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NMR spectroscopy and antioxidant activity of flavanones and flavones isolated from *Chromolaena tacotana* (Klatt) R.M. King & H. Rob.

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Abstract

Chromolaena tacotana is considered as a source of flavonoids. Here we examined the content and antioxidant properties of flavones and flavanones from the leaves of the plant. Four flavonoids, including (Cta) 5, 4' dihydroxy-7-methoxy flavanone, (Ctb) 3,5,3'-trihydroxy-7,4'-dimethoxyflavone; (Ctc), 3,4'-dihydroxy -5,7- dimethoxyflavanone; and (Ctd) 4'-hydroxy-5,7-dimethoxyflavanone, were isolated from leaves extracts, were identified by their NMR spectroscopic data, and then free radical scavenging activities of the flavonoids were assessed against DPPH. The antioxidant activity for the flavanone Ctb was the highest even compared to that of quercetin, with IC50 of 6.27 µg/mL and 8.67 µg/mL respectively. The flavanones Cta, Ctc and Ctd presented a lowest activity against free radicals as expected according to their molecular substituents and the position within the structure. Data obtained from this study support the ethnomedicinal use of the leaves of *C. tacotana* for an antioxidant purpose.

Key words: *Chromolaena tacotana*, flavanones, flavones, Free radical scavenging activity.

Introduction

Flavonoids are a large group of natural polyphenolic compounds with biological properties that are structure dependent [1,2]. These compounds are well known as active ingredients in multiple plant sources, both food and medicinal for their beneficial effects on human health [3].

Several flavonoids are antioxidants, it depends upon the configuration, substitution, total number of hydroxyl groups between others that influence radical scavenging and metal ion chelation ability [1].

Chromolaena genus has been considered as a source of flavonoids with potential medicinal in prevention and treatment of chronic diseases associated with oxidative stress [4–7], however, the specific medicinal properties of *Chromolaena tacotana*, commonly called "sanalotodo" are not completely studied. *Ch. tacotana* is a species recognized by the content of flavonoids as 3,5,4'-tryhidroxy -7- methoxy flavone, 3,5,8- trihydroxy -7,4 '-dimethoxy flavone, 5,4'- dihydroxy -7-methoxyflavanonol, and 5,7,3 ', 4' - tetrahydroxy- 3-methoxyflavone, this latter flavonoid with the best response in antioxidant activity with an IC50 of 2.51 mg/L by DPPH and 2.13 mg/L by ABTS assay, and all of them with cytotoxic activity against breast cancer MDA-MB-231 cells [5,8].

The aim of the present study is to continue investigating aerial parts for *C. tacotana*, to isolate and identify flavonoids not reported before and to examine their antioxidant activity.

Methods

Plant material and flavonoids isolation: The *C. tacotana* plant was collected from Villa de Leyva, Boyacá, Colombia and taxonomy identification was performed by National Herbarium as COL595376.

The chemicals used for isolation were Merck's analytical reagents. 794 g of dried and ground leaves were subjected to Soxhlet extraction with dichloromethane (DC) CH_2Cl_2 to remove the content of fats and chlorophylls, next 134 g from that total extract named (DC-EI) was flocculated with methanol (MeOH): water (1:1), and after, the aqueous portion was extracted with CH_2Cl_2 and concentrated in vacuum. This second dichloromethane extract was named (DC-EII) and it was used to obtain the flavonoids. 20 g from DC-EII were separated by column chromatography with Silica gel (40-60 µm) and RP18 (20-40 µm), the flavonoids were isolated using a mixture of $CHCl_3$: MeOH in a ratio of 9.8: 0.2 for Cta and Ctb and crystallization was performed with nHexane. The compounds Ctc y Ctd were isolated by RP18 chromatography with MeOH: H_2O (7:3) and with MeOH respectively, and their crystallization were carried out with MeOH.

Identification (see table 1) was carried out by means of UV (nm) spectra taken on a Jenway 6405 UV-VIS spectrophotometer with displacement reagents (AcONa, MeONa and H_3BO_3). Mono and two-dimensional 1H NMR and 13C NMR spectra taken on a Bruker 300MHz spectrophotometer.

