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Identification and Characterization of *Bauhinia* Species by Spectroscopic and Spectrometric Fingerprints

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Identificação e Caracterização de Espécies de *Bauhinia* por Impressões Digitais Espectroscópicas e Espectrométricas

Resumo: Algumas espécies de plantas do gênero *Bauhinia* são plantas medicinais com relevantes propriedades diuréticas, digestivas, anti-inflamatórias, analgésicas, sendo popularmente usadas no tratamento da hipertensão e do diabetes. As plantas deste gênero são conhecidas como "pata-de-vaca" devido ao formato de suas folhas; essa similaridade morfológica entre espécies e/ou subespécies torna sua diferenciação taxonômica um desafio. Buscando uma ferramenta auxiliar para discriminar espécies de *Bauhinia*, as impressões digitais espectroscópicas e espectrométricas dos extratos das folhas foram comparadas usando a análise de componentes principais (PCA). As amostras foram diferenciadas de acordo com o tipo (comercial ou não comercial), região de origem e espécie. A análise indicou que existem diferenças de perfil químico entre as amostras comerciais e não comerciais possivelmente originadas pela mistura de diferentes espécies de *Bauhinia* nas amostras comerciais. Desta forma, a PCA é uma ferramenta auxiliar na autenticação de amostras comerciais e não comerciais, rastreamento de origem e classificação taxonômica. Além disso, a atividade antirradicalar foi estatisticamente superior para amostras não comerciais ($p<0,05$), sugerindo que essa atividade diminui durante o armazenamento na prateleira.

Palavras-chave: *Bauhinia*; pata-de-vaca, perfil metabolômico, propriedades farmacológicas, comparação de qualidade, identificação taxonômica.

Abstract

Some species of plants of the genus *Bauhinia* are medicinal plants with relevant pharmaceutical properties such as diuretic, digestive, anti-inflammatory, analgesic and are popularly used to treat hypertension and diabetes. They are widely known as “cow’s foot” (“pata-de-vaca”) due to the shape of their leaves; this morphological similarity among species and/or subspecies makes their taxonomic differentiation a challenge. Seeking for an auxiliary tool to discriminate *Bauhinia* species, spectroscopic and spectrometric fingerprints of extracts of *Bauhinia* leaves were compared using principal component analysis (PCA). Samples were distinguished according to their type (commercial or non-commercial), geographic origin, and specie. Multivariate data analysis indicated that there are differences between the chemical profile of commercial and non-commercial samples possibly due to the mixture of different species of *Bauhinia* in commercial samples. Thus, PCA may help to authenticate commercial and non-commercial samples, to trace their origin and indicate taxonomic classification. Additionally, the antiradical activity was statistically higher for non-commercial samples ($p<0.05$), suggesting that this activity diminishes during shelf storage.

Keywords: Bauhinia; cow’s foot, metabolomic profiling, pharmacological properties, quality comparison, taxonomic identification.

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Identification and Characterization of *Bauhinia* Species by Spectroscopic and Spectrometric Fingerprints

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1. Introduction

Among the plants with therapeutic properties, it is worth highlighting species of *Bauhinia* genus (*Fabaceae* family, *Caesalpiniaceae* subfamily). With approximately 300 species found mostly in the southern hemisphere, these plants are found *in natura* in the form of shrubs or small trees, with some species popularly known as "cow's foot" ("pata-de-vaca") due to the morphological characteristics of their leaves.[1]

The interest in this genus comes from traditional folk knowledge that uses these plants for diuretic, digestive, skin healing, anti-inflammatory, and analgesic purposes and to treat hypertension and diabetes.[1-3] In Brazil, the most widely used species against hypoglycemia and diabetes are *B. candicans* and *B. forficata* Link.[4,5]

According to Soares and co-workers[4], *B. candicans* is used synonymously to indicate the subspecies *pruinosa* of *B. forficata* L.[4] Additionally, medicinal properties have been reported for *B. bauhinioides, B. cheilantha, B. holophylla, B. microstachya, B. rufa* and *B. ungulate*.[6]

A comparative study with *B. forficata*, *B. candicans*, and *B. variegata* was performed by Soares and Scarminio who used multivariate analysis

and mixture designs to verify the authenticity of the species based on fingerprints obtained by high performance liquid chromatography (HPLC).[4] Through Principal Component Analysis (PCA), species were discriminated into four groups and the authors suggested that *B. candicans* was different from other species; therefore, it could not be regarded as synonymous of *B. forficata* as affirmed earlier. In addition, PCA discriminated the species due to different polar compounds.

Furthermore, Ferreres and co-workers[7] concluded that the species *B. forficata* and *B. forficata* subsp. *pruinosa* had different chemical composition.[7] Likewise, commercial samples sold as *B. forficata* showed large difference in chemical composition from the certified samples. This may suggest that the species sold were different from that declared in the label or otherwise a more proper control quality is needed to guarantee the safety of this medicinal plant to public health.

The distinction among species of *Bauhinia* is a challenge due to the similar morphology of their leaves. The use of the incorrect species may not have the expected therapeutic efficacy or may cause unexpected or undesirable pharmacological effects. Thus, quick, and low-cost auxiliary tools to assist with the correct identification of these plants are necessary since current taxonomic classifications are not infallible, especially concerning the differentiation between species and/or subspecies that have subtle morphological differences.[5-8]

Taking this into consideration, the aim of the current study was to perform an exploratory analysis of commercial and certified non-commercial samples of plants belonging to the *Bauhinia* genus. To accomplish this, the fingerprints obtained by spectroscopic and spectrometric methods were analyzed using chemometric tools. Additionally, since the plants of this genus have relevant antioxidant properties, all samples had their antiradical activity evaluated as an easy and straightforward way to verify possible differences in the antiradical capacity of the species.

2. Experimental

2.1. Samples

Bauhinia samples were purchased in local markets of Campo Mourão-Paraná (PR) and Guarapuava-PR, Brazil. Only two out of seven commercial samples had their content identified in the label as *Bauhinia forficata* and the others only mentioned the popular name cow's foot (Table 1).

