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Article

Polyphenol Profile and Quantitative Assessment of the Flavonoid Kaempferitrin in Wild and Cultivated Brazilian Amazonian *Uncaria guianensis* (Rubiaceae)

Djavan da Paixão,[a] Rodolfo S. Barboza,[a] Ligia M. M. Valente,*[,a] Matheus O. Souza,[a] Antonio C. Siani,[b] Rita C. A. Pereira,[c] Blanca Gallo[d] and Luis A. Berrueta[d]

[a]Instituto de Química, Universidade Federal do Rio de Janeiro, Av. Athos da Silveira Ramos, 149, C. T., Bl. A, 21941-909 Rio de Janeiro-RJ, Brazil

[b]Instituto de Tecnologia em Fármacos, Fundação Oswaldo Cruz, R. Sizenando Nabuco, 100, 21041-250 Rio de Janeiro-RJ, Brazil

[c]Embrapa Agroindústria Tropical, R. Dra. Sara Mesquita, 2270, 60511-110 Fortaleza-CE, Brazil

[d]Departamento de Química Analítica, Facultad de Ciencia y Tecnología, Universitad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), P.O. Box 644, 48080 Bilbao, Spain

The Amazonian Rubiaceae species *Uncaria guianensis* (UG) is locally used as anti-inflammatory, antitumor, antidiabetic, anti-ulcers, and others. The phenolic content of its leaves is characterized by the great predominance of the flavonoid kaempferol-3,7-*O*-(α)-L-dirhamnoside (kaempferitrin). The present study quantitatively evaluates the kaempferitrin content in the leaves and branches of cultivated and wild UG specimens collected in different locations of the Brazilian Amazon rainforest by employing high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD). Besides, the understanding of the polyphenol profile performed by electron spray ionization is deepened by tandem mass spectrometry analysis (ESI-MS/MS), using a previously approached leaf UG extract, and the flavonoid quercetin-3,7-*O*-(α)-L-dirhamnoside was first isolated from UG. All samples showed quite similar qualitative polyphenol profiles. Kaempferitrin in UG ranged from 1.1 to 1.9 mg 100 mg^{-1} for dry leaves of adult wild plants, 0.3 to 0.7 mg 100 mg^{-1} for dry leaves of cultivated young plants and 0.00 to 0.04 mg 100 mg^{-1} for dry branches of adult wild plants. Besides suggesting the distribution of kaempferitrin in the species, these results reinforce this flavonol as a suitable chemical marker for UG leaves and the products derived from them.

Keywords: *Uncaria guianensis*, Rubiaceae, cat's claw, polyphenolic compounds, kaempferitrin, chemical marker

Introduction

The genus *Uncaria* (Rubiaceae) contains about 60 species distributed in the tropical areas of Southeast Asia, Africa and Central and South America.[1,2] It is represented in Central and South America by two species: *U. tomentosa* (Willd.) DC and *U. guianensis* (Aubl.) Gmel.[3] Popularly known as cat's claw, "*unha-de-gato*" (Brazil) or "*uña de gato*" (Spanish America), both of them have been indistinctly used for the same purposes in traditional medicine, such as to treat gastritis, gastric ulcers, cancer, arthritis, asthma and inflammatory conditions.[4-6]

Previous chemical studies of *U. guianensis* revealed indole and oxindole alkaloids (low content),[7-11] proanthocyanidins,[12] flavonoids, and chlorogenic acid,[11,13] triterpenoid glycosides and sterols.[13-15] *In vitro* and clinical studies using a decoction of *U. guianensis* bark have corroborated its traditional use as an anti-inflammatory and antioxidant.[12,16] Bioassay-guided fractionation of the EtOH extract from the *U. guianensis* bark using a yeast-based assay for deoxyribonucleic acid (DNA)-damaging agents led to two weak but selectively active oxindole alkaloids.[17] Quinovic acid glycosides were also correlated with the observed anti-inflammatory activity of the species.[14] The leaves of *U. guianensis* showed antibacterial activity[15] while the bark slowed antitumor effects.[18]

*e-mail: valente@iq.ufrj.br

In previous works we have reported that the EtOH extract from the *U. guianensis* leaves showed anti-inflammatory and anti-allergic activities[19] and that the leaf EtOH:H_2O 1:1 extract showed a decrease in dengue virus infection.[20] We have also reported that the flavonol kaempferitrin (**5**) (kaempferol-3,7-*O*-(α)-L-dirhamnoside) was isolated for the first time from the *Uncaria* species detected in leaves and branches of *U. guianensis* but not in *U. tomentosa*.[21] Kaempferitrin has been reported as an active compound against several biological targets.[22-26]

Despite the medicinal uses and bioactivities of *U. guianensis*, there is currently no effective chemical marker for this species, although kaempferitrin (**5**) has already been proposed to play this role.[21] This study aimed to evaluate the content of kaempferitrin in the leaves and branches of cultivated and wild samples of *U. guianensis*, collected in different locations (and seasons) of the Brazilian Amazon rainforest to validate this flavonol diglycoside as a chemical marker of polyphenol-based leaf extracts of this species.

Experimental

Reagents

Methanol (MeOH), ethanol (EtOH), chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-hexane, acetic acid (AcOH), formic acid (HCOOH) and acetonitrile (CH_3CN), all analytical grade reagents, were purchased from Vetec (Rio de Janeiro, Brazil). CH_3CN high-performance liquid chromatography (HPLC) grade, MeOH HPLC grade and MeOH-d_4 (CD_3OD) were purchased from Tedia (Rio de Janeiro, Brazil). Milli-Q grade water was obtained from Merck system (Darmstadt, Germany). Rutin was purchased from Sigma-Aldrich (St. Louis, USA). Aluminum chloride ($AlCl_3$) was purchased from Riedel-de-Haën (Seelze, Germany). Rutin, diphenylboric acid-β-ethylamino ester (NP) and polyethylene glycol-4000 (PEG) were purchased from Sigma-Aldrich (St. Louis, USA).

