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Original Article

Foliar phenolic compounds of ten wild species of Verbenacea as antioxidants and specific chemomarkers

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Abstract

The family Verbenaceae hosts important species used in traditional medicine of many countries. The taxonomic controversies concerning the specific delimitation of several of its species make it difficult to guarantee the botanical origin of herbal preparations based on species of this family. To contribute to the development of both specific chemomarkers and a quality control tool to authenticate the botanical origin of herbal preparations of Verbenacea species, we determined the foliar HPLC-DAD phenolic profiles and the antioxidant properties of 10 wild species of this family occurring in Mexico. The contents of phenols and flavonoids varied significantly among species. *Priva mexicana* showed the highest levels of total phenolics (53.4 mg g^{-1} dry tissue) and *Verbena carolina* had the highest levels of flavonoids (17.89 mg g^{-1} dry tissue). Relevant antioxidant properties revealed by antiradical and reducing power were found for the analyzed species. These properties varied significantly in a species-dependent manner. The phenolic compounds accumulated were flavones and phenolic acids. Flavones were the only type of flavonoids found. The results of a cluster analysis showed that the compounds were accumulated in species-specific profiles. The phenolic profiles are proposed as valuable chemomarkers that can become a useful tool for the quality control concerning the botanical origin of herbal medicinal preparations based on the species analyzed. In addition, phenolic profiles could contribute importantly to solve the taxonomic controversies concerning species delimitation in the family Verbenaceae.

Keywords: Verbenacea, antioxidant activity, chemomarkers, flavones, phenolic profiles.

Compostos fenólicos das folhas de dez especies selvagem de Verbenaceae como antioxidantes e quimiomarcadores específicos

Resumo

A família Verbenaceae compreende importantes espécies utilizadas na medicina popular de muitos países. As dificuldades taxonômicas relativas à delimitação específica de muitas das suas espécies face difícil a verificar a origem botânico das preparações herbales baseadas nas espécies desta família. Para fazer uma contribuição ao desenvolvimento de indicadores taxonômicos e dum método de controle de qualidade para verificar a origem botânico de preparações herbales das espécies de Verbenaceae, os perfis fenólicos, obtidos pares HPLC-DAD, e as atividades antioxidantes das folhas de 10 espécies selvagens Mexicanas desta família foram determinados. Os conteúdos dos compostos fenólicos totais e dos flavonoides foram significativamente diferentes entre as espécies. *Priva mexicana* apresentou a maior quantidade de compostos fenólicos totais (53.4 mg g^{-1} amostra seca) e *Verbena carolina* apresentou a maior quantidade de flavonoides (17.89 mg g^{-1} amostra seca). Verifica-se importantes propriedades antioxidantes, como os resultados dos ensaios da capacidade antiradical e do poder redutor indicaram. As propriedades antioxidantes foram significativamente diferentes entre as espécies. Verificou-se que os compostos fenólicos conteúdos nas folhas das espécies analisadas foram só flavonas e ácidos fenólicos. Os resultados das análises de agrupamento provarãn que os perfiles fenólicos foram espécie-específicos. Estes perfis podem ser considerados como indicadores químicos da qualidade relativa à origem botânico de preparações medicinais baseadas nas espécies analisadas e podem fazer importantes contribuições para a delimitação específica na família Verbenaceae.

Palavras-chave: Verbenaceae, actividade anti-oxidante, indicadores taxonómicos, flavonas, perfis fenólicos.

1. Introduction

The Verbenaceae family is a taxonomically difficult group of plants. There are many controversies concerning the circumscription of several of its genera, species, and even the own family. Considering what was regarded as subfamily Verbenoideae, now considered as family Verbenaceae, and excluding the genera *Callicarpa*, *Clerodendron*, and *Tetraclea* (now forming part of the family Lamiaceae), Rzedowski and Calderón de Rzedowski (2002) reported about 1000 species belonging to the family Verbenaceae, which occur in template and tropical regions worldwide, mainly in the American continent.

Many species of Verbenacea are used as important folk remedies for the treatment of several human health disorders, like some kinds of cancer and hypertension (Ghisalberti, 2000; Manica-Cattani et al., 2009). Due to the medicinal importance or toxic effects of several species of Verbenaceae, efforts have been made to develop markers that allow distinguishing among species. Some of these efforts have focused on anatomical studies (Calzada-Sánchez et al., 2014; Passos et al., 2009).

Plant phenolic compounds are important natural antioxidants (Shin et al., 2015) that have beneficial effects on human health. For several groups of plants, profiles of these compounds have been reported as worthy chemomarkers because they were species-specific, like *Equisetum* (Veit et al., 1995), *Pinus* (Almaraz-Abarca et al., 2006), and *Salvia* (Kharazian, 2014). The species-specific condition of phenolic profiles could be of taxonomic relevance. In addition, the profiles can represent a quality control tool concerning the botanical origin of plant-based medicinal preparations, as adulteration is a global latent risk for traditional herbal preparations, as documented by Ahmad et al. (2009).