Leaves of non-commercial plants were also collected in different cities of Paraná state (southern Brazil): Campo Mourão, Guarapuava, Irati, Paranavaí, Prudentópolis, and in the botanical garden of Copel in Faxinal do Céu. All non-commercial plant species were identified and certified by the botanical museum of Curitiba-PR, Brazil. A summary of the characteristics and origin of the *Bauhinia* samples are shown in Table 1.

After collection all non-commercial samples were dried at room temperature for 4 days and subsequently storage at -5 °C in plastic bags protected from light and humidity to avoid microbial degradation and decomposition of metabolites.[9,10]

2.2. Extraction

The dried leaves of each sample were crushed and macerated with methanol in a 1:15 ratio (10.0 g / 150.0 mL^{-1}) for 24 h, followed by filtration under vacuum. Three further steps of extraction with fresh solvent were carried out to exhaustive metabolite extraction. The solvent was removed by evaporation at 45 °C under reduced pressure.[11]

2.3. Analytical methods

2.3.1. Ultraviolet and visible spectroscopy (UV–Vis) analysis

UV-Vis analyses were performed in a Femto® 800XI spectrophotometer. The spectra were acquired within 200 and 800 nm, with the addition of 2 nm s^{-1}. For this purpose, solutions of methanolic extract with a concentration of 250.0 mg L^{-1} were prepared, and measurements were carried out in a 1 cm quartz cuvette with 3.0 mL of solution. For more information about UV-Vis data, see supplementary material.

2.3.2. Infrared spectroscopy (FTIR) analysis

Fourier transform infrared analyses were performed in a Bio-Rad® FTS 3500GX spectrophotometer. The spectra were recorded in the 4000-400 cm^{-1} range in emission mode with 32 scans and 4 cm^{-1} resolution. For this, 1.0 mg

Table 1. Description of the *Bauhinia* species

Sample Code	Type	Origin	Specie	Brand	Flower Color
CG1	Commercial	Guarapuava	Unknown	Brand A	Unknown
CG2	Commercial	Guarapuava	*B. forficata*	Brand B	Unknown
CCM	Commercial	Campo Mourão	Unknown	Brand C	Unknown
CT	Commercial	Turvo	Unknown	Brand D	Unknown
CRS1	Commercial	Rio Grande do Sul	*B. forficata*	Brand E	Unknown
CM	Commercial	Maringá	Unknown	Brand F	Unknown
CRS2	Commercial	Rio Grande do Sul	Unknown	Brand G	Unknown
NPvBvR	non-commercial	Paranavaí	*B. variegata* Linn	-	Pink
NCMBvB	non-commercial	Campo Mourão	*B. variegata* Linn	-	White
NCMBvR	non-commercial	Campo Mourão	*B. variegata* Linn	-	Pink
NFCBfB	non-commercial	Faxinal do Céu	*B. forficata* Link	-	White
NFCBR	non-commercial	Faxinal do Céu	Unknown	-	Pink
NFCBB	non-commercial	Faxinal do Céu	Unknown	-	White
NPrBfpB	non-commercial	Prudentópolis	B. *forficata* Link . subsp pruinosa (Vogel) Fortunato & Wunderlin	-	White
NIBvR	non-commercial	Irati	*B. variegata* Linn	-	Pink
NIBcB	non-commercial	Irati	*B. cheilanta* (Bong.) Steud.	-	White

*Sample Codes: Sample type was classified as commercial or *non-commercial* according to whether they had been purchased from local markets or directly collected from nature. Origin refers to the city of collection in Paraná state. Plant species was mentioned only when specified in the label of commercial samples or identified by a botanical curator for *in nature* non-commercial samples. Brand was identified by letters only for commercial samples. The flower color was only reported for *non-commercial* samples which had flowers at the collection time. CG: commercial samples from Guarapuava. CCM: commercial sample from Campo Mourão. CT: commercial samples from Turvo. CRS: commercial samples from Rio Grande do Sul. CM: commercial sample from Maringá. NPvBnR: non-commercial sample from Paranavaí. NCMBv: non-commercial samples from Campo Mourão. NFCB: non-commercial samples from Faxinal do Céu. NPrBfpB: non-commercial sample from Prudentópolis. NIB: non-commercial samples from Irati

of dry extract was weighed and homogenized with 1.0 g of solid KBr in an agate mortar.[12] The full details about infrared data, are available in supplementary material.

2.3.3. LC-MS/MS and-ESI-MS/MS analyses

ESI-MS fingerprints and mass exact data were acquired in a 7.2T LTQ FT Ultrahigh mass spectrometer (Thermo Scientific®, Bremen, Germany) equipped with a chip-based direct infusion nanoelectrospray ionization source (Advion BioSciences®). Data acquisition was performed along the *m/z* 50-800 range using the software Xcalibur® 2.0. Fingerprints of the samples were obtained by direct injection of 5.0 µL of samples into the electrospray source in negative mode (ESI-MS). Electrospray source and mass spectrometer analytical conditions were the following: capillary voltage -3.55 kV; cone, -30 V; source and desolvation temperature 150 and 350°C. All ESI-MS data are available in supplementary material.

Chromatographic analysis was carried out on an UPLC mass spectrometer (Waters Acquity, Milford, MA, USA) coupled with a TQD Acquity mass spectrometer (Micromass - Waters®), with an ESI source. The chromatographic separation of the components was performed using a Waters Acquity BEH C18 (50 mm × 2.1 mm i.d., 1.7 mm) column at 30 °C. Water with 0.1 % of formic acid (solvent A) and acetonitrile (solvent B) was used as mobile phase at a flow rate of 0.2 mL min $^{-1}$. The initial condition was 95% A and 5 % B with a linear gradient changing to 100 % B in 9 min, maintaining this condition until 10 min, then returning to the initial condition and stabilizing line base until 12 min. Nitrogen and argon were used as nebulizer and collision gases, respectively. The electrospray ionization was carried in the negative ion mode under the following conditions: capillary, -1.55 kV; cone, -160 V; source temperature and desolvation

temperature, 270 °C and 350 °C, respectively. Argon was used as collision gas, and the collision energy for induced dissociation (CID) was 25 eV. The chemical composition of the extracts was proposed using Xcalibur software®.

