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Article

Kaempferitrin from *Uncaria guianensis* (Rubiaceae) and its Potential as a Chemical Marker for the Species

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Uncaria tomentosa (Willd.) DC. e *U. guianensis* (Aubl.) Gmel., conhecidas como unha-de-gato, são trepadeiras lenhosas nativas das florestas tropicais amazônica e central-americana. As espécies contêm, em diferentes proporções, alcalóides indólicos e oxindólicos, triterpenóides glicosilados, esteróides e proantocianidinas. *U. tomentosa* é quimicamente identificada pelo perfil e conteúdo de alcalóides oxindólicos, ao passo que *U. guianensis* não possui um marcador químico efetivo. Nesse trabalho descreve-se o isolamento de canferol-3,7-O-(α)-dirramnosídeo (canferitrina) pela primeira vez em espécies do gênero *Uncaria*. A triagem para essa substância em folhas, galhos ou cascas das duas espécies por CCD e CLAE-DAD-EM demonstrou a presença de canferitrina apenas nas folhas e galhos de *U. guianensis*, numa proporção cerca de trinta e seis vezes maior nas folhas do que nos galhos. Estes resultados revelaram a seletividade da *U. guianensis* em produzir o flavonóide glicosilado, sugerindo esta substância como um marcador químico potencial para a espécie.

Uncaria tomentosa (Willd.) DC. and *U. guianensis* (Aubl.) Gmel., known as cat's claw, are large woody vines native to the Amazonian and Central American rainforests. The species contain, in different proportions, indole and oxindole alkaloids, triterpenoid glycosides, sterols and proanthocyanidins. *U. tomentosa* can be chemically identified by its oxindole alkaloid profile and content, whereas *U. guianensis* has no satisfactorily established chemical markers. This work describes, for the first time, the isolation of kaempferol-3,7-O-(α)-dirhamnoside (kaempferitrin) in *Uncaria* species. Screening for this compound in leaves, stems or bark of both species through TLC and HPLC-DAD-MS showed the presence of kaempferitrin only in the leaves and stems of *U. guianensis*, at a ratio almost thirty six times greater in the leaves than in the stems. These results reveal the selectivity of *U. guianensis* to produce this bioactive flavonoid glycoside, and suggest this compound as a potential chemical marker for the species.

Keywords: *Uncaria guianensis*, *Uncaria tomentosa*, Rubiaceae, kaempferitrin, cat's claw

Introduction

The genus *Uncaria* (Rubiaceae) contains 34 species distributed among the tropical areas of Southeast Asia, Africa and Central and South America.[1] It is represented in Central and South America by two species: *U. tomentosa* and *U. guianensis*.[2] These species, known as cat's claw, *unha-de-gato* (Brazil), *uña de gato*, *garabato* etc., are large woody vines that have been used medicinally by indigenous peoples to treat several diseases for at least two thousand years.[3,4]

From the genus *Uncaria* there have so far been isolated over 150 compounds with a predominance of alkaloids. Other classes of isolated compounds include terpenoids and terpenoid glycosides, flavonoids (specially flavanols and flavonols) and coumarins.[5,6] The South American species *U. tomentosa* and *U. guianensis* present some morphological differences[2,7] and *U. tomentosa* can be further identified by its oxindole alkaloid profile and content.[8] On the other hand, these alkaloids are present in *U. guianensis* at very low concentration[9,10] and no satisfactory chemical markers have been established for this species so far.[11]

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Previous chemical studies of *U. guianensis* revealed indole and oxindole alkaloids (whole plant),[9] proanthocyanidins (bark),[12] flavonols (bark)[13] and triterpenoid glycosides (bark).[13,14] *In vitro* and clinical studies using a decoction of *U. guianensis* bark have corroborated its traditional use as an anti-inflammatory and an antioxidant.[12,15] Bioassay-guided fractionation of the EtOH extract of *U. guianensis* bark using a yeast-based assay for DNA-damaging agents lead to two weakly but selectively active oxindole alkaloids.[16] The EtOH extract of the leaves of *U. guianensis* showed anti-inflammatory and anti-allergic activities.[17]

The present work describes the isolation of kaempferol-3,7-*O*-(α)-dirhamnoside (kaempferitrin) from the leaves of *U. guianensis* and the screening for this compound in the EtOH extracts from the leaves, stems or bark of *U. guianensis* and *U. tomentosa* through TLC and HPLC-DAD-MS techniques.

