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Article

Chemotaxonomic Significance of Volatile Constituents in *Hypenia* (Mart. ex Benth.) R. Harley (Lamiaceae)

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A análise multivariada da composição química dos óleos essenciais de treze espécies de *Hypenia* indicou a presença de dois grupos de óleos em relação às seções botânicas das amostras. O primeiro grupo (grupo I) incluiu as três espécies da seção *Densiflorae* em adição a *H. subrosea* e *H. aristulata*, o qual foi caracterizado pelo maior percentual de α-muurolol (5,85 ± 3,08%). No grupo II, com oito espécies da seção *Laxiflorae*, os principais constituintes discriminantes foram o (E)-cariofileno (7,09 ± 4,88%), germacreno D (18,1 ± 11,4%) e o biciclogermacreno (6,65 ± 1,19%). Todos os óleos essenciais apresentaram predominantemente sesquiterpenos, tais como espatulenol (4,5-31,6%), óxido de cariofileno (2,2-14,4%) e selin-11-en-4α-ol (0-34,8%). Os agrupamentos foram idênticos quando utilizada a análise multivariada baseada nos esqueletos carbônicos dos constituintes químicos ou de 18 caracteres morfológicos das folhas das espécies.

Multivariate analysis of essential oil compositions of thirteen *Hypenia* species revealed the presence of two taxonomic clusters. Cluster I included three species belonging to section *Densiflorae* in addition to *H. subrosea* and *H. aristulata*, and showed the highest percentages of α-muurolol (5.85 ± 3.08%). In Cluster II, which contained eight species belonging to section *Laxiflorae*, the major discriminant constituents were (E)-caryophyllene (7.09 ± 4.88%), germacrene D (18.1 ± 11.4%), and bicyclogermacrene (6.65 ± 1.19%). All essential oils showed a predominance of sesquiterpenes, such as spathulenol (4.5-31.6%), caryophyllene oxide (2.2-14.4%) and selin-11-en-4α-ol (0-34.8%). Furthermore, identical clusters were revealed by multivariate analysis of chemical constituents based on carbon skeletons, as well as on 18 morphological leaf characters of the species studied.

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

Introduction

The Lamiaceae family includes approximately 258 genera and 7193 species. Genera such as *Salvia* and *Scutellaria* have a wide and cosmopolitan distribution, although lamiaceous plants are especially abundant in the Mediterranean region.[1,2] In Brazil and other Cerrado areas of eastern South America, the Lamiaceae family is mainly represented by the subtribe Hyptidinae. It is characterized by sternotribic flowers with stamens held in the compressed lower lip of the corolla, which forms an explosive pollination mechanism.[2] A total of nine genera of the neotropical subtribe Hyptidinae are now recognized. *Hypenia* (Mart. ex Benth.) R. Harley was recently separated from *Hyptis* Jacq. section *Hypenia* based largely on number of chromosomes and morphological aspects.[2]

Hypenia contains 27 recognized species on the basis of lax or dense inflorescences including sections *Densiflorae* Benth. and *Laxiflorae* Benth.[2,3] *Hypenia* species are usually found in oligotrophic and sandy soils with high levels of aluminum, and are distributed over some regions of Venezuela, Bolivia, Paraguay and southern Brazil. In Brazil, they are more common in Cerrado regions where a greater diversity and endemism may be found. *Hypenia* species are aromatic and are frequently reported in Brazilian Cerrado for their ethnobotanical use, such as the infusion or decoction

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of leaves in the treatment of the flu, common cold and other respiratory diseases.[3,4] Moderate radical scavenging and antioxidant activities of methanol extracts of the leaves and stems of *H. salzmannii* (Benth.) R. Harley are also reported.[5]

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.[3] These difficulties may be partly attributed to the small number of specimens deposited in the herbarium. For example, *H. paradisi* has been collected in only two field trips and *H. concinna* Benth. is known only from the type species.[2,3] Since all of them are morphologically and anatomically similar, it is important to find alternative methods of interspecific chemical identification in order to complement analyses of floral traits.

Therefore, this research investigates the chemical constituents of essential oils of thirteen unknown species of Brazilian *Hypenia*, thus contributing to future taxonomic studies of the genus. We analyzed disability data, as well as species considered rare in Brazil.[6] In light of the possible chemotaxonomic significance of the oils, results from the chemical analysis were compared with leaf anatomy and taxonomy. For this purpose, essential oils from individuals of representative populations were evaluated by a gas chromatography coupled with mass spectrometer (GC-MS). To study chemical variability, compounds in oil samples and morphological data were submitted to multivariate analysis for determination of taxa distribution patterns and identification of oil constituents, which may be distinguished among the groups of species.