2.3.4. Determination of antiradical capacity

Antiradical capacity was estimated by the spectrometric method that uses the stable radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) in accordance to Rufino *et al*. with some modifications.[13]

The reaction was conducted by direct dilution in a quartz cuvette with the addition of a fixed volume (2.5 mL) of the DPPH working solution (absorbance= 0.7 at 515 nm). The test was performed varying the volume of each sample, and the addition of methanol to the final volume of 3.0 mL.

For every sample, an analytical calibration curve was made with the radical scavenging percentage RS %= 100×(Ai–Af)/Ai) versus the concentration of crude extract, where Ai is the absorbance of the solution composed by 2.5 mL of the DPPH working solution and 0.5 mL of methanol.

The final absorbance (Af) was measured after 30min of reaction between the DPPH radical and the natural antioxidants in *Bauhinia* extracts. Antiradical activity of each extract was reported as EC_{50} value, which represents the necessary sample concentration needed to a 50 % reduction of the initial DPPH working solution absorbance.

2.3.5. Statistical analysis

PCA was applied to all data of FTIR, UV-Vis, and ESI-MS fingerprints.[14] The UV-Vis data was normalized from 0 to 1. In this case, PCA was applied to a 16 x 288 data matrix being the variables the different wavelength from 250 to 490 nm in each column and the cases corresponding to each *Bauhinia* sample. The software used was Statistica® version 7.0.

For multivariate analysis of FTIR spectra, the data were pretreated by normalization of 0 to 1, smoothing of the base line by Fourier transform with 10 % of cut of the relative frequencies to experimental noise and application of the second derivative to all remaining spectra. The PCA was applied to a 16 x 3641 data matrix assembled with each row corresponding to one *Bauhinia* samples and each column corresponding to the second derivative of the % transmittance of different wavenumber. The software used was MATLAB® version 7.8.0 (R2009a).

ESI-MS raw data was submitted to a variable selection process by cross-validation for an easy and better interpretation without loss of important information. Thus, 136 variables were discarded, and a new correlation matrix was set without any pre-treatment. PCA was applied to a 16 x 224 data matrix using the abundance of each ion (*m/z*) of the fingerprint as variables and each *Bauhinia* sample as cases. The software Statistica® version 7.0 was used.

One-way ANOVA followed by post hoc Fisher test with 95 % statistical significance ($p< 0.05$) was applied to the results of antiradical activity tests. Statistica® version 7.0 was used for calculations.

3. Results

3.1. Multivariate analysis of UV-Vis and IR fingerprints

Most UV-Vis spectra of *Bauhinia* extracts showed the same absorption profile with intense absorption within the range of 250 to 410 nm. The most intense bands occurred at around λ_{max} 270, and 350 nm (see supplementary material). This absorption profile may indicate the presence of flavonoids that generally have two absorption maxima, one occurring between 240 and 285 nm and another between 300 and 400 nm. For example, in flavonols Band I (absorption due to B-ring) is usually within 300 - 380 nm and Band II (absorption due to A-ring) within 240-280 nm.[1,10,15]

Figure 1 shows the grouping of *Bauhinia* samples resulting from PCA of UV-Vis spectra. Altogether, the first and second principal components explain 96.20 % of the variability of the extracts. It is possible to verify that both components discriminated the samples according to their type (commercial or non-commercial) and according to the plant species.

The first component was responsible for discriminating non-commercial samples according to their *Bauhinia* species. Most samples in the positive PC1 axis were identified as *B. variegata* (with the exception of NIBcB, which belongs to the species *B. cheilanta*, and NFCBR, with no identified specie), while those situated at the

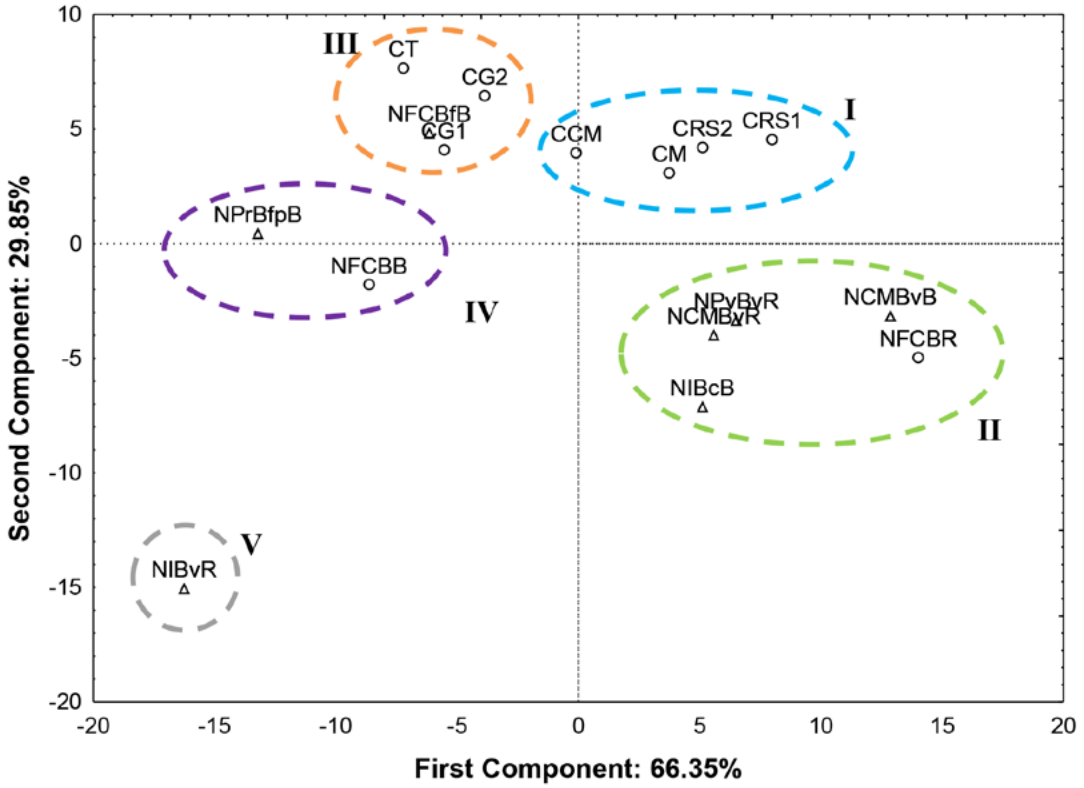

Figure 1. *Bauhinia* grouping by PCA of UV-VIS fingerprints from 250 to 490 nm

negative PC1 axis were identified as *B. forficata* (with the exception of NIBvR, which belongs to the species *B. variegata*). It can be noticed that non-commercial samples from the same geographic region were separated due to their *Bauhinia* species. For example, samples from Irati-PR (NIBvR and NIBcB) were classified into different groups, and samples from Faxinal do Céu-PR (NFCBfB, NFCBB, and NFCBR) were distributed into different groups. This may indicate that the non-commercial samples from Faxinal do Céu-PR belong to different species or varieties of *Bauhinia*.

