to rule out the existence of chemicals that could contaminate the isolated GT fractions. To dislodge leaf surface material, the adhesive tape was secured on the table with the adhesive side facing up. The adaxial surfaces of individual fully expanded fresh leaves (*i.e.*, 4 – 5 cm long, 1.5 – 2.0 cm wide) of *F. campestris*, known to have a higher density of GTs,[8] were laid on the adhesive tape. Leaves were gently rubbed, especially along the veins where GTs are more concentrated,[8] and rapidly removed by pulling from their petioles. This procedure was previously optimized by checking the tape and peeled leaves under light microscopy, corroborating that GTs were dislodged with almost no interference of epidermal cells. The tapes with the material of 20 peeled leaves were submerged in a *Petri* dish containing 20 ml of a modified gland isolation buffer[26] containing 50 mM *Tris*·HCl, 200 mM d-sorbitol, 20 mM sucrose, 14 mM 2-sulfanylethanol, 10 mM KCl, 0.5 mM KH_2PO_4, 0.6% (*w/v*) methylcellulose, and 1% (*w/v*) polyvinylpyrrolidone (PVP), pH = 7.15 at room temperature (*ca.* 20 °C). After shaking for 1 min, adhered components (hereafter called starting material) were gently and completely removed from the submerged tape with the help of a paint brush. The resulting mixture (gland isolation buffer + starting material) was transferred to 50 ml falcon tubes and trichomes were liberated from the sticky components by vortexing for 1 min. The mixture was then filtered consecutively through 100 and 80 μm metal meshes in order to exclude the larger sized leaf debris. Meshes were washed with 5 ml of gland isolation buffer and the resulting mixture was centrifuged at 1000 *g* for 5 min. The supernatant, containing small debris and dissolved sticky components (as inspected through light microscopy after spinning at 5000 *g* for 5 min), was discarded. The pellet, containing capitate and filiform shaped trichomes together with some small plaques of sticky components, was re-suspended in 10 ml of gland isolation buffer and further vortexed for 30 s. The suspension was then filtered through a 43 μm metal mesh that retained remaining debris and filiform trichomes. From this step onwards, and in order to facilitate the washing and passage of GTs through the meshes, ice-cold gland isolation buffer without methyl cellulose and PVP was used. GTs, with an average diameter of 30 μm, were collected on a 25 μm metal mesh which allowed smaller debris to pass through. The collected trichomes were washed at least five times on the mesh, and then transferred to a 10 ml centrifuge tube by washing the mesh placed up-side down on top. The tubes were vortexed for 10 s and centrifuged at low speed. GTs were collected from the bottom of the tubes and immediately used for light microscopy inspection, hemocytometer counting and/or phytochemical analysis.

Light Microscopy

Epidermal layers from whole leaves were manually removed, mounted in 50% glycerine, and inspected through light microscopy. Adhesive tapes with attached starting material obtained from fresh adaxial leaf

surfaces were placed up-side down on a slide containing a drop of 50% glycerine and inspected with no need of a cover lid. In some instances, and for visualization purposes, fresh leaves were rinsed with ethanol in order to remove bulk resins deposited on their surface.

Samples from the different steps of the GT isolation method were immediately placed on slides, covered with lids, and observed under light microscopy without any further treatment.

Some materials were fixed in FAA and embedded in paraffin. Sections (7 – 10 μm thick) were cut and stained with safranin combined with fast green.[8] Micrographs were obtained using a digital video microscopic camera with its corresponding software (MotiCam 1.3 MP). Hemocytometer counting was performed in a *Newbauer* chamber filled with isolated gland fractions previously diluted to a final volume of 1 ml with gland isolation buffer. Results were expressed as means ± SE.

Phytochemical Analysis

The occurrence of metabolites was analyzed in the following samples: whole leaves (intact leaves), leaf surfaces (adaxial, abaxial, dislodged leaf surface materials (*i.e.*, starting material), leaf rinses, peeled leaves (in which adaxial and abaxial trichomes and surface secreted products have been removed with adhesive tapes), isolated GTs and in the different steps of GT isolation.

(–)-Hamanasic Acid A ((–)-HAA)

Samples were extracted by vortexing with 10 × volumes of ethyl acetate. After centrifuging at 3000 *g* and evaporating at 40 °C under N_2 flow, (–)-HAA was identified and quantitatively measured in ethyl acetate phases by means of 2D-TLC.[1][8][46] The resulting pellets from investigated fractions were dried in order to obtain their dry weights. Means ± SE from triplicates were expressed as mg (–)-HAA g^{-1} DW.

GC/MS Analysis was also performed in the polar phase of isolated GTs previously partitioned with hexane (see Compounds **1** and **2**, *Experimental Section*). Since (–)-HAA is not commercially available, (–)-HAA used as standard was obtained by purification from *F. campestris* leaves as already described.[1][46][47]

Compounds **1** *and* **2**

Leaves of *F. campestris* (120 g) were extracted with water (2 l) for 24 h at 22 °C and partitioned with ethyl acetate (20% *v/v*). The organic phase was dried at 30 °C under a N_2 flow, yielding green syrup. The resulting ethyl acetate extracts (*ca.* 1 g) were next fractionated by silica gel CC (1.5 cm i.d., 47 cm; 50 g silica gel) eluted at a flow rate of 5 ml min^{-1} using chloroform/ethyl acetate/methanol (5:1:0.25; *Solvent I*). The collected 1 min fractions were subjected to TLC using the same solvent and detected under UV_{254} illumination and with I_2 vapors. As described in *Silva et al.*,[1] (–)-HAA was purified from a bioactive fraction (*Frs. 34 – 48*). The accompanying compounds from a non-bioactive fraction (*Frs. 6 – 24*) were subjected to preparative TLC using hexane/acetone (70:30) as running solvent. The separated band (R_f = 0.6) was scrapped from the plaque and eluted from the silica gel with pure acetone, yielding *ca.* 8 mg (0.007% DW basis) of a colorless substance. Compound identification was based on GC/MS analysis and ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), DEPT, COSY, HMBC, and HSQC spectra, and by comparison to previously reported data. The scrapped band afforded a mixture of two compounds identified as 4-hydroxyacetophenone (**1**) and 2-hydroxy-5-methoxyacetophenone (**2**), in a concentration relation of 3:1.