Antioxidant activity: The antioxidant activity of the flavonoids was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as previously described [9] with some modifications. The absorbance was measured at 515 nm in a Thermo Scientific 4001/4 spectrophotometer after 10 min of reaction between the samples Cta-Ctd or DC-EII and the 0.8 mM DPPH methanolic solution (1:5), all of them prepared to different dilutions in series in a range of 5 to 500 µg/mL. The mixture 80% methanol: DPPH methanolic solution was used as the control. The test was performed by triplicate. The free radical scavenging effect was established for each dilution and the IC50 values for substances were calculated by using the corresponding linear regression equations.

Results and Discussion

Flavonoids Isolated from the Leaves of C. tacotana: Four uncommon flavonoids were isolated from the leaves of C. tacotana and described as follow:

5,4' -dihydroxy –7 -methoxy flavanone (Cta): White solid, eluted in the fractions with $CHCl_3$: MeOH 9.8: 0.2, Rf 0.57; crystallized from nHexane, Mp 145 °C, soluble in $CHCl_3$. UV nm in MeOH: 285; plus AcONa: 285 confirms OH substituted at C7 plus MeONa 295, 365 and plus $AlCl_3$ confirms OH free in C5. From 1H NMR spectral data (see table 1), appears that the compound is a flavanone (signals at 2.36, 3.09 and 5.34 ppm) with a methoxyl group at C7 and one ring B with OH in para position.

13C JMOD NMR data match with these reported before for the compound 5,4' -dihydroxy –7 -methoxy flavanone [4].

3,5,3'-trihydroxy-7,4'-dimethoxyflavone (Ctb): Yellow powder slightly soluble in Me2CO, soluble in DMSO, melting point of 231 to 232 °C, Rf of 0.58 (silica gel, CHCl3: MeOH 9.5: 0.5) reveals yellow spot with NH3 vapours. The UV nm: MeOH 256; 376; plus MeONa 275; 437 (OH in C3, not in 4'); plus AcONa 256; 376 (not free OH in C7) plus HBO3 256; 374 (not two OH in ortho); plus AlCl3 270; 424 (OH in C5 and/or C3), plus AlCl3 plus HCl 270; 425 (confirms OH in C5 and/or C3). According 1H and 13C NMR spectral data (see table 1), the compound is a flavone with two methoxyl groups, one at C7 and one ring B with two carbon atoms oxygenated and confirm OH in C5. The spectroscopic data match with those reported in the literature for the compound with a molecular formula C17H14O7 termed as 3,5,3'-trihydroxy-7,4'-dimethoxyflavone, also known as Ombuin (Fig 1), [10–12].

3,4'-dihydroxy-5,7-dimethoxyflavanone (Ctc): White solid crystallized from MeOH, melting point 217 °C, soluble in acetone and DMSO. UV nm data in MeOH 290 (0.772); plus AcONa 290 (0.772) not free OH in C7; plus MeONa 290 (0.760), 360 (0.317); other UV spectra equal to the original with MeOH. 1H NMR (see table 1) and the 13C NMR (see table 1 and, supplementary table 1). These data indicate that the compound is a flavanone with OH free in C3 (signal at 72.90 ppm 13C NMR) two methoxy groups in ring A and ring B substituted in para (signals at 7.31 and 6.79 ppm in the 1H NMR) (Fig 2).

4'-hydroxy-5,7-dimethoxyflavanone (Ctd): Crystalline solids from MeOH, Mp 180-181 °C, Rf 0.38 (Si-gel in CHCl3: MeOH 9.8: 0.2), soluble in acetone UV nm: 283 (0,655), sh 315 (0,192): plus MeONa 285 (0,558); sh 320 (0,208), 400 (0,239) OH in ring B; plus AcONa, plus H3BO3, plus AlCl3 do not present changes, spectra equal to that of the compound with MeOH, indicates that there is no free OH in C5, C7 nor in ortho position in rings A and B. 1H NMR data (see table 1) analysis indicates that the compound is a flavanone (CH2 45.3, OCH 78.95 ppm) with two methoxy groups in ring A (55.17 and 55.25 ppm) and an OH in ring B in position para (6.92 and 7.41 ppm), which is confirmed with the study of the connectivity's shown in the 2D NMR spectra (Fig 3), whose summary data are shown in the supplementary table 2.