Results and Discussion

Despite the great diversity of *Hypenia* species in Brazilian Cerrado areas, the composition of volatile compounds is only known for *H. salzmannii*.[7] In our study, essential oil compositions were obtained from thirteen species in the inflorescence phenophase, of which three belonged to section *Densiflorae* (*H. brachystachys*, *H. marifolia*, *H. paradise*) and ten belonged to section *Laxiflorae* (*H. sphaerocephala*, *H. durifolia*, *H. crispata*, *H. reticulata H. macrosiphon*, *H. macrantha*, *H. aristulata*, *H. subrosea* and *H. niquelandiensis*). The provenance and voucher specimens are shown in Table S1, at the Supplementary Information (SI).

All *Hypenia* species investigated contained essential oils ranging from 0.01 to 0.13% based on dry weight (Table S2). The low oil yields were in agreement with those reported for *H. salzmannii*, which suggests that *Hypenia* may be a species-poor genus when compared to their rich oil allies, like *Hyptis*.[2,7] A total of 85 compounds were identified, accounting for 88-100% of volatile constituents in the oil samples, and a total of 29 compounds presented an average ≥ 0.5%, accounting for 77-100% of sampled data. Essential oil compositions revealed a predominance of sesquiterpenes (41.7-97.5%). High contents of oxygenated sesquiterpenes were present in most species, although hydrocarbons were majority (44.1-54.0%) in a few taxa belonging to section *Laxiflorae*. Apart from *H. brachystachys* and *H. marifolia,* which showed significant levels of terpenes, aromatic and aliphatic esters (other constituents; 15.44 and 15.00%, respectively), all the other species had lower levels of such compounds (< 4.17%).

Essential oil compositions of all *Hypenia* species contained (E)-caryophyllene, δ-cadinene, spathulenol and caryophyllene oxide. The most abundant constituents were: spathulenol (11.27-31.55%), which showed high average values, with the exception of *H. marifolia* and *H. niquelandiensis* (average value 4.86 ± 0.52%); caryophyllene oxide (6.10–14.38%), with the exception of *H. niquelandiensis* (2.17%); and selin-11-en-4α-ol (4.39-34.80%), with the exception of *H. niquelandiensis* (2.12%) and *H. marifolia* (absent). Germacrene D and bicyclogermacrene were the main constituents in species from section *Laxiflorae*. All of these results are in agreement with previously described *H. salzmannii* oils (*Laxiflorae*), which had high levels of (E)-caryophyllene, germacrene D and bicyclogermacrene.[7] Moreover, *Hypenia* essential oils showed a wide range of minor constituents.

Despite the fact that the sampling sites featured slightly different soil composition and texture, canonical redundancy analysis revealed no significant correlation between edaphic factors and essential oil chemovariations (data not shown). This result suggests that *Hypenia* oils were genetically rather than environmentally influenced. Thus, volatile variations may contribute to chemotaxonomic or phylogenetic relationships within the genus. In fact, essential oil polymorphism can help to identify the taxonomic relationships of several Lamiaceae genera, as well as to examine intraspecific variability by processing more than one population *per* taxon.[8]

In order to assess the use of oil constituents for identifying taxonomic relationships among species, multivariate analysis by principal component analysis (PCA) and nearest neighbour complete linkage cluster-analysis (Ward's method)[9] were performed with oil constituent levels ≥ 0.5% (13 samples × 27 variables = 351 data). Figure 1 shows the relative position of the taxa through axial representation based on PCA results. The first PCA accounts for *ca.* 26% of the total variance and separates samples well above the 97% confidence level of the species *H. aristulata*, *H. paradise* and *H. subrosea* from *H. niquelandiensis*. All