Plant material

The leaves (L) and branches (B) from wild adult specimens of *U. guianensis* (UG) were collected in three different locations of the Brazilian Amazon rainforest: Juruena, Mato Grosso (MT) state (12º50' S, 58º55' W; 277 m elevation) in June of 2001 (UGL-MT); Manaus, Amazonas (AM) state (3º05' S, 60º00' W; 55 m elevation) in September of 2007 (UGL-AM and UGB-AM); and Rio Branco, Acre (AC) state (9º58' S, 67º48' W; 143 m elevation), two sets collected from the same specimen in September and October 2008 (UGL-ACSept and UGL-ACOct). The specimen from Mato Grosso state was identified by the botanist Pierro Delprete and a voucher was deposited in the Central Herbarium of the Universidade Federal do Mato Grosso, Brazil, under No. 24715. The specimens from Acre and Amazon states were identified by the botanist Mário Gomes and the vouchers were deposited in the Herbarium of the Universidade Federal do Rio de Janeiro, Brazil, under Nos. RFA36973 and RFA37499, respectively. The samples from Pará (PA) state were purchased at the Ver-o-Peso Herbal Market in Belém (UGL-PA and UGB-PA). The two cultivated leaf samples of *U. guianensis* were previously obtained in the following way:[9] one was generated from an *in vitro* 45 day plantlet cultivation that was transferred to a 72 cell tray with the appropriated substrate and after 15 days transplanted to a greenhouse and collected after 8 months (UGL-C8); the other one corresponds to an *in vivo* cultivation from seeds collected from a wild adult specimen of *U. guianensis* in the municipality of Boca do Acre, Amazonas (AM) state, germinated for 60 days in a greenhouse, transplanted, kept there for 6 months and then transferred to the field and collected after 24 months (median part of the shoot and anatomic structures fully developed) (UGL-C24). Legal access of the Brazilian genetic heritage component was properly registered in the SisGen platform under code AE8FE55.

Thin layer chromatography analyses

The thin layer chromatography (TLC) analyses were performed by applying 10 μL of MeOH solutions (c = 25 mg mL^{-1}) of the samples in precoated silica-gel 60F254 (Merck, Darmstadt, Germany), with mobile phases: (a) $CHCl_3$/acetone/HCOOH 7.5:1.6:0.8 v/v/v and (b) EtOAc/HCOOH/AcOH/H_2O 100:11:11:27 v/v/v both for monitoring the choice of extraction solvent and (b) for the isolation procedures; (c) EtOAc:MeOH 7:1 v/v for monitoring the solid phase extraction (SPE) procedures. UV irradiation at 254 nm and NP/PEG in MeOH followed by UV irradiation at 365 nm were used to visualize the spots.[27] The polyphenolic compounds were detected by bright yellow, orange, green and blue colors with NP/PEG.

HPLC-DAD-ESI-MS/MS analysis

The high-performance liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS/MS) analysis was carried out in an Alliance 2695 Waters (Waters Corp., Milford, USA) equipment with a quaternary pump, autosampler, column oven, diode array detector

and equipped with a Phenomenex Luna C18(2) column (Phenomenex, Torrance, USA) (3 μm, 150 × 4.6 mm) with a Waters Nova-Pack C18 guard column (4 μm, 10 × 3.9 mm) (Waters Corp., Milford, USA). A previously reported gradient program was employed: AcOH:H_2O (0.5:99.5 v/v) (phase A) and MeOH (phase B). The applied elution conditions were as follows: 0-2 min, 0% B isocratic; 2-6 min, linear gradient from 0 to 15% B; 6-12 min, 15% B isocratic; 12-17 min, linear gradient from 15 to 20% B; 17-35 min, 20% B isocratic; 35-90 min, linear gradient from 20 to 35% B; 90-136 min, 35% B isocratic; and finally, washing and reconditioning of the column was performed. The diode array detector was set at an acquisition range of 250-600 nm and phenolic compound monitoring was performed at 280, 320 and 370 nm. The EtOH:H_2O 1:1 extract (3.36 mg) previously obtained from the leaves of *U. guianensis*[20] was dissolved in 1 mL of H_2O:MeOH:AcOH 69:30:1 v/v/v. The injection volume was 50 μL. The online MS were obtained on a Micromass (Waters Corp., Milford, USA) quattro micro triple quadrupole mass spectrometer coupled to the exit of the diode array detector and equipped with a Z-spray electrospray ionization (ESI) source. A flow of 70 μL min^{-1} from the DAD eluent was directed to the ESI interface using a flow-splitter. Nitrogen was used as the desolvation gas at 300 ºC and a flow rate of 450 L h^{-1}, and no cone gas was used. A potential of 3.2 kV was used on the capillary for positive ion mode and 2.6 kV for negative ion mode. The source block temperature was held at 120 ºC. Full scan mass spectra (MS) within the *m/z* range of 50-1000 were performed in the positive mode at different cone voltages (15, 30 and 45 V) and in the negative mode at −30 V. MS/MS product ion spectra in positive mode were recorded using argon as collision gas at $1.5.10^{-3}$ mbar and under different collision energies in the range of 10-40 eV and optimized cone voltages for each compound. The optimum cone voltages were those that produced the maximum intensity for protonated molecule $[M + H]^+$ and protonated aglycone ion $[Y_0]^+$ in the previous MS experiments. The flavonoid and aglycone fragment ions were designed according to the nomenclature proposed by Ma *et al.*[28] and Dommon and Costello.[29] Phenolic standards were supplied as previously described.[30,31] All stock standard solutions (in concentrations ranging from 300 to 2700 μg mL^{-1}, depending on each phenolic compound), were prepared in MeOH. All were stored at 4 ºC in darkness.