Free radical scavenging by DPPH. Between flavonoids evaluated, the flavone Ctb was the most antioxidant compound with the IC50 value of 6.15 µg/mL, even than quercetin used as a positive control (Fig 4) which is in accordance with the presence of OH in C3 of the flavonols [13,14]. The other flavanones presented the lowest inhibition of free radicals as expected (see table 2), according their structures (Fig 5).

Conclusion

In addition to the flavonoids already determined from leaves of *Chromolaena tacotana*, another four, but uncommon flavonoids (Cta) 5,4'- dihydroxy-7-methoxy flavanone, (Ctb) 3,5,3'-trihydroxy-7,4'-dimethoxyflavone; (Ctc), 3,4'-dihydroxy -5,7-dimethoxyflavanone; (Ctd) 4'-hydroxy-5,7-dimethoxyflavanone were now isolated and identified spectroscopically. The flavonoid Ctb showed the major antioxidant activity with IC50 6.27 µg/mL, better even than quercetin. The production of this large number of flavonoids and their structural variety advocate the species *Chromolaena tacotana* as a plant with good potential for studies of anticancer activity and support the ethnomedicinal use for protecting against cellular damage by oxidative stress.

Acknowledgments

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References

1. Kumar S, Pandey A, Chemistry and Biological Activities of Flavonoids: An Overview. Scientific World Journal. 162750, 2013. doi: 10.1155/2013/162750.
2. Thomas CM, Wood RC, Wyatt JE, Pendleton MH, Torrenegra RD, et al. Anti-Neoplastic Activity of Two Flavone Isomers Derived from Gnaphalium elegans and Achyrocline bogotensis. PLoS One. 7: e39806, 2012. doi: 10.1371/journal.pone.0039806.

3. Panche AN, Diwan AD, Chandra SR. Flavonoids: An overview. Journal of Nutritional Science. 5: e47, 2016. doi:10.1017/jns.2016.41.
4. Torrenegra RD and Rodríguez OE, Chemical and biological activity of leaf extracts of Chromolaena leivensis. Natural Products communications. 6: 947–50, 2011. doi :10.1177/1934578x1100600706.
5. Rodriguez J, Gómez LM, Gutierrez AC, Mendez-Callejas G, Reyes AI, Tellez LC, Rodriguez OE, Torrenegra RD. Chromolaena tacotana (Klatt) R.M. King & H. Rob. Source of Flavonoids with Antiproliferative and Antioxidant Activity. Indian Journal of Science and Technology. 11 1–7, 2018. doi:10.17485/ijst/2018/v11i19/122872.
6. Srinivasa KR, Chaudhury PK, Pradhan A. Evaluation of anti-oxidant activities and total phenolic content of Chromolaena odorata. Food and Chemical Toxicology. 48: 729–732, 2010. doi: 10.1016/j.fct.2009.12.005.
7. Phan TT, Hughes MA, Cherry GW. Effects of an aqueous extract from the leaves of Chromolaena odorata (Eupolin) on the proliferation of human keratinocytes and on their migration in an in vitro model of reepithelialization. Wound Repair Regen. 9: 305–13, 2002. doi:10.1046/j.1524-475X.2001.00305.x
8. Ray AS, Joardar N, Mukherjee S, Rahaman CH, Sinha SP. Polyphenol enriched ethanolic extract of Cajanus scarabaeoides (L.) Thouars exerts potential antifilarial activity by inducing oxidative stress and programmed cell death. PLoS One. 13: e0208201, 2018). doi:10.1371/journal.pone.0208201.
9. Kedare SB and Singh RP. Genesis and development of DPPH method of antioxidant assay. Journal of Food Science and Technology. 48: 412–422, 2011. doi:10.1007/s13197-011-0251-1.
10. Berim A, Gang DR. Methoxylated flavones: occurrence, importance, biosynthesis, Phytochemistry Reviews. 15: 363–390, 2016. doi:10.1007/s11101-015-9426-0.
11. Buckingham J and Munasinghe VRN. Dictionary of Flavonoids, CRC Press, 1st Ed, 2015 ISBN 9781466554344.
12. Swaminathan M, Chee CF, Chin SP, Buckle JC, Rahman NA, Doughty SW, Chung LY. Flavonoids with M1 muscarinic acetylcholine receptor binding activity. Molecules. 19: 8933–8948, 2014. doi:10.3390/molecules19078933.
13. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. The Journal of Nutritional Biochemistry. 13: 572-584, 2002. doi:10.1016/S0955-2863(02)00208-5.
14. Dudonné S, Vitrac X, Coutière P, Woillez M, Mérillon JM. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. Journal of Agricultural and Food Chemistry. 57: 1768–74, 2009. doi:10.1021/jf803011r.

