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Chemical composition of essential oils of peltate glandular trichomes from leaves and flowers of *Lepechinia floribunda* (Lamiaceae)

JULIA L. CAMINA*[1], JOSÉ S. DAMBOLENA[1,2], JULIO A. ZYGADLO[1,2] and LORENA ASHWORTH[1]

Summary: The knowledge of the chemical composition of different plant organs provides valuable information for clarifying the functionality of secondary metabolites. Essential oils are secondary metabolites produced and stored in secretory structures on leaves, flowers, and steam. Many studies have shown that the chemical composition of the essential oil can differ depending on the plant organ and the secretory structures even if they are at the same organ. Such differences may be the consequence that organs also differ in type and density of glandular structure. Moreover, the same type of glandular structure can change its chemical composition depending on its position within the same organ. However, no study has been evaluating the chemical composition of the essential oil from the same glandular structure in leaves and fertile floral whorls. The aim of this work was to characterize the chemical composition of essential oils of peltate glandular trichomes from anthers and leaves of *Lepechinia floribunda*. We found a lower richness of chemical compounds and higher relative abundance of monoterpene hydrocarbons on anthers than leaves. Leaves showed an increased relative abundance of oxygenated sesquiterpenes. Such differences probably respond to a high tissue-specificity expression of genes in different plant organs.

Key words: Anther, glandular trichome, Lamiaceae, leaf, *Lepechinia floribunda*, terpenes.

Resumen: Composición química del aceite esencial de tricomas glandulares peltados en hojas y flores de *Lepechinia floribunda* (Lamiaceae). El conocimiento de la composición química del aceite esencial producido por distintos órganos en las plantas, brinda información para conocer las funciones de dichos compuestos. Los aceites esenciales son metabolitos secundarios producidos y almacenados en diferentes estructuras glandulares en hojas, flores y tallos. La composición química del aceite esencial difiere entre órgano de la planta y entre estructuras de secreción, incluso ubicadas en un mismo órgano. Tales diferencias podrían ser consecuencia del distinto tipo y densidad de estructuras glandulares en cada órgano. El aceite esencial del género *Lepechinia* ha sido escasamente estudiado, sin embargo su uso etnobotánico pone en evidencia propiedades medicinales y antisépticas. Hasta la fecha varios estudios han evaluado la composición química del aceite esencial en distintas partes de las plantas o de la misma estructura glandular en diferentes posiciones dentro de las mismas. Sin embargo, no se han encontrado estudios que evalúen la composición química del aceite esencial del mismo tipo de estructura glandular en hojas y verticilos florales fértiles. El objetivo de este trabajo fue caracterizar la composición química del aceite esencial proveniente de tricomas glandulares peltados de anteras y hojas de *Lepechinia floribunda* (Lamiaceae). Se encontró menor riqueza de compuestos químicos y un incremento en la abundancia relativa de monoterpenos hidrogenados en el aceite esencial de anteras que en hojas, mientras que en hojas hubo mayor abundancia de sesquiterpenos oxigenados. Estos cambios en la composición química probablemente respondan a la alta especificidad tisular en la expresión de genes que existe para cada órgano.

Palabras clave: Antera, hoja, Lamiaceae, *Lepechinia floribunda*, terpenos, tricoma glandular.

[1] Instituto Multidisciplinario de Biología Vegetal, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Córdoba (UNC), (5000) Córdoba, Argentina. Telefax (54) 351-433-1056/2104
[2] Instituto de Ciencia y Tecnología de los Alimentos (ICTA), Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba (UNC), Argentina
*juliacamina@yahoo.com.ar - 54-351-4331056/2104

Introduction

Lamiaceae is one of the greatest families of Angiosperms and most of species are aromatics (Li *et al*., 2016). The genus *Lepechinia* is exclusive from America and it is composed by 43 species with mainly an Andean distribution, ranging from California, USA to Buenos Aires, Argentina (Drew & Sytsma, 2012). The essential oils of *Lepechinia* have many medicinal and antiseptic properties (Stashenko *et al.,* 1999; Malagón *et al.,* 2003; Acevedo *et al.,* 2005; Arias Toledo *et al.,* 2010; Ruiz *et al.,* 2015), but compared to other genus of Lamiaceae, its chemical composition has been scarcely studied (Bozin *et al.,* 2006; De Martino *et al.,* 2009).