HPLC-DAD analyses

High-performance liquid chromatography coupled to diode array detection (HPLC-DAD) analyses were performed in a PerkinElmer series 200 equipment with a quaternary pump, autosampler, and vacuum degasser (PerkinElmer, Shelton, USA). The DAD was set to an acquisition range of 190-600 nm at a spectral acquisition rate of 88 scans s^{-1}. The data were gathered using TotalChrom Workstation software (PerkinElmer, Shelton, USA). Prior to the injection, the samples were filtered through a 0.45 mm Chromafil R Xtra PVDF membrane (Macherey-Nagel, Dueren, Germany). Kaempferitrin analyses were performed with a reversed-phase C18 column Lichrocart Lichrospher (5 μm, 250 × 4.6 mm) (Merck, Darmstadt, Germany). Elutions were performed in a gradient mode at a 0.8 mL min^{-1} flow: 10 min 10% solvent B (CH_3CN) in solvent A (H_2O with 0.1% HCOOH, pH 3), 10-23 min 10-40% solvent B in A and finally 12 min 40% solvent B in A. An equilibration period of 20 min was used between the runs. Kaempferitrin monitoring was performed at 265 nm and the injected volume was 20 μL. Semipreparative HPLC-DAD was performed with an RP-18 Inertsil Prep-ODS column (10 μm, 250 × 6 mm) (GL Sciences Inc., Tokyo, Japan) coupled to a SupelguardTM LC-18 precolumn (5 μm, 20 × 4.0 mm) (Merck, Darmstadt, Germany) in isocratic elution mode with CH_3CN:H_2O 12:88 v/v (H_2O with 0.1% HCOOH, pH 3). A washing step with 100% CH_3CN was included at the end of the run. The flow rate was 1.4 mL min^{-1}, the injection volume was 190 μL (sample solution = 10 mg mL^{-1}), and the column oven temperature was set to 30 ºC. The runs were monitored at 280 nm.

Isolation and identification of the major flavonols from *Uncaria guianensis* leaves

The EtOH:H_2O 1:1 extract (13.48 g) previously obtained from the leaves of *U. guianensis*[20] was solubilized in MeOH/H_2O 9:1 v/v (1 L) and partitioned with *n*-hexane (10 × 1 L). Part of the dried defatted fraction of the extract (4.00 g) was submitted to a reversed phase C-18 silica gel 40-63 μm chromatographic column (Merck, Darmstadt, Germany) (45 mm diameter × 15 cm phase height) using EtOH:H_2O 1:3 v/v as the mobile phase yielding 33 subfractions that were pooled together according to their similar TLC profile. Compound **3** (2.6 mg) was isolated from sub-fraction 10-12 (53.3 mg) by semipreparative HPLC-DAD procedure. Compound **5** (kaempferitrin) (116.2 mg) was isolated from subfraction 13-17 (226.7 mg) after keeping it overnight in a refrigerator (4 ºC), followed by centrifugation and separation of the supernatant to yield a pure yellowish solid. The chemical structures of the isolated compounds were elucidated based on UV (from HPLC-DAD), ^1H nuclear magnetic resonance (NMR) for kaempferitrin (**5**) and 1D and 2D NMR techniques

for compound **3**. All NMR spectra were recorded on a Bruker DRX-400 (400 MHz) spectrometer (Bruker Corp., Billerica, USA) in CD_3OD and TMS as internal standard. Chemical shifts (δ) are given in ppm and coupling constants (J) are given in Hz. The data were compared to those from the literature.[21,32,33]

Quercetin-3,7-*O*-(α)-L-dirhamnoside (**3**)

Yellowish solid; λ_{max} 255, 355 nm; ^{1}H NMR (400 MHz, CD_3OD) δ 7.38 (d, 1H, *J* 2.0 Hz, H-2'), 7.36 (dd, 1H, *J* 2.0, 8.1 Hz, H-6'), 6.94 (d, 1H, *J* 8.1 Hz, H-5'), 6.75 (d, 1H, *J* 1.8 Hz, H-8), 6.49 (d, 1H, *J* 1.8 Hz, H-6), 5.56 (br s, 1H, 3-*O*-Rh-1''), 5.40 (br s, 1H, 7-*O*-Rh-1'''), 4.24 (dd, 1H, *J* 1.9, 3.2 Hz, H-2''), 4.02 (br s, 1H, H-2'''), 3.77 (dd, 1H, *J* 3.9, 9.1 Hz, H-3'''), 3.67 (br d, 1H, *J* 1.9 Hz, H-3''), 3.56-3.68 (m, 1H, H-5'''), 3.40-3.52 (m, 2H, H-4'' and H-4'''), 3.30-3.39 (m, 1H, H-5''), 1.26 (d, 3H, *J* 6.0 Hz, 7-*O*-Rh-CH_3), 0.92 (d, 3H, *J* 6.0 Hz, 3-*O*-Rh-CH_3); ^{13}C NMR (150 MHz, CD_3OD) δ 179.0 (C-4), 163.8 (C-7), 163.2 (C-5), 158.3 (C-2 and C-9), 150.2 (C-3'), 146.7 (C-4'), 136.7 (C-3) 123.1 (C-1'), 122.9 (C-6'), 117.1 (C-2'), 116.6 (C-5'), 107.6 (C-10), 103.7 (C-1''), 100.7 (C-6), 100.1 (C-1'''), 95.8 (C-8), 73.4 (C-5''), 73.0 (C-4'''), 72.3 (C-3'''), 72.1 (C-2''), 72.0 (C-2'''), 71.4 (C-3'' and 5'''), 18.2 (7-*O*-Rh-CH_3), 17.8 (3-*O*-Rh-CH_3).

