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CHEMICAL SCIENCES

Levels of phenylpropanoids and iridoids in extracts and infusions of *Verbena minutiflora*

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Abstract: Herein we report for the first time the levels of phenylpropanoids and iridoids in extracts and infusions of *V. minutiflora* consumed in Brazil to treat urinary and infectious disorders. An *in house* validation study demonstrated good accuracy and precision to determine the bioactive compounds in *V. minutiflora* by HPLC-DAD. Phenylpropanoids varied in the extracts (leaves 139.70 to 221.20 mg g^{-1}, flowers 106.43 to 227.22 mg g^{-1}, stems 42.18 to 56.48 mg g^{-1}). Verbascoside occurred in higher concentration in extracts of leaves (87.66 – 136.16) mg g^{-1} and flowers (58.12 – 148.96) mg g^{-1} than in stems (19.24 – 24.62) mg g^{-1}. Iridoids in extracts were as follows: leaves (46.60 – 54.79) mg g^{-1}, flowers (55.88 – 93.87) mg g^{-1} and stems (40.05 to 61.74) mg g^{-1}. High levels of iridoids (314.70 – 415.10) µg mL^{-1}, phenylpropanoids (1996.39 – 2674.13) µg mL^{-1} and verbascoside (1029.38 – 1456.42 µg mL^{-1}) in infusions support the popular consume of *V. minutiflora*.

Key words: medicinal plants, Verbenaceae, Gervão, analytical validation, Verbascoside, Hastatoside.

INTRODUCTION

Medicinal plants are used since ancient times and continue to attract worldwide attention because of their benefic effects on human health (Pan et al. 2015). However, the efficacy and safety of many species are not well established and researches about the chemical, pharmacological and toxicological properties of medicinal plants are essential to ensure their quality (Souza-Moreira et al. 2008).

The Verbenaceae family includes about 100 genera and 250 species distributed around the globe, in temperate, tropical, and subtropical areas. Among them, representative species of *Verbena* are popularly used for the treatment of fever, diarrhea, gastrointestinal disorders and some sexually transmitted diseases. Further indications are: diuretic, expectorant, anti-rheumatic, anti-inflammatory, antibacterial, antifungal, analgesic, antinociceptive, neuroprotective effects and antioxidant (Souza et al. 2005, El-Hela et al. 2010, Schönbichler et al. 2013, Hernández et al. 2000, Casanova et al. 2008, Calvo et al. 1998, Deepak & Handa 2000, Speroni et al. 2007, Calvo 2006, Rehecho et al. 2011, Braga et al. 2012, Lai et al. 2006, Bilia et al. 2008). *Verbena officinalis* L. is a typic species of *Verbena* and its main classes of metabolites are phenylpropanoids (verbascoside, isoverbascoside and β-OH-verbascoside), iridoids (hastatoside and verbenalin) and flavonoids (luteolin and apigenin) (Figure 1) (El-Hela et al. 2010, Schönbichler et al. 2013, Bilia et al. 2008, Castro-Gamboa & Castro 2004).

Verbena minutiflora occurs in southern Brazil and is popularly known as "gervão". The species is often used in folk medicine as infusions for the treatment of infections and urinary disorders. However, there is a

