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## Chemotaxonomic Markers in Essential Oils of *Hypenia* (Mart. ex Benth.) R. Harley

Maria T. Faria,<sup>a,b</sup> Deomar P. Costa,<sup>c</sup> Eliane C. Vilela,<sup>c</sup> Dalva G. Ribeiro,<sup>a</sup>  
Heleno D. Ferreira,<sup>b</sup> Suzana C. Santos,<sup>c</sup> José C. Seraphim<sup>d</sup> and Pedro H. Ferri<sup>\*,c</sup>

<sup>a</sup>Instituto de Biologia, Universidade de Brasília, CP 4451, 70910-900 Brasília-DF, Brazil

<sup>b</sup>Instituto de Ciências Biológicas, <sup>c</sup>Instituto de Química and <sup>d</sup>Instituto de Matemática e Estatística,  
Universidade Federal de Goiás, CP 131, 74001-970 Goiânia-GO, Brazil

O Cerrado Central brasileiro é considerado o centro de origem e dispersão de *Hypenia* (Lamiaceae: subtribe Hyptidineae) na América do Sul. O gênero tem sido mantido sem divisão, ou subdividido em duas ou cinco seções taxonômicas, baseadas quase exclusivamente a partir de um conjunto limitado de características florais. Neste trabalho, uma delimitação química interespecífica é descrita a fim de complementar as análises das características botânicas. Análise multivariada dos óleos essenciais de dez espécies na fase vegetativa suporta a divisão taxonômica do gênero em duas seções. Grupo I incluiu quatro espécies da seção *Laxiflorae* em adição à *H. irregularis*, os quais foram caracterizados pelos mais altos percentuais de espatulenol e óxido de cariofileno. Grupo II conteve cinco espécies da seção *Densiflorae* com  $\alpha$ -cadinol,  $\alpha$ -muuroleno e  $\alpha$ -muurolol como constituintes principais. Cadinanos e germacranos/aromadendranos parecem ser as classes marcadoras quimiotaxonômicas nas seções *Densiflorae* e *Laxiflorae*, respectivamente. Além disso, análise de redundância canônica não indicou correlação significante entre fatores edáficos com quimiovariação nos óleos essenciais, sugerindo que em *Hypenia* os óleos são constituídos por substâncias constitutivas e não induzidas pelo ambiente.

Central Brazilian Cerrado is regarded as the center of origin and dispersion of *Hypenia* (Lamiaceae: subtribe Hyptidineae) in South America. The genus has either remained undivided or been divided into two or seven taxonomic sections, based almost exclusively on a limited range of floral features. Interspecific chemical delimitation to complement analyses of botanical traits is described. Multivariate analysis of essential oils of ten species in the vegetative stage supports the taxonomic division in two sections. Cluster I included four species belonging to section *Laxiflorae* in addition to *H. irregularis*, which were characterized by the highest percentages of spathulenol and caryophyllene oxide. Cluster II contained five species belonging to section *Densiflorae* with  $\alpha$ -cadinol,  $\alpha$ -muurolene and  $\alpha$ -muurolol as major constituents. Cadinanes and germacranes/aromadendranes seem to be chemotaxonomic markers of sections *Densiflorae* and *Laxiflorae*, respectively. Furthermore, redundancy analysis revealed no significant correlation between edaphic factors and oil chemovariations, suggesting that *Hypenia* oils consist of constitutive rather than induced compounds.

**Keywords:** *Hypenia*, essential oil, chemical variability, chemotaxonomy, multivariate analysis

### Introduction

In Cerrado areas throughout Brazil and parts of Eastern South America, the recently redefined and amplified Lamiaceae family is mainly represented by the neotropical subtribe Hyptidineae, tribe Ocimeae, whose patterns of floristic and taxonomic variation have resulted in endemic genera and a large number of new species.<sup>1-3</sup> Furthermore,

it is characterized by sternotribic flowers whose stamens are held in the compressed lower lip of the corolla, forming an explosive pollination mechanism.<sup>4,5</sup>

Nine genera of the Hyptidineae have been identified, apparently in two separate clades: one represented by *Eriope* Humb. & Bonpl. ex Benth., *Hypenia* (Mart. ex Benth.) R. Harley and *Eriopidion* (Harley) and the other represented by *Hyptis* Jacq., together with *Peltodon* Pohl, *Marsypianthes* Mart. ex Benth., *Rhaphiodon* Schau., the only one restricted to Central America,

\*e-mail: pedro@quimica.ufg.br

*Asterohyptis* Epling and the genus *Hyptidendron* Harley.<sup>1,3</sup> *Hypenia* was separated from *Hyptis* section *Hypenia* based largely on the number of chromosomes, pollen surface and stem anatomy, as well as by traditional characters.<sup>1,4</sup> In addition, it represents a monophyletic group based on nuclear and plastid DNA.<sup>3</sup>

Its twenty seven recognized species have remained taxonomically undivided or have been distributed into sections *Densiflorae* Benth. and *Laxiflorae* Benth., based on lax or dense inflorescence.<sup>4,6</sup> Although Briquet<sup>7</sup> initially proposed seven sections, some species have now been assigned to the genus *Eriope*.<sup>5</sup> They are usually found in oligotrophic and sandy soils with high aluminum contents and low nutrient availability, and are spread over some regions of Venezuela, Bolivia, Paraguay and Southern Brazil. A new and unique species representative of the genus *Hypenia* in Mexico has recently been described.<sup>8</sup> In Brazil, they are more common in Cerrado regions which hold greater diversity and endemism.<sup>1,4</sup> Similarly to the correlated genus *Hyptis*, *Hypenia* species are aromatic and are frequently cited in Brazilian Cerrado for their ethnobotanical use, such as the infusion or decoction of leaves in the treatment of flu, common cold and other respiratory diseases.<sup>9</sup> Moderate radical scavenging and antioxidant activities of methanol extracts of leaves and stems of *H. salzmännii* (Benth.) R. Harley have also been reported.<sup>10</sup>

