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Original Article

Phytochemical analysis of *Vernonanthura tweedieana* and a validated UPLC-PDA method for the quantification of eriodictyol

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ABSTRACT

Vernonanthura tweedieana (Baker) H. Rob., Asteraceae, is used in the Brazilian folk medicine for the treatment of respiratory diseases. In this work the phytochemical investigation of its ethanol extracts as well as the development and validation of an UPLC-PDA method for the quantification of the eriodictyol from the leaves were performed. The phytochemical study for this species lead to the identification of ethyl caffeate, naringenin and chrysoeriol in mixture, eriodictyol from leaves, and the mixture of 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-propan-1-one and evofolin B, apigenin, the mixture of caffeic and protocatechuic acid and luteolin from stems with roots, being reported for the first time for *V. tweedieana*, except for eriodictyol. The structural elucidation of all isolated compounds was achieved by ^{1}H and 2D NMR spectroscopy, and in comparison with published data. An UPLC-PDA method for quantification of the eriodictyol in leaves of *V. tweedieana* was developed and validated for specificity, linearity, precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ), accuracy and robustness. In this study, an excellent linearity was obtained (r^2 = 0.9999), good precision (repeatability RSD = 2% and intermediate precision RSD = 8%) and accuracy (average recovery from 98.6% to 99.7%). The content of eriodictyol in the extract of leaves of *V. tweedieana* was 41.40 ± 0.13 mg/g. Thus, this study allowed the optimization of a simple, fast and validated UPLC-PDA method which can be used to support the quality assessment of this herbal material.

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Introduction

Natural products, as well as medicinal plants, are considered as a good source for the development of medicines and drugs and as a tool for the discovery of innovative molecular patterns (Alvim et al., 2006; Balunas and Kinghorn, 2005; Cragg et al., 2009; Cragg and Newman, 2013).

Belonging to the Asteraceae family, *Vernonanthura tweedieana* (Baker) H. Rob. is known in Brazil as "assapeixe" and "mata-pasto" and their leaves are popularly used for the treatment of respiratory diseases (Cabrera and Klein, 1980; Trevisan et al., 2012; Zanon, 2006; Zanon et al., 2008). Extracts of this species have demonstrated antibacterial (Díaz et al., 2008), immunomodulatory (Petri et al., 2008), antioxidant (Zanon, 2006), antinociceptive and antiedematogenic (Trevisan et al., 2012), antifungal (Portillo et al., 2005) and allelopathic (Olguin et al., 2005) activities. Among the secondary metabolites already described for *V. tweedieana* are lupeol, α- and β-amyrin, β-sitosterol, stigmasterol (Díaz et al., 2008; Portillo et al., 2005; Zanon, 2006; Zanon et al., 2008), α-spinasterol (Trevisan et al., 2012; Zanon et al., 2008), eriodictyol (Rossato et al., 2011; Zanon, 2006), 6-cinnamoyloxy-1-hydroxyeudesm-4-en-3-one (Portillo et al., 2005) and palmitic acid (Díaz et al., 2008).

For the evaluation of quality of raw materials and phytomedicines is fundamental to know and to standardize the content of their main components (Bara et al., 2006). Since herbal extracts contain many different chemical components, techniques with high sensibility and selectivity are mandatory to their adequate quality control. In the context, ultra performance liquid chromatography (UPLC) has shorter run time and higher sensitivity and resolution when compared to HPLC and is an elegant technique for detection and screening of complex analytes in a large number of samples (Steinmann and Ganzera, 2011; Walter and Andrews, 2014).

In order to better explore the chemical composition of *V. tweedieana*, ethanol extracts from stems with roots and from leaves

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(the latter being prepared according to the popular use) were prepared and chemically fractioned, conducting to the identification of its main compounds. Additionally, considering the importance of quality control of *V. tweedieana* leaves due to its popular use, a new and simple UPLC-PDA method for quantification of its major compound (eriodictyol) was developed and validated in this work.

Materials and methods

Chemicals

The solvents used in chromatographic procedures were *p.a.* grade: *n*-hexane, dichloromethane, ethyl acetate, acetone, methanol (Vetec® Química Fina, Rio de Janeiro, RJ, Brazil). For UPLC-PDA analyses were used acetonitrile HPLC grade (Tedia® Brazil, Rio de Janeiro, RJ, Brazil), formic acid 85% *p.a.* grade (Vetec® Química Fina, Rio de Janeiro, RJ, Brazil) and the water was obtained by Millipore® Milli Q (Bedford, MA, USA) water purification system. All the solutions prepared for UPLC analyses were filtered through a 0.22 μm membrane and sonicated before use. For TLC analyses plates of silica gel 60 (SiliCycle®, Quebec, QC, Canada) and silica RP C_{18} (Macherey-Nagel®, Düren, NRW, Germany) were used. For NMR analyses methanol-d_4 and acetona-d_6 (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) were used.

Plant material

Vernonanthura tweedieana (Baker) H. Rob., Asteraceae, was collected during blooming season in Florianópolis (27°33′54.8″S 48°25’48.0″W and 27°35′54.7″S 48°31′00.3″W), Santa Catarina, Brazil in April 2013, and identified by Professor Dr. Ademir Reis. A voucher specimen was deposited in the Herbarium RB (RB 612274), at the Jardim Botânico do Rio de Janeiro, Rio de Janeiro/RJ, Brazil.