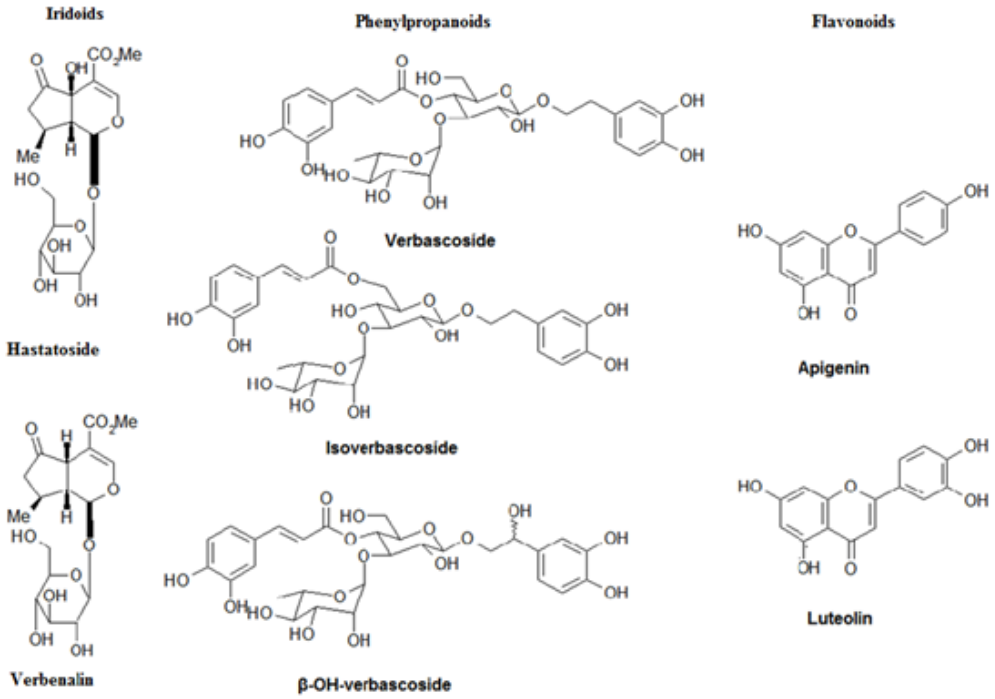

Figure 1. Main compounds found in *Verbena* species.

lack of scientific studies about the chemical composition or pharmacological activity of *V. minutiflora*. In previous work, we reported the presence of verbascoside, isoverbascoside and hastatoside in ethanol extracts and infusions of aerial parts (leaves, flowers, and stems) of *V. minutiflora* (Figure 1) (Soares et al. 2015). Since the pharmacological activity of *Verbena* is mostly associated with the presence of verbascoside and its derivatives, in this research we aimed to determine the levels of phenylpropanoids and iridoids in extracts and infusions of *V. minutiflora*. The plant material was collected during four consecutive years to verify the quantitative variability of these metabolites. Additionally, an *in house* validation study was performed according to international and national guidelines for foods and beverages to ensure the reliability of the quantitative results (ANVISA 2003, AOAC 2012). This is the first study about the quantitative composition of specific bioactive compounds in *V. minutiflora*.

MATERIALS AND METHODS

Plant material, extracts and infusions

V. minutiflora (cadastro SisGen n° A9406D5) was collected in Guarapuava, Paraná state, southern Brazil (25 23'08.57 ''S, 51 ° 26'50 63. ''W; altitude 1,115 m). The species was identified and classified by Osmar Ribas Santos, curator of the Museu Botânico Municipal in Curitiba/PR/BR. A voucher specimen was deposited in the referred herbarium under No. 359683. Samples were collected at the same location for four consecutive years from 2009 to 2012 and always in November (spring) when the plant has many flowers and leaves (Soares et al. 2015).

The extracts and infusions of *V. minutiflora* were prepared as described in Soares et al. (2015). The aerial parts of the plant (leaves, flowers and stems), collected in 2009, 2010, 2011 and 2012 were dried at room temperature and separately macerated in ethanol (plant material/ethanol 1:20 w/v) under stirring at room temperature. Dried ethanolic extracts of leaves, flowers and stems were obtained after solvent evaporation.

The infusions of *V. minutiflora* were prepared with 10 g of leaves and 200 mL of boiling water. The capped mixture was maintained for 20 minutes, then cooled and filtered.

HPLC-DAD analyses

Extracts and infusions were analyzed by HPLC (Waters 600 Controller) with UV diode array detection (Waters 2996) (HPLC-DAD). Chromatography was performed on a Waters XT Terra MS C18 column (250 mm × 4.6 mm, 5 µm) maintained at 26 ° C and with pre-column at the same stationary phase. The mobile phase flow rate was 0.8 mL min^{-1} and consisted of an aqueous formic acid solution at pH 3.2 (solvent A) and acetonitrile (solvent B). The linear gradient was performed according to Bilia et al. (2008). The elution started with 87% of solvent A and a linear gradient was carried out until 85% of solvent A in 10 min. After this, a second linear gradient was stablished up to 25% of solvent A in 20 minutes. This mobile phase composition was held for 6 minutes and then the percentage of solvent A was gradually decreased to 5% in 2 minutes and remained unchanged for 2 minutes (total chromatographic running time of 30 minutes). Afterward, the initial elution condition was established in one minute and maintained by 10 minutes for column equilibration before subsequent injection. The UV-VIS spectra for chromatographic peaks were recorded between 220 and 500 nm and the chromatograms were monitored at 240, 330 and 350 nm according to previous reports by Bilia et al. (2008). Each sample was injected in triplicate.

Validation study

Verbascoside 86.87% HWI Analytik GMBH Solutions Pharma (Germany) was used as an analytical standard representative of the class of phenylpropanoids while geniposide 98% (Sigma-Aldrich) was used for iridoids. Three samples of *V. minutiflora*, leaves 2010, flowers 2011 and stems 2012 were randomly selected to evaluate all validation parameters. Stock solutions of verbascoside and geniposide were made in methanol at 500 µg mL^{-1}. For all validation steps and subsequent application of the chromatographic method to determine phenylpropanoids, methanolic solutions of extracts of leaves, flowers and stems of *V. minutiflora* were made at concentrations 2.300, 2.200 and 6.200 µg mL^{-1}, respectively. On the other hand, to determine iridoids, methanolic solutions of extracts of leaves, flowers and stems of *V. minutiflora* were made at concentrations 8.200, 8.100 and 6.100 µg mL^{-1}, respectively.

