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An Unprecedented High Content of the Bioactive Flavone Tricin in *Huperzia* Medicinal Species Used by the Saraguro in Ecuador

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The flavone tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) is considered to be a selective potent inhibitor of different cancer cell lines and a potential colorectal cancer chemopreventive agent. In this paper we describe a reliable UHPLC-UV-ESIMS method for the determination of tricin in *Huperzia* plants used in the traditional medicine of the Saraguro community living in Southern Ecuador. An unusually high amount of tricin was found in *H. brevifolia* and *H. compacta*, which exceeded the content of this flavone determined so far in other plants.

Keywords: Ecuador, Medicinal plants, *Huperzia*, Flavonoids, Tricin, UHPLC-UV-ESIMS.

Several studies have evidenced that the naturally occurring flavone tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) is one of the most bioactive flavonoids [1]. Its broad spectrum of biological activities includes antioxidant, antiradical [2,3], anti-inflammatory, antiviral [4], antihistaminic [5], and cancer cell inhibitory [6,7] properties. More important, *in-vivo* studies have showed that dietary tricin supplementation inhibits inflammation-related mouse colon carcinogenesis, suggesting a potential use of this compound for clinical trials of colorectal cancer chemoprevention and for the treatment of human intestinal polyps [8,9]. Tricin was first isolated from a rust-resistant variety of wheat leaves (*Triticum dicoccum* L. var. Khapli) [10a] and, later, in cereal grains, rice, barley, oats and maize, in the husks of winter wheat [6a], and in some other plants [1, 10b]. However, the rather low concentration of tricin in different natural sources [6a, 11], in addition to the high production cost, has prejudiced its use in preclinical studies and for commercial use. There is, therefore, a demand for new, rich sources of this flavone.

In this paper we report a robust analytical UHPLC-UV-MS method for the characterization and quantification of tricin in the extracts of *H. compacta* (Hook.) Trevisan, *H. crassa* (Humb. & Bonpl. ex Willd.) Rothm., *H. espinosana* B. Øllg., *H. brevifolia* (Grev. & Hook.) Holub, and *H. kuestery* B. Øll. These plants are used by the Saraguro healers in Ecuador to prepare psychoactive ritual preparations and intestinal purgative remedies. No quantitative determination has so far been carried out on extracts of *Huperzia* spp. The aerial parts of each plant were exhaustively extracted with aqueous MeOH; then, after removing alkaloids by exposure to 2% aqueous H_2SO_4, the total flavonoid mixture (TFF) was isolated through separation on a short C-18 SPE cartridge, followed by preparative TLC (see Experimental). TFF was analysed by UHPLC-UV-ESIMS and tricin was identified in the chromatogram by its UV absorption band, the $[M-H]^-$ ion at *m/z* 329 in the negative ESI-MS, and the coelution with an authentic sample; it was quantified by integration of the area under the peak and interpolation from a calibration curve, showing complete linearity in the concentration range of interest.

H. brevifolia and *H. compacta* showed the highest contents of flavonoids and also the highest relative amount of tricin based on the dried plant; these values were quite low in *H. crassa* (Table 1). The amounts of tricin found in dried *H. brevifolia* and *H. compacta* largely exceeded those reported so far for other natural sources [6a, 11]. The high production of tricin in Ecuadorian *Huperzia* could be explained in terms of environmental factors. These plants grow at high altitudes, near the equatorial line, being thus exposed to high quantities of plant tissue damaging UV rays; therefore, only the plants that have managed to produce high amounts of protective compounds, such as flavonoids, are able to grow and reproduce.

Table 1: Weight (g) of flavonoid fractions and tricin from different *Huperzia* plants.

Dried plant (g)	NAF[a]	EFF[b]	TFF[c]	Tricin[d]
H. kuestery (38.7)	4.7	0.79	0.24 (0.61)	0.11 (46; 0.28)
H. brevifolia (21.9)	5.9	2.32	1.04 (4.76)	0.60 (58; 2.77)
H. espinosana (48.1)	4.6	1.30	0.65 (1.35)	0.19 (29; 0.39)
H. crassa (200.0)	15.6	2.65	1.72 (0.86)	0.04 (2.3; 0.02)
H. compacta (200.0)	18.1	12.51	6.88 (3.44)	1.14 (17; 0.57)

[a]NAF: non-alkaloid fraction (g); [b]EFF: estimated total enriched flavonoid fraction from SPE of NAF (g); [c]TFF: estimated total flavonoid fraction from TLC (% on dried plant); [d]estimated total amount (g) of tricin (% on TFF and on dried plant, estimated by UPLC-UV-MS analysis).