Table 1: UV nm, 1H NMR and 13C NMR spectral data for the flavonoids.

Compound	UV nm	1H NMR	13C NMR	Supplementary data
5,4' -dihydroxy – 7 -methoxy flavanone **(Cta)**	In MeOH: 285; plus AcONa: 285, plus MeONa 295, 365, and plus AlCl3.	1H NMR 300Mz, CDCl3, δ ppm: 2.36 m(H), 3.09 m(H), 3.61 s (3 H), CH3O 5.34 m(H), 6.04 (d, 3 Hz), 6.07(d, 3 Hz) 6.90(d, 7 Hz) 7.34 (d, 7 Hz) 12.05 s OH C5.	13C JMOD NMR, 75 Mz, δ ppm (phase): 43.33 (-), 55.65, 79.13, 94.40, 95.15, 103.27(-), 115.62, 126.12, 130.68 (-), 156.26 (-), 163.03 (-), 164.26 (-), 168.15 (-), 196.23 (-).	N/A
3,5,3'-trihydroxy-7,4'-dimethoxyflavone **(Ctb)**	In MeOH 256; 376; plus MeONa 275; 437 plus AcONa 256; 376, plus HBO3 256; 374; plus AlCl3 270; 424, plus AlCl3 plus HCl 270; 425	1H NMR 300 Mz, (DMSO d6), δ ppm (#H), (m, JHz) 3.83 (CH3O), 3.835 (CH3O), 6.30 (1H), (d, 2 Hz), 6.66 (1 H), (d, 2 Hz), 7.07(1 H), (d, 8.8 Hz), 7.65 (d, 8.8 Hz), 7.68, (1 H), 12.05	13C NMR, 75 Mz, (DMSO d6), δ ppm: 56.01 (CH3O); 56.41 (CH3O); 92.28; 97.89; 104.39; 112.08; 115.03; 120.38; 123.66; 136.81; 146.49; 147.13; 149.86; 156.50; 160.70;165.36; 176.41(C=O).	N/A
3,4'-dihydroxy-5,7-dimethoxyflavanone **(Ctc)**	In MeOH 290 (0.772); plus AcONa 290 (0,772) ,plus MeONa 290 (0.760), 360 (0.317); other U.V spectra equal to the original with MeOH.	1H NMR (300 MHz, DMSO) δ 9.58 (s, 1 H), 7.31 (d, 8.4 Hz, 2H), 6.79 (d, 8.4 Hz, 2 H), 6.27 - 6.14 (m, 2H), 5.31 (d, 4.7 Hz, 1H), 5.01 (d, 11.4 Hz, 1H), 4.38 (dd, 11.4, 4.7 Hz, 1H), 3.80 (s, 3H), 3.39 (s, 3H)	13C NMR (75 MHz, DMSO) δ 190.89; 166.12; 164.35; 162.05; 158.12; 129.87; 128.13; 115.32; 103.94; 93.96, 93.38; 82.95; 72.90; 56.39; 56.23	For 1H NMR and 13C NMR additional data see **Supplementary table 1**
4'-hydroxy-5,7-dimethoxyflavanone **(Ctd)**	In acetone 283 (0,655), sh 315 (0,192): plus MeONa 285 (0,558); sh 320 (0,208), 400 (0,239); plus AcONa, plus H3BO3, plus AlCl3 do not present changes, spectra equal to that of the compound with MeOH	1H NMR (300 MHz, Acetone) δ ppm (#H) (m) 8.66s, (1H) (s); 7.41(2CH) (d J= 8.5 Hz) 6.92 (2CH) (d J= 8.5Hz); 6.19 (CH) (d 2.1 Hz); 6.16 (CH) (d 2.1 Hz); 5.42 (CH) (dd J= 13.0 Hz, 2.9 Hz); 3.86 (3 H), (s); 3.83 (3 H) ,(s) 3.01 (CH) (dd J= 16.3 Hz and 13.0 Hz); 2.60 (dd J= 16.3 Hz and 2.9 Hz).	13C NMR (75 MHz, Acetone) δppm (type of carbon): 187.63 (=C), 165.74 (=C), 165.74 (=C), 164.96 (=C), 162.29 (=C), 157.71 (=C), 130.25 (=C), 128.04 (=CH), 115.22 (=CH), 105.71 (=C), 93.47 (=CH), 92.60 (=CH), 78.95 (=CH), 55.25 (CH3), 55.17 (CH3), 45.30 (CH2).	For 1H NMR and 13C NMR additional data see **Supplementary table 2**

