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Avances en Química, 8(3), 145-151 (2013)
Artículo científico

Isolation and characterization of (+)-mellein, the first isocoumarin reported in *Stevia* genus

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Recibido: 06/08/2013 **Revisado**: 28/11/2013 **Aceptado**: 18/12/2013

Resumen

Del extracto acetónico obtenido de las hojas y ramas de la *Stevia lucida* Lagasca fueron aislados los derivados fenólicos: (+)-meleina **[1]**, hispidulina **[2]**, pectolinaringenina **[3]** e isosakuranetina **[4]**. Estos compuestos se caracterizaron sobre la base de estudios espectroscópicos, incluyendo experimentos de RMN uni- y bi-dimensionales. La revisión de la literatura indica que las isocumarinas son compuestos poco frecuentes en las plantas superiores. Esto da relevancia a su descubrimiento en el género *Stevia*

Palabras clave: *Stevia*, isocumarinas, flavonoides, (+)-meleina, hispidulina, pectolinaringenina, isosakuranetina

Abstract

From the acetone extract obtained of leaves and stems of *Stevia lucida* Lagasca were isolated the following phenolic derivatives: (+)-mellein **[1]**, hispidulin **[2]**, pectolinaringenin **[3]** and isosakuranetin **[4]**. These compounds were characterized on the basis of spectroscopic studies, including 1D- and 2D-NMR experiments. The literature review indicated that isocoumarins are rather rare compounds in higher plants. This gives importance to their discovery in the *Stevia* genus.

Keywords: *Stevia*, isocoumarins, flavonoids, (+)-mellein, hispidulin, pectolinaringenin, isosakuranetin

Introduction

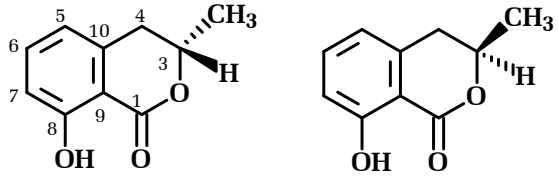
The genus *Stevia* (family Asteraceae, tribe Eupatoriae) has approximately 230 species. Its geographic distribution range extends from the southwestern of United States to central Argentina, through Central America, South American Andes and the highlands of Brazil[1]. Though taxonomically *Stevia* is one of the most distinctive genera in Asteraceae, its chemistry is not very uniform; most species contain sesquiterpene lactones, longipinene derivatives, diterpenes and a wide variety of aromatic compounds like chromanes, benzofuranes and flavonoids[2,3]. In the present work, we report the isolation and identification of an isocoumarin characterized as (+)-mellein **[1]** and three flavonoids from leaves and stems of *Stevia lucida* Lagasca.

The isocoumarins are aromatic compounds mainly found in fungi from genera such as *Aspergillus*, *Ceratocystis*, *Cladosporium*, *Fusarium*, *Penicillium* and many other ones[4-6]; this compounds occur, in a more limited extension in other natural sources including bacteria[7,8], lichens[9], liverworts[10], higher plants[11-16], insects[17,18] and marine sponges[19]. Isocoumarins and 3, 4-dihydroisocoumarins have shown to possess a broad spectrum of biological and pharmacological properties such as serine protease inhibitors[20], anti-oxidative qualities[16], hepatoprotective effects[13] and anti-inflammatory[11], antiplasmodial[7], antifungal[14], antimicrobial[8,15], antiangiogenic[21] and antitumoral activities[8,22,23], among any others[4,6].

The best-known 3,4-dihydroisocoumarin is mellein, a metabolite originally isolated in 1933 from the fungus *Aspergillus melleus*[24]. Several years later, Blair and Newbold[25] determined its structure and Arakawa *et al.*[26] established its absolute configuration which shown to be 8-hydroxy-3(*R*)methyl-3,4-dihydroisocoumarin; since this metabolite is denominated (-)-(*R*)-mellein, although, in the past, it was also called ochracin (Fig. 1). Its enantiomer, (+)-(*S*)-mellein is also known as a natural product [27].