The occurrence of isolated compounds was performed taking into account both, their low concentration in *F. campestris* whole leaves (*ca.* 0.007% DW basis) and their capacity of being concentrated in the hexane phase from hexane/methanol/water partition. The total isolated GTs (*ca.* 0.3 g FW) from 40 fully expanded fresh leaves were vortexed with a mixture of 4.5 ml methanol and 1.5 ml water followed by the addition of 15 ml hexane. After vortexing for 2 min and centrifuging at 3000 *g*, the hexane phase was evaporated at 40 °C under N_2 flow.

Compounds **1** and **2** were identified by means of GC/MS, using the same pure isolated compounds as standards. The presence of **1** and **2** was also studied in the records of mass spectra from secretory duct resins manually collected from fresh tissue.[8]

1-(4-Hydroxyphenyl)ethanone (**1**). *LRI* standard (*DB-5*, 60 m, 0.25 mm, 0.25 μm, ramp temperature): 1475 ± 0, *n* = 3. ^{1}H-NMR ($CDCl_3$): 2.57 (*s*, Me(8)); 6.91 (*d*, *J* = 8.6, H–C(2,6)); 7.80 (*d*, *J* = 8.6, H–C(3,5)). ^{13}C-NMR ($CDCl_3$): 26.4 (C(8)); 115.6 (C(2,6)); 129.9 (C(1)); 131.2 (C(3,5)); 161.2 (C(4)); 198.0 (C(7)). GC/MS (EI, 70 eV): 136 (37, M^+), 121 (100), 93 (34), 65 (20). HR-MS: 136.0519 ($[M + H]^+$, $C_8H_9O_2^+$; calc. 136.0524).

1-(2-Hydroxy-5-methoxyphenyl)ethanone (**2**). *LRI* standard (*DB-5*, 60 m, 0.25 mm, 0.25 μm, ramp temperature): 1502 ± 0, *n* = 3. ^{1}H-NMR ($CDCl_3$): 2.57 (*s*, Me(8)); 3.93 (*s*, Me(9)); 7.53 – 7.56 (*m*, H–C(6)); 7.53 – 7.56 (*m*, H–C(4)); 6.95 (*d*, *J* = 8.0, H–C(3)). ^{13}C-NMR ($CDCl_3$): 26.3 (C(9)); 56.2 (C(7)); 109.9 (C(4)); 114.0

(C(3)); 124.4 (C(6)); 130.1 (C(1)); 146.8 (C(2)); 150.8 (C(5)); 197.8 (C(8)). GC/MS (EI, 70 eV): 166 (51, M^+), 151 (100), 123 (26), 108 (9), 77 (5), 65 (6). HR-MS: 166.0621 ($[M + H]^+$, $C_9H_{11}O_3^+$; calc. 166.0624).

Glycine Betaine

Glycine betaine localization was assessed as previously reported.[2] Briefly, a micro-method for extract preparations was applied which included sample grinding (70 mg FW) in a mixture of 1 ml of chloroform and 0.4 ml of methanol/water (1:1), boiling during 30 s, vortexing, and centrifuging. The identification and concentration of glycine betaine was determined in the supernatants as described by *Piazza et al.*[2]

Volatiles

In order to complete the characterization of total plant volatiles previously reported for *F. campestris* using HS-SPME,[1] the same dry plant material (collected in spring 2007 and stored at −20 °C) was subjected to hydrodistillation, and the composition of the resulting essential oils was analyzed.

In addition, and since GTs have to be isolated from fresh leaves, new plant material was harvested in the summer of 2014. The collected material was pooled and divided into three batches. From the first batch, 40 fresh leaves were used for isolating GTs; 70 g (FW) of the second batch were subjected to hydrodistillation,[1] and 70 g of the third batch were air dried (19.3 g DW left) and also subjected to hydrodistillation. Extractions through hydrodistillation were performed in a *Clevenger*-type apparatus, with a separated extraction chamber, during 3 h. The essential oils were dried over anhydrous sodium sulphate (30 g l^{-1}) and stored at −20 °C until analysis.

Another portion of the fresh leaves harvested in summer 2014 was used to study the localization of volatiles using non-polar extracts obtained by hexane partition. Analysis of volatile profiles in the samples was performed through TLC, and their identification was achieved through GC/MS as described in *General, Experimental Section*. In solvent extracted samples, volatiles were assumed to be those compounds whose *LRI*s values fall in the same range as those detected in the essential oils obtained through hydrodistillation (*i.e.*, ≤ 1714, see *Results and Discussion*).

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201700511.

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Author Contribution Statement

Leonardo A. Piazza: Isolation of glandular trichomes, identification and quantification of compounds, manuscript writing. *Daniela López*: Purification and quantification of HAA, extractions of volatiles, manuscript reviewing. *Mariana P. Silva*: Volatiles extraction, manuscript reviewing. *Marisa J. López Rivilli*: Identification and quantification of compounds, GC/MS analysis. *Mónica G. Tourn*: Light microscopy. *Juan J. Cantero*: Manuscript reviewing and discussion. *Ana L. Scopel*: Manuscript writing and discussion.

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