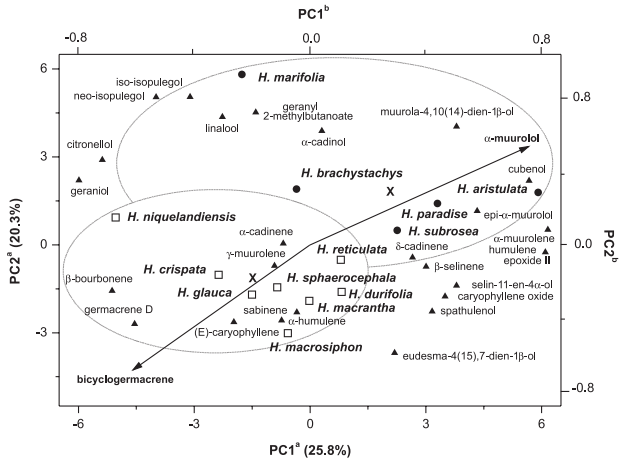

Figure 1. Biplot from PCA of *Hypenia* spp. essential oil to whose cluster it belongs: I (□); II (●). [a]Axes refer to scores from the samples. [b]Axes refer to loadings from oil constituents represented as shaded triangles (Table S2) and discriminant variables are highlighted as vectors from the origin. Crosses represent cluster centroids and values between parentheses refer to the explained variance on each principal component.

of them are grown in Serra dos Veadeiros (GO, Brazil) and showed the highest α-muurolol contents. The second PCA distinguishes ($p < 0.005$) seven species belonging to section *Laxiflorae* mainly due to highest contents of bicyclogermacrene, germacrene D, and (E)-caryophyllene of *H. marifolia* and *H. brachystachys* (*Densiflorae* section), which are described as monoterpene-rich.

Therefore, two types of essential oils were identified. Cluster I revealed the three species from section *Densiflorae* (in addition to *H. subrosea* and *H. aristulata*) which were characterized ($p < 0.008$) by the highest percentages of α-muurolol (5.85 ± 3.08%). Cluster II revealed eight species of section *Laxiflorae* containing germacrene D (18.1 ± 11.4%), (E)-caryophyllene (7.09 ± 4.88%) and bicyclogermacrene (6.65 ± 1.19%) as the main discriminant constituents ($p < 0.03$). Percentages of oil constituents in clustered taxa are shown in the SI (Table S3).

The constituent data were grouped according to carbon skeletons in order to assimilate the overall trend in volatile leaf oils and to decrease the uncontrolled factors affecting quantitative variations (Table S4). As regards the volatile constituents, PCA/cluster analysis on carbon skeletons showed identical differences among these taxa (Figures S1 and S2). Cluster I indicated significant ($p < 0.005$) results concerning the presence of cadinane (23.1 ± 10.4%), copaane (1.9 ± 4.3%), as well as occurrences of isolongifolane, bisabolane and farnesane. Cluster II revealed significant ($p < 0.006$) results for germacrane (19.6 ± 11.5%), bicyclogermacrane (7.0 ± 1.2%) and bourbonane (2.0 ± 1.9%) as the main biosynthetic class (Figures S1 and S2), as well as occurrences of tricyclane, pinane, cedrane and silphiperfolane derivatives (Table S5).

Notwithstanding morphological similarities among *Hypenia* spp., differences were observed in their leaf morphology. According to Boeger *et al.*,[10] leaves are one of the most exposed plant organs, which makes them directly influenced by environmental changes. Therefore, they are important elements for the study of a species or of plant communities. Thus, eighteen morphological leaf characters were analyzed in all taxa and coded as independent characters (states present or absent), as recommended by Sneath and Sokal (Table S6).[11] Multiple correspondence analysis on such taxonomic characters distinguished ($\chi^2 = 8.0, 5.0$; degrees of freedom, $DF = 1, 1$; $p < 0.025$) clusters I (II) based on the presence or absence of: crystals in small-caliber leaves; starch grains in the pith; and parallel striations on leaf cuticles (Figure S3). Occurrence of calcium oxalate crystals has been related to the mechanical support and protective action against herbivory.[12] On the other hand, ornamental cuticles have been associated with leaf impermeability and sunlight reflecting, which constitute two important adaptive characteristics of plants in Cerrado regions.[13]

The result most relevant to our study is the agreement between the three assessment procedures (based on chemical and morphological analyses) used for dividing *Hypenia* into two major groups of species with identical contents. In fact, canonical discriminant analysis (CDA) on chemical data confirmed *a priori* cluster groups. An axial representation of CDA results discriminated over 99.9% of the two groups based only on the contents of α-muurolol and bicyclogermacrene (predictor variables). Discriminant function analysis explained the overall variability (F-test value = 43.198; $DF = 2$ and 10; $p < 0.0001$). It was also possible to make an accurate prediction of total well-classification in the original clusters by cross-validation or Jackknife approach.[14] These techniques consider a slightly reduced number of samples from the parent data set, estimate parameters from each of these modified data sets, and then calculate the precision of predictions for the samples previously removed by the resulting models.[15] All similarities between sampled oil constituents and morphological characters are shown in the dendrogram in Figure 2.