All commercial *Bauhinia* samples had positive scores in the second principal component and were separated from non-commercial samples, except for NFCBfB. By analyzing both components together, the formation of five distinct groups was observed. Group I contained commercial samples from Campo Mourão-PR, Maringá-PR and Rio Grande do Sul-RS, Brazil. Only the commercial samples CG2 and CRS1 were clearly identified in the package label as *B. forficata* while the other samples were identified as cow´s foot. Group II was made of non-commercial samples from Campo Mourão, Paranavaí, Irati and Faxinal do Céu. Group III contained commercial samples from Guarapuava, Turvo and one non-commercial sample of *B. forficata*. This suggests that CT and CG1 are also *B. forficata* species. It should be highlighted that as the flowers were not extracted, the flower color of each plant was not important for classification in this analysis. See for instance that group II contains plants with white and pink flowers.

Another interesting point is that the commercial samples gave intermediate scores in the first component. They were located between the natural samples of groups II and IV, mostly belonging to *B. variegata* and *B. forficata* species, respectively. On one hand, this may indicate that the commercial samples are composed of a mixture of species of *Bauhinia* and that group III was formed mainly by leaves of *B. forficata*, while group I was bulkily formed by leaves of *B. variegata*. On the other hand, although raw UV-Vis data were normalized before multivariate analyses, one needs to consider possible differences in the concentrations of natural compounds in both types of *Bauhinia* samples which results in different amount of absorption.

In the same way as observed for UV absorption, the IR spectra of *Bauhinia* samples showed a similar

profile with vibrational bands centered at 3300 cm^{-1} (broad and intense) due to O-H stretching, 2850-2960 cm^{-1} due to $C_{sp}{}^{3}$-H asymmetrical and symmetrical stretching. Multiple bands within 1620- 1680 cm^{-1} correspond to stretches of C=O and C=C; this altogether with medium bands around 625 cm^{-1} (ring bend) are indicative of aromatic rings. Also, bands between 1000 and 1200 cm^{-1} correspond to C-O stretch band.[15,16]

The multivariate analysis of these data resulted in the groupings observed in Figure 2. This time the first and second principal components had a cumulative variance of 55.37 %.

The most straightforward grouping is the separation of non-commercial from commercial samples; samples with scores above 0.8 are commercial *Bauhinia* samples while those with scores below 0.8 are non-commercial samples (exception CT).

The second principal component separated the samples by region of origin. *Bauhinia* samples with positive scores in PC2 were from Midwest Paraná and Rio Grande do Sul, while samples with negative PC2 scores were from northern Paraná. Because the commercial samples were grouped with non-commercial samples for group I, we suggested that commercial samples are composed of a mixture of *Bauhinia* species. This is reinforced by the fact that CG2 and CRS1 were identified as *B. forficata* while NCMBvB was identified as *B. variegata*. Possibly, a mixture of *Bauhinia* species occurs during collection and processing due to the morphological similarities among the leaves of *Bauhinia* species.

Group II was formed by CCM and CM samples. Separation of groups I and II may be due to different climatic conditions, which affect metabolite production. It is worth mentioning that the climate of the Midwest region of Paraná is similar to some regions of Rio Grande do Sul, which may contribute to the grouping of the commercial samples from these two locations. Other factors that possibly affect the quality of commercial samples are shelf time and even handling of the plant since the time of collection to consumption.