(-)-Mellein is a common metabolite in fungi and molds[4-6] and particularly in endophytic fungi associated with higher plants[28,29]. Occasionally, it has been isolated from some higher plants such as *Ficus formosana* (Moraceae)[30] and *Garcinia bancana* (Clusiaceae)[31] and also from marine

organisms[32]. On the other hand, it is particularly notable the presence of this compound in insects in which acts as a pheromone[33] and as a defense substance[34]. (+)-Mellein has been isolated from various fungi[4,6], but to the best of our knowledge, to date it has not been reported in higher plants.



(+)-mellein **(-)-mellein**

Fig. 1 Chemical structures of (+)-mellein and (-)-mellein

The chemical and biological interest of these compounds is clearly evident in the numerous syntheses described in the literature, which have been addressed by several research groups. The first reported synthesis led to the racemic mixture (±)-mellein[35,36]; however, more recently have been described stereoselective synthesis, in which are developed various ingenious routes for preparation of both enantiomers[37-40]. (-)-Mellein has been recognized for its antibacterial, phytotoxic, larvicide and fungicide activities[41-43], and also because it acts as an inhibitor of HCV protease enzyme[44] and prostaglandin synthesis[45]. (+)-Mellein is a potent phytotoxic[46] and neurotoxic[47]; in addiction its insecticidal activity against *Calliphora erytrocephala* is remarkably effective[48].

Materials and Methods

General

Melting points were determined with a Fisher-Johns instrument and they have not been corrected. Optical activity was measured in $CHCl_3$ on 60 Hz-Steeg & Reuter G.m.b.H. polarimeter. UV spectra were obtained in a Perkin-Elmer spectrophotometer, Lambda 3B, using quartz cells with 1 cm thick and methanol (Merck-Uvasol) as solvent. IR spectra were recorded on a Perkin-Elmer FT-1725X spectrophotometer as KBr pellets. ^{1}H-, ^{13}C- and two-dimensional NMR spectra were acquired with a Bruker-Avance DRX-400 instrument, using $CDCl_3$ or DMSO-d_6 as solvents. Mass spectra were recorded on a Hewlett-Packard Mass Spectrometer, model 5930A (70 eV). TLC were developed on 0.25 mm layers of silica gel PF 254 (Merck) and spots were visualized by spraying with a mixture *v*/*v* CH_3COOH-H_2O-H_2SO_4 (20:4:1) and then heating with air flow at 100 °C for few minutes. VCC was performated with silica gel Merck 60 (63-200 μm, 70-230 mesh). Size-exclusion chromatography columns were packed with Sigma Sephadex LH-20.

Plant material

Plant material (leaves and stems) was collected at "Páramo de la Negra, Municipio Rivas Dávila, Estado Mérida, Venezuela". Species was identified as *Stevia lucida* Lagasca by Eng. Juan A. Carmona Arzola, Department of Pharmacognosy and Organic Medicaments, Faculty of Pharmacy and Bioanalysis, University of Los Andes; a voucher specimen (J. M. Amaro-Luis & P. Chacón, N° 2332) was deposited at the Herbario MERF of this faculty.

Extraction

Dry not crushed leaves and stems (≅ 4.0 Kg) were exhaustively extracted with ethanol in a sohxlet. The solution obtained was filtered and concentrated *in vacuum* on a rotary evaporator, to afford a crude extract (970 g), which was preadsorbed on silica gel and extracted successively with petroleum ether, acetone and methanol, exhaustively in each case. Acetone-solution was concentrated under reduced pressure to dryness and a brown residue (≅ 270 g) was obtained.