Experimental

General experimental procedures

NMR spectra were recorded on a Bruker DRX-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer in DMSO-d_6/drops D_2O with TMS as internal standard. Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz. The TLC analyses were made using pre-coated silica-gel 60F_{254} (Merck), mobile phase EtOAc/HCOOH/HOAc/H_2O 100:11:11:27 and UV irradiation (254 and 326 nm) and NP/PEG reagent followed by UV at 365 nm to visualize the spots.[18] The HPLC analyses were performed on a Shimadzu HPLC system (Kyoto, Japan) composed of a system controller SCL-10Avp, SIL-10ADvp auto injector, two LC-10ADvp pumps, DGU-12A degasser, SPD-M10Avp diode array detector and equipped with a reverse-phase C18 column (Lichrocart Lichrospher 5 µm, 250 × 4.6 mm i.d.). Elutions were performed in a gradient elution mode at a 0.8 mL min^{-1} flow: 10 min 10% solvent B (MeCN) in solvent A (H_2O with *ca.* 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 10 min was used between the runs. The diode-array detector was set at an acquisition range of 200-600 nm at a spectral acquisition rate of 156 scans s^{-1} (peak width 0.2 min). Flavonol monitoring was performed at 280 nm and 320 nm. The diode-array detector was coupled to a Micromass ZQ single quadrupole mass spectrometer. The conditions for positive ionization mode in the electrospray probe were: capillary 3.0 kV, cone 50 V, extractor 3 V, RF lens 1 V, source temperature 100 °C and 250 °C desolvation temperature. Acquisition was obtained in the *m/z* 100-700 range. Retention times, UV spectra and MS ions (and their relative abundances) in comparison to those of the isolated kaempferitrin were evaluated.

Plant material

The specimens used in this work were collected in three different locations in the Brazilian Amazon rainforest. *U. guianensis* was collected in Juruena, Mato Grosso (MT) state (12°50' S, 58°55' W; 277 m elevation) and in Manaus, Amazonas state (AM) (3°05' S, 60°00' W; 55 m elevation). The specimen from Mato Grosso was identified by the botanist Pierro Delprete (New York Botanic Garden) and a voucher deposited in the Central Herbarium of the Universidade Federal do Mato Grosso, Brazil, under No. 24715. A voucher specimen from the Amazonas state sample was deposited at the Centro de Biotecnologia da Amazônia, in Manaus. *U. tomentosa* was collected in the valley of the Juruá river, Acre state (AC) (7°40' S, 72°38' W; 192 m elevation); for voucher data see Miranda *et al*.[19]

Isolation of kaempferitrin

Leaves of *U. guianensis* from MT were exhaustly extracted with EtOH as previously described.[17] The ethanol extract (2 g) was partitionated between CH_2Cl_2/H_2O to yield an insoluble yellowish solid (27 mg) from the aqueous fraction.

Screening for kaempferitrin in U. tomentosa and U. guianensis

Dried and milled leaves and stems of *U. guianensis* from AM, bark of *U. guianensis* from MT and leaves and bark of *U. tomentosa* from AC (5 g of each sample) were exhaustedly and ultrasonically extracted with EtOH. The solvent was evaporated at low pressure and the dried extracts were partitioned with *n*-hexane and MeOH/H_2O 9:1. The EtOH extract (5 g) previously obtained from the leaves of *U. guianensis* from MT were also partitioned in a similar way. Aliquots of 10 µL of the MeOH solutions (c = 25 mg mL^{-1}) of the *n*-hexane and MeOH/H_2O fractions were compared to the isolated kaempferitrin (c = 1 mg mL^{-1}) by TLC. The MeOH/H_2O fractions and the isolated kaempferitrin solutions all at c = 1 mg mL^{-1} were filtered through 0.45 µm nylon membranes and injected (20 µL) into the HPLC system. MeOH/H_2O fractions of bark of *U. guianensis* and leaves and bark of *U. tomentosa* were further subjected to Sephadex LH-20 cc eluted sequentially with *n*-hexane/CH_2Cl_2 1:4, CH_2Cl_2/acetone 3:2 and MeOH, in order to concentrate the polyphenolic compounds and thus better visualize the presence (or not) of kaempferitrin. The MeOH subfractions thus obtained (c = 1 mg mL^{-1} in MeOH) were submitted to HPLC-DAD-MS analysis using the same conditions as described above.

kaempferol-3,7-O-(α)-dirhamnoside (kaempferitrin)