The chemical composition of essential oils of the genus *Lepechinia* has been studied in less than a half of the 43 *Lepechinia* species (Drew & Sytsma, 2012). Overall the essential oils of this genus are composed mainly by terpenes, although the identity and kind of them are highly variable. Three groups of species were identified based on the mayor compound of the essential oils. Species like *L. meyenii* (Walp.) Epling, *L. schiedeana* (Schltdl.) Vatke, *L. mutica* (Benth.) Epling, *L salviaefolia* (Kunth) Epling, *L. betonicifolia* (Lam.) Epling, *L. Salviifolia* (Kunth) Epling, *L. rufocampii* Epling & Mathias, and *L. Calycina* (Benth.) Epling, produce essential oils mostly conformed by monoterpenes (Lawrence & Morton, 1979; Ciccio *et al*., 1999; Eggers *et al.,* 2001; Malagón *et al.,* 2003; Caballero-Gallardo *et al.,* 2011; Brand *et al.,* 2016; Ruiz *et al.,* 2015). While species like *L. graveolens* (Regel) Epling, *L. paniculata* (Kunth) Epling, *L. bullata* (Kunth) Epling and *L. floribunda* (Benth.) Epling, show dominance of sesquiterpene compounds (Velazco-Negueruela *et al.,* 1994; Eggers, 2000; Arze *et al.,* 2009; Valarezo *et al.,* 2012). The third group formed by *L. urbanii* (Briq.) Epling, *L. caulesens* (Ortega) Epling, *L. chamaedryoides* (Balb.) Epling, *L. vulcanicola* J. R. I. Wood, *L. radula* (Benth.) Epling and *L. conferta* (Benth.) Epling have essential oils composed by equivalent proportions of mono and sesquiterpene compounds (Valenzuela *et al.,* 1992; Zanoni & Adams, 1991; Acevedo *et al.,* 2005; Borges *et al.,* 2006; Brand *et al.,* 2016; Morocho *et al.,* 2017).

At intraspecific level the chemical composition of the essential oil can vary by seasonal changes, by effect of different extrinsic factors, throughout the life stages of individuals and, or given a particular life stage, among vegetative and reproductive structures (Turner *et al.,* 2000; Glas *et al.,* 2012). Essential oils are produced and stored in specialized structures that allow the plant to make use of their biological functions in the right place at the right time (Fahn, 2000). Structures such as glandular trichomes can be found on approximately 30% of all vascular plants, and these trichomes are the main sources of production and secretion of essential oils in Lamiaceae (Glas *et al.,* 2012; Rehman, 2016). Plants have many kinds of glandular trichomes, and it is currently known that different kind of trichomes have distinct biological functions as they produce different secondary metabolites (reviewed by Glas *et al.,* 2012). The Lamiaceae species are well known for their often densely haired aromatic leaves (Metcalfe & Chalk, 1950). The presence of both glandular (peltate and capitate) and non-glandular trichomes is a characteristic feature of this family (Maffei & Codignola, 1990). Glandular trichomes are widely distributed over the aerial reproductive and vegetative organs and they are the source of aromatic, volatile oils and terpenes (Bhatt *et al*., 2010). Peltate glandular trichomes comprise a basal and stalk cell and a head composed by several secretory cells, which are surmounted by a large sub-cuticular storage cavity (Turner *et al.,* 2000). Peltate trichomes have been found on anthers of flowers and on leaves of some species of Lamiaceae like *Leonotis leonurus* L. R. Br., *Leonurus sibiricus* L., *Orthosiphon stamineus* Benth. and *Prostanthera gilesii* B. J. Conn & T. C. Wilson (Ascensão *et al.,* 1995; Moyano *et al.,* 2003; Keng & Siong, 2006; Conn & Wilson, 2015). Despite the presence of peltate glandular trichomes in different plant organs, little is known about the chemical composition of their essential oils and therefore about their functional variability (Ascensão *et al.,* 1995; Moyano *et al.,* 2003; Keng & Siong, 2006; Conn & Wilson, 2015). Many studies have shown that the chemical composition of the essential oil differs among plant organs (e.g. Angioni *et al.,* 2006; Borges *et al.,* 2006; Marzoug *et al.,* 2011), and such differences may be consequences that organs also differ in the type and density of glandular structures (e.g. Maffei & Sacco, 1987). Moreover, the same type of glandular structure can change its chemical composition depending on the position of the organ within the plant and the position of the glandular structure within the same organ (Maffei *et al*., 1989; Rohloff, 1999; Stešević *et al*., 2016). Thus, as example, the chemical composition of the same glandular structure can ever vary among different position within a flower. Up to date, no study has evaluated the chemical composition of the essential oils from the same type of glandular trichome in leaves and flower fertile whorls.