Kaempferitrin (kaempferol-3,7-*O*-(α)-L-dirhamnoside) (**5**)

Yellowish solid; λ_{max} 264, 343 nm; ^{1}H NMR (400 MHz, CD_3OD) δ 7.78 (d, 2H, *J* 8.7 Hz, H-2' and 6'), 6.93 (d, 2H, *J* 8.7 Hz, H-3' and 5'), 6.71 (d, 1H, *J* 1.9 Hz, H-8), 6.45 (d, 1H, *J* 1.9 Hz, H-6), 5.56 (d, 1H, *J* 1.9 Hz, 7-*O*-Rh-1'''), 5.40 (d, 1H, *J* 1.9 Hz, 3-*O*-Rh-1''), 4.26 (dd, 1H, *J* 1.9, 3.4 Hz, H-2''), 4.03 (d, 1H, *J* 1.9, 3.4 Hz, H-2'''), 3.84 (d, 1H, *J* 3.4, 9.3 Hz, H-3'''), 3.72 (dd, 1H, *J* 3.4, 9.3 Hz, H-3''), 3.59-3.62 (m, 1H, 5'''), 3.52 (t, 1H, *J* 9.3 Hz, H-4'''), 3.48 (t, 1H, *J* 9.4 Hz, H-4''), 3.30-3.37 (m, 1H, H-5''), 1.26 (d, 3H, *J* 6.4 Hz, 7-*O*-Rh-CH_3), 0.93 (d, 3H, *J* 5.6 Hz, 3-*O*-Rh-CH_3).

Total flavonoid content

The total flavonoid content was determined spectrophotometrically using a method based on the formation of a flavonoid-aluminum complex.[34] Aliquots of 5 mL of the extracts were transferred to a 25 mL volumetric flask, and the volume was adjusted with MeOH:H_2O 7:3 v/v. A 1 mL aliquot was then transferred to a 10 mL volumetric flask, 1 mL of a 5% MeOH solution of $AlCl_3$ was added, and the volume was adjusted with MeOH. After 30 min the absorbance was measured at 425 nm in a Beckman DU-70 spectrophotometer (Beckman General Lab. Equip., Los Angeles, USA). The concentration of the total flavonoid (expressed as μg of flavonoids (rutin mg^{-1}) of the plant material) was calculated with reference to a rutin external calibration curve built from five different concentrations presenting suitable linearity ((coefficient of determination (R^2) = 0.9994) in the range of 10-350 μg mL^{-1}. All tests were carried out in triplicate.

Development of the extraction method

Effect of the extraction solvent

In test tubes containing 500 mg of milled leaves of *U. guianensis* from MT (undefined particle size), 5 mL of the solvent system (MeOH, EtOH:H_2O 1:1 v/v, EtOH and H_2O) were added. The mixtures were ultrasonicated for 5 min, and centrifuged. The supernatants were transferred to a 25 mL volumetric flask; the volume was adjusted with MeOH:H_2O 1:1 v/v, and the total flavonoid content was determined. The solvents were then evaporated under low pressure, the dry extracts were weighed, and the TLC profiles were analyzed. All tests were carried out in triplicate.

Determination of the extraction conditions

U. guianensis milled leaves from MT were sieved to particle size ≤ 0.177 mm and used in subsequent tests. To arbitrate the best extraction time, test tubes containing 500 mg of powdered leaves from MT and 5 mL of EtOH:H_2O 1:1 v/v were submitted to different times of extraction assisted by ultrasound (10, 20, 30 and 40 min). The tubes were then centrifuged, the supernatants were separated, and the solvent was evaporated to determine the yields. This procedure was carried out in duplicate. The number of extraction cycles was determined in test tubes containing 500 mg of powdered leaves from MT and 10 mL of EtOH:H_2O 1:1 v/v. The mixture was ultrasonicated for 20 min, centrifuged, and the supernatant was transferred to a 25 mL volumetric flask. This procedure was repeated eight times by adding the same volume of the fresh solvent system to the residual plant material. The supernatant from each extraction was separated into different 25 mL volumetric flaks, and the volume was adjusted with EtOH:H_2O 1:1 v/v. Then, a 1 mL aliquot of each solution was transferred to different 10 mL volumetric flasks. The volume was adjusted with MeOH, and the total flavonoid content was determined. The test was carried out in triplicate.

Development of the clean-up SPE method

Choice of the SPE solvent system

The previously obtained EtOH:H_2O 1:1 extract of

U. guianensis leaves from MT[20] was used to develop the clean-up SPE method. First, 5 mL aliquots of the dried extract solutions (25 mg mL^{-1} in MeOH:H_2O, EtOH:H_2O and CH_3CN:H_2O all 1:9 v/v) were deposited on three different SPE cartridges (Supelco C-18, 500 mg, 3 mL) (Merck, Darmstadt, Germany), which had been preconditioned with 5 mL of MeOH followed by 5 mL of Milli-Q H_2O. The cartridges were then independently eluted with 5 mL of each sequence of the solvent system tested: organic solvent: H_2O 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 v/v and then pure organic solvent. The resulting eluates were evaporated under low pressure and the residues were submitted to TLC. Next, two separate experiments were run to determine the appropriate EtOH:H_2O proportion: (*i*) using the *U. guianensis* MT leaf extract and (*ii*) using the isolated kaempferitrin. In both experiments, the cartridges were preconditioned as described above. Aliquots (5 mL) of the extract solution (25 mg mL^{-1} in EtOH:H_2O 1:1 v/v) and of the kaempferitrin EtOH/H_2O 1:1 v/v solution (0.519 mg mL^{-1}) were deposited onto two different SPE cartridges and the samples eluted sequentially with 5 mL of EtOH:H_2O 1:1, 3:2, 7:3 v/v and EtOH and EtOH:H_2O 1:1, 3:2 v/v and EtOH, respectively. The fractions from the *U. guianensis* extract were monitored by TLC and those from the standard kaempferitrin were submitted to HPLC-DAD.