Amorphous yellowish solid; HPLC-UV λ_{max}/nm: 230, 264 and 343; HPLC-MS: *m/z* (rel. int.) $[M+1]^+$ 579 (27), 433 (39), 287 (100); ^{1}H NMR (DMSO-d_6/drops D_2O, 400 MHz): δ 6.46 (d, *J* 2.4 Hz, H-6); 6.79 (d, *J* 2.0 Hz, H-8); 7.80 (d, *J* 8.8 Hz, H-2' and 6'); 6.93 (d, *J* 8.8 Hz, H-3' and 5'); 5.30 (d, *J* 1.6 Hz, 3-O-Rh-1''); 3.99 (dd, *J* 1.6 and 3.2 Hz, H-2''); 3.47 (dd *J* 3.2 and 8.8 Hz, H-3''); 3.15 (t, *J* 8.8 Hz, H-4''); 3.13 (m, H-5''); 0.81 (d, *J* 5.6 Hz, 3-O-Rh-CH_3); 5.56 (d, *J* 1.6 Hz, 7-O-Rh-1'''); 3.84 (dd, *J* 1.6 and 3.4 Hz, H-2'''), 3.64 (dd, *J* 3.4 and 9.2 Hz, H-3'''); 3.31 (t, *J* 9.2 Hz, H-4'''); 3.43 (m, H-5'''); 1.13 (d, *J* 6.4Hz, 7-O-Rh-CH_3); ^{13}C NMR (DMSO-d_6/drops D_2O, 100 MHz): δ 157.8 (C-2), 134.5 (C-3), 178.0 (C-4), 160.9 (C-5), 99.4 (C-6), 161.7 (C-7), 94.6 (C-8), 156.1 (C-9), 105.8 (C-10), 120.7 (C-1'), 130.7 (C-2' and 6'), 115.4 (C-3' and 5'), 160.1 (C-6'), 101.9 (3-O-Rh-C-1''), 70.0 (C-2''), 70.3 (C-3''), 71.1 (C-4''), 70.7 (C-5''), 17.5 (3-O-Rh-CH_3), 98.4 (7-O-Rh-C-1'''), 69.8 (C-2'''), 70.2 (C-3'''), 71.6 (C-4'''), 70.0 (C-5'''), 17.9 (7-O-Rh-CH_3).

molecular ion M+1 at *m/z* 579 (27%) and fragment ions of *m/z* 433 (loss of one rhamnose, 146 a.m.u.) and *m/z* 287 (aglycone) in the MS (relative abundance proportions: 27:39:100, respectively). ^{1}H and ^{13}C NMR (400 MHz and 100 MHz respectively, DMSO-d_6/drops D_2O) analyses with the help of the correlation on the COSY, HMQC and HMBC spectra and comparison of their spectral data with those already reported,[20,21] confirmed the structure of kaempferol-3,7-*O*-(α)-dirhamnoside (kaempferitrin) (Figure 1) for this compound.

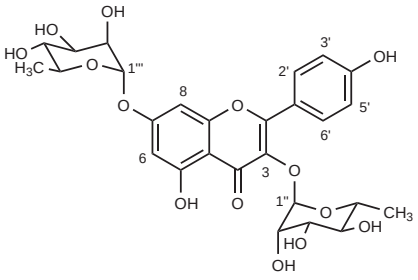

Figure 1. Structure of kaempferitrin.

Results and Discussion

HPLC-DAD-MS analysis of the isolated solid revealed the presence of a major compound (*ca*. 90% purity) absorbing at UV λ_{max}/nm: 230, 264 and 343, with a pseudo-

Screening for kaempferitrin in leaves, stems or bark of *U. guianensis* and *U. tomentosa* through HPLC-DAD-MS techniques (Figures 2 and 3) and TLC revealed the presence of this compound only in the leaves and stems of *U. guianensis* at a ratio almost thirty six times greater in the