Isolation and identification of the constituents

The acetone extract was preadsorbed on silica gel and chromatographed (VLC) over silica gel 60, eluting with hexane and EtOAc in mixtures of increasing polarity. Fractions of 500 mL were collected and combined according to the TLC characteristics to afford twelve major fractions (A-L). Combined fractions C [12-14, eluted with hexane-EtOAc (4:1)], E [21-24, eluted with hexane-EtOAc (7:3)] and H [37-48, eluted with hexane-EtOAc (1:1)] were purified by repeated flash chromatography, size-exclusion chromatography on Sephadex LH-20 or preparative TLC to furnish compounds **[1]** [(+)-mellein], **[2]** (hispidulin), **[3]** (pectolinaringenin) and **[4]** (isosakuranetin) [Fig. 6].

(+)-Mellein **[1]**: Purification of major fraction C, which was carried out on preparative TLC plates eluted with mixtures hexane-CH_2Cl_2 (4:1), provided a resinous pale pink solid (≅ 10 mg), m.p. = 52-54 °C; $[\alpha]_D$ = + 92° (c, 1.14, MeOH). UV (CH_3OH), λ_{max} (nm).: 243, 311. IR (KBr), $\nu_{max.}$ (cm^{-1}): 3355 (-OH), 3060 (=C-H), 1676 (C=O), 1619 (C=C), 760 (=C-H). ^{1}H NMR (Table 1). ^{13}C NMR (Table 1). HR-MS: *m/z* 178.0651 [M^+].

Hispidulin **[2]**: From combined fractions 21-24 [E] precipitated a yellow solid (≅ 15 mg), which was purified by filtration over Sephadex LH-20; its chromatographic behavior was typical of a flavonoid. m.p. = 285-287 °C (decomposition). UV, $\lambda_{max.}$ (nm) : (CH_3OH) 272, 333; (NaOMe) 274, 321*sh*, 387; ($AlCl_3$) 298, 352 ; ($AlCl_3$/HCl) 298, 350. IR, $\nu_{max.}$ (cm^{-1}): 3338 (OH), 1652 (C=O), 1610 (C=C), 1251 and 1179 (C-O). ^{1}H NMR (Table 1).

^{13}C NMR (Table 1). EI-MS, [*m/z*, (% rel. int.)]: 300 (68.96) [M^+], 285 (53.50), 282 (38.81), 257 (47.46), 254 (9.37).

Pectolinaringenin **[3]**: This compound precipitated as an impure yellow solid from combined fractions 37-48 [H]; purification was achieved by preparative thin layer chromatography, eluted with hexane-EtOAc (4:1) (developed 2x); crystallization from mixtures EtOAc/hexane provided pure yellow needles (≅ 120 mg); m.p. = 211-213 °C. UV, $\lambda_{max.}$: (CH_3OH) 214, 274, 330; (NaOMe) 274, 367; ($AlCl_3$) 298, 352 ; ($AlCl_3$/HCl) 297, 348. IR, $\nu_{max.}$ (cm-^{1}): 3330 (OH), 1661 (C=O), 1609 (C=C), 1382 and 1186 (C-O). ^{1}H NMR (Table 1). ^{13}C NMR (Table 1).

Isosakuranetin **[4]**: Liquid recovered after filtration of major fraction H (37-48) was concentrated to dryness and the residue subjected to dry silica gel column chromatography yielding a pale yellow solid; purification on Sephadex LH-20 column and subsequent crystallization in methanol gave yellow flakes; m.p. = 194-196 °C. UV, $\lambda_{max.}$: (CH_3OH) 248, 276, 309. ^{1}H NMR (Table 1). ^{13}C NMR (Table 1).