Selectivity

The selectivity of the HPLC-DAD method was assessed by comparison of retention times and UV-DAD spectra of analytical standards verbascoside and geniposide with the chromatographic peaks assigned to verbascoside and its structurally related derivatives and to iridoids in all samples of aerial parts of *V. minutiflora*.

Linearity

To assess the linearity of the method, an analytical curve was built in the concentration range of 40 to 200 µg mL^{-1} for verbascoside and of 50 to 500 µg mL^{-1} for geniposide. Each analytical curve was made with seven levels of concentration, each concentration in triplicate.

A linear regression at 95% confidence level was adjusted to the experimental data (peak area and concentration). The linearity of the model was assessed by applying an analysis of variance and a lack-of-fit test (F_{lof}) at 95% confidence level.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limits of detection and quantification were calculated from parameters of the linear equation: LOD = *(3 × SD)/m* and LOQ = *(10 × SD)/m*, where *SD* is the standard deviation of intercept of the analytical curve and *m* is the slope of the analytical curve (Araujo 2009).

Precision

The precision was estimated for repeatability and the intermediate precision. To this, samples of leaves 2010, flowers 2011, and stems 2012 were prepared in triplicate. The repeatability was evaluated by injecting each sample in different periods of a single day (intra-day precision). Intermediate precision was studied by injection of each sample during five consecutive days (inter-day precision). Relative standard deviations *RSD* (%) were calculated as estimates of intermediate precision and repeatability.

Accuracy

The accuracy of the HPLC-DAD method was assessed by recovery tests. Extracts of leaves 2010, flowers 2011 and stems 2012 were spiked by the addition of standards in three concentration levels: verbascoside (50, 120 and 180 μg mL^{-1}) and geniposide (80, 200 and 400 μg mL^{-1}). Recovery rates were used as accuracy estimates and were calculated by the equation $Recovery\ (\%) = [(C_1 - C_2)/C_3] \times 100$ where C_1 is the concentration of analyte in the fortified sample, measured by the HPLC-DAD method, C_2 is the concentration of the analyte in the unfortified sample, and C_3 is the concentration of analyte added to the sample.

Statistical analyses

All statistical analyses were conducted at the 95% confidence level using the statistical software Minitab version 16.2.2.

RESULTS AND DISCUSSION

Validation of the HPLC-DAD method to determine Verbascoside and its derivatives and iridoids in extracts and infusions of *Verbena minutiflora*

Firstly, in the present study we carried out an *in house* validation to determine verbascoside and other phenylpropanoids and also iridoids in extracts and infusions of aerial parts of *V. minutiflora*. Validation parameters such as selectivity, linearity, Limit Of Detection (LOD), Limit Of Quantification (LOQ), precision and accuracy were checked according to the recommendations of ANVISA (2003) and AOAC (2012).

The selectivity of the HPLC-DAD method to determine verbascoside in *V. minutiflora* samples was confirmed by comparing retention times and UV-DAD absorption profile for both verbascoside standard and peak assigned to verbascoside in samples. Under the chromatographic conditions set, verbascoside had a retention time of 20.1 min and showed two absorption bands centered at 330 and 227 nm (Figure 2). All samples showed minor chromatographic peaks with close retention time and similar UV-DAD absorption profile to verbascoside. These chromatographic peaks were assigned to phenylpropanoid derivatives of verbascoside, which were previously observed by LC-ESI-MS/MS (similar fragmentation pattern as verbascoside) and are commonly reported in *Verbena* plants (Bilia et al. 2008). Thus, the HPLC-DAD method can be considered selective for quantitation of verbascoside and its analogues phenylpropanoids. Furthermore, the analytical method allowed differentiating among verbascoside analogues, making it possible to determine the verbascoside content and the total content of phenylpropanoids in *V. minutiflora* samples.