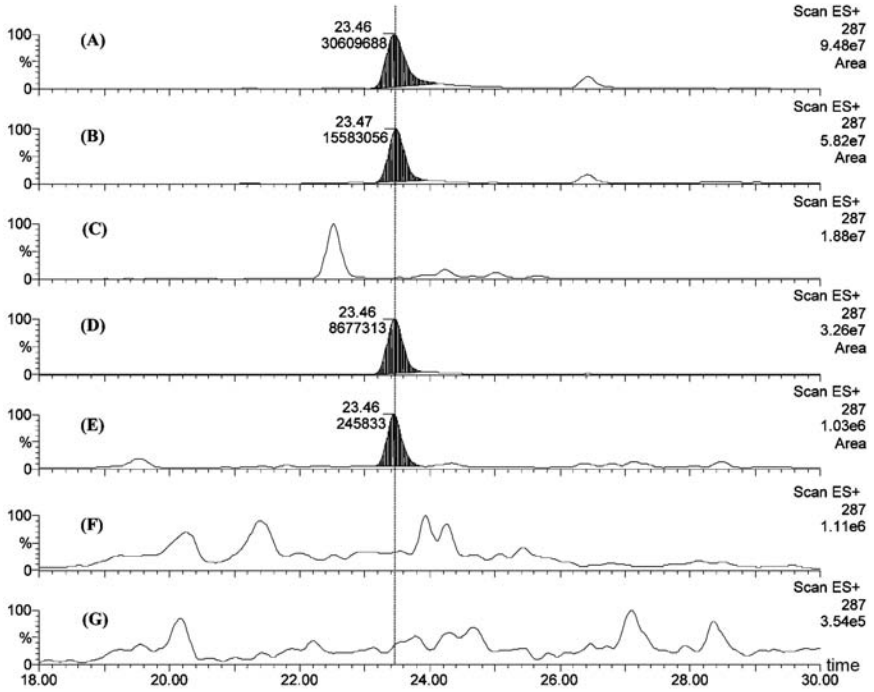

Figure 2. HPLC-MS fragmentograms (extracted ion *m/z* 287) of isolated kaempferitrin (A) and MeOH/H_2O fractions from: leaves of *Uncaria guianensis* from Mato Grosso (B), bark of *Uncaria guianensis* from Mato Grosso (C), leaves of *Uncaria guianensis* from Manaus (D), stems of *Uncaria guianensis* from Manaus (E), leaves of *Uncaria tomentosa* (F), bark of *Uncaria tomentosa* (G). The area and retention time of kaempferitrin are indicated at the top of each peak.

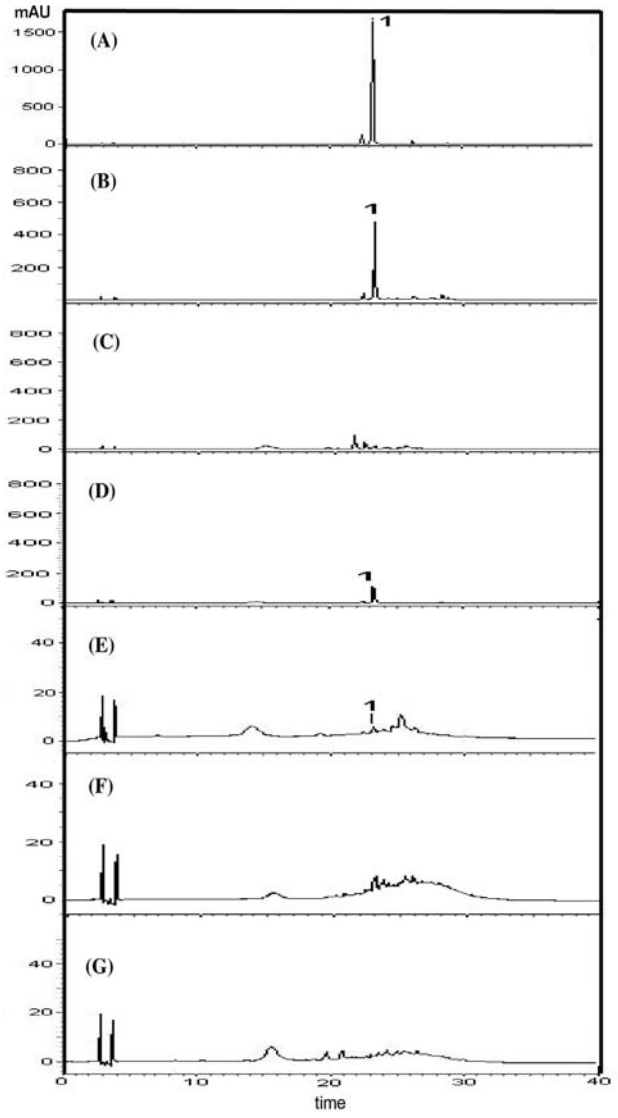

Figure 3. HPLC-UV profiles (320 nm) of (A) isolated kaempferitrin (1, t_R = 23.27 min) and MeOH/H_2O fractions from: (B) leaves of *Uncaria guianensis* from Mato Grosso (B), bark of *Uncaria guianensis* from Mato Grosso (C), leaves of *Uncaria guianensis* from Manaus (D), stems of *Uncaria guianensis* from Manaus (E), leaves of *Uncaria tomentosa* (F), bark of *Uncaria tomentosa* (G).

leaves than in the stems. Kaempferitrin was not found in the bark of the analyzed specimen of *U. guianensis*. Leaves and bark of *U. tomentosa* did not present kaempferitrin.

In addition to reporting for the first time the isolation of kaempferitrin from the *Uncaria* species and of a flavonoid glycoside from the leaves of *Uncaria guianensis*, the present study describes the selective production of this compound in leaves and stems of *U. guianensis*; a result that contributes to the chemotaxonomy of the *Uncaria* species and also could be a useful chemical tool in the differentiation of the leaves of *U. guianensis* from those of *U. tomentosa*. Furthermore, diverse bioactivities are reported for the isolated kaempferitrin;[22-24] a fact that may be connected to some of the traditional uses and pharmacological properties of *U. guianensis*.