Lepechinia floribunda is a perennial subshrub distributed from Bolivia to central-east Argentina (Epling, 1938). The plant surface is coated with capitate and peltate glandular trichomes, has white, hermaphroditic and tubular flowers. Each flower has four purple anthers with peltate glandular trichomes (Camina, Pers. Obs.). Previous studies on the chemical composition of the essential oils of *L. floribunda* have shown four groups of terpenes: monoterpene hydrocarbons, where camphene was the most representative compound; oxygenated monoterpenes with borneol and 1,8-cineole as the most abundant compounds; sesquiterpene hydrocarbons with β-caryophyllene and γ-cadinene as mayor compounds and oxygenated sesquiterpenes where ledyl acetate, guaiol and β-eudesmol were the most abundant compounds (Velasco-Negueruela *et al.,* 1994; Viturro *et al.,* 2002; Fuselli *et al.,* 2008; Arze *et al.,* 2009). The aim of this work was to determine for the first time the chemical composition of the essential oils from glandular peltate trichomes on leaves and anthers of *Lepechinia floribunda*.

Materials and Methods

In October 2014 we selected three healthy (without herbivory or florivory) individuals of *L. floribunda* from a natural population located in the "Reserva Hídrica Natural Municipal Los Manantiales" (31º 10' 21,3''S, 64º 20' 47,5''O; Río Ceballos, Córdoba, Argentina). Selected plants were of similar size separated by at least 10 m. Two or three flowering branches per plant (Fig. 1A) were cut early in the morning when the corolla of the flower was just opening in order to avoid pollinator visitation. Branches were maintained in a styrofoam bucket with water for 2 hours until the essential oils extraction. Images of peltate glandular trichome in anthers and leaves were taken on the fresh material by a magnifying glass (Leica EZ5, range zoom 1 to 5 with 1x objective and 10x eyepieces) and reflection confocal microscopy (OLYMPUS LEXT OLS4000, lens:MPLAPONLEXT20, zoom X1, scanning mode: XYZ step scan + Color). These images were edited by Adobe Photoshop CS6 (Fig. 1). The essential oil extraction was performed in the laboratory under a magnifying glass (Leica EZ5, zoom: 50x). We used a filter paper of 0.5 mm cut as an isosceles triangle, simulating a needle (Schleicher and Schuell, 0859 type, 90 mm) to break the trichome cuticle and absorb by capillarity the essential oil. Special care was taken to avoid contact the filter paper with any other structures. Anthers of *L. floribunda* have only peltate trichomes (Fig. 1 B-D) and leaves have also non-glandular trichome and capitate trichomes (Fig.