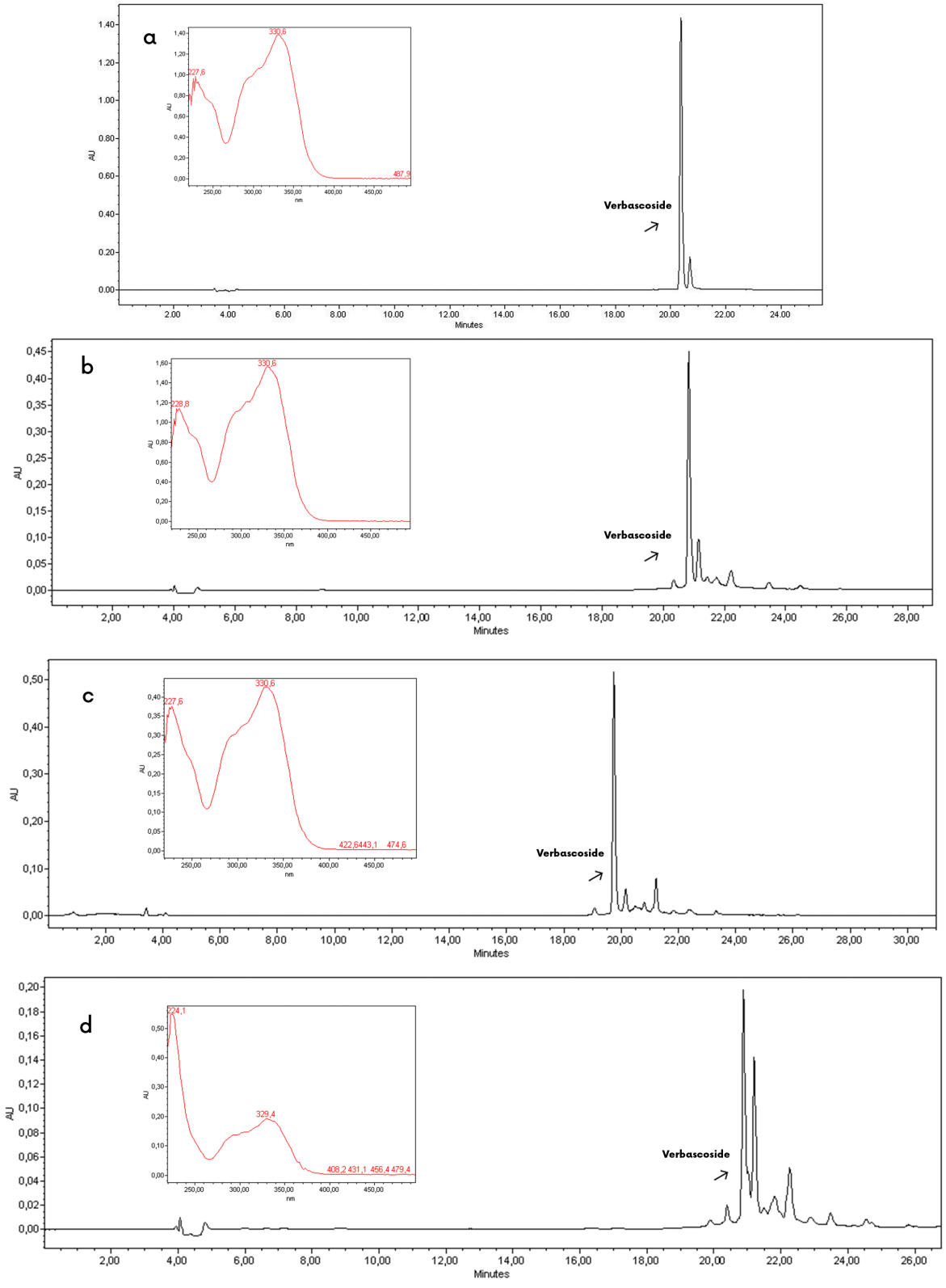

Figure 2. Chromatograms at 330 nm and UV-DAD spectra for peak at 20.1 minutes: (a) Verbascoside standard at 500.0 µg mL^{-1} and Extracts of (b) Leaves 2010 (c) Flowers 2011 (d) Stems 2012 of *Verbena minutiflora.*

Table I. Linear regression parameters for quantitation of Verbascoside (phenylpropanoids) and Geniposide (iridoids) by HPLC-DAD.

Verbascoside					
Regression		***Lack-of-fit***		***r***	***R^2***
$F_{regression}$	*p-value*	F_{lof}	*p-value*	0.996	99.2
2518.5	0.000	0.30	0.902		
Linear regression coefficients ± SE				$t_{observed}$	*p-value*
Intercept: -199443 ± 58062				3.43	0.003
Slope: 20936 ± 417				50.18	0.000
Geniposide					
Regression		***Lack-of-fit***		***r***	***R^2***
$F_{regression}$	*p-value*	F_{lof}	*p-value*	0.998	99.6
3998.4	0.000	5.16	0.008		
Linear regression coefficients ± SE				$t_{observed}$	*p-value*
Intercept: 278769 ± 63734				4.37	0.000
Slope: 13415 ± 212				63.23	0.000