Some species of Verbenaceae have been analyzed to determine their phenolic composition and some biological activities, like *Verbena officinalis* L. (Calvo et al., 1997), *Lantana camara* L. (Wollenweber et al., 1997), *Vitex polygama* Cham. (Gonçalves et al., 2001), and *Lampaya medicinalis* Phil. (Morales and Paredes, 2014). Phytochemical studies with taxonomical interest are few and have been based mainly on the essential oil composition (Sena Filho et al., 2012; Satyal et al., 2016). Despite the important studies already done, there are many other species of Verbenaceae to analyze for their phenol composition and antioxidant activity. The present study focused on determining the foliar phenolic profiles and antioxidant activity of *Verbena gracilis* Desf., *V. carolina* L., *V. bipinnatifida* Nutt., *V. menthifolia* Benth., *Lantana camara* L., *Phyla nodiflora* (L.) Greene, *Aloysia gratissima* (Gill. et Hook) Tronc., *Bouchea prismatica* (L.) Kuntze, *Priva mexicana* (L.) Pers., and *Lippia umbellata* Cav., occurring in Mexico, to assess their potential as specific chemomarkers.

2. Material and Methods

2.1. Plant material

Leaves of adult flowering plants of 10 species of Verbenaceae were collected from natural populations of Durango, Mexico. Sampling sites are described in Table 1. Voucher specimens were deposited at the Herbarium CIIDIR. For each single species, all leaves of four individuals were combined and three pools of samples were formed and analyzed separately. The dried and ground leaves were kept in plastic bags, in darkness, and stored at room temperature until analysis.

2.2. Preparation of extracts

For each pool of samples, phenols were extracted from 4 g of dry and ground leaves by maceration in 40 mL of 80% ethanol (v/v) for 24 h, shaking at 100 rpm, in darkness, at room temperature. The extracts were centrifuged (5000 rpm, 10 min, at room temperature) and the supernatants decanted. Aliquots were used in the HPLC-DAD analysis and in the antioxidant assays.

2.3. Total phenolics

The concentrations of total phenolics were determined using Folin-Ciocalteu reagent, according to Falleh et al. (2011). The phenolic contents were calculated from a standard curve of gallic acid (A_{760nm}, slope = 104.190, *y* axis crossing point = - 0.0093, correlation coefficient r = 0.9985). Total phenolic concentrations were expressed as milligrams gallic acid equivalents per gram of dry tissue (mg GAE g^{-1} dt).

Table 1. Collection sites for 10 species of Verbenaceae from Mexico.

Curatorial number	Species	Location	Latitude (N)	Longitude (W)	Altitude (m)	Date
45018	*Verbena gracilis*	Durango	24° 03' 04"	104° 36' 41"	1,876	June 30/2014
45307	*Phyla nodiflora*	Durango	23° 57' 40"	104° 35' 05"	1,877	July 9/2014
45308	*Verbena carolina*	Vicente Guerrero	23° 44' 58"	103° 58' 48"	1,933	Aug 8/2014
45310	*Lantana camara*	Durango	24° 00' 47"	104° 23' 33"	1,951	Aug 8/2014
45311	*Verbena bipinnatifida*	Nombre de Dios	23° 47' 43"	103° 50' 48"	2,212	Aug 15/2014
45309	*Aloysia gratissima*	Durango	24° 00' 43"	104° 26' 02"	1,860	Aug 15/2014
45315	*Bouchea prismatica*	Durango	24° 00' 43"	104° 26' 02"	1,860	Sept 26/2014
45314	*Verbena menthifolia*	Durango	24° 00' 43"	104° 26' 02"	1,860	Sept 26/2014
45378	*Priva mexicana*	Vicente Guerrero	23° 48' 43"	105° 52' 14"	2,263	Oct 2/2014
45313	*Lippia umbelata*	Tamazula	25° 26' 02"	106° 56' 28"	1,507	Oct 9/2014

2.4. Total flavonoids

Flavonoid contents were determined by using $AlCl_3$, according to Falleh et al. (2011), from a standard curve of apigenin (A_{425nm}, slope = 111.111, *y* axis crossing point = - 0.0021, correlation coefficient r = 0.9988). Flavonoid contents were expressed as milligrams apigenin equivalents per gram of dry tissue (mg AE g^{-1} dt).

2.5. HPLC-DAD analysis

Phenolic compositions were obtained from a gradient method previously described (Campos and Markham, 2007), with a Perkin Elmer Series 200 HPLC system and a Perkin Elmer Brownlee Analytical C18 column (4.6 × 250 mm, 5 µm). Water adjusted to pH 2.5 with orthophosphoric acid was solvent A and acetonitrile was solvent B. Solvents were mixed according to the following gradient: starting with 100% A, decreasing to 91% over the next 12 min, to 87% over the next 8 min, and to 67% over the next 12 min, and to 57% until the end of the 60 min analysis. Chromatograms were registered at 260 nm. Fifty microliters of samples were injected. The flow rate used was 0.8 mL min^{-1}. The analyses were carried out at 25°C. Spectral data for all peaks were accumulated in the range of 200 to 400 nm using diode-array detection (Perkin Elmer Series 200). Structural information was obtained by comparisons of retention times (RT) and UV spectra of resolved compounds with those of reference compounds (apigenin: RT: 59.62, λ_{max}: 269, 335; chlorogenic acid: RT: 28.16, λ_{max}: 243sh, 293sh, 325), as well as with the principles of the UV theory developed by Campos and Markham (2007) for flavonoids and phenolic acids. The foliar phenol profile of each species was made up of all compounds present in the respective HPLC-DAD chromatogram, treating each compound as a single chemical character.