Extraction and partition

The fresh leaves (2.6 kg) and stems with roots (5.9 kg) were extracted by maceration with ethanol 75% at room temperature, for 56 days. The extractive solutions were filtered through paper; the solvent was removed under reduced pressure and used to re-extract the plant material. The resulting crude extracts from leaves (ELF, 172 g) and from stems with roots (ESR, 291.6 g) after solvent removal were kept in a desiccator *in vacuo*.

Both crude extracts were partitioned with increasing polarity solvents, yielding *n*-hexane (17.9 g), dichloromethane (CH_2Cl_2, 3.6 g), ethyl acetate (EtOAc, 10.4 g) and the aqueous residual (104.3 g) fractions from ELF, and *n*-hexane (10.9 g), CH_2Cl_2 (4.2 g), EtOAc (2.5 g) and the aqueous residual (244.9 g) fractions from ESR.

Phytochemical study

The CH_2Cl_2 (3.6 g) fraction from partition of ELF was subjected to silica gel (230–400 *mesh*) liquid chromatography under vacuum (*flash*-LCV) and eluted in a gradient system consisting of increasing concentrations of CH_2Cl_2 in *n*-hexane (0–70%), followed by acetone in CH_2Cl_2 (0–80%) and followed by methanol (MeOH) in acetone (0–100%), yielding seventeen subfractions (LF1–LF17). Pooled subfractions LF7 and LF8 (676.6 mg) was also subjected to *flash*-LCV, eluted in a gradient system consisting of increasing concentrations of CH_2Cl_2 in *n*-hexane (40–70%), followed by acetone in CH_2Cl_2 (0–70%) and followed by MeOH in acetone (0–100%), yielding sixteen subfractions, named LF7-8A to LF7-8P. Subfraction LF7-8F (368.7 mg) was subjected to silica gel column chromatography (CC) and eluted in a gradient system consisting of increasing concentrations of acetone in hexane (25–100%) followed by MeOH (100%), yielding ten subfractions, named LF7-8F*I* to LF7-8FX, in which the fraction LF7-8F*VIII* corresponds to compound **1** (37.3 mg).

The EtOAc (10.4 g) fraction from partition of ELF was subjected to *flash*-LCV, eluted in a gradient system consisting of increasing concentrations of CH_2Cl_2 in hexane (30–70%), followed by acetone in CH_2Cl_2 (0–80%) and followed by MeOH in acetone (0–100%), yielding eighteen subfractions (LF18–LF35). Subfraction LF25 (319 mg) was subjected to silica gel CC eluted in a gradient system consisting of increasing concentrations of CH_2Cl_2 in hexane (80–95%), followed by acetone in CH_2Cl_2 (0–70%) and followed by MeOH in acetone (0–100%), resulting eleven subfractions (LF25A–LF25K). The subfraction LF25G (22 mg) was fractionated through Sephadex LH-20 with acetone as eluent, yielding seven subfractions, named LF25G*I* to LF25G*VII*, which fraction LF25G*V* corresponds to the mixture (3.4 mg) of compounds **2** and **3**. Subfraction LF26 (2079.3 mg) was subjected to silica gel CC eluted in a gradient system consisting of increasing concentrations of CH_2Cl_2 in hexane (80%), followed by acetone in CH_2Cl_2 (0–30%) and followed by MeOH in acetone (0–50%), until water in MeOH (0–10%), resulting seventeen subfractions (LF26A-LF26Q). Pooled subfractions LF26K and LF26L (262.1 mg) was subjected to silica gel CC, eluted in a gradient system consisting of increasing concentrations of acetone in CH_2Cl_2 (15–70%) followed by MeOH in acetone (0–100%), yielding nine subfractions, named LF26K-L*I* to LF26K-L*IX*, which fraction LF26K-L*IV* corresponds to compound **4** (104.7 mg).

The CH_2Cl_2 (4.2 g) fraction from partition of ESR was subjected to *flash*-LCV and eluted in a gradient system consisting of increasing concentrations of CH_2Cl_2 in hexane (0–70%), followed by EtOAc in CH_2Cl_2 (0–80%) and followed by MeOH in EtOAc (0–70%), until water in MeOH (0–50%), yielding eighteen subfractions (SR1–SR18). The subfraction SR10 (112 mg) was subjected to silica gel CC, eluted in a gradient system consisting of increasing concentrations of acetone in CH_2Cl_2 (3–70%) followed by MeOH in acetone (0–100%), yielding twelve subfractions, named SR10A to SR10L. The subfraction SR10E (19.7 mg) was fractionated through Sephadex LH-20 with acetone as eluent, yielding six subfractions, named SR10E*I* to SR10E*VI*, which fraction SR10E*VI* corresponds to the mixture (0.8 mg) of compounds **5** and **6**.

The EtOAc (2.5 g) fraction from partition of ESR was subjected to *flash*-LCV, eluted in a gradient system consisting of increasing concentrations of EtOAc in CH_2Cl_2 (0–70%) and followed by MeOH in EtOAc (0–100%), yielding seven subfractions (SR19-SR25). The subfraction SR21 (135.9 mg) was subjected to silica gel CC, eluted in a gradient system consisting of increasing concentrations of acetone in CH_2Cl_2 (3–70%) followed by MeOH in acetone (0–100%), yielding fifteen subfractions, named SR21A to SR21O. The subfraction SR21H (16.7 mg) was also subjected to silica gel CC, eluted in a gradient system consisting of increasing concentrations of MeOH in CH_2Cl_2 (5–100%), resulting six subfractions, named SR21H*I* to SR21H*VI*. The subfraction SR21H*V* (9.6 mg) was subjected to preparative RP C_{18} TLC, eluted with water-MeOH (30:70, v/v) allowing the isolation of compounds **4** (4.9 mg) and **7** (0.7 mg). The subfraction SR21K (21.3 mg) was fractionated through Sephadex LH-20 with acetone as eluent, yielding eight subfractions, named SR21K*I* to SR21K*VIII*, which fraction SR21K*V* corresponds to the mixture (11 mg) of compounds **8** and **9**, and the fraction SR21K*VII* corresponds to the compound **10** (1 mg).