The selectivity of the method was also evaluated for the class of iridoids. Although Geniposide does not occur in the samples of *V. minutiflora* under investigation, we have previously identified the iridoid hastatoside as a bioactive compound of these samples of *V. minutiflora* (Soares et al. 2015) and Geniposide and hastatoside are structurally related. Under the analytical chromatographic conditions established, these compounds showed the same retention time at 9.8 min. Additionally, both compounds have identical UV-DAD absorption profile with absorption maximum at 240 nm (Figure 3). In view of these facts, geniposide iridoid was considered an appropriate standard to determine iridoids in *V. minutiflora*. Consequently, the proposed chromatographic method showed adequate selectivity for iridoids analysis in the investigated samples and the content of iridoids may be reported as geniposide equivalents.

Linearity was evaluated on a calibration curve built with methanolic standard solutions of verbascoside in the range of 40 to 200 μg mL^{-1} and standard solutions of geniposide in the range of 50 to 500 μg mL^{-1}. To evaluate the suitability of the linear model, an analysis of variance and a lack-of-fit test (F_{lof}) were performed. The results (Table I) indicate that the linear model is appropriate to establish the relationship between the area of the chromatographic peak of verbascoside and its concentration, and between the area of the chromatographic peak of geniposide and iridoid concentration. Values for F_{lof} were not significant at the 95% confidence level ($p > 0.05$). This fact is also highlighted by the *F* values of the regressions which were highly significant ($p = 0.000$) at the same confidence level.

The analytical calibration curve was linear for verbascoside in the range of concentrations studied and may be represented