The botanical keys of the two *Hypenia* sections show that the characters used for their distinction derived almost exclusively from a limited range of floral features.<sup>6</sup> These difficulties may be partly attributed to probable hybridization between sympatric species, as in *Eriope*, in addition to the small number of specimens deposited in a herbarium.<sup>5</sup> For example, *H. paradisi* were, until recently, collected in just two field trips.<sup>4,11,12</sup> Since all of them are morphologically and anatomically similar, it is important to find alternative methods of interspecific chemical identification in order to complement floral trait analyses.

Recent studies described the chemical constituents of essential oils from thirteen Brazilian flowering *Hypenia* species, as well as compared the chemotaxonomic significance of essential oil chemovariations with botanical taxonomy by means of multivariate analysis, to determine taxa distribution patterns into two taxonomical sections.<sup>12</sup>

The results obtained for the chemical composition of essential oils of ten *Hypenia* taxa are now reported, including four new species, all of which were located in Central Brazilian Cerrado in the vegetative stage. Intraspecific variability was also verified by processing more than one population *per* taxon for three species.

## Results and Discussion

The Central Brazilian Cerrado region is regarded as the center of origin and dispersion of *Hypenia* in South America.<sup>5</sup> Despite the great diversity of species that live in these areas, essential oil composition is only known from flowering samples.<sup>12</sup> The lack of phenophase influence on chemovariation may be partly attributed to the small number of specimens from wild populations and which are deposited in the herbarium.<sup>13</sup> In addition, all of the *Hypenia* species are morphologically similar and floral traits are very important for their botanical identification.<sup>4,6</sup> In our study, *Hypenia* species were collected from distinct physiognomic sites in which the soil features are representative of the Cerrado fertility gradient.<sup>14</sup> The main soil features that showed the highest variations for the species under investigation were clay, silt, pH, Al<sup>3+</sup>, cationic exchange concentration (CEC), organic matter and macronutrients (K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, P).

Essential oil compositions were obtained from ten species in the vegetative stage, six of which belonged to section *Densiflorae* (*H. chapadensis* (Hcha), *H. inelegans* (Hine), *H. irregularis* (Hirr), *H. jorgeana* (Hjor), *H. paradisi* (Hpar) and *H. pruinosa* (Hpru)) and four of which belonged to section *Laxiflorae* (*H. calycina* (Hcal), *H. macrantha* (Hmac), *H. puchra* (Hpuc) and *H. rupestre* (Hrup)), following Epling's classification.<sup>6</sup> As regards plant collection, *H. chapadensis*, *H. jorgeana*, *H. puchra* and *H. rupestre* are currently undescribed species in the genus. A list of the taxa investigated, as well as their provenance and vouchers, are shown in the Supplementary Information (SI) section (Table S1).

Unlike their rich essential oil allies, such as *Hyptis*,<sup>5</sup> vegetative *Hypenia* species showed low essential oil yields (0.04 ± 0.02%) and failed to show significant differences in relation to flowering species,<sup>12</sup> which suggests that *Hypenia* may be an oil-poor genus. A total of eighty five oil constituents was identified (Table 1) and nearly half presented an average > 0.5%, accounting for 86-97% of the sampled data.

Essential oil compositions revealed a predominance of sesquiterpenes (43.9-100%), particularly high contents of sesquiterpene hydrocarbons in most species (23.4-83.9%). Oxygenated sesquiterpenes were the majority only in *H. irregularis* (Hirr, 74.7%). With the exception of population Hpru1 of *H. pruinosa*, which showed significant levels of aromatic compounds and esters (other constituents; 53.1%), all the other samples had lower levels of such compounds (< 10%).

Essential oil compositions of all *Hypenia* species contained  $\alpha$ -copaene (range of 0.74-12.5%, average value of 3.20%) and (*E*)-caryophyllene (1.99-27.2%,

**Table 1.** Percentages of essential oil constituents from *Hypenia* species<sup>b</sup> according to Epling's classifications