2.6. Free radical scavenging activity

The DPPH* method described by Yang et al. (2008) was used to evaluate the free radical scavenging activity. A standard curve of DPPH* (A_{523nm}, slope = 0.0309, *y* axis crossing point = 0.0019, correlation coefficient r = 0.9996) was used to estimate the DPPH* concentration (µg mL^{-1}) in the reaction medium. Antiradical activities were expressed in terms of EC_{50} (concentration of extract needed to decrease by 50% the initial concentration of DPPH*) in micrograms per milliliter (µg mL^{-1}). Quercetin and epicatechin were used as reference samples and assayed in the same manner.

2.7. Total antioxidant capacity (TAC)

The TAC of each extract was evaluated according to Prieto et al. (1999). The absorbance of the reaction mixtures was registered at 695 nm. The TAC values were calculated from a standard curve of ascorbic acid (A_{695nm}, slope = 4.213, *y* axis crossing point = 0.02365, correlation coefficient r = 0.998) and expressed as milligrams ascorbic acid equivalents per mL (mg AAE mL^{-1}). Quercetin and epicatechin were analyzed as reference samples in the same manner.

2.8. Iron reducing power (RP)

The RP values were calculated by using the method described by Yang et al. (2008). For each sample, a graph of absorbance at 700 nm vs. increased extract concentrations was constructed to calculate the extract concentration providing 0.5 of absorbance (defined as IC_{50}). RP was expressed as micrograms per milliliter (µg mL^{-1}). Quercetin and epicatechin were analyzed in the same manner as references.

2.9. Data analysis

Each assay was made for three independent samples of each item. Data were subjected to an analysis of variance ($p \leq 0.05$) and means were separated by Tukey test. Correlations between different parameters were carried out with Pearson test, using the SPSS Statistics 17.0 computer program. The foliar phenolic profiles were constructed with all compounds resolved in the respective HPLC-DAD chromatograms; each compound representing a single chemical attribute. To determine the species-specific condition of the foliar phenolic profiles, a presence-absence matrix, formed by all individual samples vs. all resolved compounds (10 samples vs. 47 phenolic compounds) was analyzed using Cluster Analysis (Paired Group algorithm, Jaccard similarity measure) from Past 1.43 (Hammer et al., 2001).

3. Results

3.1. Phenolic and flavonoid contents

The foliar phenolic and flavonoid contents estimated for each species are displayed in Table 2. *Priva mexicana* showed the highest levels of total phenolics (53.4 mg g^{-1} dt) and *L. camara* and *A. gratissima* accumulated the lowest levels (14.6 and 15 mg g^{-1} dt, respectively). The levels of foliar flavonoids ranged from 6.6 to 17.8 mg g^{-1} dt for *L. camara* and *V. carolina*, respectively. Flavonoids accounted for between 22 and 84% (*Lippia umbelata* and *Aloysia gratissima*, respectively*)* of total phenolics.

3.2. Antioxidant properties and their correlations with phenolic and flavonoid contents

The values of the antiradical activities of foliar extracts of the analyzed species are shown in Table 2. The highest activity was found for *P. mexicana* (EC_{50} = 1.68 µg mL^{-1}) and the lowest ones for *A. gratissima* (EC_{50} = 3.98 µg mL^{-1}) and *V. bipinnatifida* (EC_{50} = 3.97µg mL^{-1}). The reducing power values are shown in Table 2. The lowest extract concentration needed to reach an absorbance value of 0.5 at 700 nm was that of *L. umbelata* (1.34 µg mL^{-1}), thus, this species displayed the highest reduction power. TAC values are shown in Table 2, the highest capacity was revealed by *V. menthifolia* (6.24 mg mL^{-1}) and the lowest by *A. gratissima* (2.08 mg mL^{-1}).

The kinetic behavior (the monitoring of changes in absorbance at a given wavelength as the result of the reduction of the oxidant by the extracts) of all the antioxidant assays was highly related to phenolic and

Table 2. Total phenolics, total flavonoids, DPPH• scavenging capacity (EC_{50}), iron reducing power (IC_{50}), and total antioxidant capacity (TAC) of 10 species of Verbenacea from Mexico.