SPE separation recovery analysis

An aliquot (5 mL) of a kaempferitrin EtOH:H_2O 1:1 v/v solution (103.8 μg mL^{-1}) was deposited onto a preconditioned SPE cartridge and then eluted with 5 mL of EtOH:H_2O 1:1 v/v. The fractions from the sample application and elution were combined and submitted to HPLC-DAD, and the recovered amount of kaempferitrin was calculated. The test was carried out in triplicate.

Validation of the HPLC-DAD method

The parameters were validated following Resolution 166/2017 of the Brazilian Agency for Sanitary Surveillance (ANVISA).[35] The linearity was determined for an external calibration curve established at 5, 40, 80, 135 and 195 μg mL^{-1} obtained from a stock solution of kaempferitrin (0.519 mg mL^{-1} in MeOH), from triplicate injections in the HPLC-UV. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from triplicate injection of the dilution solvent (EtOH:H_2O 1:1 v/v) in the HPLC to evaluate the baseline noise at the retention time of kaempferitrin. LOD and LOQ were determined by signal to noise rates of three and ten times, respectively. The repeatability of the response of the HPLC instrument was evaluated through the injection of six replicates of kaempferitrin MeOH solutions at three different concentrations: 10, 40 and 60 μg mL^{-1}. The intermediate precision of the instrument was evaluated by injecting the same kaempferitrin MeOH solutions twice a week for three weeks (n = 6). The specificity and selectivity were evaluated using an EtOH:H_2O 1:1 extract of *U. tomentosa* leaves free of kaempferitrin[21] and a similar sample fortified with kaempferitrin at a known concentration in MeOH (25.95 μg mL^{-1}), injected in triplicate into the HPLC system. The absence of a peak at the retention time of kaempferitrin combined with the UV spectrum profiles of the HPLC peaks in the retention region of this target compound was diagnostic for these analyses. The accuracy was evaluated by injecting nine replicates of the same kaempferitrin fortified EtOH:H_2O 1:1 extract of *U. tomentosa* leaf MeOH solution (25.95 μg mL^{-1}) followed by quantification of kaempferitrin.

Quantitative determination of kaempferitrin in *Uncaria guianensis* samples

Leaves, branches, and bark were separated. The leaves and branches were milled and sieved to ≤ 0.177 mm particle size and 250 mg of each material were transferred to test tubes and ultrasonically extracted with 10 mL EtOH:H_2O 1:1 v/v for 20 min. The mixtures were centrifuged, and the supernatants were separated. The extraction procedure was repeated six times, and the supernatant of the samples were combined. All extractions were performed in triplicate. Next, 5 mL of each extract were transferred to a preconditioned SPE-RP18 cartridge and eluted with 5 mL of EtOH:H_2O 1:1 v/v. Both fractions from the sample application and the elution were combined and submitted to HPLC-DAD analysis. The amount of kaempferitrin was expressed as mg 100 mg^{-1} of the dry plant material.

Results and Discussion

HPLC-DAD-ESI-MS/MS polyphenol profile of the previously obtained leaf EtOH:H_2O 1:1 extract of *Uncaria guianensis*

The selective presence of kaempferitrin in the *U. guianensis* leaves collected in the state of Mato Grosso has previously been established by HPLC-DAD-MS upon an anti-inflammatory and anti-allergic EtOH extract.[21] This observation was subsequently corroborated by TLC and ^{1}H NMR profiles of the anti-dengue type 2 EtOH:H_2O 1:1 extract of leaves from the same *U. guianensis* specimen.[20] In the present study, this bioactive hydroalcoholic leaf extract was analyzed by HPLC-DAD-ESI-MS/MS to

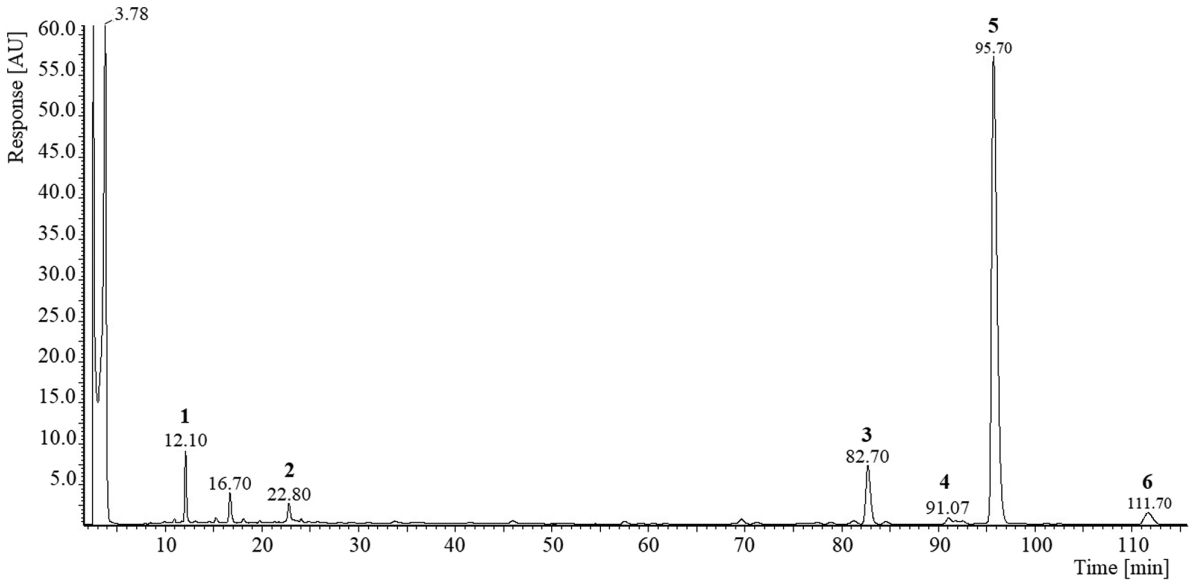

Figure 1. HPLC-DAD chromatogram (sum of absorbances at all wavelengths) of the previously obtained EtOH:H_2O 1:1 extract[20] from *Uncaria guianensis* leaves. 3,4-Dihydroxybenzoic acid (**1**)*, 5'-caffeoylquinic acid (**2**)*, quercetin-3,7-*O*-(α)-L-dirhamnoside (**3**), quercetin-monorhamnoside (**4**), kaempferol-3,7-*O*-(α)-L-dirhamnoside (kaempferitrin) (**5**), kaempferol-monorhamnoside (**6**) (*identified with standard).

advance in the details of the polyphenolic composition of *U. guianensis* leaves.