Constituent	RI <sup>a</sup>	<i>Laxiflorae</i> section					<i>Densiflorae</i> section									
		Hcal1	Hcal2	Hmac	Hpuc <sup>d</sup>	Hrup <sup>d</sup>	Hcha <sup>d</sup>	Hine	Hirr	Hjor1 <sup>d</sup>	Hjor2 <sup>d</sup>	Hpar	Hpru1	Hpru2	Hpru3	
1 Heptanal	897	–	–	–	–	–	–	–	–	–	0.52	–	–	–	0.37	–
2 Tricyclene	923	–	–	–	0.48	–	–	–	–	–	–	–	–	–	–	–
3 $\alpha$ -Pinene	929	–	–	–	1.51	–	–	–	–	–	–	–	–	0.49	–	–
4 (2 <i>E</i> )-Heptenal	949	–	–	–	–	–	–	–	–	–	0.34	–	–	–	1.09	–
5 $\beta$ -Pinene	972	–	1.80	–	3.18	–	–	–	–	–	–	–	–	0.58	–	–
6 Myrcene	986	–	–	–	0.78	–	–	–	–	–	–	–	–	–	–	–
7 $\rho$ -Cymene	1020	–	–	–	2.33	–	8.74	–	0.76	1.87	2.18	1.42	–	1.63	–	–
8 Limonene	1024	–	–	–	–	–	0.82	–	–	–	–	–	–	–	10.3	–
9 $\beta$ -Phellandrene	1025	–	2.24	–	4.41	–	–	–	–	–	–	–	–	–	–	–
10 1,8-Cineole	1028	–	–	–	6.50	–	–	–	–	0.93	0.96	–	–	–	–	–
11 $\gamma$ -Terpinene	1054	–	–	–	2.58	–	–	–	–	–	–	–	–	–	–	–
12 Acetophenone <sup>c</sup>	1063	–	–	–	–	–	–	–	–	–	–	–	–	21.4	–	–
13 Linalool	1096	–	–	0.35	1.77	–	0.52	–	1.10	–	–	–	–	1.14	–	0.48
14 <i>trans</i> -Thujone	1113	–	–	–	–	–	1.49	–	–	–	–	–	–	–	–	–
15 (2 <i>E</i> )-Nonen-1-al	1155	–	–	–	–	–	–	–	–	0.66	–	–	–	–	–	–
16 $\alpha$ -Copaene <sup>c</sup>	1374	1.86	12.5	0.99	1.14	4.38	3.01	1.66	1.13	2.48	2.90	0.74	1.75	1.66	8.61	–
17 Geranyl acetate	1381	1.13	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18 $\beta$ -Bourbonene	1382	2.33	2.08	2.01	–	2.52	2.06	1.45	1.39	1.29	1.50	2.17	1.20	1.72	1.37	–
19 $\beta$ -Elemene	1389	1.48	–	1.17	–	2.44	3.72	–	–	–	–	–	1.11	–	–	–
20 Methyl eugenol	1400	–	–	–	–	–	–	–	–	–	–	–	0.91	–	–	–
21 Longifolene	1407	–	–	–	–	–	–	–	–	–	–	–	0.64	–	–	–
22 $\alpha$ -Gurjunene	1412	–	–	–	0.66	–	–	–	–	–	–	–	–	–	–	–
23 $\beta$ -Funebrene	1415	–	–	–	–	–	–	–	–	1.36	1.64	–	–	–	–	–
24 ( <i>E</i> )-Caryophyllene <sup>c</sup>	1417	5.39	27.2	4.33	12.4	8.07	3.88	9.01	3.71	3.69	4.45	3.81	1.99	2.01	4.36	–
25 $\beta$ -Copaene	1427	–	–	0.37	–	–	–	0.66	–	–	–	–	–	–	–	–
26 $\alpha$ - <i>trans</i> -Bergamotene	1434	–	–	–	–	–	–	0.66	–	–	–	–	1.39	–	1.74	–
27 $\alpha$ -Guaiene	1436	–	–	–	–	–	–	–	–	–	–	–	0.58	–	–	–
28 Aromadendrene	1437	–	–	–	1.65	–	–	–	–	–	–	–	–	–	–	–
29 Neryl propanoate <sup>c</sup>	1450	–	–	–	–	–	–	–	–	–	–	–	12.8	–	–	–
30 $\alpha$ -Humulene	1451	1.64	2.35	1.15	1.34	1.20	2.47	6.13	1.03	1.35	1.63	2.67	–	–	3.07	–
31 Geranyl acetone <sup>c</sup>	1451	–	–	–	–	–	–	–	–	2.34	2.81	1.70	–	1.60	3.62	–
32 <i>allo</i> -Aromadendrene	1458	–	–	–	–	–	–	–	–	–	–	–	–	–	3.89	–
33 <i>trans</i> -Cadina-1(6),4-diene <sup>c</sup>	1474	–	–	–	–	–	–	2.77	1.49	3.70	2.99	2.74	1.55	–	–	–
34 $\gamma$ -Gurjunene	1474	–	–	–	–	–	1.88	–	–	–	–	–	–	3.84	0.73	–
35 $\gamma$ -Muurolene <sup>c</sup>	1479	18.9	–	33.8	0.72	–	4.08	12.5	5.91	7.71	8.71	23.5	2.39	6.17	7.20	–
36 $\gamma$ -Himachalene	1484	–	–	0.31	–	–	–	0.59	–	1.39	1.50	1.10	–	–	–	–
37 Germacrene D <sup>c</sup>	1484	–	27.2	–	–	38.7	–	–	–	–	–	–	–	–	–	–
38 $\beta$ -Selinene <sup>c</sup>	1484	–	2.78	–	–	–	2.12	–	–	–	–	–	–	2.65	5.07	2.13
39 $\delta$ -Selinene <sup>c</sup>	1493	–	–	–	18.2	–	3.04	–	–	–	–	–	–	3.65	6.23	5.81
40 Indipone	1493	–	–	–	–	–	–	–	–	3.56	3.44	–	–	–	–	–
41 Bicyclogermacrene <sup>c</sup>	1495	10.3	1.80	19.6	–	6.55	–	6.56	4.66	3.08	3.62	9.06	–	–	–	–
42 $\alpha$ -Muurolene <sup>c</sup>	1497	–	–	–	–	0.74	4.00	0.95	–	7.80	9.33	3.68	1.94	5.32	2.21	–
43 Germacrene A	1503	–	–	0.90	–	0.86	2.80	–	–	–	–	–	1.06	–	–	–
44 $\gamma$ -Cadinene <sup>c</sup>	1511	1.21	1.74	0.98	0.67	–	1.34	1.97	1.10	2.96	3.14	3.71	2.19	4.35	1.62	–
45 <i>trans</i> -Cycloisolongifol-5-ol	1514	–	–	–	5.37	–	–	–	–	–	–	–	–	–	–	–
46 Cubebol	1514	–	–	–	–	2.16	–	0.50	–	–	–	–	–	–	–	–
47 <i>trans</i> -Calamenene <sup>c</sup>	1520	–	–	–	–	–	2.29	–	–	4.96	5.58	–	–	3.42	–	–
48 $\delta$ -Cadinene <sup>c</sup>	1521	2.18	4.39	1.49	1.50	5.27	–	4.73	3.00	–	–	4.26	5.96	–	7.58	–