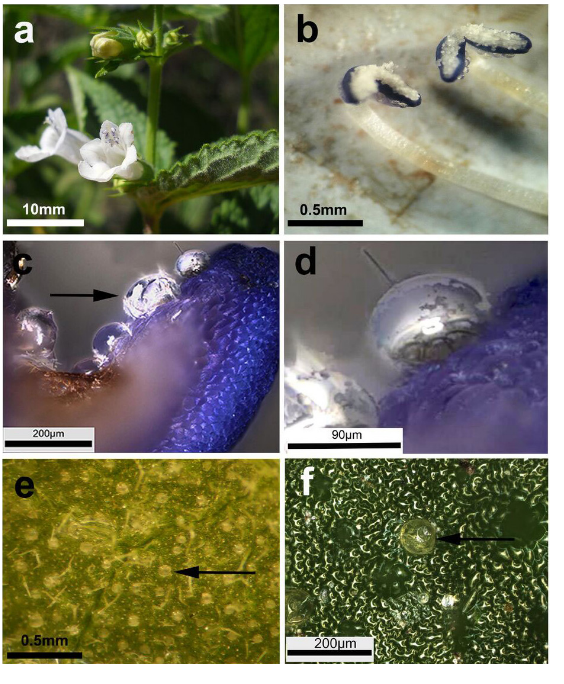

Fig. 1. A: Branch with flower showing the anthers. **B**: Peltate glandular trichomes on anthers. **C**: Peltate glandular trichome (arrow) on anther. **D**: Detail of a peltate glandular trichome on anthers. **E**: Peltate glandular trichomes (arrow) on the abaxial surface of a leaf. **F**: Detail of peltate glandular trichome (arrow) on the abaxial surface of a leave.

1E, F). However, as peltate trichomes are bigger (approximately 90 μm) than other type of trichome, and they are easily to view under magnifying glass, we were sure that any other structure was touched during the extraction of the essential oil. For each plant, we collected the essential oil of all peltate trichomes of all anthers of three to five flowers (total 70-200 trichomes per plant) and ten peltate trichomes from the adaxial surface of three to four apical leaves (total 30-40 trichomes per plant). Following this protocol, each plant is a replicate. Each fragment of filter paper was placed in an Eppendorf at -18ºC until processing. The extraction of the essential oil from the filter paper was made with 10 μl of hexane per Eppendorf. Extractions were performed inside Eppendorf tubes.

The identifications of chemical compounds was made through their mass pattern fragmentations obtained by gas chromatography-mass spectrometry (GC-MS Perkin Elmer 600), equipped with a capillary column DB-5 (60 m x 0.25mm i.d. and 0.25 μm coating thickness). Chromatography conditions were as follows: oven temperature profile of 60 °C to 240 °C by 2 °C/min; helium was the carrier gas with a constant flow of 0.9 ml/min and 70 eV ion source. The injector was operated in splitless mode at 250 °C, same as the detector temperature. The volatile compounds were identified by comparing their retention indices (RI) determined on the basis of an homologous series of n-alkane hydrocarbons (C8–C25), mass spectra and pure reagents (SIGMA, USA) with those of mass spectral databases from the Wiley library and NIST 98 MS Library and with bibliography (Adams, 2007).

To compare the chemical composition of essential oils of glandular trichomes between leaves and anthers, four groups of terpenes were considered: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpenes hydrocarbons and oxygenated sesquiterpenes. The mean relative abundance of each group, and the mean relative abundance of the main compounds of the essential oil (those that had more than 5% of relative abundance) were compared between leaves and anthers by a *t-test* with Welch correction to correct the degree of freedom when the homogeneity of variance assumption was not met (Zar, 1999). All statistical analyses were performed by R software, version 3.3.1 (R Development Core Team).

Results

Lepechinia floribunda has between three to eight peltate glandular trichomes per theca, and on average 9.82 ± 2.21 per anther. Because each flower has four anthers, there are in total between 30-48 peltate trichomes per flower. Peltate glandular trichomes were located on the ventral face of the anthers (Fig. 1B-D), and they remain intact until a mechanical disruption by some external agent, e.g. a floral visitor makes contact with anthers breaking the cuticle of the trichomes and thus releasing their content.

The essential oil from peltate glandular trichomes of leaves had 19 compounds (Table 1), among them, 1,8-cineole, β-caryophyllene, ledol and an undefined oxygenated sesquiterpene were the most abundant compounds (Fig. 2). In contrast, the essential oil of peltate glandular trichomes of anthers had only 12 compounds (Table 1), all found also in the leaves. Moreover, in contrast to leaves the relative abundances of these compounds in anthers were rather equitable since there were no dominant compounds (Fig. 2). The seven compounds absent in the essential oil of anthers were found in low relative abundance in leaves, excepting guaiol and aromadendrene which were found in non-negligible amounts (Table 1).