Table 2: The IC_{50} values for antioxidant activity, equation of the line and correlation coefficient of the isolated flavonoids from DC-EII from leaves of *C. tacotana*.

FLAVONOID/EXTRACT	EQUATION	R^2	IC_{50}	$1/IC_{50}$
Quercetine	y = 5.7566x + 16.75	0.948	8.670	0.115
DC-EII	y = 0.9493x + 23.777	0.8725	30.380	0.033
Ctb	y = 3.7515x + 33.798	0.9732	6.272	0.159
Cta	N/A	……	>500	……
Ctc	N/A	……	>500	……
Ctd	N/A	……	>500	……

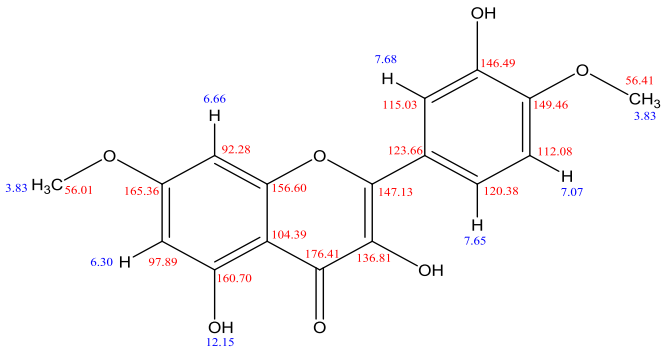

Figure 1. Molecular structure of the compound (**Ctb**), 3,5,3'-trihydroxy-7,4'-dimethoxyflavone with carbon and hydrogen assignment.