Results and Discussion

High resolution EI-MS in conjunction with analysis of ^{1}H-NMR and ^{13}C-NMR spectral data (Table 1) of **[1]** allowed to establish the molecular formula $C_{10}H_{10}O_3$. Analysis of its ^{1}H-NMR spectrum indicated that in the molecule exists a 1,2,3-trisubstituted benzene ring, confirmed by the presence of signals for three aromatic protons that make up a typical ABX system; this couple pattern is particularly detectable in the ^{1}H,^{1}H-COSY spectrum (Fig. 2) {double doublet [δ_H: 7.40 (J ≅ 8.4 and 7.6 Hz) (H-6)] which it is coupled to a doublet at δ_H: 6.89 (J ≅ 8.4 Hz) (H-5) and to other double doublet at δ_H: 6.69 (J ≅ 7.6 and 1.2 Hz) (H-7)}.

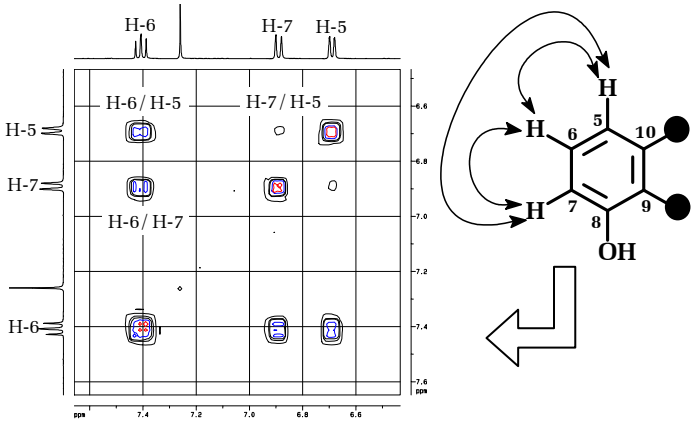


Fig. 2 ^{1}H,^{1}H-COSY spectrum of aromatic ABX coupled system.

Through HSQC spectrum (Fig. 3) was possible to locate the signals of those carbons that support these three aromatic protons [δ_C: 118.0 (C-5); δ_C: 136.2 (C-6) and δ_C: 116.4 (C-7)].

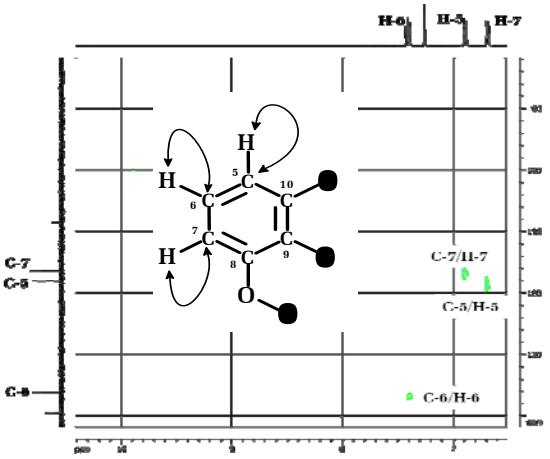


Fig. 3 HSQC spectrum (ABX aromatic coupled system region)

The ^{1}H NMR spectrum also shows a sextet (J ≅ 7.2 Hz) at δ_H: 4.73, assigned to an aliphatic oxymethine hydrogen (H-3) coupled to the protons of an adjacent methylene [δ_H: 2.93, *d* (J ≅ 7.2 Hz) (H-4)] and a secondary methyl [δ_H: 1.53, *d* (J ≅ 7.2 Hz) (H-11)]. These data let propose other fragment of the molecule (Fig. 4) consisting of three sp^3 carbons, whose NMR signals [δ_C: 76.2 (>CH-O-; C-3), δ_C: 34.7 (-CH_2-; C-4) and δ_C: 20.9 (-CH_3; C-11)] were unambiguously assigned through their HSQC correlations.