Table 1. continuation

Constituent	RI <sup>a</sup>	<i>Laxiflorae</i> section					<i>Densiflorae</i> section									
		Hcal1	Hcal2	Hmac	Hpuc <sup>d</sup>	Hrup <sup>d</sup>	Hcha <sup>d</sup>	Hine	Hirr	Hjor1 <sup>d</sup>	Hjor2 <sup>d</sup>	Hpar	Hpru1	Hpru2	Hpru3	
49 Zonarene	1524	–	–	–	–	–	–	–	–	–	–	–	–	0.36	–	0.50
50 $\alpha$ -Cadinene	1535	–	–	–	–	–	–	–	–	–	–	1.08	–	–	–	
51 $\alpha$ -Calacorene <sup>c</sup>	1539	–	–	–	–	–	1.84	–	–	3.73	3.79	2.76	–	2.60	–	
52 Italicene epoxide	1550	0.67	–	–	–	–	–	–	–	–	–	–	–	–	–	
53 Germacrene B	1555	0.71	1.81	0.58	–	–	0.99	10.3	–	–	–	–	–	–	–	
54 Caryophyllenyl alcohol	1568	–	–	–	–	–	1.26	–	–	–	–	–	–	–	–	
55 Caryolan-8-ol <sup>c</sup>	1569	–	–	–	–	–	–	–	–	5.00	4.72	–	–	–	–	
56 Spathulenol <sup>c</sup>	1581	30.4	3.50	11.1	9.56	6.25	3.13	7.63	47.8	3.21	3.33	3.79	–	4.07	6.61	
57 <i>trans</i> -Sesquisabinene hydrate	1581	–	–	–	–	–	4.85	–	–	1.73	–	–	2.77	1.81	–	
58 Caryophyllene oxide <sup>c</sup>	1584	12.5	6.29	4.07	6.28	7.44	1.37	5.19	13.4	2.13	4.18	4.13	–	6.04	3.88	
59 Globulol <sup>c</sup>	1589	–	–	1.87	10.1	5.20	–	–	–	1.42	–	–	0.68	–	3.32	
60 ( <i>E</i> )-Dihydroapofarnesol	1590	–	–	–	–	–	–	–	–	–	–	–	–	7.80	–	
61 Guaiol	1600	–	–	–	–	–	–	–	–	1.17	–	–	–	–	–	
62 Ledol	1601	–	–	–	–	–	–	–	–	–	–	–	–	–	1.84	
63 Humulene epoxide	1608	0.86	–	0.90	–	–	1.86	2.62	1.51	1.05	–	–	–	–	0.98	
64 Junenol	1616	–	–	–	–	–	–	2.09	–	–	–	–	–	–	–	
65 $\alpha$ -Corocalene	1621	–	–	–	–	–	–	–	–	0.91	–	–	–	–	–	
66 1- <i>epi</i> -Cubenol <sup>c</sup>	1627	–	–	–	0.41	–	1.08	–	1.45	3.96	2.49	1.54	0.89	2.46	3.63	
67 Muurolo-4,10(14)-dien-1 $\beta$ -ol	1627	–	–	–	–	–	–	4.98	–	–	–	–	–	–	–	
68 $\gamma$ -Eudesmol	1631	–	–	–	–	–	–	–	–	0.62	–	–	–	–	–	
69 <i>epi</i> - $\alpha$ -Cadinol	1637	–	–	–	1.28	–	–	–	–	–	–	8.90	2.88	–	7.27	
70 <i>allo</i> -Aromadendrene epoxide	1637	–	–	–	–	–	–	–	1.06	–	–	–	–	–	–	
71 Selina-3,11-dien-6 $\alpha$ -ol	1638	–	–	1.06	–	–	–	1.01	–	–	–	–	–	–	–	
72 $\alpha$ -Muurolol <sup>c</sup>	1641	–	–	1.98	–	–	4.45	3.72	1.66	8.88	10.5	–	–	6.71	–	
73 Cubenol <sup>c</sup>	1645	–	–	0.77	–	0.99	2.25	1.48	1.17	3.68	4.40	2.59	–	2.24	2.11	
74 Pogostol	1654	–	–	8.80	–	–	–	–	–	–	–	–	–	–	–	
75 $\alpha$ -Cadinol <sup>c</sup>	1654	3.27	–	–	1.47	–	–	8.07	3.18	4.34	5.92	11.2	6.28	7.09	7.37	
76 Selin-11-en-4 $\alpha$ -ol	1660	–	2.31	–	–	4.53	27.1	–	–	–	–	0.37	–	–	–	
77 <i>cis</i> -Calamenen-10-ol	1661	–	–	–	–	–	–	–	–	0.55	–	–	–	–	–	
78 14-Hydroxy-9- <i>epi</i> -( <i>E</i> )-caryophyllene	1670	1.41	–	–	0.56	–	–	–	1.46	–	–	–	–	–	–	
79 Cadalene	1672	–	–	–	–	–	0.84	–	–	2.81	2.70	1.92	–	2.32	–	
80 Mustakone <sup>c</sup>	1676	2.25	–	–	–	–	–	–	–	–	–	–	–	–	–	
81 Eudesma-4(15),7-dien-1 $\beta$ -ol <sup>c</sup>	1685	1.54	–	1.46	–	2.69	–	1.22	1.97	–	–	–	–	–	–	
82 Eudesm-7(11)-en-4-ol	1694	–	–	–	–	–	0.68	0.68	–	–	–	–	–	–	–	
83 Benzyl benzoate	1759	–	–	–	–	–	–	–	–	–	–	–	0.60	–	–	
84 Hexahydrofarnesyl acetone <sup>c</sup>	1843	–	–	–	–	–	–	–	–	–	1.61	–	9.58	–	2.72	
85 ( <i>5E,9E</i> )-Farnesyl acetone <sup>c</sup>	1915	–	–	–	–	–	–	–	–	–	–	–	7.87	–	3.45	
Monoterpene hydrocarbons		–	4.04	–	12.7	–	9.56	–	0.76	1.87	2.18	1.42	1.07	11.94	–	
Oxygenated monoterpenes		–	–	0.35	10.9	–	2.01	–	1.10	0.93	0.96	–	1.14	–	0.48	
Sesquiterpene hydrocarbons		46.0	83.9	67.7	38.3	72.9	40.4	60.3	23.4	52.8	56.9	63.2	30.4	44.7	50.8	
Oxygenated sesquiterpenes		52.9	12.1	32.0	35.0	27.1	48.1	38.7	74.7	37.7	35.5	32.6	13.5	38.2	37.0	
Others		1.13	–	–	–	–	–	–	–	3.86	4.42	1.70	53.1	3.06	9.8	
Oil yield / %, m/m		0.04	0.04	0.03	0.06	0.05	0.06	0.03	0.02	0.04	0.03	0.05	0.05	0.09	0.03	
Total identified		99.9	99.9	100	90.9	100	99.9	99.0	99.9	96.3	100	98.8	98.2	96.5	98.1	