Significant differences were found in the composition of the essential oils from peltate glandular trichomes from anthers and leaves. As a general pattern, the essential oil from anthers had a higher relative proportion of monoterpenes, while leaves had a higher relative proportion of sesquiterpenes (Table 1). The essential oils from anthers had a higher relative proportion of monoterpenes hydrocarbon (t = 6.04, P = 0.021, Fig. 2) and a lower relative proportion of oxygenated sesquiterpenes compared to leaves (t = -5.5, P = 0.0061, Table 1). There were no significant differences in the relative proportion of oxygenated monoterpenes and sesquiterpene hydrocarbons between anthers and leaves (t = -0.953, P = 0.4 and t = -1.19, P = 0.325, respectively, Table 1). Among monoterpene hydrocarbons only the relative proportion of α-pinene, camphene, β-terpinene and limonene were significantly higher in anthers than in leaves (t=2.039, P=0.0338; t=3.99, P=0.048; t=2.039, P=0.0338; t=2.07, P=0.0071, respectively, Fig. 2). Additionally, the relative proportion of ledol and the unidentified oxygenated sesquiterpene did not differ statistically between anthers and leaves; however their abundance tends to be higher in leaves. Finally, it is

Table 1. Mean relative abundance (% ± SD) and richness of chemical compounds of the essential oil of peltate glandular trichomes from anthers and leaves of *Lepechinia floribunda*. Compounds are listed in order of elution from DB-5 column. RI: Retention Index. 1-Monoterpenes hydrocarbons, 2-Oxygenated monoterpenes, 3-Sesquiterpenes hydrocarbons and 4-Oxygenated sesquiterpenes. Different letters above means value show significant differences in the relative abundance of a compound between anthers and leaves.

RI DB-5	Compounds	Anther	Leaf	Methods of identification
936	α-Pinene[1]	8.6±5.07[a]	1.28±0.76[b]	GCMS.Co
953	Camphene[1]	5.61±1.34[a]	2.56±1.30[b]	GCMS
982	β-Terpinene1	15.53±4.79[a]	1.12±0.47[b]	GCMS
1034	Limonene[1]	17.51±2.66[a]	0.36±0.36[b]	GCMS.Co
1038	1,8-Cineole[2]	12.27±3.62[a]	13.03±6.66[a]	GCMS
1185	Borneol[2]	14.43±2.56[a]	17.91±4.7 [a]	GCMS
1204	Terpineol[2]	-	0.77±0.39	GCMS
1294	Bornyl Acetate[2]	-	1.22±0.34	GCMS
1438	β-Caryophyllene[3]	8.72±2.23[a]	9.61±2.96[a]	GCMS
1458	Aromadendrene[3]	-	3.18±0.31	GCMS
1475	α-Humulene[3]	2.65±0.44[a]	1.56±0.49[a]	GCMS
1510	Ledene[3]	0.34±0.61[a]	0.61±0.32[a]	GCMS
1573	Nerolidol[4]	-	0.75±0.37	GCMS
1602	Spathulenol[4]	0.52±0.91[a]	1.25±0.24[a]	GCMS
1608	Caryphyillene Oxide[4]	-	0.96±0.62	GCMS
1618	Guaiol[4]	-	1.54±2.67	GCMS.Co
1622	Unidentified Oxygenated Sesquiterpene[4]	9.38±7.53[a]	33.07±15.26[a]	GCMS
1633	Ledol[4]	3.82±2.45[a]	8.88±14.72[a]	GCMS
1684	α-Eudesmol[4]	-	0.25±0.43	GCMS
	Identified compounds	90.62	66.93	
	Monoterpene Hydrocarbons	47[a]	5[b]	
	Oxygenated Monoterpenes	27[a]	33[a]	
	Sesquiterpenes Hydrocarbons	12[a]	15[a]	
	Oxygenated Sesquiterpenes	14[a]	47[b]	