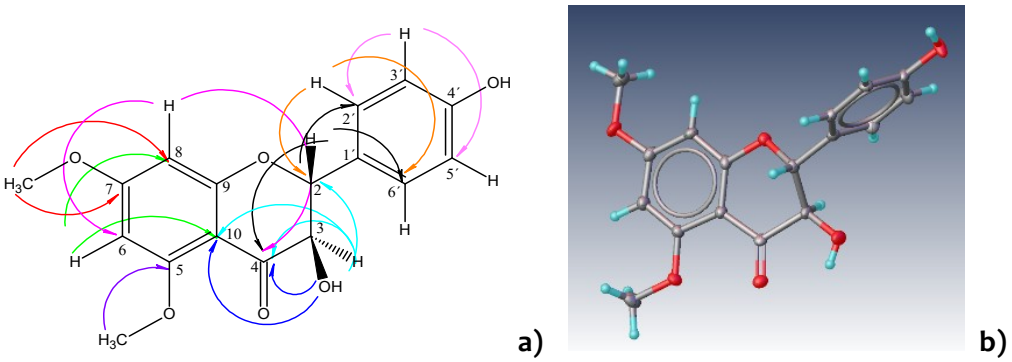

Figure 2. a) Molecular structure of 3,4'-dihydroxy-5,7-dimethoxyflavanone (**Ctc**) with the assignment for H and C in NMR spectra. **b)**. Structure of the flavanone displaying the orientation of the rings was determined by X-ray diffraction analysis. Data collection, cell refinement, and data reduction: MSC/AFC6S diffractometer control software (Molecular Structure Corp., The Woodlands, TX). Program used to solve structure: SHELXS97; program used to refine structure: SHELXL97; molecular graphics: SHELXTL-PC (G.M Sheldrick, Institute of Inorganic Chemistry Göttingen, Germany).

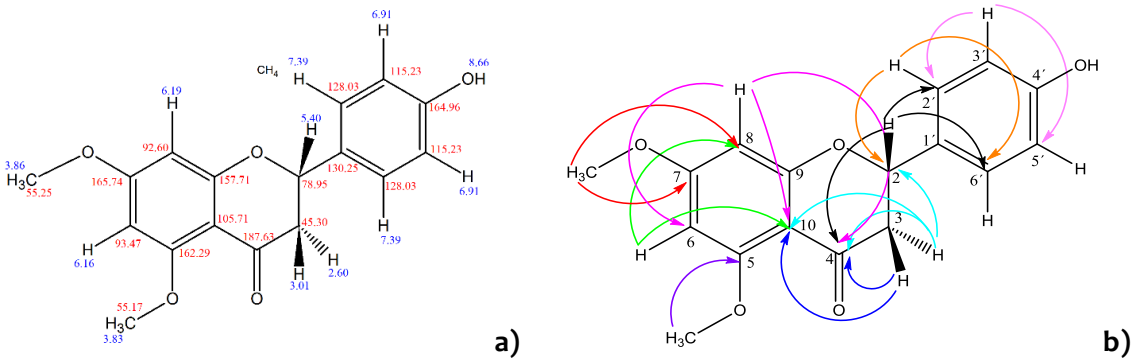

Figure 3. a) Molecular structure of 4'-hydroxy-5,7-dimethoxyflavanone with the assignment for H and C in NMR spectra**. b)** H-C connectivity's observed in the 2D NMR spectrum for (**Ctd**)

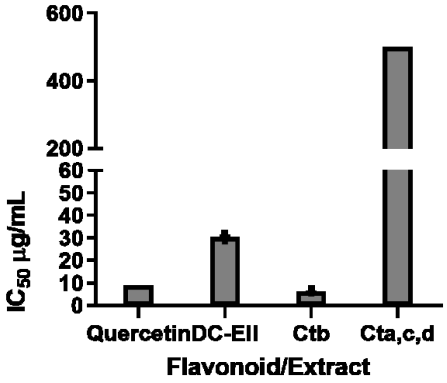

Figure 4. The IC_{50} values of flavonoids, extract and positive control through the DPPH assay. **Ctb** resulted in the flavonoid with the highest antioxidant activity. Analysis and graphic made in GraphPad Prism 6.0 software