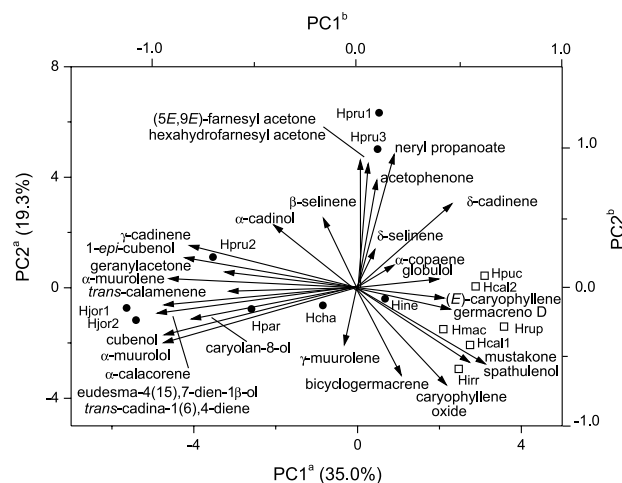
<sup>a</sup>Retention index; <sup>b</sup>*Hypernia* samples: Hcal = *H. calycina*; Hcha = *H. chapadensis*; Hine = *H. inelegans*; Hirr = *H. irregularis*; Hjor = *H. jorgeana*; Hmac = *H. macrantha*; Hpar = *H. paradisi*; Hpru = *H. pruinosa*; Hpuc = *H. puchra*; Hrup = *H. rupestre*; <sup>c</sup>selected for PCA/HCA analyses; <sup>d</sup>new species; –: not detected.

average value of 6.74%). The most abundant constituents were spathulenol (0-47.8%, 10.1%), caryophyllene oxide (0-13.4%, 5.50%) and germacrene D (0-38.7%, 4.71%), which showed high values in species from section *Laxiflorae* with the addition of *H. irregularis*. However,  $\alpha$ -cadinol (0-11.2%, 4.16%) and  $\alpha$ -muurolene (0.95-9.33%, 2.57%) were the main constituents in species from section *Densiflorae* with the exception of *H. irregularis*.  $\gamma$ -Muurolene (0-33.8%, 9.40%), (*E*)-caryophyllene and bicyclogermacrene (0-19.6%, 4.66%) showed high contents in both botanical sections. These results are in agreement with previous studies as regards flowering *Hypenia* essential oils,<sup>12</sup> although they differ in the low content of selin-11-en-4 $\alpha$ -ol (vegetative samples: 0-27.1%, 2.45%; flowering samples: 0-34.8%, 9.76%) and a variety of minor constituents. High contents of (*E*)-caryophyllene and derivatives in *Hypenia*, which are known to prevent herbivory regardless of the developmental stages in question,<sup>15</sup> suggest that these essential oil constituents may provide *Hypenia* with selective advantages over native species in terms of habitat adaptation and consequently of high endemism.