The elucidation of the compounds was achieved by ^{1}H and 2D (HSQC and HMBC) NMR spectroscopy, in Bruker® *Ascend600* (600 MHz for H, 125 MHz for C), and in comparison with published data.

Ethyl caffeate (**1**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) *J* (Hz), 7.16 (1H, d, *J* = 2.0, H-2), 6.87 (1H, d, *J* = 8.2, H-5), 7.04 (1H, dd, *J* = 8.2; 2.0, H-6), 7.54 (1H, d, *J* = 15.9, H-7), 6.27 (1H, d, *J* = 15.9, H-8), 4.18 (2H, q, *J* = 7.2, H-1′), 1.27 (3H, t, *J* = 7.2, H-2′), 8.17 (1H, br, OH-3) and 8.41 (1H, br, OH-4). ^{13}C NMR (acetone-d_6, 150 MHz,

TMS) δ_C (ppm), 127.5 (C-1), 115.2 (C-2), 146.4 (C-3), 148.7 (C-4), 116.3 (C-5), 122.3 (C-6), 145.2 (C-7), 115.8 (C-8), 167.4 (C-9), 60.5 (C-1′) and 14.7 (C-2′).

Naringenin (**2**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) J (Hz), 5.45 (1H, dd, J = 12.9; 3.0, H-2), 3.17 (1H, dd, J = 17.1; 12.9, H-3a), 2.74 (1H, dd, J = 17.1; 3.0, H-3b), 5.95 (1H, d, J = 2.1, H-6), 5.96 (1H, d, J = 2.1, H-8), 7.39 (2H, d, J = 8.5, H-2′, H-6′), 6.90 (2H, d, J = 8.5, H-3′, H-5′) and 12.17 (1H, s, OH-5). ^{13}C NMR (acetone-d_6, 150 MHz, TMS) δ_C (ppm), 80.0 (C-2), 43.5 (C-3), 197.2 (C-4), 166.8 (C-5), 96.4 (C-6), 161.8 (C-7), 95.9 (C-8), 164.5 (C-9), 103.2 (C-10), 130.7 (C-1′), 129.0 (C-2′, C-6′), 116.4 (C-3′, C-5′) and 158.7 (C-4′). This compound was identified in mixture with compound **3**.

Chrysoeriol (**3**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) J (Hz), 6.69 (1H, s, H-3), 6.26 (1H, d, J = 2.0, H-6), 6.55 (1H, d, J = 2.0, H-8), 7.63 (1H, d, J = 2.1, H-2′), 7.01 (1H, d, J = 8.3, H-5′), 7.60 (1H, dd, J = 8.3; 2.1, H-6′), 4.00 (3H, s, OCH_3-1″) and 13.00 (1H, s, OH-5). ^{13}C NMR (acetone-d_6, 150 MHz, TMS) δ_C (ppm), 164.9 (C-2), 104.5 (C-3), 182.9 (C-4), 163.3 (C-5), 99.7 (C-6), 165.0 (C-7), 94.7 (C-8), 158.7 (C-9), 105.2 (C-10), 123.6 (C-1′), 110.7 (C-2′), 148.8 (C-3′), 151.3 (C-4′), 116.4 (C-5′), 121.4 (C-6′) and 56.6 (C-1″). This compound was identified in mixture with compound **2**.

Eriodictyol (**4**): ^{1}H NMR (methanol-d_4, 600 MHz, TMS), δ_H (ppm) J (Hz), 5.28 (1H, dd, J = 12.8; 3.0, H-2), 3.06 (1H, dd, J = 17.2; 12.8, H-3a), 2.69 (1H, dd, J = 17.2; 3.0, H-3b), 5.88 (1H, d, J = 2.2, H-6), 5.90 (1H, d, J = 2.2, H-8), 6.91 (1H, m, H-2′), 6.78 (1H, m, H-5′) and 6.79 (1H, m, H-6′). ^{13}C NMR (methanol-d_4, 150 MHz, TMS) δ_C (ppm), 80.7 (C-2), 44.2 (C-3), 197.8 (C-4), 165.6 (C-5), 97.2 (C-6), 168.6 (C-7), 96.2 (C-8), 164.9 (C-9), 103.5 (C-10), 131.8 (C-1′), 114.7 (C-2′), 146.1 (C-3′), 147.1 (C-4′), 116.6 (C-5′) and 119.2 (C-6′).

3-Hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-propan-1-one (**5**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) J (Hz), 7.34 (2H, s, H-2, H-6), 3.17 (2H, d, J = 6.2, H-8), 3.91 (2H, m, H-9) and 3.91 (6H, s, OCH_3-3, OCH_3-5). ^{13}C NMR (acetone-d_6, 150 MHz, TMS) δ_C (ppm), 129.3 (C-1), 107.0 (C-2, C-6), 148.5 (C-3, C-5), 142.0 (C-4), 198.2 (C-7), 41.8 (C-8), 58.6 (C-9) and 56.9 (OCH_3-3, OCH_3-5). This compound was identified in mixture with compound **6**.