Species and standards	Total phenolics (mg GAE g^{-1} dt)	Total flavonoids (mg AE g^{-1} dt)	EC_{50} (µg mL^{-1})	IC_{50} (µg mL^{-1})	TAC (mg AAE mL^{-1})
Lantana camara	14.67 ± 0.38a	6.66 ± 0.16a	3.18 ± 0.02g	5.49 ± 0.19d	2.32 ± 0.08ab
Aloysia gratissima	15.04 ± 0.15a	12.61 ± 0.27d	3.98 ± 0.05i	9.21 ± 0.12g	2.08 ± 0.02a
Verbena bipinnatifida	16.50 ± 0.12b	14.32 ± 0.20f	3.97 ± 0.01i	8.18 ± 0.31f	2.66 ± 0.10bc
Phyla nodiflora	16.55 ± 0.25b	12.35 ± 0.31d	1.81 ± 0.01ba	3.12 ± 0.24b	2.54 ± 0.19b
Verbena gracilis	16.59 ± 0.62b	8.60 ± 0.36b	3.87 ± 0.09h	12.72 ± 0.43h	2.31 ± 0.03ab
Bouchea prismatica	24.34 ± 0.22c	10.72 ± 0.19c	2.77 ± 0.03f	4.24 ± 0.06c	4.48 ± 0.17f
Verbena carolina	32.45 ± 0.61d	17.89 ± 0.54g	2.60 ± 0.04e	3.84 ± 0.03bc	4.25 ± 0.22ef
Lippia umbelata	40.65 ± 0.14e	8.99 ± 0.08b	1.95 ± 0.04x10^{-1}c	1.34 ± 0.02a	3.33 ± 0.21d
Verbena menthifolia	47.68 ± 0.53f	13.85 ± 0.27f	2.09 ± 0.01d	6.03 ± 0.22d	6.24 ± 0.16g
Priva mexicana	53.46 ± 0.46g	13.24 ± 0.25e	1.68 ± 0.04x10^{-1}a	1.71 ± 0.13a	3.93 ± 0.04e
Quercetin			5.29 ± 0.39j	9.27 ± 0.23g	8.23 ± 0.21h
Epicatechin			14.33 ± 0.29k	7.17 ± 0.14e	2.14 ± 0.03c

GAE: Gallic acid equivalents; AE: Apigenin equivalents; AAE: Ascorbic acid equivalents. The values represent the mean and standard deviation of three independent analysis. Different letters in the same column mean significant differences ($p < 0.05$).

flavonoid contents ($0.9823 < r < 0.999$). However, the correlation analysis revealed lower associations between the antioxidant properties and the phenolic and flavonoid contents in the different samples (0.0033 between total phenolics and reduction power < Pearson correlation value < 0.77917 between total phenolics and DPPH scavenging activity). This means that the antioxidant properties did not increase in parallel to the increase of the levels of total phenolics and total flavonoids in the samples. Extracts having the highest contents of total phenolics or total flavonoids did not always display the highest antioxidant properties, neither the samples having the lowest contents of total phenolics or total flavonoids displayed the lowest antioxidant properties (Table 2).

3.3. Phenolic composition

According to the UV theory developed by Campos and Markham (2007), based on of the number of absorption bands; intensity and shape of bands; as well as on the number, position and shape of shoulders in the UV spectra, it is possible to determine the types of phenolic compounds and some OH- and glycoside-substitutions in their structures. Under our extraction conditions, flavones were the only flavonoids found in the foliar tissues of the analyzed species of Verbenacea. Retention time and λ_{max} for 47 phenolic compounds found are shown in Table 3. Compound **3** was suggested to be chlorogenic acid because its RT (28.37 min) and spectral data (λ_{max}: 243sh, 293sh, 325) coincided with the respective RT and λ_{max} of this reference compound. Compound **39** was suggested as eupafolin (6-methoxy-5,7,3',4'-tetrahydroxyflavone) based on its spectral data (λ_{max}: 253sh, 270, 345), which corresponded to those reported for this compound (λ_{max}: 253.3, 269.9, 345) by Wang et al. (2013). A total of 37 flavones were found: eupafolin (compound **34**), one tricetin glycoside, one diosmetin glycoside, seven chrysoeriol glycosides, nine scutellarein glycosides, seven apigenin glycosides, and eleven luteolin glycosides. The other phenolics found were chlorogenic acid (compound **3**) and other nine phenolic acids. Chlorogenic acid was found only in the leaves of *A. gratissima*. The phenolic profiles of the analyzed species varied from two phenolic compounds in *Lantana camara* to 12 in *Verbena menthifolia.* The chromatograms of each species, showing the UV spectra of some of the major compounds, are displayed in Figure 1. All profiles were species-specific. Except for *V. gracilis*, each species accumulated at least two unique compounds: *P. nodiflora*, compounds **34** and **40**; *V. carolina*, compounds **11** and **14**; *L. camara*, compounds **41** and **47**; *V. bipinnatifida*, compounds **4**, **24**, **32**, **33**, **38**, **42**, and **46**; *A. gratissima*, compounds **3** and **16**; *B. prismatica*, compounds **5**, **20**, **23**, and **36**; *V. menthifolia*, compounds **17**, **30**, **31**, and **37**; *P. mexicana*, compounds **1**, **12** and **35**; and *L. umbelata*, compounds **8**, **18**, **21**, and **45** (Table 3). Figure 2 depicts the dendrogram revealing the species-specific condition of phenolic profiles. The profiles of *V. gracilis* and *V. carolina* were the most similar, with a Jaccard similarity value of 0.6 (Jaccard similarity index of 1 means equality).