These results indicate the presence of two *Hypenia* sections due to remarkable differences in morphological characters and essential oil compositions. Furthermore, we concluded that the sectional delimitation of *H. subrosea* and *H. aristulata* in the *Laxiflorae* section should be revised. Differences in volatile constituents among *Hypenia* spp. may be useful for understanding phylogenetic relationships, especially considering that its species are not easily identified.

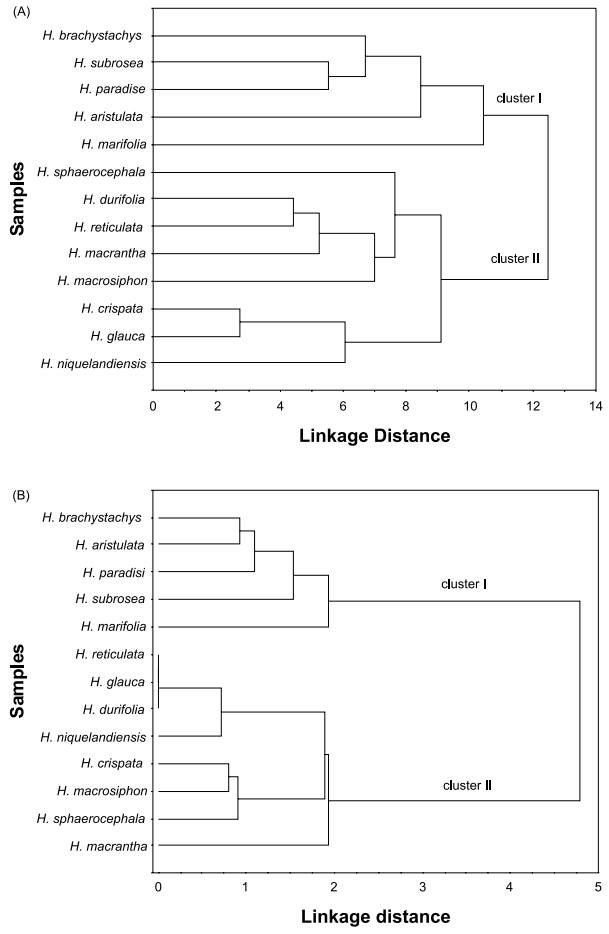

Figure 2. Dendrogram representing the similarity relationships among *Hypenia* spp. based on (A) essential oil constituents or (B) taxonomic leaf characters to whose cluster it belongs: I and II.

Conclusions

Essential oil analysis and the morphological and anatomical leaf characteristics of thirteen *Hypenia* species found in central Brazilian Cerrado areas (GO, Brazil) revealed high polymorphism, which may be related to genetic influences. Furthermore, the two clusters of constituents were in agreement with the division of species into two taxonomic sections.

Experimental

Plant material

Hypenia spp. inflorescence samples were collected between May 2006 and November 2007 in Goiás State, Brazil. The specimens were identified by Dr. Raymond M. Harley, and voucher specimens were deposited at the UFG herbarium (conservation unit of Universidade Federal de Goiás, Goiás State, Brazil). A list of the taxa investigated as well as provenance and voucher specimens are shown in Table S1.

To assess essential oil chemical compositions, 5-10 individuals of each species originated from 2-3 populations were pooled and dried at room temperature for 7 days at 30 °C until constant weight was achieved. After having been powdered, 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_2SO_4, 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 samples were collected at a depth of 20 cm in all sampling sites and were collected around each population and 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 analysis. The pH was determined in a 1:1 soil/water volume ratio. Ca, Mg and Al were extracted with KCl 1 mol L^{-1}, whereas P, K, Zn, Cu, Fe and Mn were extracted with Mehlich solution. Organic matter, cationic exchange capacity (CEC), potential acidity (H + Al), and soil texture were determined by the usual methods.[16]