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Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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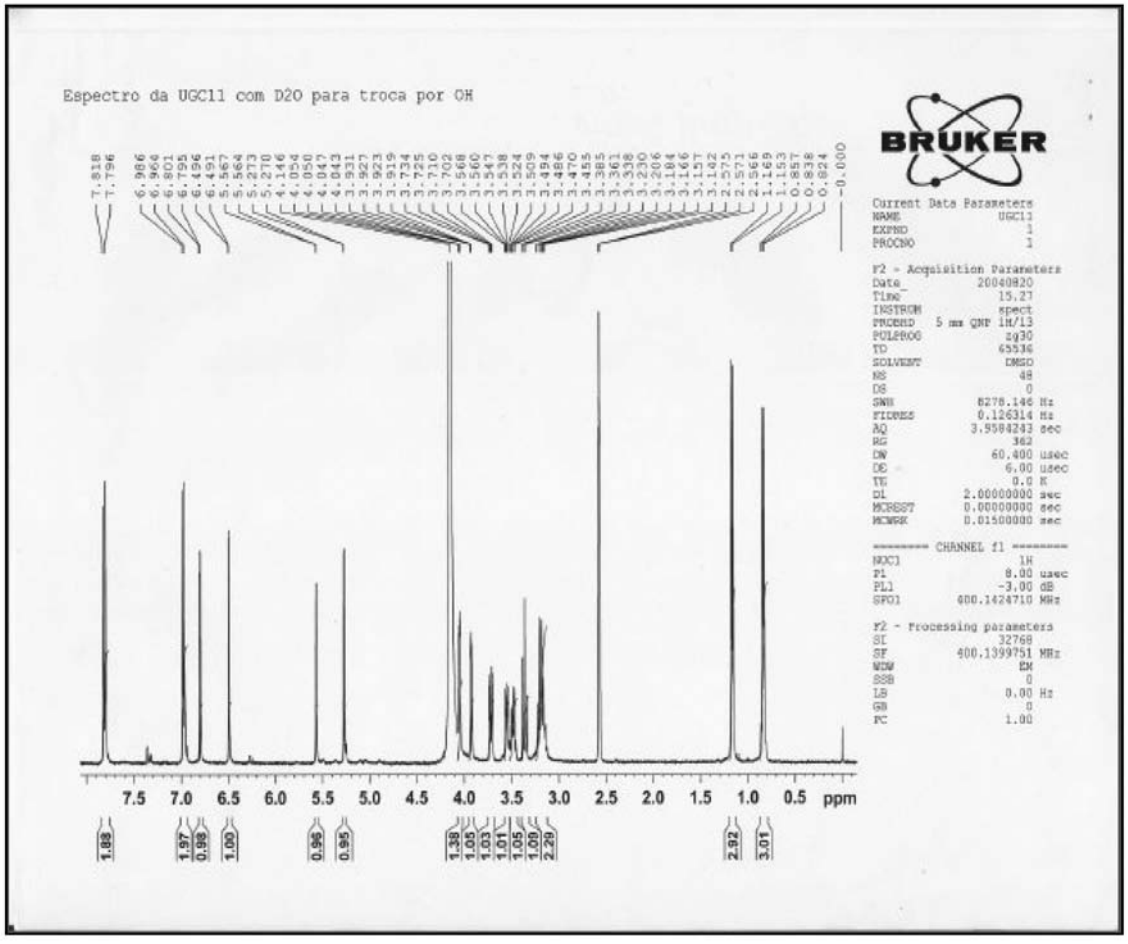

Figure S1. [1]H NMR spectrum (400 MHz), in DMSO-d_6/drops D_2O and TMS as internal standard of kaempferitrin.