4. Discussion

The analyzed species of Verbenacea accumulated important levels of total phenolics and flavonoids. Significant ($p \leq 0.05$) species-dependent variations in both contents were found (Table 2). Considering the foliar total phenolics, seven groups of species were formed. *Lantana camara* and *A. gratissima* could not be discriminated one from each other according to their levels of accumulated total phenolics, the same was found for *V. bipinnatifida*, *P. nodiflora* and *V. gracilis*. However, the other five species accumulated significantly different total phenolic contents. *Priva mexicana* showed similar levels of total phenolics

Table 3. Retention time (RT) and spectral data (λ_{max}) for the phenolic compounds found in the leaves of 10 species of Verbenaceae.

Number of compound	RT[a] (min)	λ_{max} (nm)	Proposed types of phenolic compounds	Species
1	21.50 ± 0.00	243sh, 295sh, 330	Phenolic acid	*Priva mexicana*
2	23.42 ± 0.00	248sh, 297sh, 330	Phenolic acid	*Priva mexicana* *Lippia umbelata*
3	28.37 ± 0.34	243sh, 293sh, 325	Chlorogenic acid	*Aloysia gratissima*
4	31.94 ± 0.00	234sh, 266, 335	Methoxytricetin derivative	*Verbena bipinnatifida*
5	32.12 ± 0.00	249sh, 269, 336	Luteolin glucuronide	*Bouchea prismatica*
6	32.87 ± 0.29	268, 335	Apigenin-7-*O*-glycoside	*Priva mexicana* *Bouchea prismatica*
7	33.59 ± 0.27	255sh, 282, 345	6-Hydroxyluteolin glycoside	*Bouchea prismatica* *Verbena menthifolia*
8	32.44 ± 0.00	254, 267sh, 345	Luteolin-7-*O*-glycoside	*Lippia umbelata*
9	33.19 ± 0.11	254, 267sh, 348	Luteolin-7-*O*-glycoside	*Lippia umbelata* *Aloysia gratissima*
10	34.24 ± 0.24	249sh, 289sh, 329	Phenolic acid	*Priva mexicana* *Phyla nodiflora* *Verbena menthifolia*
11	34.61 ± 0.00	282, 330	Scutellarein glycoside	*Verbena carolina*
12	35.01 ± 0.00	280, 333	Scutellarein glycoside	*Priva mexicana*
13	35.97 ± 0.23	255, 267sh, 347	Luteolin-7-*O*-glycoside	*Priva mexicana* *Verbena menthifolia* *Lippia umbelata*
14	35.49 ± 0.00	279, 332	Scutellarein glycoside	*Verbena carolina*
15	36.10 ± 0.28	249sh, 289sh, 330	Phenolic acid	*Priva mexicana* *Verbena menthifolia* *Lippia umbelata*
16	35.95 ± 0.00	267, 336	Apigenin-7-*O*-glycoside	*Aloysia gratissima*
17	37.22 ± 0.00	252sh, 277, 341	6-hydroxyluteolin derivative	*Verbena menthifolia*
18	36.84 ± 0.00	253, 268sh, 350	Luteolin-7-*O*-glycoside	*Lippia umbelata*
19	37.05 ± 0.23	254, 267sh, 348	Luteolin-7-*O*-glycoside	*Aloysia gratissima* *Bouchea prismatica* *Lippia umbelata*
20	37.72 ± 0.00	232sh, 254sh, 283, 344	6-Hydroxyluteolin-7-*O*-glycoside	*Bouchea prismatica*
21	38.10 ± 0.00	248sh, 288sh, 327	Phenolic acid	*Lippia umbelata*
22	38.76 ± 0.00	267, 337	Apigenin-7-*O*-glycoside	*Priva mexicana* *Verbena bippinatifida*
23	39.23 ± 0.00	297sh, 314	Phenolic acid	*Bouchea prismatica*
24	39.42 ± 0.00	267, 336	Apigenin-7-*O*-glycoside	*Verbena bipinnatifida*
25	39.29 ± 0.26	269, 331	Scutellarein derivative	*Verbena carolina* *Verbena gracilis*
26	39.51 ± 0.08	270, 335	Apigenin-7-*O*-glycoside	*Verbena menthifolia* *Verbena bipinnatifida*
27	39.99 ± 0.15	251sh, 287sh, 330	Phenolic acid	*Priva mexicana* *Bouchea prismatica*
28	40.63 ± 0.20	267, 335	Apigenin-7-*O*-glycoside	*Verbena bipinnatifida* *Bouchea prismatica*
29	40.04 ± 0.16	271, 331	Scutellarein derivative	*Verbena carolina* *Verbena menthifolia* *Verbena gracilis*
30	40.41 ± 0.00	252sh, 275, 343	6-hydroxyluteolin derivative	*Verbena menthifolia*

[a]Values represent the mean and standard deviation of at least three independent analysis.