Despite the fact that the sampling sites showed a relatively significant difference as regards soil chemical composition and texture, canonical redundancy analysis (RDA) revealed no correlation between edaphic variables and essential oil chemovariations. A significance test with an unrestricted Monte Carlo permutation technique (999 permutations) found Fischer's *F*-ratio for the first canonical axis (*F*-value = 1.087; *p* = 0.439) and for all canonical axes (*F* = 1.040; *p* = 0.394), giving signs that patterns did arise by chance.<sup>16</sup> A strong but non-significant intersite correlation (*R* = 0.995; first RDA axis, *p* = 0.085; trace statistics, *p* = 0.054) was observed between clay, silt, Al<sup>3+</sup>, Mn<sup>2+</sup> and Zn<sup>2+</sup> contents and *H. calycina*, *H. jorgeana* and *H. pruinosa* collected at different sampling sites. These results suggest that *Hypenia* oils were genetically rather than environmentally influenced, in agreement with previous studies that reported genetic control in essential oil variations of flowering *Hypenia* species.<sup>12</sup> Therefore, essential oil chemovariations in the vegetative stage may also contribute to chemotaxonomic or phylogenetic relationships within the genus. Similar results were described for species of *Hypericum* L. (Hypericaceae) from different locations and phenophases. Even though no major differences were observed in essential oil compositions within each species, the range of dominant constituents supports the sectional separation of species.<sup>17</sup>

To assess the use of essential oil constituents in identifying taxonomic relationships among species, multivariate analysis by principal components analysis (PCA) and nearest neighbor complete linkage cluster

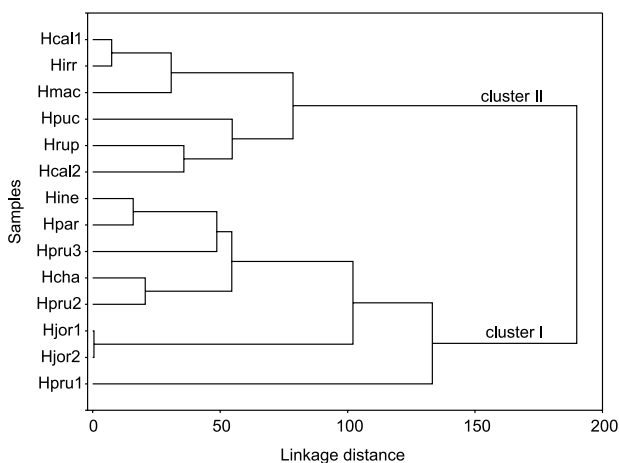
analysis were initially performed with oil constituent levels  $\geq 0.5\%$  (14 samples  $\times$  41 variables = 574 pieces of data). After the variable modeling which aimed to stabilize the hierarchical structure (final matrix, 14  $\times$  28 = 392), the relative position of the PCA based taxa is shown in Figure 1. The first PC accounts for 35.0% of total variance and separates populations of *H. calycina* (Hcal1 and Hcal2), *H. irregularis* (Hirr), *H. macrantha* (Hmac), *H. puchra* (Hpuc) and *H. rupestre* (Hrup) above the 97% confidence level from other specimens according to the highest contents of spathulenol, germacrene D, (*E*)-caryophyllene and caryophyllene oxide. The second PC (19.3% of total variation) distinguishes (*p* < 0.005) *H. pruinosa* populations (Hpru1, Hpru2 and Hpru3) mainly because of the highest contents of neryl propanoate and  $\alpha$ -cadinol.



**Figure 1.** PCA biplot of *Hypenia* species essential oil, according to the clusters they belong to: I (□); II (●). <sup>a</sup>Axes refer to scores from the samples. <sup>b</sup>Axes refer to loadings from oil constituents represented as vectors from the origin. Values between parentheses refer to the explained variance on each principal component. *Hypenia* samples: Hcal = *H. calycina*; Hhcha = *H. chapadensis*; Hhine = *H. inelegans*; Hhrr = *H. irregularis*; Hhjr = *H. jorgeana*; Hhmac = *H. macrantha*; Hhpar = *H. paradisi*; Hhpru = *H. pruinosa*; Hhpu = *H. puchra*; Hhru = *H. rupestre*.

Therefore, two types of essential oils were identified. Cluster I revealed four species of the section *Laxiflorae*, in addition to *H. irregularis*, which were characterized (*p* < 0.009) by the highest percentages of spathulenol (18.1  $\pm$  17.4%), (*E*)-caryophyllene (10.2  $\pm$  8.90%) and caryophyllene oxide (8.34  $\pm$  3.76%). Cluster II revealed five species of the section *Densiflorae* containing  $\alpha$ -cadinol (6.29  $\pm$  3.23%),  $\alpha$ -muurolene (4.40  $\pm$  2.93%),  $\alpha$ -muurolol (4.28  $\pm$  4.15%) and  $\gamma$ -cadinene (2.66  $\pm$  1.06%) as the main constituents. Figure 2 shows the similarities between taxa in terms of Euclidean distances, all of which originated from the cluster analysis via PC scores.