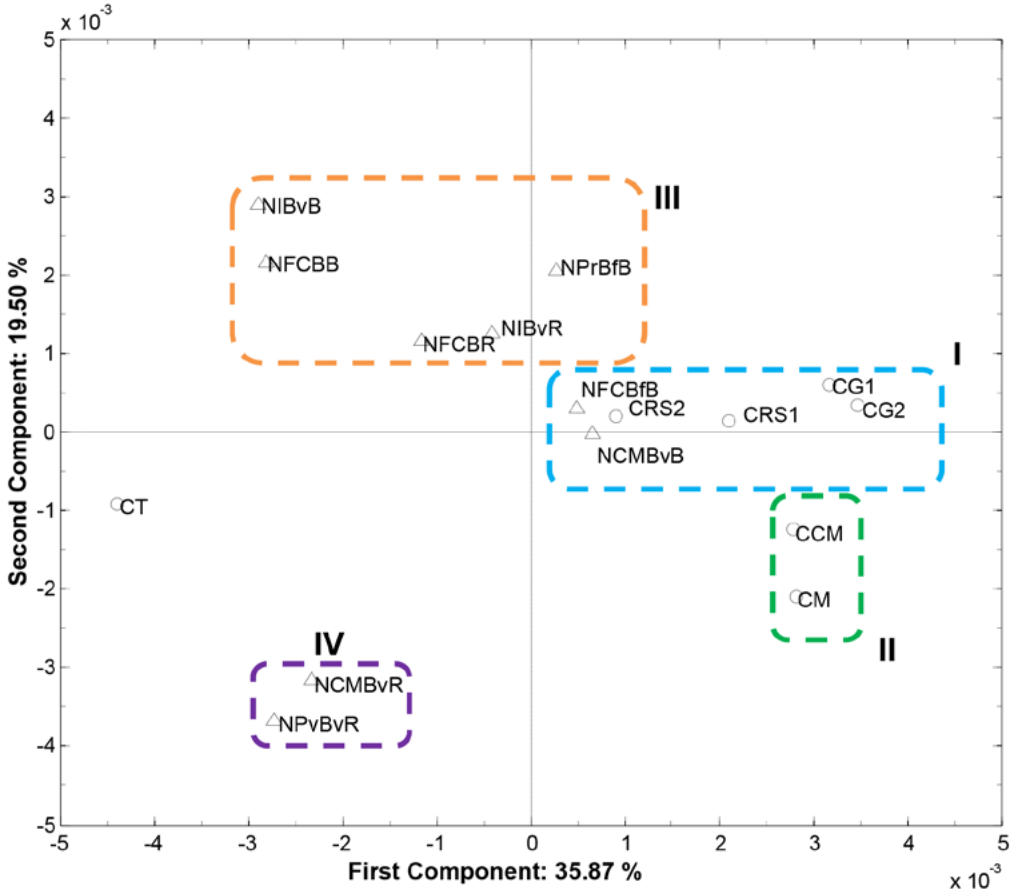

Figure 2. *Bauhinia* grouping by PCA of IR fingerprints from 490 to 3900 cm^{-1}

The samples in group III include only non-commercial *Bauhinia* samples from the Midwest region of Paraná, while group IV includes those from northern region. In this way, the analysis of the spectroscopy data at the infrared region aligned with the PCA, allowed discriminating *Bauhinia* samples mainly by their type (commercial or non-commercial) and the region of origin.

3.2. Multivariate analysis of ESI-MS fingerprints and tentative identification of compounds in leaves of *Bauhinia* species by LC-MS/MS and Exact Mass Data

To verify if ESI-MS fingerprints would allow discrimination of *Bauhinia* samples, PCA was applied to these data (Figure 3). The first and second principal components showed a cumulative variance of 49.14 %.

Once again, PCA displayed a clear distinction between non-commercial and commercial samples. Group I and II were totally made of non-commercial samples, while group III was composed of commercial samples. Therefore, the first component reveals that there are differences between commercial and non-commercial samples originated by different chemical profile in ESI(-)-MS.

The second component was responsible for discriminating non-commercial samples according to their geographic origin. For example, *Bauhinia* samples collected from northern Paraná were separated in the positive axis of PC2, while those from the middle west region of Paraná showed negative scores for the same component. Moreover, the second component partially segregated the samples belonging to different *Bauhinia* species; those classified in group I were all *B. variegata*. Only the sample NIBvR of *B. variegata* was classified in group II, indicating the same behavior observed by the chemometric analysis of UV-Vis and FTIR fingerprints (Figure 1 and Figure 2, respectively).

The other samples of group II were of different or of unknown species and the ESI-MS fingerprint of this group showed a difference in their chemical composition. Again, the scores of commercial samples in the second component may uncover a mixture of *Bauhinia* species in the commercial package since these are located in the middle of the score graph with the samples certified by Municipal Herbarium of Curitiba-PR. It is important to highlight that commercial package of CG2 and CRS1 samples are labeled as *B. forficata*. Due to the intermediate score of these samples, it is possible to suggest that the chemical profiles of

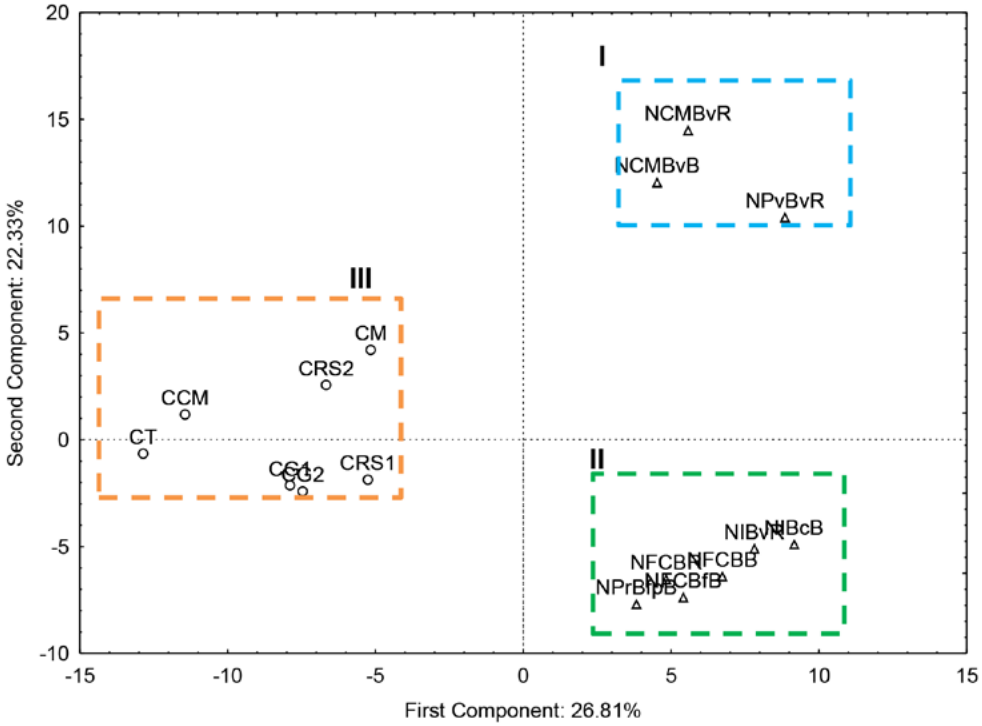

Figure 3. *Bauhinia* grouping by PCA of ESI-MS fingerprints within 60 to 800 m/z

the commercial samples were similar to species *B. forficata* and *B. variegata* (NFCBfB and NIBvR).

Multivariate analysis of the ESI-MS data made it possible to correlate the samples according to their type, that is, it was possible to determine if the sample was collected directly in the wild or if it was purchased in the market.

An attempt to identify the compounds in the methanolic extracts was performed using LC-MS/MS analysis and exact mass measure (Table 2). The results were compared with those available in the scientific literature about the chemical composition of *Bauhinia* genus.[7,17]

Ion fragmentations were analyzed and correlated with data reported. In addition, exact mass data observed by LC-MS/MS, the molecular formula was obtained, and the data was compared with compounds already reported for this genus. This attempt at identification resulted in a suggestion of 18 compounds (Table 2).