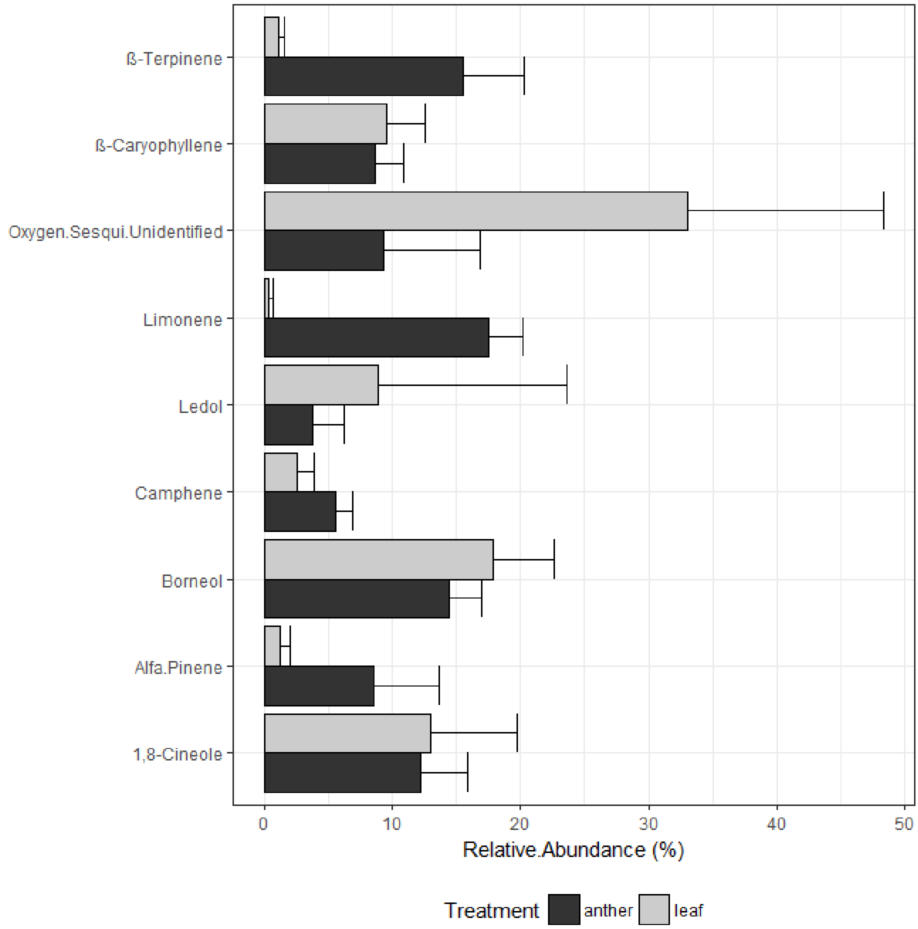

Fig. 2. Mean relative abundance (% ± SD) of the main chemical compounds of the essential oil of peltate glandular trichomes from anthers and leaves of *Lepechinia floribunda.*

notable that from the seven compounds absent in the essential oil from anthers (but present in leaves), four of them were oxygenated sesquiterpenes (Table 1).

Discussion

Much progress has been performed in order to disentangling the ecological function of chemical compounds produced in different organs of plants (Dobson *et al.,* 1999; Anderson *et al.,* 2002; Marín-Loaiza & Céspedes, 2007; Borghi *et al.,* 2017). Many studies have characterized the chemical composition of the essential oil of flowers and leaves in different species of the aromatic Lamiaceae (Werker *et al.*, 1985; Ascensão *et al.,* 1995; Borges *et al.,* 2006; Touati *et al.,* 2011), nonetheless essential oils from glandular trichomes located on reproductive organs has rarely been analyzed so far (Spring & Bienert, 1987; Duke & Paul, 1993; Gopfert *et al*., 2005). We found that the composition of the essential oils from peltate glandular trichomes located on anthers differ from those located on leaves.