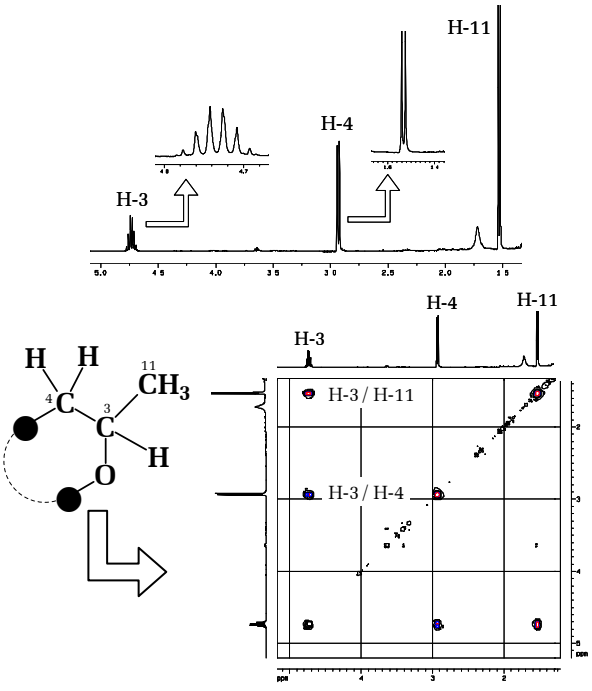


Fig.4 ^{1}H NMR and ^{1}H,^{1}H-COSY spectra in aliphatic oxymethine hydrogen region

Table1. Chemical shifts in ^{1}H and ^{13}C-NMR spectra of compound [**1**]-[**4**]

Position	δ_C for ^{13}C NMR (100 MHz) spectra				δ_H (*multiplicity*, J in Hz) for ^{1}H NMR (400 MHz) spectra			
	[1]	**[2]**	**[3]**	**[4]**	**[1]**	**[2]**	**[3]**	**[4]**
1	170.0	-	-	-	-	-	-	-
2	-	163.8	163.3	79.8	-	-	-	5.49 (*dd*, 13.0,3.0)
3	76.2	102.3	103.0	43.5	4.73 (*sx*, 7.2)	6.78 (*s*)	6.96 (*s*)	3α (*dd*, 17.0, 13.0) 3β (*dd*, 17.0, 3.0)
4	34.7	182.1	182.1	197.1	2.93 (*d*, 7.2)	-	-	-
5	118.0	152.7	152.7	165.3	6.89 (*d*, 8.4)	-	-	-
6	136.2	131.3	131.4	96.9	7.40 (*dd*, 8.4, 7.6)	-	-	5.97 (*d*, ≅ 1)
7	116.4	157.3	157.3	167.4	6.69 (*dd*, 7.6, 1.2)	-	-	-
8	162.3	94.2	94.3	95.9	-	6.59 (*s*)	6.71 (*s*)	5.97 (*d*, ≅ 1)
9	108.4	152.4	152.4	164.3	-	-	-	-
10	139.5	104.0	104.1	103.9	-	-	-	-
11	20.9	-	-	-	1.53 (*d*, 7.2)	-	-	-
1’	-	121.2	122.8	131.9	-	-	-	-
2’	-	128.4	128.3	114.8	-	7.93 (*d*, 12.5)	8.12 (*d*, 10.0)	6.99 (*d*, 8.0)
3’	-	115.9	114.5	128.9	-	6.92 (*d*, 12.5)	7.19 (*d*, 10.0)	7.48 (*d*, 8.0)
4’	-	161.1	162.3	161.0	-	-	-	-
5’	-	115.9	114.5	128.9	-	6.92 (*d*, 12.5)	7.19 (*d*, 10.0)	7.48 (*d*, 8.0)
6’	-	128.4	128.3	114.8	-	7.93 (*d*, 12.5)	8.12 (*d*, 10.0)	6.99 (*d*, 8.0)
-OCH_3 (6)	-	59.9	55.5	-	-	3.74 (s)	3.85 (*s*)	-
-OCH_3 (4’)	-	-	59.9	55.6	-	-	3.95 (*s*)	3.82 (*s*)
-OH (5)	-	-	-	-	-	13.08 (*s*)	13.13 (*s*)	-
-OH (8)	-	-	-	-	11.03 (*s*)	-	-	-