Table 3. Continued...

Number of compound	RT[a] (min)	λ_{max} (nm)	Proposed types of phenolic compounds	Species
31	40.79 ± 0.00	252sh, 275, 342	6-hydroxyluteolin derivative	*Verbena menthifolia*
32	40.93 ± 0.00	236sh, 253sh, 266, 343	Chrysoeriol glycoside	*Verbena bipinnatifida*
33	41.42 ± 0.00	236sh, 253sh, 265, 343	Chrysoeriol glycoside	*Verbena bipinnatifida*
34	41.49 ± 0.00	253, 270, 345	Eupafolin	*Phyla nodiflora*
35	41.22 ± 0.00	250sh, 289sh, 330	Phenolic acid	*Priva mexicana*
36	41.48 ± 0.00	254, 266sh, 347	Luteolin-7-*O*-glycoside	*Bouchea prismatica*
37	42.00 ± 0.00	289sh, 329	Phenolic acid	*Verbena menthifolia*
38	42.11 ± 0.00	236sh, 251sh, 266, 343	Chrysoeriol glycoside	*Verbena bipinnatifida*
39	47.82 ± 0.25	274, 330	Scutellarein derivative	*Verbena carolina* *Verbena menthifolia* *Verbena gracilis*
40	45.20 ± 0.00	253, 270sh, 344	Diosmetin glycoside	*Phyla nodiflora*
41	45.20 ± 0.00	272, 328	Scutellarein derivative	*Lantana camara*
42	45.57 ± 0.00	236sh, 251, 266, 342	Chrysoeriol glycoside	*Verbena bipinnatifida*
43	45.74 ± 0.00	254, 273, 344	Diosmetin glycoside	*Phyla nodiflora*
44	46.88 ± 0.26	269, 331	Apigenin-7-*O*-glycoside	*Verbena menthifolia* *Lippia umbelata*
45	48.09 ± 0.00	252sh, 267, 345	Chrysoeriol glycoside	*Lippia umbelata*
46	48.56 ± 0.00	266, 331	Apigenin-7-*O*-glycoside	*Verbena bipinnatifida*
47	48.63 ± 0.00	268, 329	Scutellarein derivative	*Lantana camara*

[a]Values represent the mean and standard deviation of at least three independent analysis.

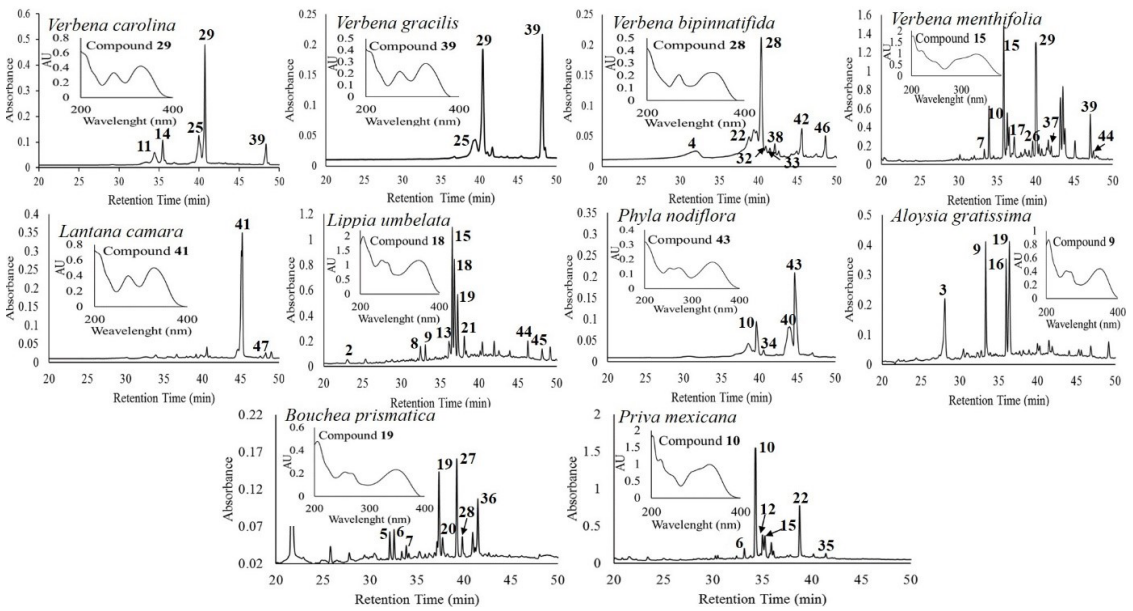

Figure 1. HPLC chromatograms (registered at 260 nm) and absorption UV spectra (obtained from 200 to 400 nm) of some of the major foliar phenolic compounds of 10 species of Verbenaceae. The number of compounds corresponds to those of Table 3.

(53.4 mg g^{-1} dt) to other species of Verbenacea with antioxidant properties, such as *Aloysia triphylla* (L'Hér.) Britton (52 mg g^{-1} dw) (Ranilla et al., 2010).