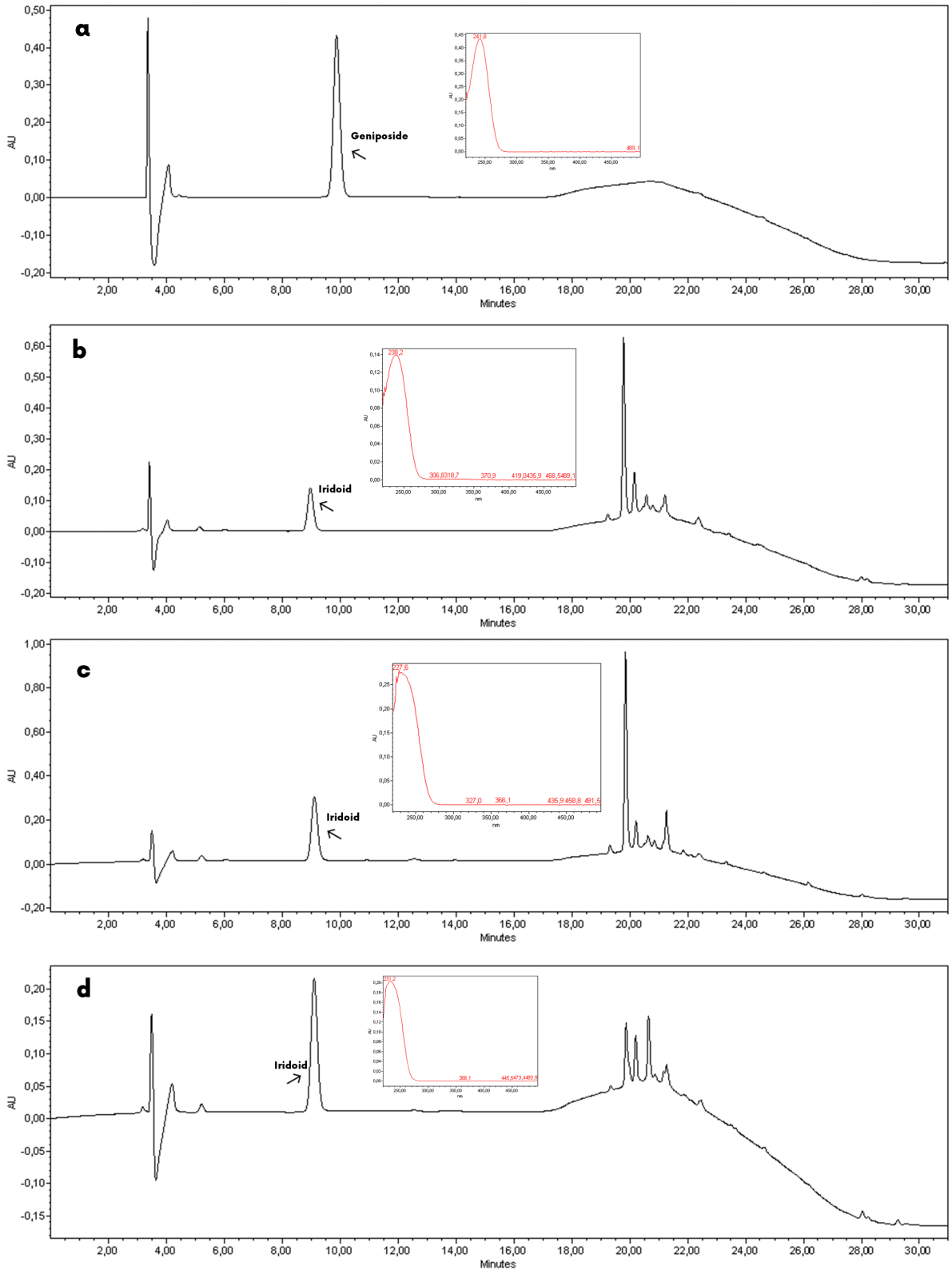

Figure 3. Chromatograms at 240 nm and UV-DAD spectra for peak at 9.8 minutes: (a) Geniposide standard at 500.0 µg mL^{-1} and Extracts of (b) Leaves 2010 (c) Flowers 2011 (d) Stems 2012 of *Verbena minutiflora.*

Table II. Precision (Repeatability and Intermediate precision, *i)* and accuracy tests (Recovery percentage, *Rec%*) for quantitation of verbascoside, phenylpropanoids and iridoids (as geniposide equivalent) in aerial parts of *Verbena minutiflora*.

Precision*				Accuracy				
Samples		*Repeatability*			Verbascoside		Geniposide	
					Concentration µg mL^{-1}	Rec%	Concentration µg mL^{-1}	Rec%
Verbascoside	Leaves 2010	2.71	9.55	**Leaves 2010**	50	107.14	80	88.91
	Flowers 2011	1.79	6.05		120	84.96	200	92.23
	Stems 2012	1.12	4.41		180	75.73	400	102.74
Total Phenylpropanoids	Leaves 2010	2.55	7.99	**Flowers 2011**	50	102.23	80	76.07
	Flowers 2011	1.54	6.47		120	75.74	200	106.11
	Stems 2012	0.57	5.48		180	76.74	400	112.89
Iridoids	Leaves 2010	4.11	4.69	**Stems 2012**	50	81.18	80	90.51
	Flowers 2011	3.36	4.39		120	77.93	200	103.86
	Stems 2012	4.58	4.87		180	114.22	400	114.33

by the equation: $A_{(verbascoside)} = -199443 + 20936 \times verbascoside\ concentration$. The analytical calibration curve for geniposide was also linear over the concentration range studied and can be represented by the equation: $A_{(geniposide)} = 278769 + 13415 \times geniposide\ concentration$.

The determination coefficients (R^2) showed that the linear regression explained 99.2% and 99.6% of the data variation for verbascoside and geniposide, respectively. Then, only 0.8% (verbascoside) and 0.4% (geniposide) refer to residues or random errors. The observed correlation coefficients (r) of 0.996 and 0.998 for verbascoside and geniposide, respectively are within the recommended limits ($r > 0.99$) by the national regulatory agency ANVISA (2003) and by AOAC (2012).

To evaluate if the proposed method would make it possible to determine verbascoside and iridoids at the concentrations that these bioactive compounds usually occur in *Verbena* samples, limits of detection and quantification