Solvent used: **[1] (**$CDCl_3$); **[2] (**DMSO-d_6); **[3] (**DMSO-d_6); **[4] (**$CDCl_3$)

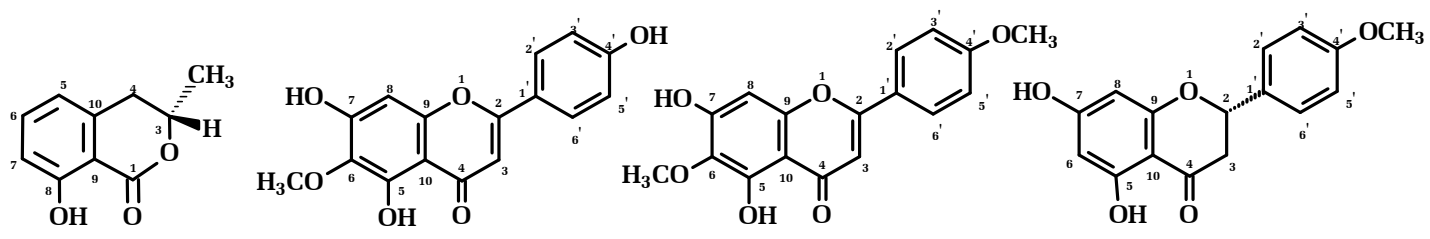


Fig. 6 Structures of compounds **[1]** [(+)-mellein], **[2]** (hispidulin), **[3]** (pectolinaringenin) and **[4]** (isosakuranetin)

The peak to lower field in the ^{13}C NMR spectrum was assigned to a carbonyl group and its chemical shift, δ_C: 170.0, is consistent with a carbonyl ester group (C-1) and not with a ketone; consequently this carbonyl must be bonded to oxygen in C-4. On the other hand, in the benzene nucleus one of the substituents is a hydroxyl group and the chemical shift of its hydrogen [δ_H: 11.03, *s* (-O<u>H</u>),] is consistent with a phenolic hydroxyl proton chelated by a carbonyl group. This permit to conclude that the carbonyl must also be bonded to benzene nucleus and integrated to a second ring, conforming a δ-lactone. The detection in the $^1H,^1H$-COSY spectrum of a long range correlation between H-4 and the aromatic proton H-5, confirm that the methylene group (C-4) is bound to carbon C-10, adjacent to C-5 (Fig. 5). Consequently, the preceding analysis indicates that structure of compound **[1]** corresponds to the 8-hydroxy-3-methyl-3,4-dihydro-1*H*-2-benzopyran-1-one; this structure is assigned in the scientific literature to mellein[25] and since the isolated compound is dextrorotatory, the configuration in C-3 is *S*, corresponding to (+)-mellein[27].