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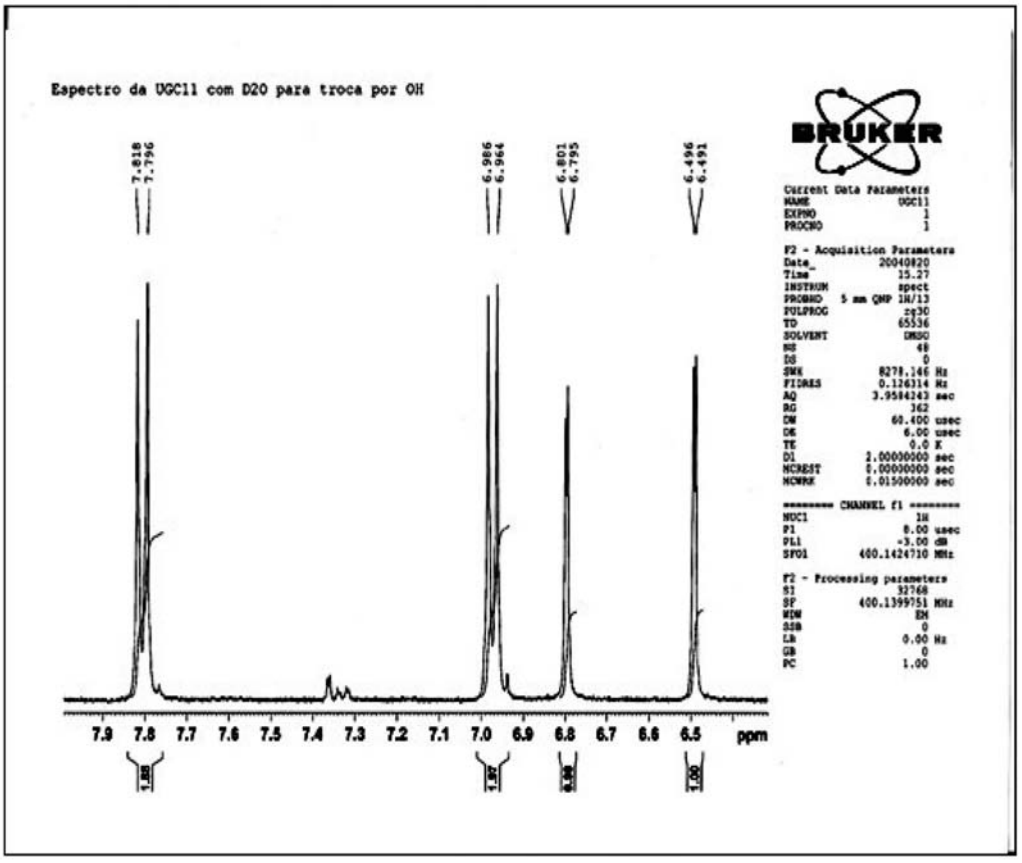

Figure S2. ^{1}H NMR spectrum (400 MHz), in DMSO-d_6/drops D_2O of the aromatic proton signals of kaempferitrin.

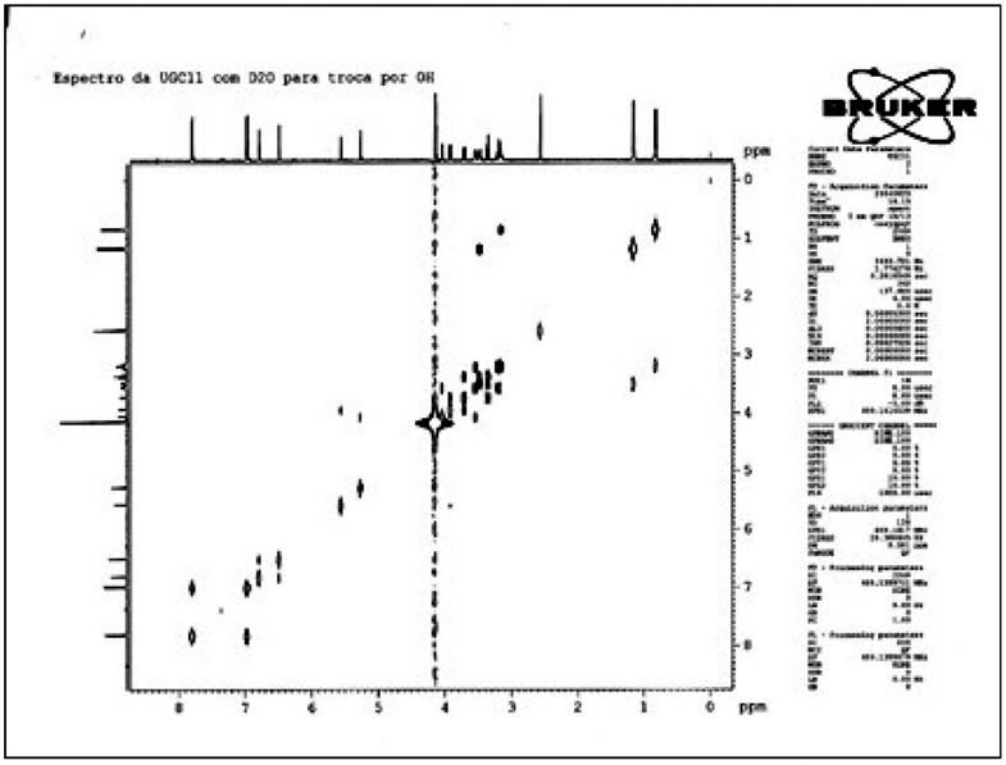

Figure S3. COSY spectrum (400 MHz), in DMSO-d_6/drops D_2O and TMS as internal standard of kaempferitrin.