Evofolin B (**6**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) J (Hz), 7.60 (1H, d, J = 2.0, H-2), 6.84 (1H, d, J = 8.3, H-5), 7.66 (1H, dd, J = 8.3; 2.0, H-6), 4.80 (1H, dd, J = 8.5; 5.2, H-8), 4.23 (1H, m, H-9a), 3.71 (1H, m, H-9b), 6.99 (1H, d, J = 2.0, H-2′), 6.74 (1H, d, J = 8.1, H-5′), 6.80 (1H, dd, J = 8.1; 2.0, H-6′), 3.88 (3H, s, OCH_3-3) and 3.82 (3H, s, OCH_3-3′). ^{13}C NMR (acetone-d_6, 150 MHz, TMS) δ_C (ppm), 130.5 (C-1), 112.3 (C-2), 148.2 (C-3), 152.1 (C-4), 115.3 (C-5), 124.5 (C-6), 198.2 (C-7), 55.9 (C-8), 65.5 (C-9), 129.1 (C-1′), 112.8 (C-2′), 148.5 (C-3′), 146.6 (C-4′), 115.9 (C-5′), 121.9 (C-6′), 56.3 (OCH_3-3) and 56.4 (OCH_3-3′). This compound was identified in mixture with compound **5**.

Apigenin (**7**): ^{1}H NMR (methanol-d_4, 600 MHz, TMS), δ_H (ppm) J (Hz), 6.60 (1H, s, H-3), 6.21 (1H, d, J = 2.1, H-6), 6.46 (1H, d, J = 2.1, H-8), 7.85 (2H, d, J = 8.9, H-2′, H-6′) and 6.93 (2H, d, J = 8.9, H-3′, H-5′). ^{13}C NMR (methanol-d_4, 150 MHz, TMS) δ_C (ppm), 166.3 (C-2), 102.9 (C-3), 183.9 (C-4), 163.2 (C-5), 100.2 (C-6), 166.1 (C-7), 95.0 (C-8), 159.5 (C-9), 105.3 (C-10), 123.3 (C-1′), 129.6 (C-2′, C-6′), 117.1 (C-3′, C-5′) and 162.7 (C-4′).

Caffeic acid (**8**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) J (Hz), 7.16 (1H, d, J = 2.0, H-2), 6.87 (1H, d, J = 8.2, H-5), 7.03 (1H, dd, J = 8.2; 2.0, H-6), 7.55 (1H, d, J = 15.9, H-7) and 6.27 (1H, d, J = 15.9, H-8). ^{13}C NMR (acetone-d_6, 150 MHz, TMS) δ_C (ppm), 127.6 (C-1), 115.2 (C-2), 146.3 (C-3), 148.7 (C-4), 116.4 (C-5), 122.5 (C-6), 146.1 (C-7), 115.9 (C-8) and 168.3 (C-9). This compound was identified in mixture with compound **9**.

Protocatechuic acid (**9**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) J (Hz), 7.53 (1H, d, J = 2.0, H-2), 6.90 (1H, d, J = 8.3, H-5) and 7.48 (1H, dd, J = 8.3; 2.0, H-6). ^{13}C NMR (acetone-d_6, 150 MHz, TMS) δ_C (ppm), 123.2 (C-1), 117.6 (C-2), 145.5 (C-3), 150.6 (C-4), 115.8 (C-5), 123.7 (C-6) and 167.7 (C-7). This compound was identified in mixture with compound **8**.

Luteolin (**10**): ^{1}H NMR (acetone-d_6, 600 MHz, TMS), δ_H (ppm) J (Hz), 6.57 (1H, s, H-3), 6.25 (1H, d, J = 2.1, H-6), 6.53 (1H, d, J = 2.1, H-8), 7.50 (1H, d, J = 2.3, H-2′), 7.00 (1H, d, J = 8.4, H-5′), 7.46 (1H, dd, J = 8.4; 2.3, H-6′) and 13.00 (1H, s, OH-5). ^{13}C NMR (acetone-d_6, 150 MHz, TMS) δ_C (ppm), 165.3 (C-2), 104.2 (C-3), 182.9 (C-4), 163.3 (C-5), 99.6 (C-6), 164.9 (C-7), 94.7 (C-8), 158.9 (C-9), 105.3 (C-10), 123.7 (C-1′), 114.1 (C-2′), 146.6 (C-3′), 150.2 (C-4′), 116.6 (C-5′) and 120.1 (C-6′).

Ultra performance liquid chromatography optimized conditions

The UPLC analyses were carried out in a Waters® *Acquity UPLC* system equipped with photo diode array (PDA) detection, an auto sampler and column oven. The chromatographic separation was achieved using a Waters® *Acquity UPLC BEH C_{18}* (2.1 mm × 50 mm i.d., 1.7 μm) at 40 °C (±2 °C). The injection volume was 2 μl and the flow rate was kept constant at 0.4 ml min^{-1}. The mobile phase consisted of a 7 min gradient system combining 0.1% aqueous formic acid adjusted to pH 3.0 (solvent A) and acetonitrile (solvent B) as follows: 0–1 min gradient of 90–80% A (curve 7); 1–5 min 80–55% A (curve 7); 5–6 min 55–35% A (curve 9); 6–7 min 35–90% A (curve 9). Data were processed using the *Empower 3* (Waters®) software. The chromatograms were recorded at wavelength of 287 nm while the UV spectra were monitored over a range of 400–200 nm. Peaks were identified by comparing retention times and UV spectra with reference standards.