Experimental

General procedures: TLC analysis was performed on Merck RP-18 F254 glass backed plates (250 µm thickness). Spots were detected under UV light (254 and 366 nm) and, additionally, by staining with a 0.5% solution of vanillin in H_2SO_4-EtOH (4:1), followed by gentle heating at 100ºC. For preparative chromatographic separations SUPELCO DSC-18 SPE cartridges (60 mL/10 g) were used. UHPLC analyses were carried on a JASCO-X-LC-3185PU instrument, connected to a PDA UV X-LC 3110MD detector set at 350 nm and a Thermo Scientific LTQ XL HESI spectrometer. Column: Agilent Poroshell 120 EC-C18 (2.7 µm). MeOH and H_2O (Optima LC/MS, Fisher Chemical) as solvents for UHPLC analysis. NMR spectra were recorded on an NMR Brucker CXP 200 MHz.

Plant material: Aerial parts of *H. compacta*, *H. espinosana*, *H. brevifolia*, and *H. kuestery* were collected in July and December 2009 in the Andean highlands (*Paramo*) at Sunin (17694877E, 594739N; 3128m a.s.l.), while *H. crassa* was collected at Inguera and Aguarongo (17700781E, 9586086N, 3478m a.s.l.), where the

Saraguro healers practice their own collections. The plants were identified by Bolivar Merino, curator of the Universidad Nacional de Loja Herbarium. Voucher specimens (PPNIc-02 for *H. co*.; Ly-HK-001 for *H. k*.; PPNIc-08 for *H. e*.; PPNIc-10 for *H. b*.; PPNIc-05 for *H. cr*.) have been deposited at the Herbarium of the Universidad Tecnica Particular de Loja (UTPL).

Isolation of the flavonoid fraction (FF) for UHPLC-UV-ESIMS analysis: Air-dried (35°C) and milled aerial parts of each plant were repeatedly extracted at room temperature with a mixture of MeOH-H_2O, 90:10, until a negative response to Dragendorff's reagent. By concentration under vacuum of the combined extracts to about half volume, a precipitate was formed that was removed. The remaining dense aqueous solution (A) was then acidified with 2% aqueous H_2SO_4 and filtered to give 2 fractions: an acidic aqueous solution (B) and a solid precipitate (C), free of alkaloids (NAF, non alkaloid fraction), that was recovered by filtration on a sintered glass Buchner filter. Solution B was washed with *n*-hexane; subsequently, the separated aqueous phase was alkalized by addition of aqueous NH_3 and the solution was extracted with $CHCl_3$ to recover the alkaloid fraction, which was submitted to another investigation. After adding a few drops of aqueous NH_3 to neutrality, a sample (1 g) of each NAF was taken in MeOH-H_2O, 4:1, and a little insoluble material was removed by centrifugation; subsequently, the supernatant was separated on a short reversed phase SPE cartridge. Three fractions were obtained: fraction 1, enriched in flavonoids and sugars (EFF), eluted with 100 mL of MeOH-H_2O, 4:1; fraction 2, consisting of triterpenes and chlorophyll, eluted with 100 mL of MeOH; and fraction 3, consisting of chlorophyll and lipids, eluted with acetone (100 mL). Subsequently, a sample (20 mg) of each EFF, dissolved in MeOH, was separated on an analytical RP-18 TLC plate. After elution with MeOH-H_2O, 4:1, the band of the stationary phase containing flavonoids, revealed by the characteristic fluorescence under UV light, was removed from the glass support, and the total flavonoid fraction (TFF) was recovered by elution with MeOH and analyzed by UHPLC-UV-ESIMS. In a separate experiment a sample (4 mg) of tricin was chromatographed on a TLC plate under the same conditions. Recovery of the flavonoid was >95%.

UHPLC-UV-ESIMS analysis of total flavonoid fractions (TFF): Quantification of tricin in* Huperzia *plants. A sample (5 μL) of a TFF solution of tricin in MeOH (at the concentration of 1 mg/mL) was analysed by UHPLC-UV-ESIMS according to a gradient of H_2O-MeOH from 90:10 to 50:50 over 2 min, then to 30:70 over 18 min, then to 0:100 over 5 min, then back to the initial mixture over 10 min. Tricin was a well resolved peak with a retention time of 15.55 min. To estimate the concentration of tricin in each FF and then on a dried sample of each *Huperzia* species (Table 1) a calibration line was plotted using tricin at different concentrations. A stock solution (300 mg/mL MeOH) was prepared, using a pure sample of tricin, identical with the literature [11b, 12], obtained by repetitive recrystallization of the TFF from *H. brevifolia*. Then, standard solutions of 5, 10, 50, 100, 500 and 1000 ppm tricin were prepared by dilution of the stock solution with MeOH. Each solution was analyzed 3 times by UPLC in order to determine the accuracy and repeatability of the method (inter-assay precision). The limit of detection (LD) and limit of quantification (LQ) of tricin were 18.853 ppm and 57.129 ppm, respectively, with the correlation coefficient $R^2 = 0.9998$. In the range analyzed, the sensitivity of the method corresponds to the slope of the calibration line y = 3386.51x-18842. The relative standard deviations for the inter-day and intra-day assays were < 5% The % tricin reported in Table 1 is the average of 3 measurements.

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