Table 2. Identification of metabolites in extracts of *Bauhinia* species by LC-MS / MS and / or exact mass data

Exact Mass *m/z* [M-H]-	Calculated Exact Mass *m/z* [M-H]-	Delta (ppm)*	MS/MS *m/z* [M-H]- (Relative Intensity)	MS/MS *m/z* [M-H]- Literature	Rt (min)	Compound
279.2327	279.1238	3.90E-04	216 (35); 103 (100); 67 (70)	279; 217; 205; 151	7.08	7'-hidroxi-abscisic acid
417.1039	417.0826	5.11E-05	417 (100); 285 (30); 152 (100)	417; 285 (100)	2.11	Kaempferol-3-O-arabinopiranoside
417.2125	417.0826	3.11E-04	417 (20); 356 (50); 284 (100); 255 (30); 193 (30)	417; 285 (100)	3.55	Kaempferol-3-O-arabinofuranoside
431.0980	431.0983	-6.96E-07	431 (100); 285 (100); 255 (90); 240 (30)	431; 285 (100)	2.71	Kaempferol-3-O-rhamnoside
433.0778	433.0775	6.93E-07	433 (100); 304 (10); 209 (10)	433; 301 (100)	2.79	Quercetin-3-O-xiloside
433.2079	433.0775	3.01E-04	433 (40); 301(100); 256 (30); 179 (30)	433; 301 (100)	3.34	Quercetin-3-O-arabinopiranoside
433.2354	433.0775	3.65E-04	326 (100); 300 (5); 239 (80); 153 (60)	433; 301 (100)	6.47	Quercetin-3-O-arabinofuranoside
447.0938	447.0933	1.12E-06	447 (100); 301 (80); 284 (30); 255 (20); 151 (30)	447; 301 (100)	3.46	Quercetin-3-O-rhamnoside
449.2028	449.0725	2.90E-04	449 (10); 316 (100); 179 (40)	449; 316 (100)	3.00	Myricetin-3-O-arabinopiranoside
449.3123	449.0725	5.34E-04	449 (30); 303 (100); 248 (40); 225 (30)	449; 316 (100)	3.48	Myricetin-3-O-arabinofuranoside
463.0885	463.0882	6.48E-07	463 (50); 316(100); 271 (20); 259 (20); 148 (20)	463; 316 (100)	3.15	Myricetin-3-O-rhamnoside
593.1518	593.1514	6.74E-07	593 (70); 447 (70); 400 (60); 284(100)	593; 285 (100)	3.16	Kaempferol-3-O-robinoside
593.2746	593.1514	2.08E-04	593 (70); 284 (100)	593; 285 (100).	3.45	Kaempferol-3-O-rutinoside
609.1466	609.1463	4.92E-07	609 (100); 300 (60)	609; 301 (100)	3.14	Quercetin-3-O-rutinoside
615.0996	615.0992	6.50E-07	615 (60); 479 (40); 317 (100)	615; 463 (100); 313 (6); 301 (30)	3.45	Quercetin-3-O-galloil-hexoside
623.1626	623.1621	8.02E-07	623 (100); 356 (70); 315 (60)	623; 315 (100)	3.50	Isorhamenetin-3-O-rutinoside
739.2106	739.2096	1.35E-06	739 (100); 591 (10); 284 (20)	739; 593 (100); 285 (7)	3.03	Kaempferol-3-O-(2-rhamnoside) rutinoside
755.2059	755.2045	1.85E-06	755 (100); 488 (10); 300 (30); 179 (20).	755; 609 (27); 591 (23); 489 (60); 301 (100)	2.85	Quercetin-3-O-rutinoside-7-rhamnoside

*Delta calculated by (Exact Mass *m/z* – Calculated Exact Mass *m/z*) / Calculated Exact Mass *m/z*

Most compounds were identified as glycosylated flavonoids derivatives of kaempferol (6 compounds), quercetin (7 compounds), and myricetin (2 compounds). Through MS/MS, it was possible to verify that the base peak of most of these molecules was related to the loss of glyosidic units. For instance, the compound Kaempferol-3-O-robinoside with $[M–H]^-$ of *m/z* 593 had a base peak at *m/z* 284 originated by the loss of 309 Daltons (Da), which corresponds to the loss of the robinoside radical (Figure 4). On the other hand, the loss of 147 Da from the molecular ion or from the deprotonated ion, which corresponds to rhaminosyl loss, originates the fragments *m/z* 446/447. In addition, the fragment *m/z* 400 may originate from the simultaneous loss of 177 Da relative to the sugar unit and 16 Da relative to the loss of oxygen of any hydroxyl group present in the structure that can be represented as $[(M–H)–177–16]^-$.

The compounds kaempferol-3-O-rutinoside and kaempferol-3-O-robinoside are diastereoisomers that differ only in the absolute configuration of carbon 2 of the radical rhaminosil (epimers). This difference means that these compounds exhibit different physical properties, which leads to different retention times (Table 2).

The most intense peak in ESI-MS/MS of quercetin-3-*O*-rhamnoside was its deprotonated molecular ion $[M–H]^-$ at *m/z* 447. The *m/z* 300/301 fragments may be formed by the loss of the glycosidic unit and can be represented as $[(M–H)–147]^-$ / $[(M)–147]^-$. The characteristic cleavage of the central ring in flavonols (1,3-cleavege) is observed by the loss of 296 Da giving the *m/z* 151 ion.