Stock solutions preparation

The quantification of eriodictyol in the samples was carried out by the external standard method. Eriodictyol (purity ≥98%, verified by Peak Purity tool in *Empower 3* software) used in this work as analytical standard was obtained from phytochemical investigation of EtOAc fraction from partition of ELF of *V. tweedieana*. The stock standard solutions of eriodictyol (1000 and 500 μg/g of solution) were freshly prepared in 0.1% aqueous formic acid–acetonitrile (1:1, v/v) mixture. The stock standard solution was then appropriately diluted in 0.1% aqueous formic acid–acetonitrile (1:1, v/v) to obtain working standard solutions.

The sample solutions analyzed by UPLC-PDA were obtained from the crude extracts ELF and ESR, resulting of maceration from leaves and from stems with roots of *V. tweedieana*, respectively. Both crude extracts, ELF and ESR, were used to prepare the stock sample solutions (1000 μg/g of solution) in 0.1% aqueous formic acid–acetonitrile (1:1, v/v). The stock sample solutions were also appropriately diluted in 0.1% aqueous formic acid–acetonitrile (1:1, v/v) to obtain working sample solutions (about 200 μg/g of solution).

These stock solutions were filtered through a 0.22 μm membrane and stored at 4 °C.

Validation of UPLC-PDA method

The UPLC-PDA method was validated in terms of specificity, linearity, precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ), accuracy and robustness (Araujo, 2009; ICH, 2005; Shabir, 2003; USP, 2007).

In order to check the specificity of the method, the purity of eriodictyol in the extract of *V. tweedieana* was verified using a PDA detector and the software *Empower 3* (Waters®).

Linearity was determined with calibration curves, which were constructed by plotting the measurements of area peak versus the concentration (μg/g of solution) of eriodictyol. Stock standard solution of eriodictyol (1000 μg/g) was diluted to seven levels concentrations, in triplicate: 1.5, 3, 7.5, 15, 20, 25 and 30 μg/g. The

results were analyzed by linear regression and the correlation coefficient (r^2) was calculated. One-way ANOVA was calculated to compare the replicates of the calibration curve (GraphPad Prism® version 6.03).

The precision of the method was assessed in repeatability (intra-day) and intermediate precision (inter-day). Repeatability was established by analyzing the relative standard deviation (RSD%) of 6 independent sample solutions determinations at 100% of the test concentration (200 μg/g). Intermediate precision was determined considering the RSD% by the analysis in sextuplicate by two analysts in three different days (analyst A performed the analysis in days one and two, while analyst B performed the analysis in day three).

The LOD and LOQ were assessed at signal-to-noise of 3 and 10, respectively, by injecting a series of dilute solutions with known concentration.

The accuracy was evaluated by the recovery test. Three different concentrations of stock standard solution (500 μg/g), corresponding the addition of 10, 14 and 18 μg/g of solution, were spiked into a known sample solution. The spiked sample, unspiked sample and corresponding added standard solutions were measured, in triplicate, under the same conditions. The recovery was calculated by the reason between the concentration of spiked sample analyzed and the theoretical spiked concentration (established by the sum of measured concentrations of unspiked sample and the standard added).

The robustness of the method was determined relative to the variation of retention time, resolution and tailing factor of the peak (USP, 2007), and concentration of eriodictyol for determinations in nominal conditions and against variations of method conditions. Experimental variables were the flow rate varied by (±) 0.1 ml min^{-1}, column oven temperature varied by (±) 10 °C and the percentage of organic modifier varied by (±) 0.05%. The results were evaluated by one-way ANOVA followed by Bonferroni post hoc test, with a confidence level of 95% (GraphPad Prism® version 6.03).

Results and discussion

Phytochemical study

The phytochemical investigation allowed characterizing ten different known phenolic compounds from ethanol extracts of *V. tweedieana*. These compounds were identified by ^{1}H and 2D NMR experiments in comparison with published data. Considering that the majority of the previously published NMR data presented some inconsistencies regarding chemical shifts (ppm) or were not in the same solvent, the new data are presented in Materials and Methods section. For compound **4** different chemical shifts of H-3a and H-3b were previously described in 2.69 ppm for both, and now assigned in 3.06 and 2.69 ppm respectively. For compound **5**, the chemical shifts for carbons C-2, C-4, C-5 and C-6 were previously published as 115.5, 152.9, 112.4 and 124.6 ppm, and now assigned respectively in 107.0, 142.0, 148.5 and 107.0 ppm. For compound **7**, the chemical shifts H-6 and H-8 previously published in 6.45 and 6.16 ppm are now assigned in 6.21 and 6.46 ppm, respectively. The compounds **8** and **9** were published before in methanol-d_4 and now acetone-d_6 was used. From CH_2Cl_2 fraction of leaves was isolated ethyl caffeate (**1**) (Uwai et al., 2008), and from EtOAc fraction was obtained naringenin (**2**) (Fatope et al., 2003) and chrysoeriol (**3**) (Lin and Kong, 2006) in mixture, and eriodictyol (**4**) (Huang et al., 2014).

From stems with roots extract, the CH_2Cl_2 fraction furnished 3-hydroxy-1-(4-hydroxy-3.5-dimethoxyphenyl)-propan-1-one (**5**) (Jones et al., 2000) and evofolin B (**6**) (Wu et al., 1995) in mixture, and from EtOAc fraction were obtained again the eriodictyol (**4**), apigenin (**7**) (Kim et al., 2014), caffeic (**8**) (Lee et al., 2012) and protocatechuic (**9**) (Liao et al., 2014) acids in mixture and luteolin (**10**) (Lin and Kong, 2006).