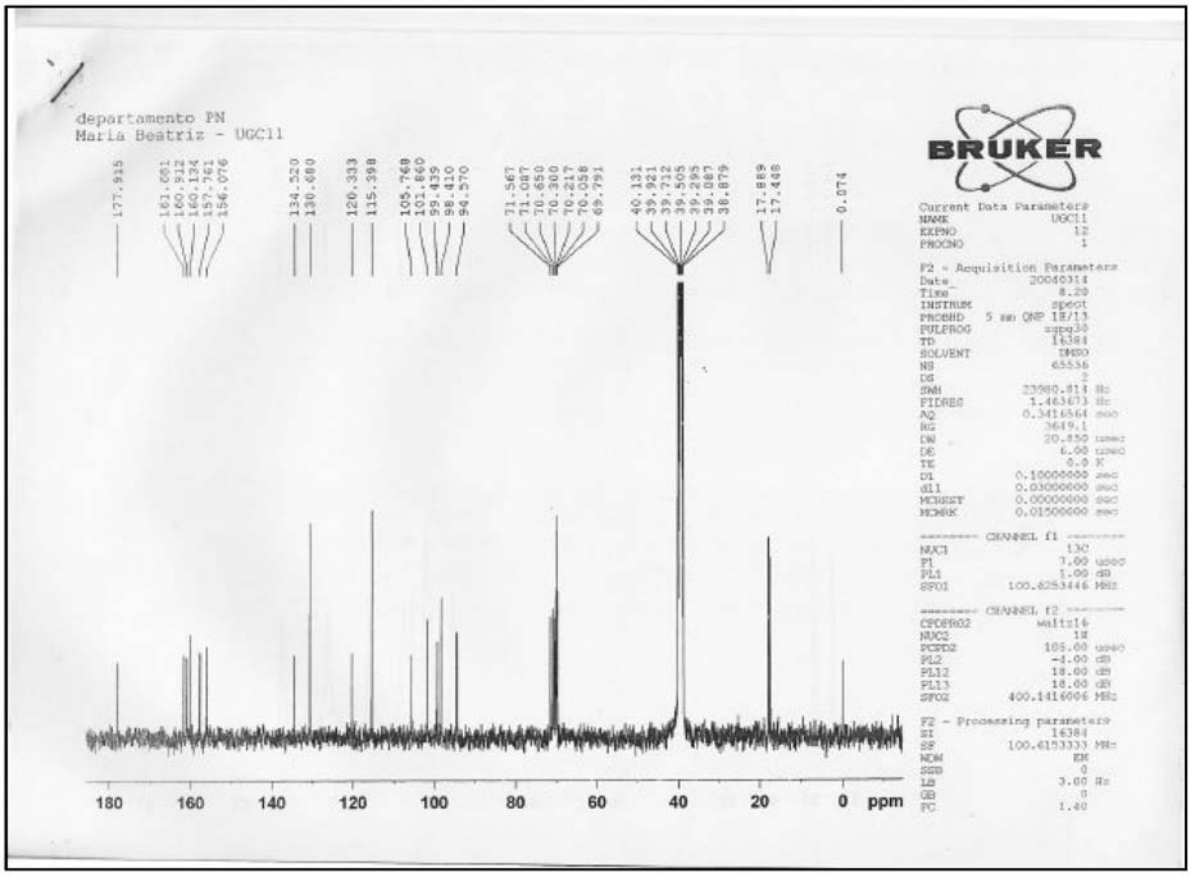

Figure S4. ^{13}C NMR spectrum (100 MHz), in DMSO-d_6/drops D_2O and TMS as internal standard of kaempferitrin.