The essential oil of peltate glandular trichomes from leaves had a significantly higher relative proportion of oxygenated sesquiterpenes than those obtained from anthers. The chemical composition of the essential oil from peltate glandular trichomes of leaves of *L. floribunda* resemble to those obtained from the leaves of *L. conferta* and *L. shiedeana,* where ledol had a high relative proportion (24.2% and 36.9% respectively) (Stashenko *et al.,* 1999; Borges *et al.,* 2006). Likewise, previous studies on another wild populations of *L. floribunda* from Córdoba province (Fester *et al.,* 1961; Viturro *et al.,* 2002; Duschatzky *et al.,* 2007), found a common pattern in chemical composition of leaves which was similar to our finding. Compounds like 1,8-cineole, limonene, borneol, camphene and β-caryophyllene has been reported as effective for the control of different kind of microorganism and against insect growth (Kessler & Baldwin, 2002; Bakkali *et al.,* 2008; Muhlemann *et al.,* 2014; Amby *et al.,* 2016). Consequently, the combination of chemical compounds found in the essential oil of peltate glandular trichomes from the leaves of *L. floribunda* would have a defensive function against herbivores and pathogens.

The essential oil of peltate glandular trichomes from anthers of *L. floribunda* has less richness of chemical compounds than peltate glandular trichomes from leaves. Moreover, the relative abundance of monoterpene hydrocarbons, specially α-pinene, camphene, β-terpinene and limonene, were ten times higher than in leaves. The lower richness of compounds observed in the essential oil of anthers trichomes was mainly due to the absence of many oxygenated sesquiterpens. A similar pattern has been previously observed in relation to the richness of compounds between the essential oil from flowers and leaves of other Lamiaceae such as *Hyssopus oficinalis* L. and *Ocimum basilicum* L. (Schulz & Stahl-Biskup, 1991; Chalchat & Özcan, 2008). In contrast, the opposite pattern has been observed in *Lavandula dentata* L., *Lepechinia conferta* and *Salvia sclarea* L. (Farka *et al.,* 2005; Borges *et al.,* 2006; Touati *et al.,* 2011). However, these studies did not allow to disentangling if the differential composition was due to a different kind of dominant trichomes at each organ or, if it was the same kind of trichome which changes its secretion products according to the its location in the plant. Here we demonstrated that chemical composition of peltate glandular trichomes varies with the location in the plant.

Interestingly, monoterpenes as camphene and limonene, found in higher relative abundance in anthers of *L. floribunda*, have been reported as compounds used by *Bombus* spp. to transmit information about rewarding floral resources to nestmates and thus promote floral constancy (Molet *et al.,* 2009). It suggests that these compounds can have a key function in the process of pollination in *L. floribunda*, as *Bombus* sp. are the most frequent floral visitors (Camina, 2018). Moreover, compounds such as camphene, β-Terpinene, 1,8-Cineole, limonene and β-caryophyllene, were founds in high proportions in anthers and they have demonstrated cytotoxic activities against floral pathogenic bacteria (Bakkali *et al.,* 2008; Junker *et al.,* 2011; Valiollahi *et al.,* 2014). Thus, the higher proportion of monoterpenes in the essential oil of anthers might act as both, pollinator attraction and microorganism defense. In Lamiaceae several developmental gland stages can occur simultaneously in a single leave, producing a short secretory phase of volatile compounds (Turner *et al*., 2000). Changes in the composition of secreted products during leaf maturation, in the same organ at different positions within the plant or in different parts of the same organ were reported (Maffei *et al*., 1989; Rohloff, 1999; Court *et al*., 1993; Hallahan, 2000). Thus, questions arise regarding to the potential changes in the composition of the essential oil from peltate glandular trichomes of the anthers and leaves of *L. floribunda*, Does its composition change throughout the maturation of the organ? Does it composition change among trichomes located at different positions at the same organ?.

In synthesis our study shows for the first time differential chemical composition (relative abundance and richness) in essential oils produced by the same type of glandular trichomes placed on different organs. These results suggest a high tissue specificity expression of genes in different organs (Stešević *et al.,* 2016). This gene specificity expression has been demonstrated in several species where genes are expressed or silenced depending on the organ (Glas *et al.,* 2012). Finally, such variation in chemical composition suggests different ecological functions for the same type of glandular trichome according to the organ.

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