In addition to revealing kaempferitrin (**5**) (kaempferol-3,7-*O*-(α)-L-dirhamnoside) as the major compound and quercetin-3,7-*O*-(α)-L-dirhamnoside (**3**) (both isolated from this extract), the present study also showed two minor flavonol monoglycosides: quercetin- and kaempferol-monorhamnoside (**4** and **6**, respectively), 3,4-dihydroxybenzoic acid (**1**) and 5'-caffeoylquinic acid (**2**) (Table 1, Figures 1 and 2). The amount and/or purity of other compounds did not allow a further structural characterization. The identification was carried out by comparing the retention time (t_R), the UV-Vis spectrum, the MS recorded in full scan and the MS/MS product ion scan mode with those of the standard analyzed under the same conditions. Compounds with no available standard had their phenolic class inferred from the UV-Vis spectra and the $[M + H]^+$ and $[M - H]^-$ ions identified in the MS full scan spectra in positive and negative modes. This approach was aided by the MS/MS product ion spectra using the $[M + H]^+$ ion as a precursor to assign the protonated aglycone $[Y_0]^+$ and the fragmentations observed in both MS/MS product ion spectra using $[M + H]^+$ or $[Y_0]^+$ as the precursor ion. The loss of 132, 146 or 162 Da would be indicative of the presence of pentose, deoxyhexose or hexose, respectively.

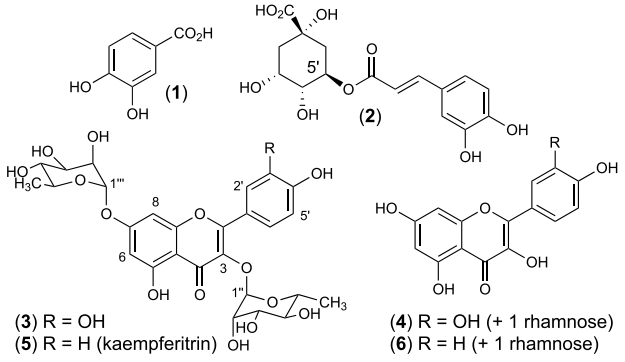

Figure 2. Structures of the isolated or characterized compounds in the previously obtained EtOH:H_2O 1:1 extract[20] from *Uncaria guianensis* leaves.

Quantitative determination of kaempferitrin in *Uncaria guianensis* samples

Aligned with the analytical background of this study, the efficiency of the ultrasound-assisted extraction[36] of kaempferitrin from the leaves of *U. guianensis* in three different solvents and one binary solvent system was assayed, to achieve the highest yield and selectivity, regarding the total flavonoid content (Table 2).

The lowest crude yield was obtained by using EtOH (9.90%), whilst all other solvents led to a similar amount of dry crude extract (14.8-16.0%). The EtOH:H_2O 1:1 mixture showed the highest selectivity concerning the flavonoid content. The comparative TLC analysis regarding the polyphenol profiles agrees with this finding (Figure S1, Supplementary Information (SI) section), supporting the choice of the binary mixture to forward the extraction and isolation of kaempferitrin.

The best time for extraction assisted by ultrasound was inferred by comparing the yields of the extraction of the leaves with EtOH:H_2O 1:1 at four different times (10, 20, 30 and 40 min). The extraction time of 20 min was

Table 1. HPLC-DAD-ESI-MS/MS data of the identified polyphenolic compounds of the previously obtained[20] EtOH:H_2O 1:1 extract from *Uncaria guianensis* leaves

Peak No.	t_R / min	Compound	λ_{max} / nm	MS^1	MS^2
				Parent ion $[M + H]^+$ *m/z*	Secondary ions *m/z*
1	12.10	3,4-dihydroxybenzoic acid (protocatechuic acid)	260, 294	155	137, 109
2	22.80	5'-caffeoylquinic acid (5-CQA)	302 (sh), 325	355	191, 163
3	82.70	quercetin-3,7-*O*-(α)-L-dirhamnoside	256, 350	595	449, 303
4	91.07	quercetin-monorhamnoside	256, 345	449	303
5	95.70	kaempferol-3,7-*O*-(α)-L-dirhamnoside (kaempferitrin)	264, 344	579	433, 287
6	111.62	kaempferol-monorhamnoside	264, 345	433	287

See Figure 1 for the correspondent chromatogram. t_R: retention time; sh: shoulder. See Figure S5 (Supplementary Information (SI) section) for MS^1 and MS^2 of kaempferitrin.

Table 2. Yield and total flavonoid content of crude extracts from *Uncaria guianensis* leaves[a] using different extraction solvents

Solvent	Yield (m/m ± SD) / %	Total flavonoid content / (μg 100 mg^{-1} ± SD)[b]
MeOH	15.96 ± 0.10	77.91 ± 1.12
EtOH:H_2O 1:1	14.85 ± 0.12	128.25 ± 0.57
EtOH	9.87 ± 0.22	30.06 ± 1.28
H_2O	15.18 ± 0.08	27.07 ± 1.17

[a]Collected in MT; [b]μg 100 mg^{-1} of dry leaves. SD: standard deviation.

chosen since no significant increase in the extract yield was observed after 20 min of sonication (21% - 10 min, 30% - 20 min and 33% - 30 and 40 min). Next, the number of extraction cycles towards the complete depletion of the flavonoid content from the *U. guianensis* leaves was determined by repeating the pre-established extraction procedure eight times (EtOH:H_2O 1:1, 20 min sonication) on the same sample and analyzing the total flavonoid content at the end of each cycle. There was no response to the presence of flavonoids from the fifth cycle; therefore, a six-cycle extraction was adopted to ensure the maximum kaempferitrin extracted thereafter.