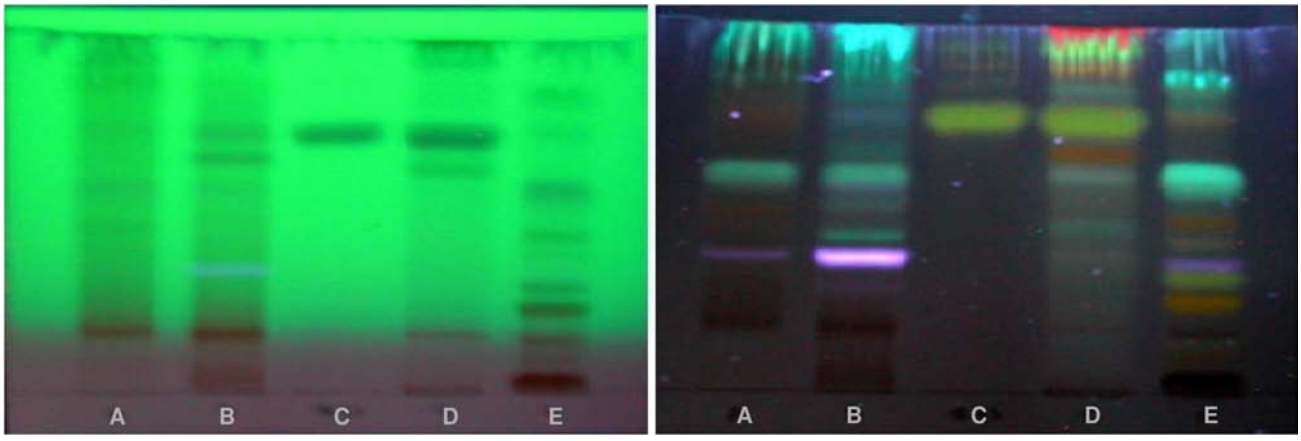

Figure S5. TLC profiles of the MeOH/H_2O fractions of: (A) *U. tomentosa* leaves (25 mg mL^{-1}); (B) *U. tomentosa* barks (25 mg mL^{-1}); (C) kaempferitrin (1 mg mL^{-1}); (D) *U. guianensis* leaves (25 mg mL^{-1}) and (E) *U. guianensis* barks (25 mg mL^{-1}); silica gel, mobile phase EtOAc/HCOOH/HOAc/H2O 100:11:11:27. The left plate under UV at 254 nm and the right plate with NP/PEG-UV at 365 nm. Digital photo.

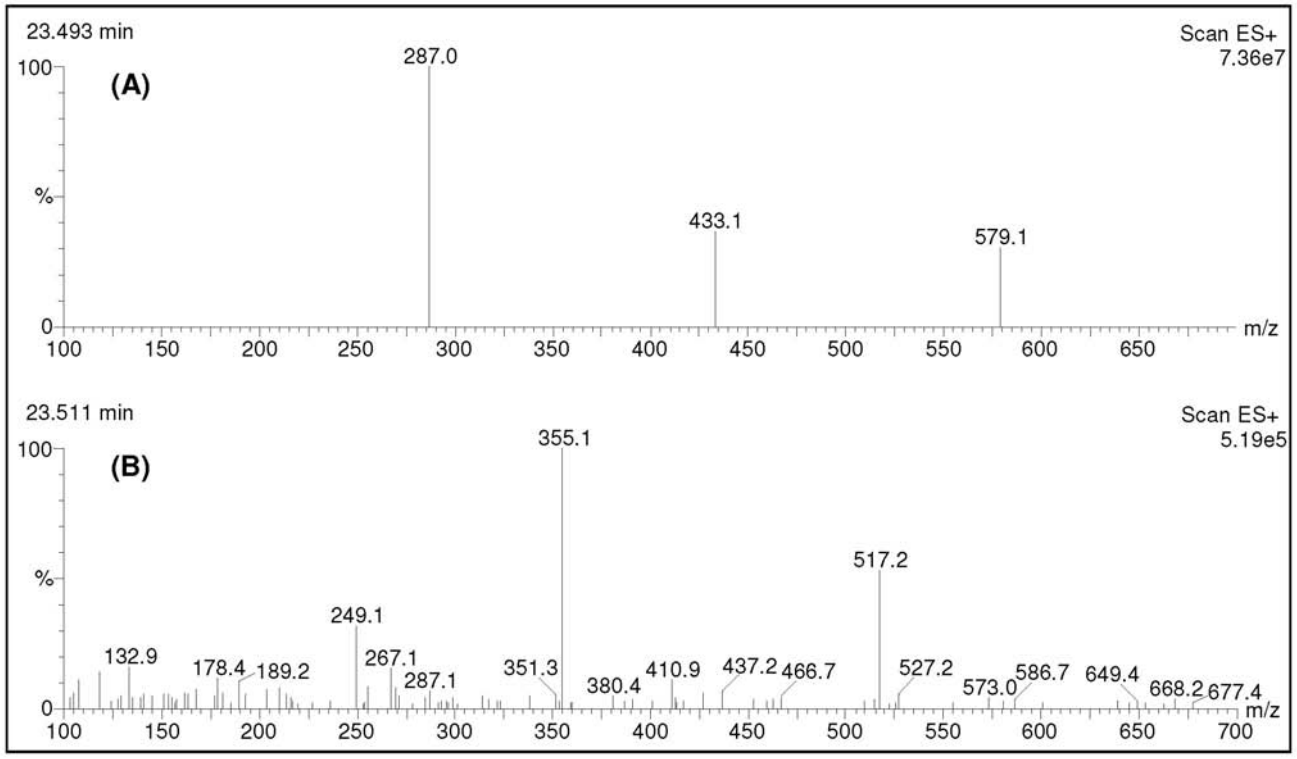

Figure S6. MS of the isolated kaempferitrin at retention time = 23.493 min (A) and MS at the same retention time in leaves of *Uncaria tomentosa* (R_T = 23.511 min) (B).