In the ESI-MS/MS spectra of myricetin -3-O-rhaminoside with $[M–H]^-$ of *m/z* 463, the base peak is the ion *m/z* 316, indicating the loss of the rhaminosidic unit $[(M–H)–147]^-$. The fragment

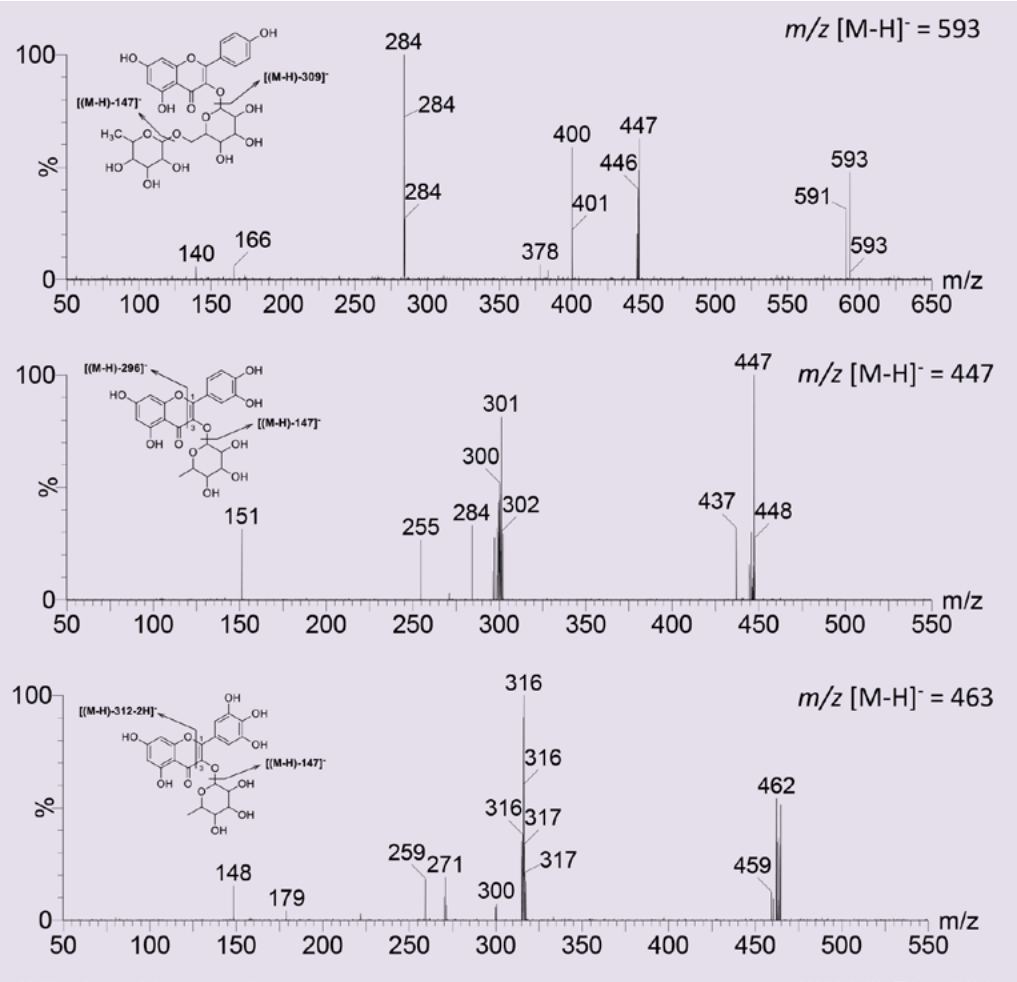

Figure 4. ESI-MS/MS and possible fragmentation of Kaempferol-3-O-robinoside (*m/z* $[M-H]^-$ 593), Quercetin-3-O-rhamnoside (*m/z* $[M-H]^-$ 447) and Myricetin-3-O-rhamnoside (*m/z* $[M-H]^-$ 463)

ion of *m/z* 148 may arise from fragmentation [(M–H)–312–2H]$^-$, where 312 Da corresponds to the cleavage of central ring.

Overall, it is possible to verify that the loss of the glycosidic unit occurs frequently, followed by the loss of the oxygen atom of carbon 3 and a central ring cleavage in flavonoids. In some flavonols was possible to check the loss of hydroxyl groups.

Ions fragments of *m/z* 284/285, 300/301, and 316/317 were observed in most ESI-MS/MS spectra of compounds derived from kaempferol (*m/z* [M–H]$^-$ 417, 431, 593, and 739), quercetin (*m/z* [M–H]$^-$ 433, 447, 609, 615, and 755), and myricetin (*m/z* [M–H]$^-$ 449 and 463), respectively, as described in Table 2. According to Ferreres and co-workers[7], the presence of the fragment ions of *m/z* 284/285 and 300/301 indicate that these flavonoids retain a glycosidic unit at position 3-O, observed by the loss of sugar units. In all identified flavonoids this behavior was verified, which agrees with data described in the literature.

3.3. Evaluation of antiradical activity

In order to better demonstrate the antiradical activity of species of *Bauhinia*, the antiradical capacity of each sample was expressed as IC_{50} (Figure 5A). The lower the IC_{50} the more potent the antiradical activity of the sample because a lower concentration of the extract is needed to inhibit 50 % of the stable radical.

From the data, it is evident that CT sample had the lowest antiradical activity, while the samples NPvBvR, NCMBvR, CM, NFCBR, and NIBcB had the best radical scavenge potential. The other samples showed intermediate antiradical activity.

Significant statistical differences (p<0.05) were found between the antiradical capacity of non-commercial and commercial samples (Figure 5B). Better antioxidant activity of the recently collected samples compared to the commercial samples may be due to the progressive degradation of the active compounds in the commercial samples due to shelf time, handling of the sample, poor quality