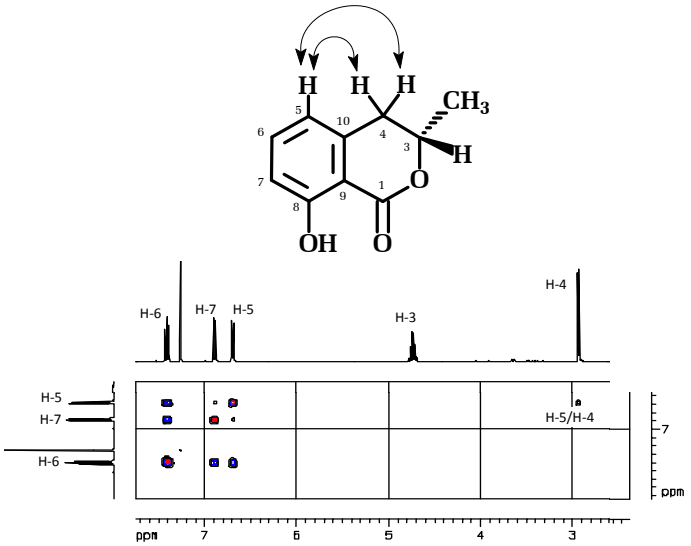


Fig.5 $^1H,^1H$-COSY spectrum in aromatic and lactonic proton region

Analysis of the ^1H-NMR and ^{13}C-NMR spectra of **[2]** (Table 1) allowed establish the number of hydrogens and carbons in the molecule, and also the degree of hybridization and the type of substitution of each carbon. These data and detection in its EI-MS of a molecular ion at *m/z*: 300 [M^+] made it possible to establish the molecular formula $C_{16}H_{12}O_6$. The presence in its IR spectrum of an absorption assignable to a cyclohexenone α,β-unsaturated ($\nu_{max.}$: 1652 $cm.^{-1}$) and observation in its UV spectrum of bands at $\lambda_{max.}$: 272 and 333 nm, confirm that the compound under study is a flavone[49]. Correlations detected in the HMBC spectrum allowed to conclude that this compound is the 5, 7, 4'-trihydroxy-6-methoxyflavone, known as hispidulin **[2]**. Its spectral data are consistent with those previously reported in the literature[50].

For a similar analysis to that performed above for hispidulin of ^1H-NMR and ^{13}C-NMR spectral data of **[3]** (Table 1), it was possible to determine its molecular formula as $C_{17}H_{14}O_6$. A subsequent detailed study of its HMBC spectrum allowed to identify with the 5,7-dihydroxy-6, 4'-dimethoxyflavone, which it is also known under the common name of pectolinaringenin **[3].** Its ^1H and ^{13}C NMR data are in good agreement with literature values[51].

Analysis of spectral data of compound **[4]** (Fig. 7) allowed to identify as a trisubstituted flavanone. In effect, its UV data (bands at $\lambda_{max.}$: 309 and 344 nm) are typical of flavanones [49] and, similarly, its ^1H and ^{13}C NMR data (Table 1) also are congruent for a flavanone [49], particularly those corresponding to carbons C-2 [δ_C: 79.8 (>C<u>H</u>-O-)] and C-3 [δ_C: 43.5 (-<u>C</u>H_2-C=O)] and the typical AMX coupled spin system of their respective hydrogens [δ_H: 5.49, *dd* (J ≅ 13.0 and 3.0 Hz) (H-2β); δ_H : 3.16, *dd* (J ≅ 13.0 and 17.0 Hz) (H-3α) and δ_H: 2.75, *dd* (J ≅ 3.0 and 17.0 Hz) (H-3β)]. A detailed study of the 1D and 2D-NMR spectra also evidenced the presence in the molecule of a 5,7-dihydroxy-substituted A-ring and a 4'-methoxy-substituted B-rig (Table I), with which it was possible to conclude that **[4]** is the 5,7-dihydroxy-4'-methoxy-flavanone, widely known by the common name of isosakuranetin **[4].** Comparison of the NMR data with those described in the literature [52], confirmed the identity of this flavanone.

Conclusions

In this paper it is reported for the first time the presence of an isocoumarin in the genus *Stevia*, which it was identified as (+)-mellein **[1]**. This result may give rise to many interpretations, taking into account that this metabolite is rare in higher plants, but is very common in endophytic fungi and molds.

The three flavonoids isolated in this study are common in the Asteraceae family, but this is the first report of isosakuranetin for the genus *Stevia* and hispidulin **[2]** in *Stevia lucida*

Acknowledgments

The authors are grateful to CDCHTA-ULA and to Venezuelan Ministry of Popular Power for Science, Technology and Innovation (MCTI), "Science Mission Program" (Grant Nº 2008000937), for financial support. Thank are also due to Eng. Juan A. Carmona Arzola, Department of Pharmacognosy and Organic Medicaments, Faculty of Pharmacy and Bioanalysis, University of Los Andes (ULA) for identification of plant material.

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