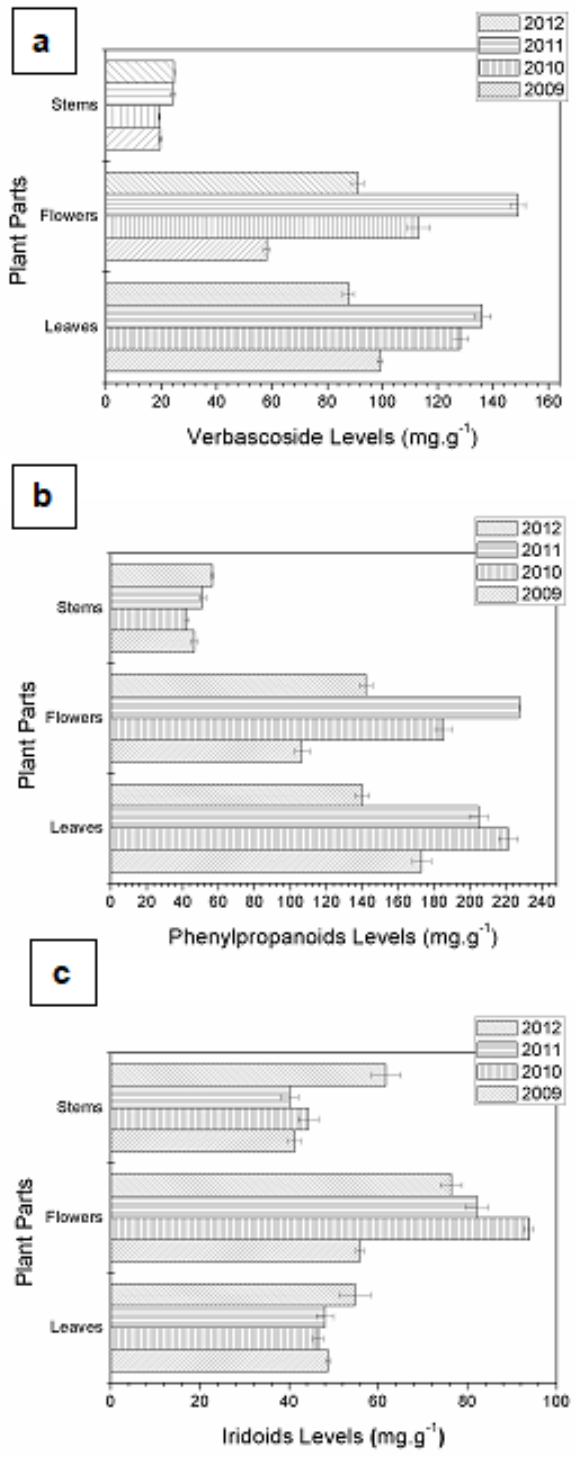

Figure 4. Annual variation of levels of Verbascoside (a), Total Phenylpropanoids (b), Iridoids (c) in aerial parts of *Verbena minutiflora.*

were estimated through parameters of the analytical curves. The LOD and LOQ values to determine verbascoside and/or its analogues were 4.09 and 13.65 μg mL^{-1}, respectively, showing that the method is suitable for the determination of phenylpropanoids in *V. minutiflora* in concentrations of ppm. The LOD and LOQ for geniposide were 14.25 and 47.50 μg mL^{-1}, respectively. It is worth mentioning that these relatively high values for iridoids are related to the wide concentration range used to calibrate iridoids in this study (50 to 500 μg mL^{-1}). LOD and LOQ calculated by curve parameters tend to be smaller when the analytical curve is set for low concentrations. This is because the slope of the curve depends upon the working concentration range and usually it is higher at lower concentrations.

Nevertheless, the HPLC-DAD method developed is suitable for determining verbascoside and its derivatives and iridoid compounds in *V. minutiflora* in amounts of parts per million, that are the levels at which these compounds occur in *Verbena* samples as previously reported by other authors (Schönbichler et al. 2013).

Precision study results for leaves 2010, flowers 2011 and stems 2012 to determine the verbascoside, phenylpropanoids and iridoids in *V. minutiflora* are shown in table II.

For the repeatability and intermediate precision *RSD* values below 15% are acceptable according to ANVISA (2003). Thus, the proposed method showed adequate precision to determine phenylpropanoids and iridoids.

The accuracy of the method was evaluated by performing the standard addition recovery tests at three levels of concentration (Table II). Recovery rates varied from 75.73% to 114.22% for verbascoside and 76.07 to 114.33% for geniposide and were within the acceptable range of 60 - 115% according to the guidelines of AOAC (2012)

and to previous reports (Brito et al. 2003). These results indicate that the proposed HPLD-DAD method has adequate accuracy for the quantitation of verbascoside and iridoids in the investigated samples.

Application of the validated method for the quantification of Verbascoside and analogues phenylpropanoids and iridoids in the aerial parts of *Verbena minutiflora*

After *in house* validation, the HPLC-DAD method was applied to all samples of extracts of leaves, flowers, and stems and to the infusions of aerial parts of *V. minutiflora*. Phenylpropanoid and iridoid contents were determined in triplicate using the same chromatographic conditions as those employed for the validation study. The results in Figure 4 are expressed as milligrams of the secondary metabolites per gram of dried extract.

All ethanolic extracts showed high levels of phenylpropanoids and iridoids. There was high variability in verbascoside and total phenylpropanoid contents for the different aerial parts. Higher levels of these bioactive compounds were extracted from leaves and flowers. Infusions were also highly concentrated in the bioactive compounds investigated: levels of iridoids varied from 314.70 to 415.10 μg ml^{-1}, of phenylpropanids from 1996.39 to 2674.13 μg mL^{-1} and of verbascoside from 1029.38 to 1456.42 μg mL^{-1}. We reported previously that infusions made from aerial parts of *V. minutiflora* had a remarkable antioxidant activity (Soares et al. 2015) which may now be related to their high levels of phenylpropanoids and iridoid glycosides.