A similar hierarchical structure emerges when it is considered the oil constituents grouped together according



**Figure 2.** Dendrogram representing similarity relationships among *Hypenia* species based on essential oil constituents, according to the clusters they belong to: I and II. *Hypenia* samples: Hcal = *H. calycina*; Hcha = *H. chapadensis*; Hine = *H. ineleigans*; Hirr = *H. irregularis*; Hjor = *H. jorgeana*; Hmac = *H. macrantha*; Hpar = *H. paradisi*; Hpru = *H. pruinosa*; Hpuc = *H. puchra*; Hrup = *H. rupestre*.

to carbon skeletons. Cluster II was clustered and cluster I was mainly split based on samples further displaced to the right of the PC-1 axis (Hrup and Hcal2), which showed a high (low) content of germacrane (bicyclogermacrane) (Figures S1 and S2 in the SI section). The analysis of variance (ANOVA) showed that cluster I revealed significant amounts of caryophyllanes ( $19.3 \pm 8.14\%$ ) and aromadendranes ( $21.7 \pm 16.3\%$ ), including tricyclane, isolongifolane and italicane as minor constituents ( $< 1\%$ ). On the other hand, cluster II revealed cadinanes ( $45.1 \pm 16.9\%$ ) as the main biosynthetic class, but with the unique occurrence of sesquisabinane, farnesane, indipane, bergamotane, cedrane, thujane and longifolane as minor constituents ( $< 1.4\%$ ). Percentages of oil constituents (or of the constituents that were rearranged according to carbon skeletons) in clustered samples are shown in the SI section (Tables S2 and S3).

Canonical discriminant analysis (CDA) on chemical data confirmed *a priori* clusters. An axial representation of CDA results discriminated the two groups based only on the contents of  $\alpha$ -cadinol and caryophyllene oxide as predictor variables. The fitted model showed high canonical correlation ( $R=0.897$ ) and significant Wilks' lambda (0.195;  $p < 0.0001$ ), which accounts for a multivariate measure of group differences over both variables.<sup>18</sup> The discriminant function explained the overall variability ( $F = 22.74$ ; degree of freedom,  $DF = 2$  and  $11$ ;  $p < 0.0001$ ) and distinguished cluster I from II due to high positive and negative scores of caryophyllene oxide and  $\alpha$ -cadinol, respectively. It was also possible to make an accurate prediction of ca. 94% well-classification in the original clusters via the cross-validation approach.<sup>18</sup> This shows that the entire group

contributes to discrimination, not only individual plants. The only predicted mismatched classification referred to a sample originating from cluster II (Hcha) which had been classified as belonging to cluster I. Such misclassification might have been caused by a lower level of  $\alpha$ -cadinol in the sample, which is characteristic of cluster I.

In terms of individual constituents or carbon skeleton contributions to the taxonomic profile, the finding according to which major terpenes such as caryophyllanes/aromadendranes and cadinanes significantly contributed to separate clusters I and II, respectively, is of particular interest. In fact, flowering *Hypenia* species from the section *Densiflorae* were distinguished based on cadinane skeleton, whereas germacrane/bicyclogermacrane separate *Laxiflorae* flowering individuals.<sup>12</sup> Apparently, germacrane (via bicyclogermacrane) were converted to aromadendranes, present in the vegetative stage as a discriminant biosynthetic group. Thus, cadinanes such as  $\alpha$ -muurolol and  $\alpha$ -cadinol could be chemotaxonomic markers in *Densiflorae* species regardless of vegetative or flowering stages, whereas germacrane/bicyclogermacrane (e.g. germacrene D and bicyclogermacrane) or aromadendrane biosynthetic pathway (e.g. spathulenol) could be chemotaxonomic markers in *Laxiflorae* species in flowering or vegetative phenophases, respectively.

Terpenes were described as chemomarkers in other genera,<sup>19</sup> such as *Helichrysum* (Asteraceae) and *Curcuma* (Zingiberaceae) and were proven to be particularly helpful in assessing the taxonomic relationships of several *Lamiaceae* genera.<sup>20</sup> In addition, qualitative and quantitative variations in essential oils during plant developmental stages were reported and chemovariations showed ecological and taxonomical significances.<sup>21</sup>

These results support the current division of *Hypenia* into two taxonomic sections due to remarkable differences in essential oil compositions. Differences in essential oil constituents among *Hypenia* sections during the developmental stages may be useful for understanding phylogenetic relationships, especially in view of the fact that its species are not easily identified and are not always found during flowering in wild populations. Moreover, the results suggest that the delimitation of *H. irregularis* in the section *Densiflorae* is more appropriate if applied to the section *Laxiflorae*. In fact, preliminary results involving a new taxonomic investigation of this species confirm the amendment from the original taxonomic section.

## Conclusions

Essential oil analyses of ten *Hypenia* species found in Central Brazilian Cerrado have revealed high chemical

polymorphism, possibly related to genetic influences. Furthermore, the two clustered samples based on multivariate analyses of essential oil chemovariations support the division of species into two taxonomic sections. Cadinane and caryophyllanes/germacranes/aromadendranes seem to be chemomarkers of sections *Densiflorae* and *Laxiflorae*, respectively.

## Experimental

### Plant material

*Hypenia* samples were collected in the vegetative stage between November 2010 and December 2011 in Goiás State, Brazil (Table S1 in the SI section). Three species were collected from different sampling sites in order to investigate the edaphic influence on oil compositions. The same sampled populations in the flowering stage were identified by one of the authors (M. T. F.) and by Raymond M. Harley, PhD, from the Royal Botanic Gardens, Kew. Vouchers were deposited at the Herbarium of Universidade Federal de Goiás (UFG), Goiás State, Brazil.