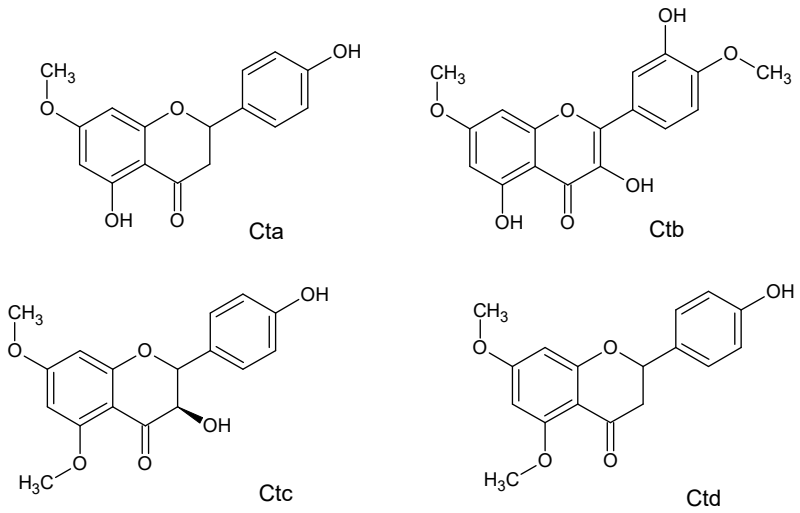

Figure 5. Molecular structures of the five flavonoids isolated from leaves of *Chromolaena tacotana*. **Cta**: 5, 4' dihydroxy-7-methoxy flavanone, **Ctb**: 3,5,3'-trihydroxy-7,4'-dimethoxyflavone; **Ctc**: 3,4'-dihydroxy -5,7-dimethoxyflavanone; and **Ctd**: 4'-hydroxy-5,7-dimethoxyflavanone.
Drawn with the MedChem Designer 5.0 program.

Supplementary Table 1. Data summary of the ^{1}HNMR, ^{13}CNMR, HMQC and HMBC spectra of the **Ctc** flavonoid, where the main direct (H-C) and long-distance (H-C-C-C-X) correlations are observed, the assignments of the C and H of the compound are also shown.

POSICIÓN	δ ^{13}C(ppm)	APT	HMQC δ^{1}H- δ^{13}C (ppm)	HMBC -δ^{1}H- δ^{13}C... (ppm)
C-2	72.90	(-) OCH	4.35-72.90	4.35-190.88;128.13;82.95
C-3	82.95	(-) OCH	4.98-82.95	4,98-190.88;129.86;72.90
C-4	190.88	(+) C=O		
C-5	162.05	(+) =C-O		
C-6	93.38	(-) =CH	6.27-93.38	6.27-166.12;162.05;103.94;93.96
C-7	166.12	(+) =C-O		
C-8	93.96	(-) =CH	6,14-93.96	6.14-164.96;103.94;93.38
C-9	158.12	(+) =C-O		
C-10	103.94	(+) = C		
C-1'	128.13	(+) =C		
C-2'	129.86	(-) =CH	7.31- 129.86	7.31-158.12;128.13;82.95
C-3'	115.32	(-) =CH	6.91-115.32	6.91-158.12;128.13;115.32
C-4'	164.96	(+) =C-O		
C-5'	115.32	(-) =CH	6.91-115.32	6.91-158.12;128.13;115.32
C-6'	129.86	(-) =CH	7.31-129.86	7.31-158.12;128.13;82.95
C5-OCH3	56.23	(-) OCH_3	3.83-56.23	3.83-166.121
C7-OCH3	56.39	(-) OCH_3	3,86-56,39	3.86-162.05

Supplementary Table 2. Data of the NMR spectra for the Ctd compound, the assignments of the C and the connectivity's obtained in the HMQC and HMBC spectra are shown.

CARBON NUMBER	δ ^{13}C(ppm)	APT	HMQC δ^{1}H- δ^{13}C (ppm)	HMBC -δ^{1}H- δ^{13}C (ppm)
C-2	78.95	(-) CH	5.40-78.95	5.40-128.03;187.63
C-3	45.30	(+) CH_2	45.30-2.60-3.01	2.60,3.01-78.95;187.36;105.71
C-4	187.63	(+) C		
C-5	162.29	(+) C		3.83-162.29
C-6	93.47	(-) CH	6.16-93.47	6.16-105.72;92.60
C-7	165.74	(+) C		3.86-165.84; 92.60
C-8	92.60	(-) CH		6.19-93.47;105.71;187.63
C-9	157.71	(+) C		
C-10	105.71	(+) C		
C-1'	130.25	(+) C		
C-2'	128.03	(-) CH		7.39-78.95;128.03
C-3'	115.23	(-) CH	6.91-115.23	6.91-128.03;115.23
C-4'	164.96	(+) C		
C-5'	115.23	(-) CH		
C-6'	128.03	(-) CH	7.39-128.03	
C5-OCH3	55.17	(+) CH_3	3.83-55.17	3.83-162.29
C7-OCH3	55.25	(+) CH_3		3.86-165.84