The foliar flavonoid contents varied significantly among all the analyzed species (Table 2), which showed higher levels (6.6 to 17.8 mg g^{-1} dt) than those reported for other species of Verbenacea, like *Lampaya medicinalis* (60.04 µg g^{-1} dt) (Morales and Paredes, 2014).

The analyzed species had antioxidant properties. A particular and significantly different antiradical activity was found for each species analyzed, except for *A. gratissima* and *V. bipinnatifida*, which expressed

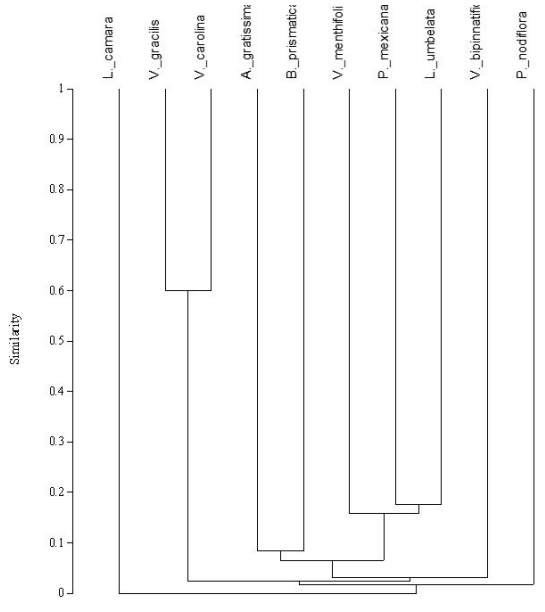

Figure 2. Results of the cluster analysis based on the comparisons of the foliar phenolic profiles of 10 species of Verbenaceae. The dendrogram was generated with the Paired Group Algorithm and the Jaccard Similarity Measure.

similar activities (Table 2). The antiradical activity of any of the analyzed species was higher than those of the standards quercetin (EC_{50} = 5.29 µg mL^{-1}) and epicatechin (EC_{50} = 14.33 µg mL^{-1}), both reported as phenolic compounds with important antiradical activity (Jiménez-Aliaga et al., 2011; Meng et al., 2015).

Significant differences in the iron reducing power were found, except between *L. umbelata* and *P. mexicana* (Table 2), which expressed similar iron reducing potentials. The activity of *A. gratissima* (9.21 µg mL^{-1}) was comparable to that of the standard quercetin (9.27 µg mL^{-1}) and the activity of seven of the analyzed species was higher than that of the standard epicatechin (7.17 µg mL^{-1}). Comparatively, all analyzed species displayed higher reducing power than the edible fruits of *Physalis alkekengi* (IC_{50} = 36.58 mg mL^{-1}) (Diaz et al., 2012).

The reducing potential of the foliar extracts of the analyzed species was also revealed by their capability to produce Mo (V) from Mo (VI) (TAC assay). The Tukey test separated the mean of each *L. umbelata*, *V. menthifolia* and *P. mexicana* in an individual group, suggesting that each of this species had a distinguishing TAC value (Table 2). All samples displayed similar or higher TAC values than the standard epicatechin (2.14 mg mL^{-1}); however, none reached the activity of the standard quercetin (8.23 mg mL^{-1}). Comparatively, the TAC value of *V. menthifolia* was 1.6-fold higher than that of the leaves of the Chihuahuan ground cherry (*Physalis subulata* Rydb.), which is 3.59 mg mL^{-1} (Medina-Medrano et al., 2015).

High correlations between the kinetic behavior of antioxidant assays and the phenolic and flavonoid contents, and unclear associations between the antioxidant activities and the phenolic and flavonoid contents in different samples have also been found by other authors (Morais et al., 2011). The current results support the proposal made by Morais et al. (2011) on the significant role of the qualitative phenolic composition, aside from the phenolic and flavonoid contents, in determining the antioxidant potentials of plant extracts.

Concerning the HPLC-DAD phenolic profiles, the results found for the analyzed species extends the groups of plants for which phenolic profiles have a species-specific condition, corroborating the proposal made by some authors on the relevance of phenolic profiles as important specific chemomarkers (Veit et al., 1995). These same authors and others (Almaraz-Abarca et al., 2013) have pointed out that under variable environmental conditions, the major changes occur in the concentrations of the individual phenolic compounds, keeping stable the qualitative composition. Thus, given the relevance of the species of Verbenaceae have in traditional medicine worldwide, the foliar phenolic patterns obtained by HPLC-DAD, which were species-specific (Figure 2), represent a chemical fingerprinting that can become an important tool of quality control regarding the specific authentication of plant-based preparations from species of Verbenaceae. The current results also suggest that the species-specific profiles found could contribute to solve taxonomic controversies concerning the establishment of specific limits in some genera of Verbenaceae, as those informed for *Lantana* (Ghisalberti, 2000).