### Essential oil extraction

To assess essential oils, 3-5 individuals of each species which originated from 2-3 local populations were pooled and dried at room temperature for seven days at 30 °C until constant weight. After powdering, the dried phytomass (5-30 g) of each sample was submitted to hydrodistillation (2 h) using a modified Clevenger-type apparatus. At the end of each distillation, the oils were collected and dried with anhydrous Na<sub>2</sub>SO<sub>4</sub>, then transferred to glass flasks where they were kept at a temperature of -18 °C. Oil yields (%) were based on the dried weight of plant samples.

### Soil analyses

Soil samples were collected at a 20 cm depth in all sampling sites and around each population. Then, they were pooled together to form a composite sample for each site. After that, they were air-dried, thoroughly mixed, and sieved (2 mm). The portion finer than 2 mm was kept for physical and chemical analyses. The pH value was determined in a 1:1 soil/water volume ratio. The cations Ca<sup>2+</sup>, Mg<sup>2+</sup> and Al<sup>3+</sup> were extracted with 1 mol L<sup>-1</sup> KCl, whereas P, K<sup>+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Fe<sup>3+</sup> and Mn<sup>2+</sup> were extracted with Mehlich solution. Organic matter, cationic exchange capacity (CEC), potential acidity (H + Al<sup>3+</sup>) and soil texture were determined by the usual methods.<sup>22</sup> Soil

parameters were ordered in an environmental data matrix with 16 variables for each sampling site. Organic matter, soil textures and other variables were arcsine and log(x + 1)-transformed, respectively.

### Essential oil analyses

Oil sample analyses were performed on a gas chromatography-mass spectrometry (GCMS) Shimadzu QP5050A instrument under the following conditions: (i) a CBP-5 (Shimadzu) fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) connected to a quadrupole detector operating in the EI mode at 70 eV with a scan mass range of 40-400 m/z at a sampling rate of 1.0 scan s<sup>-1</sup>; (ii) carrier gas: He (1 mL min<sup>-1</sup>); (iii) injector and interface temperatures of 220 and 240 °C, respectively, with a split ratio of 1:20. The injection volume was 0.4 μL (20% in hexane) and the oven temperature was raised from 60 to 246 °C with an increase of 3 °C min<sup>-1</sup>, then of 10 °C min<sup>-1</sup> to 270 °C, holding the final temperature for 5 min.

Essential oil constituents were identified by comparing their mass spectra with those from the National Institute of Standards and Technology (NIST),<sup>23</sup> and by comparing mass spectra and calculated linear retention indices (RI) with values found in the literature,<sup>24</sup> standards, commercial ylang-ylang (*Cananga odorata* (Lam.) Hook. F. & Thoms., Annonaceae), sage clary (*Salvia sclarea* L., Lamiaceae) and tea-tree (*Melaleuca alternifolia* Cheel, Myrtaceae) essential oils. Retention indices were obtained by co-injection with a mixture of linear hydrocarbons, C<sub>8</sub>-C<sub>32</sub> (Sigma, USA), and by the equation of Dool and Kratz.<sup>25</sup> Total ion chromatograms (TIC) of *Hypenia* essential oils, standards and commercial essential oils are shown in the SI section (Figures S3 to S20).

### Statistical analyses

PCA and cluster (HCA) analyses using SPAD data mining<sup>26</sup> were applied to assess the interrelationships between taxa and essential oil constituents. Nearest neighbor complete linkage technique by Benzécri algorithm was used as an index of similarity,<sup>27</sup> and hierarchical clustering was performed according to Ward's variance minimizing method.<sup>28</sup> Oil constituents with ≥ 0.5% arbitrary amounts to the chemical profiles (average values) were initially kept in the original matrix. For variable selection, the threshold of residual eigenvalues (≤ 0.70) in the data matrix was used to establish the maximum number of variables that could be removed. The variables which were effectively eliminated



revealed the highest loadings in residual eigenvalues.<sup>18</sup> Prior to the multivariate analysis, the final oil data matrix (14 samples  $\times$  28 variables = 392 pieces of data) was processed by mean centering and auto-scaling.

RDA was applied to describe the patterns of the only explained variation of interrelationships between essential oil constituents, including biosynthetic class and oil yields (14  $\times$  92 = 1288), in addition to the interspecific variations as a function of soil parameters (14  $\times$  16 = 224), treated as environmental variables.<sup>16</sup> An unrestricted Monte-Carlo permutation test (999 permutations) was used to test the eigenvalue significance of the first canonical axis as well as the sum (trace) of all canonical axes. RDA was performed in CANOCO for Windows,<sup>29</sup> and oil constituents were arcsine-transformed. Discriminant analysis using SAS CANDISC and SAS DISCRIM procedures were used to distinguish taxa and clusters on the basis of essential oil composition.<sup>30</sup> The predictive ability of canonical discriminant function was assessed by a cross-validation approach as implemented in SAS.

Multiple comparisons were established by ANOVA using SAS general linear model (GLM) procedure. All data were checked for homoscedasticity with the use of Hartley's test. Whenever heteroscedasticity was observed, the variable was angular or rank-transformed. In addition, whenever a difference in ANOVA was established, a Tukey's *post-hoc* test was performed. Results are shown by mean values and are joined by the standard deviation of independent measurements. *p*-Values below 0.05 were regarded as significant.

## Supplementary Information

Supplementary data (Tables S1-S3, Figures S1-S20) are available free of charge at <http://jbcbs.sbg.org.br> as a PDF file.

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