Morphological and anatomical analyses

The leaf variations among the thirteen specimens were recorded using a Zeiss-Axioskop light microscope and a Jeol JSM 840A scanning electron microscope operated at 10 kV. A list of two-state qualitative characters is presented in Table S5. Fully developed leaves of approximately equal tickness were selected for the study of cross-sectional anatomy. They were cut into segments and fixed for 12 h in a 2% glutaraldehyde-paraformaldehyde solution with 0.05 mol L^{-1} sodium cacodylate buffer (pH 7.2). Segments were post fixed in OsO_4-$K_3[Fe(CN)_6]$ and dehydrated in a water-acetone series. All sections were mounted on grids coated with a layer of gold (40 nm) and were viewed with a scanning electron microscope. Thicker sections of the same material were also cut, dried and stained with 0.1% basic fuchsine and 0.3% astra blue (1:3) for 3 min. Then, they were rinsed, dried again and placed under cover slips with a permanent mounting medium for light microscopy.

Leaves were also cleared and stained for paradermal viewing. Fresh leaf material was placed in a beaker containing boiling 80% (v/v) ethanol until the chlorophyll was extracted. It was then put in 10% aqueous NaOH solution and left to clear. After that, it was rinsed in distilled water and stained in 1% safranin solution. Stained

tissue was placed on a glass slide in water, covered with a cover slip and examined under the light microscope.

Chemical analyses

Oil sample analyses were performed on a GC-MS (gas chromatography coupled with mass spectrometer) Shimadzu QP5050A instrument under the following conditions: a column CBP-5 (Shimadzu) fused silica capillary column (30 m × 0.25 mm i.d., 0.25 mm 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^{-1}; carrier gas: He (1 mL min^{-1}); injector and interface temperatures of 220 and 240 °C, respectively, with a split ratio of 1:20. The injection volume was 0.4 mL (20% in hexane) and the oven temperature was raised from 60 to 246 °C with an increase of 3 °C min^{-1}, then 10 °C min^{-1} to 270 °C, holding the final temperature for 5 min. Individual components were identified by a comparison of linear retention indices,[17] which were determined by a co-injection with a C_8-C_{32} n-alkanes series,[18] co-injection with standard, ylang-ylang (*Cananga odorata* (Lam.) Hook. F. & Thoms., Annonaceae) and sage clary (*Salvia sclarea* L., Lamiaceae) essential oils,[17] mass spectra with those of the literature and a computerized NIST MS database.[17]

Statistical analyses

Principal component (PCA) and multiple correspondence (MCA) analysis were applied in order to examine the interrelationships between plant taxa, chemical constituents and leaf taxonomic characters (presence/absence status). For these procedures we used Système Portable d'Analyse des Données-SPAD software package.[19] Cluster analysis was also applied to the study of similarities between species by considering essential oil constituents or taxonomic character distributions. Nearest neighbor complete linkage technique by Benzécri algorithm was used as an index of similarity,[20] and hierarchical clustering was performed according to Ward's variance minimizing method.[9] Oil constituents with arbitrated amounts ≥ 0.5% 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 two variables, which were effectively eliminated, revealed the highest loadings in the lowest residual eigenvalues. Prior to the multivariate analysis, the final data matrix (13 samples × 27 variables = 351 data) was processed by means of auto-scaling and mean centering. Oil constituents were also grouped according to biosynthetic class. The normalized data matrix (13 samples × 21 variables = 273 data) without variable selection was submitted to multivariate analysis (Table S5).

Canonical discriminant analysis using SAS CANDISC procedure[21] was used to differentiate between taxa and clusters on the basis of oil composition. The predictive ability of canonical discriminant function was evaluated by cross-validation and Jackknife approaches as implemented in SAS statistical package.

Canonical redundancy analysis (RDA) was applied to describe the patterns of the only explained variation of interrelationships between oil composition and the inter-specific variations as a function of soil parameters, treated as environmental variables. An unrestricted Monte-Carlo permutation test (1000 permutations) was used to test eigenvalue significance of the first three canonical axes.[22] RDA was performed in CANOCO software.[23]

Multiple comparisons were established by univariate analysis of variance (ANOVA) using SAS GLM procedure.[24] All data were checked for homoscedasticity with the use of Hartley's test. This test revealed significant departures from the basic assumption for the oil components, which were arcsine and rank-transformed when necessary. Whenever a difference was established, a Tukey's *post-hoc* test was performed. Results are indicated 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 are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgements

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