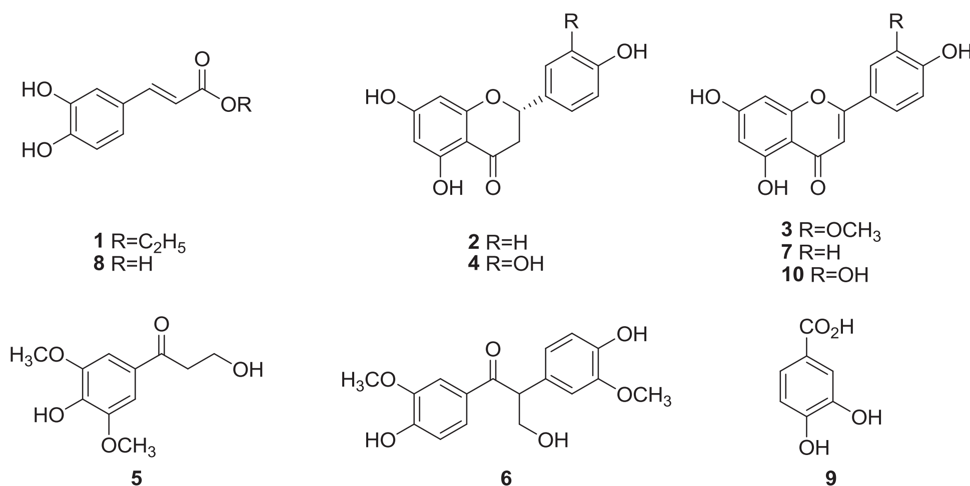
Except for eriodictyol that was previously reported for the leaves of *V. tweedieana* (Rossato et al., 2011; Zanon, 2006), all substances are described for the first time in this species. These results allow increasing the knowledge for the species *V. tweedieana*.

UPLC-PDA analyses and method validation

Both crude ethanol extracts, from leaves and from combination of stems with roots of *V. tweedieana*, were analyzed by UPLC-PDA and the chromatograms are showed in Fig. 1A, B.

The comparative analysis of the crude extracts with all isolated compounds allowed characterizing four peaks in the chromatograms: ethyl caffeate (**1**), eriodictyol (**4**), apigenin (**7**) and luteolin (**10**).

The UPLC-PDA analyses of the two crude ethanol extracts showed distinct chromatographic profiles. Eriodictyol (**4**) was the major compound in ELF, while it was not observed in the ESR chromatogram, probably because its content was not detectable, since the amount of eriodictyol isolated from ESR was very low (4.9 mg, 0.047% of the crude extract). Flavonoid content varies within individual leaves, stems or roots (Julkunen-Tiitto et al., 2015). The presence of flavonoids within different cells and cellular compartments can be related to their functions in plant environment interactions, influenced by factors as UV-light, herbivore pressure, water and nutrients (Agati et al., 2012; Fang et al., 2012; Laitinen et al., 2002).



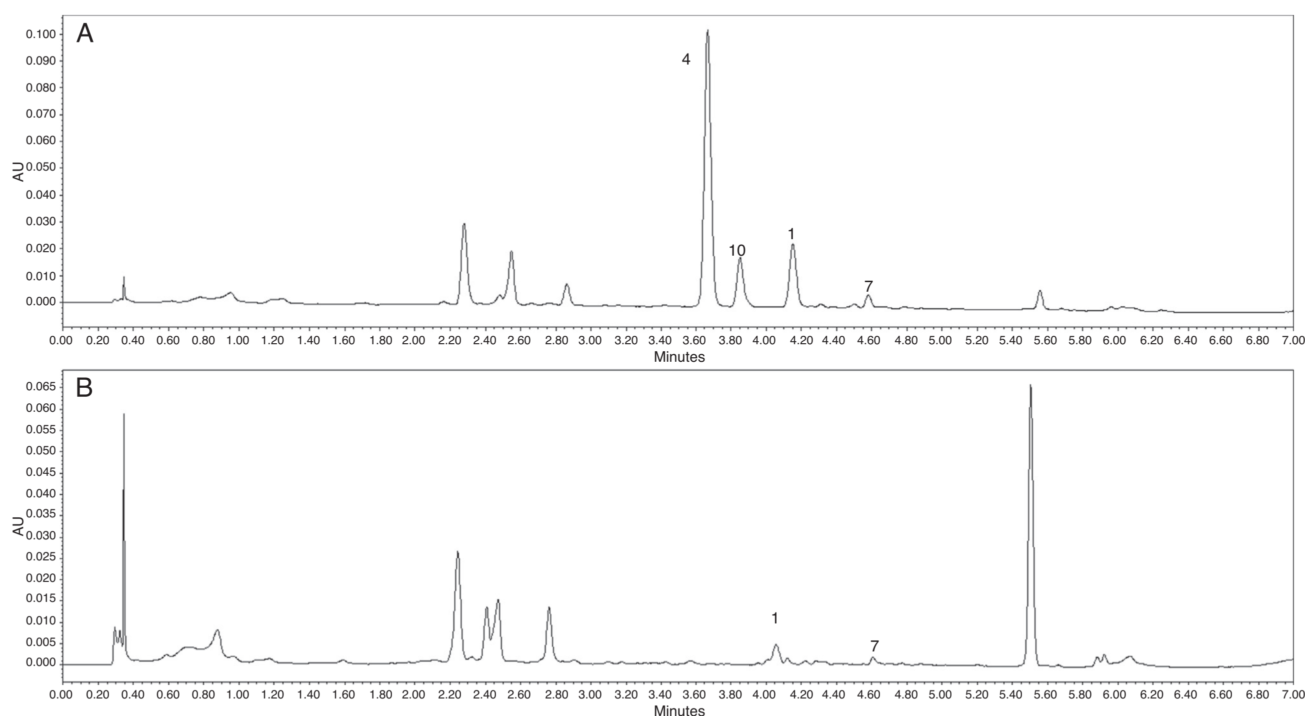


Fig. 1. UPLC chromatograms of crude ethanol extracts (200 μg/g) from leaves (A) and from combination of stems with roots (B) of *V. tweedieana*, with diode array detection at 287 nm. **1**. ethyl caffeate; **4**. eriodictyol; **7**. apigenin; **10**. luteolin. For chromatographic conditions, see Materials and methods section.