The simultaneous determination of four bioactive compounds (aucubin, hastatoside, verbenalin and verbascoside) in the methanolic extract of *Verbena officinalis* L. showed that verbascoside levels ranged between (1.32 – 10.99) mg g^{-1} and the iridoids hastatoside levels ranged between (2.84 – 7.21) mg g^{-1} and verbenalin levels ranged between (1.21 – 5.01) mg g^{-1} (Liu et al. 2012).

Phenylpropanoids protect plants against infections, ultraviolet radiation, and herbivores (Chen et al. 2002). Additionally, phenylpropanoids have been reported to possess antioxidant, anti-inflammatory and antitumor activities (Chen et al. 2002, Sá et al. 2014). For example, researches on the effect of isoverbascoside in cell line proliferation and differentiation of the tumor cells MGC 803 (human gastric cancer) showed that isoverbascoside inhibited proliferation of these cells (Chen et al. 2002). Furthermore, several biological activities have been reported for verbascoside such as anti-inflammatory, antimicrobial, antitumor, hepatoprotective and antioxidant (Deepak & Handa 2000, Carrillo-Ocampo et al. 2013, Avila et al. 1999, Funes et al. 2009, 2010, Xie et al. 2012, Alipieva et al. 2014). Then, the high levels of verbascoside in extracts and infusions of *V. minutiflora* may be the cause of the potent antioxidant activity that we reported previously for this plant (Soares et al. 2015).

Iridoid glycosides are a large group of naturally occurring monoterpenoids (cyclopentapyran monoterpenoids) which are widespread in *Apocynaceae, Scrophulariaceae, Verbenaceae, Lamiaceae, Loganiaceae and Rubiaceae* (Tiwari et al. 2008, Viljoen et al. 2012). *In vitro* and *in vivo* pharmacological studies revealed that iridoids have neuroprotective activity, anti-inflammatory, immunomodulatory, hepatoprotective, cardioprotective, anti-cancer, antioxidant, antimicrobial, hypoglycemic, antispasmodic, and purgative properties (Tundis et al. 2008, Viljoen et al. 2012). It has been reported that the iridoid geniposide isolated from plants of the *Rubiaceae* showed potent anti-inflammatory activity, demonstrating significant inhibition of the inflammatory

mediator interleukin-2 (IL-2) (Chen et al. 2009, Viljoen et al. 2012). Other studies reported that the ethanolic extract of *Gardenia jasminoides* J. Ellis (*Rubiaceae*), which contained geniposide, can reduce the risk of gastritis by reversing gastric lesions in rats and also inhibited paw edema in rats induced by carrageenan (Koo et al. 2006, Lee et al. 2009, Viljoen et al. 2012).

Two new iridoids, 3- (5- (methoxycarbonyl) -2-oxo-2H-pyran-3-yl) butanoic (verbeofflin I) and 7-hidroxidehidrohastatosideo were isolated from the aerial parts of *Verbena officinalis* L., along with three known iridoids verbenalin, 3,4-dihidroverbenalin and hastatoside (Shu et al. 2014). The iridoids brasoside and verbraside were isolated from the aerial parts of *Verbena brasiliensis* Vell. (*Verbenaceae*), which is native to South America (Ono et al. 2006).

Iridoids like verbenalin and hastatoside are commonly isolated from species of *Verbena* and are considered as chemical markers to target constituents for quality control of these medicinal plants (Shu et al. 2014).

CONCLUSIONS

This is the first report about the levels of individual active ingredients in *Verbena minutiflora* collected in southern Brazil. We showed that extracts and infusions of aerial parts of *V. minutiflora* are rich in iridoids and phenylpropanids, particularly in verbascoside and its analogues. These facts support the popularly consume of infusions of *V. minutiflora*. To accomplish the quantification of these metabolites, we first carried out an *in house* validation study of an HPLC-DAD method. It was possible to verify that the proposed HPLC-DAD method met the recommendations of national and international regulatory agencies and showed adequate selectivity, accuracy, precision, and linearity to determine the phenylpropanoid verbascoside and derivatives, and iridoids, as geniposide equivalent. In addition, the obtained detection and quantification limits demonstrated that the technique was appropriate to determine the main metabolites of *V. minutiflora* that occur in concentrations of parts per million. Consequently, the analytical method is suitable for the quality control of *V. minutiflora*.

As phenylpropanoids and iridoids were determined in *V. minutiflora* collected during four consecutive years, the data herein reported may guide studies to establish a quality standard for this plant. *Verbena minutiflora* has wide ethnopharmacological use and therefore strong appeal to become a phytotherapic and here we demonstrated that the plant is an excellent source of bioactive compounds.

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