The SPE procedure to separate the kaempferitrin, along with the minor phenolic constituents of the EtOH:H_2O 1:1 extract, from less polar and nonphenolic compounds was achieved through 5 mL of the application sample followed by 5 mL of EtOH:H_2O 1:1. This procedure was able to recover 96% ± 0.11 of kaempferitrin.

The HPLC method to quantify kaempferitrin was validated by determining the precision (intra- and inter-day variability measures). Intra-day variation (repeatability) was determined by analyzing six replicates of kaempferitrin standard solutions at three different concentrations within one day. For the inter-day variability test (intermediate precision), the same kaempferitrin solutions were analyzed twice a week for three weeks. The relative deviation taken as indicative of precision resulted in low values in both cases (RSD < 5.0%) (Table 3).

The mean recovery was 110.9% (RSD 1.95%), which can be considered satisfactory. The calibration curve presented linearity in the concentration range from 5.0 to 195.0 μg mL^{-1} (R^2 = 0.999) and the calibration equation: $y = 34851.3x + 11809.6$. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as 1.398 and 4.66 mAU, respectively, corresponding to 0.3 μg mL^{-1} (LOD) and 1.0 μg mL^{-1} (LOQ) of kaempferitrin.

The kaempferitrin contents in the cultivated and wild *U. guianensis* samples from different locations in the Amazon rainforest are expressed in mg of kaempferitrin *per* 100 mg of dry plant material (Table 4). The HPLC-DAD polyphenol profiles of the cultivated and wild leaves are shown in Figure 3 (see Figure S2 (SI section) for HPLC-DAD polyphenol profiles of the wild branches).

Table 3. Precision results[a] of the HPLC method related to repeatability and intermediate precision

Kaempferitrin concentration / (μg mL^{-1})	Repeatability		Intermediate precision	
	Mean ± SD	RSD / %	Mean ± SD	RSD / %
20	668.8 ± 1.6	0.66	669.6 ± 14.3	2.1
40	1488.6 ± 5.1	0.34	1468.7 ± 30.5	2.1
60	2276.3 ± 17.8	0.78	2276.9 ± 22.8	1.0

[a]Based on the kaempferitrin HPLC-UV peak area at 265 nm. RSD: relative standard deviation for each sample (n = 3); SD: standard deviation.

Table 4. Concentration of kaempferitrin in cultivated and wild *Uncaria guianensis* leaves and branches collected in different locations of the Brazilian Amazon rainforest

Sample	Concentration / (mg 100 mg^{-1})[a]	RSD / %
UGL-C8 (b)	0.28 ± 0.01	3.4
UGL-C24 (c)	0.69 ± 0.01	1.7
UGL-MT (d)	1.38 ± 0.04	3.1
UGL-ACOct (e)	1.13 ± 0.02	2.0
UGL-PA (f)	1.48 ± 0.02	1.0
UGB-PA (f')	< LOQ	–
UGL-ACSept (g)	1.39 ± 0.02	1.8
UGL-AM (h)	1.94 ± 0.04	2.3
UGB-AM (h')	0.04 ± 0.01	2.9

[a]mg 100 mg^{-1} of dry plant. UGL-C8 (b): *in vitro* cultivated leaves, 8 months old; UGL-C24 (c): leaves cultivated from seeds, 24 months old; UGL-MT (d): leaves from Juruena-MT; UGL-ACOct (e) and UGL-ACSept (g): leaves from Rio Branco-AC, collected in October and September 2008, respectively; UGL-PA (f) and UGB-PA (f'): leaves and branches from the Ver-o-Peso herbal market, Belém-PA, respectively; UGL-AM (h) and UGB-AM (h'): leaves and branches from Manaus-AM, respectively. RSD: relative standard deviation. LOQ: limit of quantification. See Figure 3 for the HPLC-DAD polyphenol profiles of the *U. guianensis* wild and cultivated leaves and Figure S2 (SI section) for the HPLC-DAD polyphenol profiles of the *U. guianensis* wild branches.

Kaempferitrin has been proposed as the chemical marker for *U. guianensis* based on its frequent detection and usual predominance in the leaves of this species and the absence or low and variable amount of the typical oxindole alkaloids[8,10] that appear with greater abundance in the *U. tomentosa*.[9,37] Reinforcing the assumption of kaempferitrin as a chemical marker, this work has extended prospective studies, covering a significant sampling, to verify its presence and variability in the aerial parts of *U. guianensis*; also establishing a profile that considers the minor polyphenolic constituents once a detailed approach to these compounds for this species was still lacking.

Both points required a previous assay to find suitable extraction process to extract those related to optimum yield and high selectivity to flavonoids. In the present study, the choice of extraction solvent played a crucial step achieving these goals and, not less important, in supporting the analytical method approach to kaempferitrin dosing. Together with the search for the best extraction yield, the use of the spectrophotometric assay of the total content of flavonoids at 427 nm allowed the detection of the selective extraction of flavonols based on the maximum absorption