Table 1
Linearity and sensitivity data.

Compound	UV_{max} (nm)	Range of linearity[a] (μg/g)	Linear regression equation	r^2	LOD (μg/g)	LOQ (μg/g)
Eriodictyol	287.6	1.5–30.0	$y = 16{,}211x - 277.7$	0.9999	0.02344	0.06214

r^2, correlation coefficient; LOD, limit of detection; LOQ, limit of quantification.
[a] Seven levels concentrations ($n = 3$).

Considering the use of leaves in popular medicinal preparations of *V. tweedieana* in Brazil (Trevisan et al., 2012), the crude extract of leaves was analyzed by UPLC-PDA, in order to verify the potential of eriodictyol as chemical marker for this plant extract.

The calibration curves were found to be linear in the range 1.5–30 μg/g of solution for eriodictyol.

Characteristic parameters for linearity are given in Table 1. The one-way ANOVA calculated to compare the replicates of the calibration curve showed no significant difference between groups ($F = 0.003254$; $p = 0.9968$). The linear regression equation and correlation coefficient (r^2) of 0.9999 revealed a good linearity response for the method developed, since according to the literature a coefficient of determination for standard curves greater than 0.999 indicates an evidence of acceptable fit of the data to the regression line (Shabir, 2003).

The LOD and LOQ values (Table 1), respectively 0.02344 and 0.06214 μg/g, were similar to those reported in the literature for a HPLC method for quantification of eriodictyol in *Sorghum bicolour* (Taleon et al., 2014). The peak purity, obtained by PDA detector analysis of eriodictyol was in agreement with its purity spectral profile.

The results of repeatability (intra-day variation) and intermediate precision (inter-day variation) showed RSD% values of 2% and 8%, respectively (Table 2). Analytical procedures for biological and biotechnological products can present a higher inherent variation. Natural products have high chemical complexity, and considering that all replicates performed in this work involved the preparation of new stock and work samples solutions, it can represent a source of variation. However, the data obtained for precision was acceptable and is in accordance with other analytical studies with same

Table 2
Repeatability, intermediate precision and accuracy data.

Compound	Repeatability ($n = 6$)		Intermediate precision ($n = 18$)			Accuracy		
	Analyst A		Analyst A	Analyst B		Standard added (μg)	Recovery[b] (%)	RSD
	Day 1[a]	RSD	Day 2[a]	Day 3[a]	RSD			
Eriodictyol	56.15 ± 1.06	2%	46.66 ± 0.34	53.74 ± 0.57	8%	10	99.7 ± 2.6	3%
						14	98.6 ± 1.6	2%
						18	99.5 ± 1.4	1%

RSD, relative standard deviation.
[a] Average ± standard deviation ($n = 6$). Values expressed in mg/g of crude extract.
[b] Average ± standard deviation ($n = 3$)

Table 3
Robustness data.

Variables	Levels	Parameters			
		Concentration[a] (mg/g)	t_R [a] (min)	R_S [a]	T_f [a]
Nominal conditions		42.56 ± 0.04	3.634 ± 0.002	2.937 ± 0.087	1.075 ± 0.007
Flow	0.3 ml min^{-1}	56.62 ± 0.20	4.376 ± 0.015	2.225 ± 0.021	1.143 ± 0.010
	0.5 ml min^{-1}	33.93 ± 0.05	3.106 ± 0.003	3.230 ± 0.068	1.076 ± 0.009
Column temperature	30 °C	42.50 ± 0.02	4.000 ± 0.013	2.262 ± 0.024	1.105 ± 0.007
	50 °C	42.22 ± 0.14	3.249 ± 0.017	3.180 ± 0.035	1.092 ± 0.000
Modifier content	0.05%	42.06 ± 0.06	3.583 ± 0.018	2.609 ± 0.046	1.087 ± 0.007
	0.15%	42.34 ± 0.01	3.546 ± 0.015	2.653 ± 0.038	1.070 ± 0.008

Nominal conditions are flow at 0.4 ml min^{-1}, column temperature at 40 °C and modifier content (formic acid) at 0.1%. t_R, retention time; R_S, resolution (calculated between peaks 4 and 10); T_f, tailing factor; The t_R, R_S and T_f values were calculated according US Pharmacopeia (2007).
[a] Average ± standard deviation (n = 3).

natural samples (Lim et al., 2014; Motilva et al., 2014; Ok et al., 2014; Stinco et al., 2014).

The accuracy data are given in Table 2. The average recovery of the three amount added of eriodictyol ranged from 98.6% to 99.7%, and their RSD values were less than 3.0%, characterizing good reliability and accuracy of the method.

The deliberate variations analyzed for robustness of the method are showed in Table 3.

The robustness was estimated by one-way ANOVA analysis between the nominal determination and the different levels of each variable (flow, column temperature and modifier content), and assessed referent to different parameters (concentration, retention time, resolution and tailing factor of the peak) (Fig. 2).