Flavone glycosides and phenolic acids were the only phenolic compounds found in the leaves of Verbenaceae analyzed. These two kinds of phenolic compounds have been reported as relevant antioxidants (Catarino et al., 2015; Shin et al., 2015). Our results indicated that *A. gratissima* profile was constituted by one phenolic acid (**3**), one 7-*O*-glycoside of apigenin (**16**), and two luteolin-7-*O*-glycosides (**9** and **19**) (Table 3 and Figure 1). The current results contrast with those reported by Zeni et al. (2013) for this same species occurring in Brazil, these authors found 10 phenolic acids and no flavone in the aerial parts. These contrasting results could be accounted for by the differences between the extraction conditions. Probably, some flavonoids were degraded during the boiling water-extraction procedure used by Zeni et al. (2013), as flavonoid degradation, producing phenolic acids, has been reported under boiling water conditions (Buchner et al., 2006). The chemical differences could also be the consequence of a high genetic variability between populations of the same species growing in different and contrasting environmental conditions (like those in Brazil and in northern Mexico), between which, genic flux is unlikely to occur. Furthermore, *A. gratissima* represents a complex formed by 22 specific and infraspecific taxa, whose boundaries are not well-established (Moroni et al., 2016), and needs a taxonomic revision. It is possibly that the analyzed taxa from Brazil and Mexico, both identified as *A. gratissima*, actually represent different taxa. *Aloysia*

gratissima was collected from the same location as *B. prismatica* and *V. menthifolia* (Table 1), where they are sympatric; therefore, they are exposed to the same environmental conditions. This suggests that the clearly different phenolic profiles (Figure 1) are the result of genetic specific differences, which command a species-specific sequential order in the phenolic biosynthesis, as Heller and Forkmann (1994) pointed out.

In the present study, only two glycoside derivatives of scutelarein (**41** and **47**) were found for *L. camara* (Table 3). Our results contrast with those of Wollenweber et al. (1997), who reported three quercetin derivatives (flavonols) for the leaves of this same species growing in the eastern United States. The contrasting results in the *L. camara* phenolic composition may have arisen from different extraction conditions (acetone extracts were prepared by Wollenweber et al., 1997), but may be also a chemical evidence of the high genetic and morphological variability of the species, which represents a complex rather than a single species (Ghisalberti, 2000) that deserves a taxonomic evaluation.

The closest chemical relationship (Figure 2) was found between *V. carolina* and *V. gracilis*, which accumulated only scutellarein derivatives and shared the compounds **25**, **29**, and **39** (Table 3 and Figure 1). The profile found for *V. bipinnatifida* was formed by one methoxy derivative of tricetin (**4**), four chrysoeriol glycosides (**32**, **33**, **38** and **42**), and five apigenin-3-*O*-glycosides (**22**, **24**, **26**, **28** and **46**); whereas that found for *V. menthifolia* was formed by two apigenin-7-*O*-glycosides (**26** and **44**), two scutellarein derivatives (**29** and **39**), five luteolin derivatives (**7**, **13**, **17**, **30** and **31**), and three phenolic acids (**10**, **15** and **37**). These two profiles were among the most complex recorded here (Table 3 and Figure 1), and agreed with the flavone diversity reported by Kawashty and El-Garf (2000) for other species of *Verbena*.

With 10 compounds, the *Lippia umbelata* phenolic pattern was also complex, formed by one chrysoeriol glycoside (**45**), one apigenin glycoside (**44**), five luteolin-7-*O*-glycosides (**8**, **9**, **13**, **18** and **19**), and three phenolic acids (**2, 15** and **21**) (Table 3 and Figure 1). Complex patterns (of 15 flavones) have been reported also for other species of *Lippia*, like *L. nodiflora* (L.) Michx. and *L. canescens* Kunth (Tomás-Barberan et al., 1987).

The foliar phenolic profile of *Phyla nodiflora* was formed by two glycoside derivatives of diosmetin (**40** and **43**) and eupafolin (**34**), aside from one phenolic acid (**10**). Ko et al. (2014) also reported eupafolin in the aerial parts of this same species.

Bouchea and *Priva* are less studied genera regarding their phenolic composition. The phenolic pattern found for *B. prismatica* was complex, formed by two apigenin-7-*O*-glycosides (**6** and **28**), two phenolic acids (**23** and **27**), and five luteolin glycosides (**5**, **7**, **19**, **20** and **36**) (Table 3 and Figure 1). A complex pattern was also found for *P. mexicana*, formed by one glycoside derivative of scutellarein (**12**), one luteolin-7-*O*-glycoside (**13**), two apigenin-7-*O*-glycosides (**6** and **22**), and six phenolic acids (**1**, **2**, **10**, **15**, **27** and **35**). The complex profile found for *P. mexicana* contrasts with that reported for the aerial parts of other species of *Priva*, like *P. lappulacea*, for which Braga et al. (2009) only mentioned luteolin.

5. Conclusion

The analyzed species of Verbenaceae are important sources of antioxidant phenolic compounds. Their leaves accumulate an important diversity of flavones but some phenolic acids can also be found. The species-specific phenolic profiles found for the taxa analyzed represent fingerprintings with taxonomical implications for defining specific limits in the family and can be used as a quality control tool for determining the authenticity of herbal preparations from species of Verbenaceae.

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