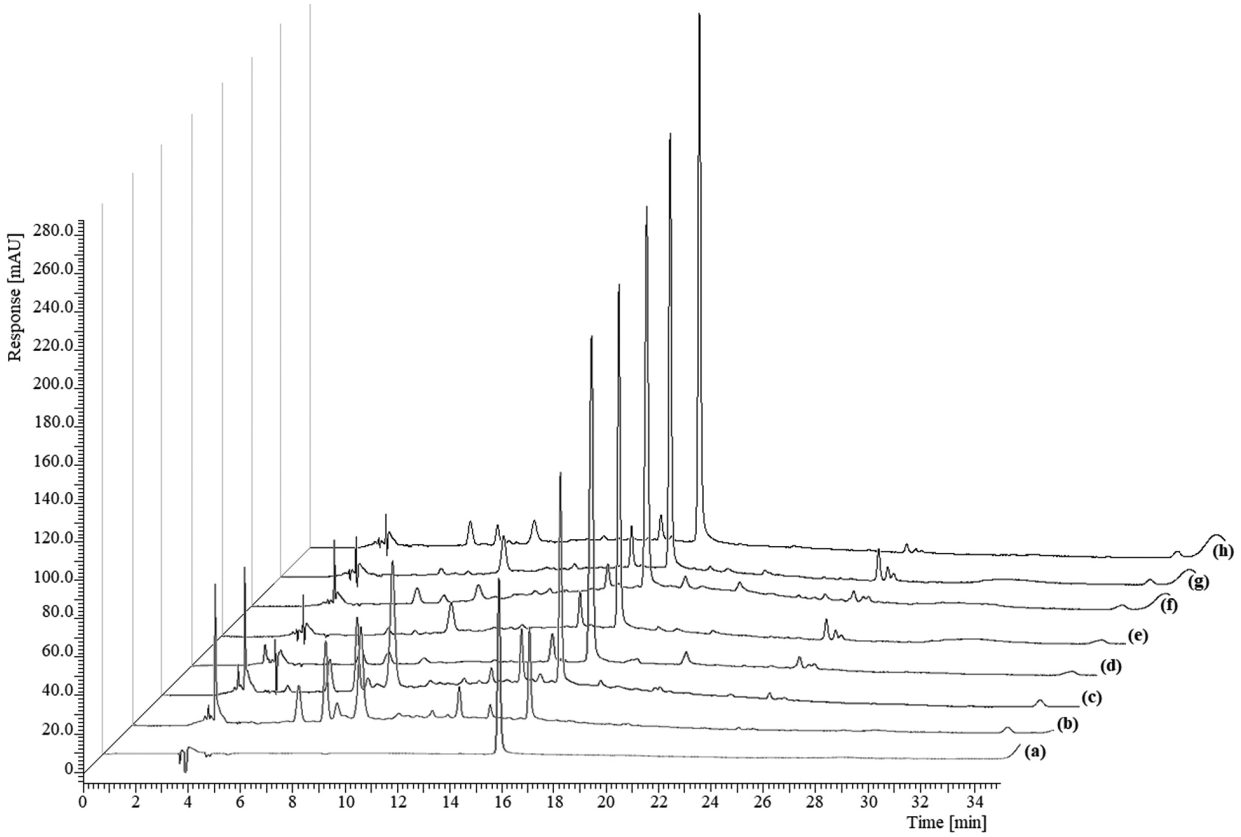

Figure 3. HPLC-DAD polyphenol profiles at 265 nm of cultivated and wild *Uncaria guianensis* leaves collected in different locations of the Brazilian Amazon rainforest. Kaempferitrin peak at retention time = ca. 15 min (a); UGL-C8 (b); UGL-C24 (c); UGL-MT (d); UGL-ACOct (e); UGL-PA (f); UGL-ACSept (g); UGL-AM (h). See Table 4 for sample codes and Figure S2 (SI section) for HPLC-DAD polyphenol profiles of the *U. guianensis* wild branches.

values of the flavonoid-aluminum complexes of most of the natural flavones and flavonols that occur in the range 390-440 nm. Besides, the plant/solvent ratio and the type, time, and extraction cycles were all standardized, and a clean-up SPE targeted method was developed. Furthermore, a quantitative HPLC-DAD method was validated as specific, precise, and accurate for the quantification of kaempferitrin, resulting in high sensitivity for low levels of quantification and detection.

When applying this tool, it was shown that kaempferitrin is present in all leaf samples tested with a slight variation in the amount found in leaves derived from adult specimens collected in the field (1.13-1.94 mg 100 mg^{-1} (Table 4, d-h)). In addition, the HPLC-DAD polyphenol profiles of these wild samples were quite similar (Figure 3). On the contrary, in the cultivated young leaves, the cultivation time seemed to influence the concentration of this flavonoid (0.28 mg 100 mg^{-1} for eight months, and 0.69 mg 100 mg^{-1} for twenty-four months (Table 4, b and c)) (Figure 3). It was also possible to preliminarily observe an amount approximately 37 times lower in the branches (Table 4) when compared to the leaves. Considering that kaempferitrin has not been previously detected in the *U. guianensis* bark,[21] these results pointed out the probable selective distribution of this compound in different parts of this species.

Conclusions

This study contributes to expanding the knowledge about the polyphenol profile and kaempferitrin content in leaves and branches of *U. guianensis*. The content of kaempferitrin was relatively uniform in the leaves of wild adult plants collected in different parts (and seasons) of the Brazilian territory. The uneven distribution found for kaempferitrin in leaves and branches confirmed some previous findings. Its content was approximately three times higher in the wild (mature) leaf than in the cultivated (young) leaf, suggesting that the ontogenesis of the plant may play a role in the biosynthesis of the kaempferitrin. The presence of kaempferitrin and other minor polyphenols visibly contrast with the profile and content of alkaloids found for this species. Overall, these results are a great help for a further step in the consolidation of kaempferitrin as a chemical marker for the leaves of *U. guianensis* and their derived products instead of the putative oxindole alkaloids.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

DP and RSB performed the chemical experiments supervised by LMMV; LMMV, BG and LAB performed the HPLC-DAD-MS/MS analysis. RCAP provided all cultivated samples; MOS supported several steps of the experiments; LMMV, RSB, ACS, BG and LAB contributed to the writing of the manuscript and its revision.

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