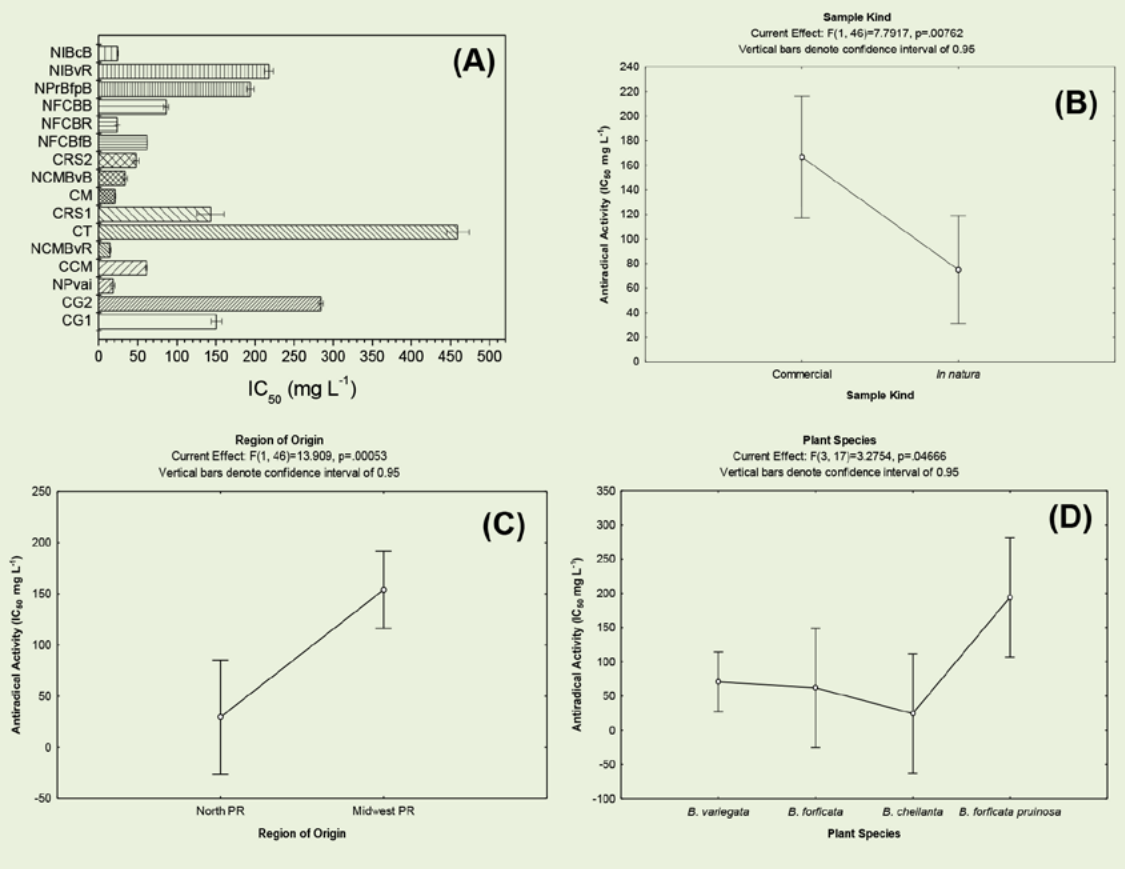

Figure 5. Antiradical activity of methanolic extracts of leaves of *Bauhinia* plants (A) and one-way ANOVA for the antiradical activity of *Bauhinia* extracts in relation to the sample type (B), region of origin (C) and plant species (D)

control of batch compliance, and other factors that can lead to decomposition.

A one-way ANOVA was applied to all non-commercial samples, considering the region of origin, within the state of Paraná, as a factor (Figure 5C). It was possible to verify significant statistical differences (p<0.05) in the antiradical activity of samples from different regions. On average, samples from the northern region of Paraná showed better antiradical capacity than samples from the Midwest. This significant difference in the antiradical potency between samples from different regions may be due to the influence of climate on the production of natural antioxidant products. As it has been demonstrated, plants from regions with higher solar radiation incidence have higher levels of phenolic compounds to protect the plant against photodamage. Other factors, such as plant age, rainfall index, altitude, and soil nutrients, also contribute to the variation in the concentration of phenolic compounds.[18]

Finally, a one-way ANOVA was applied to the antiradical activity of *Bauhinia* considering the species of each sample. In Figure 5D, it is possible to verify that on average there was no significant difference among the antioxidant activity displayed by the different species of *Bauhinia* analyzed in the current study. As the *p*-value is at the cutting-edge of significance it is plausible to highlight that, the extracts of *B. variegata*, *B. forficata* and *B. cheilanta* show a trend to a more powerful antiradical capacity than the extracts of *B. forficata pruinosa*. From this, we suggest that commercial samples of *Bauhinia* leaves must have explicit labels with the proper identification of the species of *Bauhinia* sold in the batch.

Ferreres and co-workers also verified that the antiradical activity of *B. forficata* was better than that of *B. forficata pruinosa*.[7] In addition, the samples of *B. variegata* herein analyzed demonstrated similar antiradical activity as that observed by Ahmed and co-workers.[19]

The several flavonoid glycosides identified in the extracts of *Bauhinia* (Table 2 and Figure 4) may account for the high antiradical potential observed in the samples. The antioxidant potential of flavonoids is well known, and this activity is exerted through several mechanisms such as H atom transfer, the single electron transfer and complexing ability of transition metals. Specially, kaempferol, quercetin and myricetin, both aglycones and glycosides, are potent natural antioxidants due to the presence of catechol moiety in ring B (quercetin), the presence of the 3-hydroxyl and 5-hydroxyl groups and the additional hydroxyl group in ring B (pyrogallol group) which seems to enhance further the antioxidant capacity (myricetin).[15,20-23]

4. Conclusion

Discrimination of *Bauhinia* samples according to their geographical origin and plant species was possible by PCA, which confirmed that this tool could assist in the traceability and authentication of the plant.

PCA also suggested that the plant marketed as *B. forficata* may be a mixture of *B. forficata* and *B. variegata*. In this way, multivariate analysis could be an auxiliary tool to the taxonomic classification of samples from different species of *Bauhinia* by simply recording UV or IR spectra or, when available ESI-MS fingerprints.

It was also possible to verify that the *Bauhinia* genus regardless the species had good antiradical activity, but non-commercial samples showed better activity than the commercial ones, which suggests that the shelf life of these plants should be reduced for better antiradical capacity.

Untargeted metabolomic fingerprinting through PCA of UV-VIS, FT-IR and ESI-MS data revealed that there are differences in the chemical composition of commercial and non-commercial *Bauhinia* samples. These differences had consequences to the antioxidant capacity of the extracts and may be originated due to the commercial samples being composed of a mixture of *Bauhinia* species collected from different geographical origins and packed together or to lower concentration of metabolites due to improper handling and shelf time degradation. Clearly, a more rigorous quality control to check batch compliance and proper identification of commercial *Bauhinia* species must be applied to guarantee efficacy of this medicinal plant.

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