Among robustness evaluated parameters, were not observed significant differences in the eriodictyol concentration for column temperature at 30 °C and modifier content at 0.15% variables. The parameter tailing factor was the least affected by the deliberate variations, tolerating changes of modifier content (lower and higher level), higher level of flow (0.5 ml min^{-1}) and higher level of column temperature (50 °C), with no statistical significant differences. The flow variation was the most impacting variable in the method, except for the tailing factor (Table 3, Fig. 2). Results show that all variables are critical for the method and should be carefully controlled during the analysis.

The quantitative analysis of eriodictyol in leaves crude extract from of *V. tweedieana* showed a content of 41.40 ± 0.13 mg/g (RDS = 0.31%). Eriodictyol has been described and quantified for

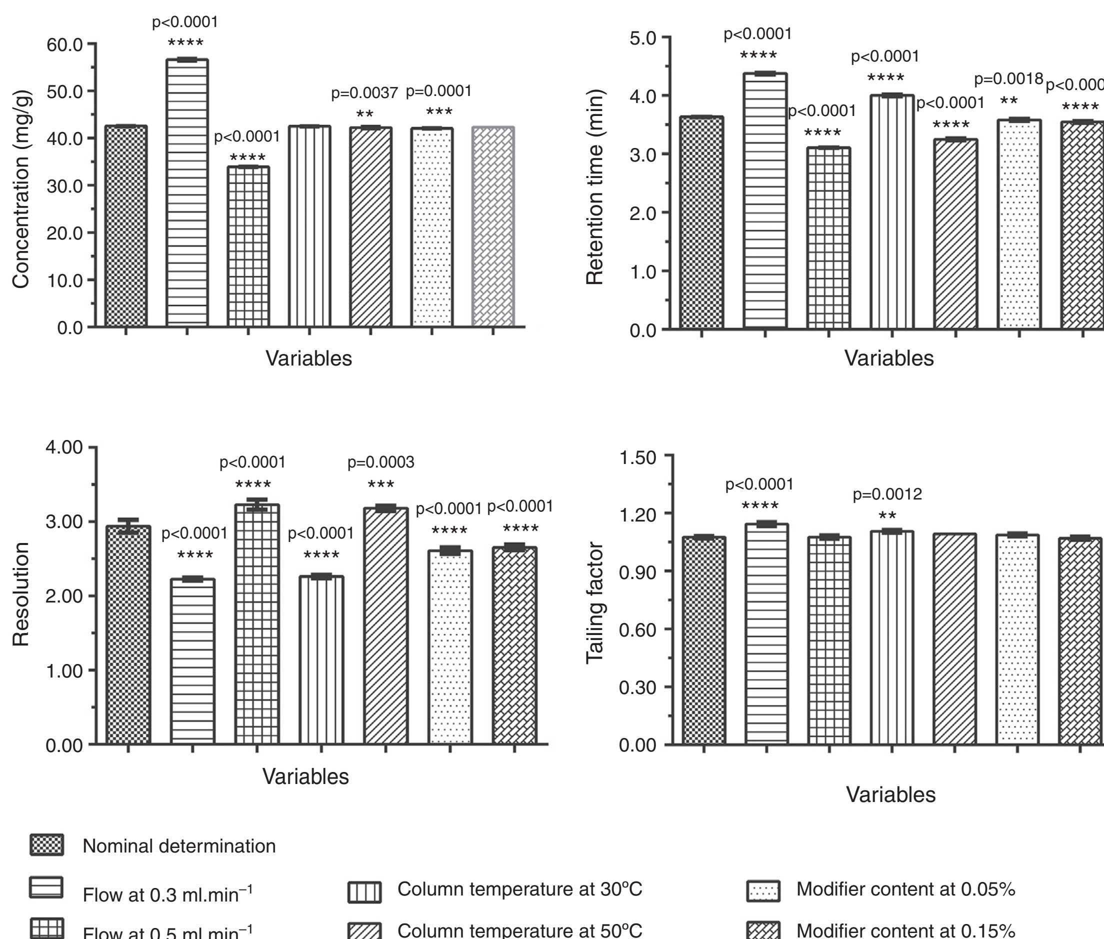


Fig. 2. Robustness evaluation of the different parameters under variables of method conditions.

some other plant extracts, but the content found in the leaves of *V. tweedieana* seems to be superior to previous published studies (Boros et al., 2010; Lin et al., 2007; Ren et al., 2008).

In summary, the phytochemical investigation allows to characterize ten different compounds, ethyl caffeate (**1**), naringenin (**2**), chrysoeriol (**3**), eriodictyol (**4**), 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-propan-1-one (**5**), evofolin B (**6**), apigenin (**7**), caffeic acid (**8**), protocatechuic acid (**9**) and luteolin (**10**), reported for the first time for *V. tweedieana* except for eriodictyol. Further, a simple and fast UPLC-PDA method for quantification of eriodictyol in crude extract from leaves of *V. tweedieana* was developed and validated, which can be used to the quality control preparations that contains *V. tweedieana*.

Authors' contributions

LALS (MSc student) contributed in collecting plant sample, phytochemical investigation, elucidation of compounds, chromatographic and validation analyses, data analyses and drafted the paper. LGF (MSc student) contributed in running the laboratory work, validation analyses and drafted the paper. FHR contributed to design and interpretation of the validated analytical conditions. ADCS (PhD student) and AB contributed to NMR analyses and elucidation of compounds. MWB designed the study, supervised the laboratory work, data analyses and contributed to critical reading and draft of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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