Secondary metabolites from *Podocalyx loranthoides*

Metabolitos secundarios de Podocalyx loranthoides

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Resumen

La investigación química de los extractos orgánicos obtenidos de las hojas de *Podocalyx loranthoides*, condujo al aislamiento de dos biflavonoides: *putraflavona y podocarpus flavona A.* Los triterpenos, 3-hidroxi-β-amirina, friedelina, pachinosol, friedelinol y estigmasterol fueron igualmente identificados, y adicionalmente una serie de hidrocarburos alifáticos tales como: tetradecano, octadecano, nonadecano y hexadecano. Se proponen las estructuras de todos los compuestos a partir de su data espectroscópica de RMN en 1D y 2D, IR, EM y por comparación con datos de la literatura.

Palabras clave: *Podocalyx loranthoides, Euphorbiaceae, triterpenoides, biflavonoides.*

Abstract

Chemical investigation of organic extracts from the leaves and stems of *Podocalyx loranthoides,* resulted in the isolation of two biflavonoids: putraflavone, and podocarpus flavone A. The triterpenes, 3-hidroxy-β-amirin, friedelin, pachynosol, friedelinol and stigmasterol were also isolated and, additionally a series of aliphatic hydrocarbons such as: tetradecane, octadecane, nonadecane, hexadecane The structures of all compounds were proposed from their spectral data (^{1}H, ^{13}C, HMQC, HMBC, IR and MS) and comparison with those reported in the literature.

Key words: *Podocalyx loranthoides, Euphorbiaceae, triterpenoids, biflavonoids*

Introduction

The family Euphorbiacea, with more of 320 genera and 8500 species is one of the largest in the tropical and subtropical area of the world (Webster et al., 1999). The Euphorbiaceae has been source of several interesting compounds showing a wide spectra of pharmacological properties such as: antihypertensive (Silva et al., 2005); cytotoxic (Roengsumran et al., 2001; Pettit et al., 2002); antinflammatory (Hohman et al., 1997, Vaisberg et al., 1989); antinociceptive (Santos et al., 1995).

We were, therefore, interested in examining the plant *Podocalyx loranthoides* Klotzsch belongs to the Euphorbiaceae family, selected as part of an ongoing research where we study the chemistry and pharmacology of the euphorbiaceae plants of Venezuela (Suárez et al., 2003, 2004, 2005, 2006). Podocalyx loranthoides grows as small tree in warm regions of Brasil, Colombia, Peru and, in the south part of Venezuela (Steyermark et al., 1999). Locally is known as «palo de agua dulce» and «reventillo». It was considered the only specie classified under the genus Podocalyx. No report on the chemical constituents of *P. loranthoides* was found except the work where we describe the isolation of the biflavones, Podocarpusflavone A and Putraflavone (Suárez et al., 2003).

Materials and Methods

Plant material

The leaves and stems of *Podocalyx loranthoides* were collected where of Sipapo river converge with

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Cuao river at Amazonas state, Venezuela in June 2000. Its identity was confirmed by Dr. Anibal Castillo, and a voucher specimen (AC2710) has been deposited in the National Herbarium of the Botanical Garden of Caracas.

EXTRACTION AND ISOLATION

Dried and powdered leaves (595.50 g) *of P. loranthoydes* were extracted via sohxlet with solvents of increasing polarity, hexane, dichloromethane, and ethyl acetate. Evaporation of the solvents under reduced pressure furnished 18.7 g, 9.2 g and 7.0 g respectively, of crude extracts. One portion of each extract was chromatographed separately over silica gel and eluted with hexane, gradually increasing the polarity with dichloromethane, ethyl acetate and then methanol. From the column chromatography of part of hexane extract (1.7), 100 fractions were collected and monitored by TLC, similar fractions were combined and purified by successive column chromatography to furnishing a series of aliphatic hydrocarbons such as: tetradecane, octadecane, nonadecane, hexadecane, which were characterized by mass spectrometry. From the dichloromethane extract (1.8 g) which was subjected to column chromatography over silica gel eluted with ($CHCl_3$: EtOAc 1:1 until 1:9 MeOH:EtOAc, were collected 25 fractions. After evaporation of the solvent and precipitation in methanol, one of the subfractions gave putraflavone (**1**) (43.0 mg) (Suárez et al., 2003). By similar procedure with different fractions of the described column chromatography, podocarpusflavone A (**2**) (50.4 mg) was isolated. The EtOAc extract (0.50 g) was subjected to a chromatography using RP-18 eluted with MeOH-H_2O (1:1) to gave also as the major components, putraflavone (**1**)(12.3 mg) (Harborne., 1986) and podocarpusflavone A (**2**) (39.1 mg).

Similar procedure was carried out with the stem extracts (560 g), the fractionation of the hexane extract (2.40 g) submitted to column chromatography afforded the triterpenes, 3-hidroxy-β-amirine (**3**) (15 mg) (Mahato and Kundu, 1994), and friedelin (**4**) (Gotlieb et al., 1985). From the EtOAc extract, the following compounds were isolated, pachynosol (**5**) (9.2 mg) (Mahato and Kundu, 1994), friedelinol (**6**) (Queiroga et al., 2000) and the steroid stigmasterol (**7**) (Holland et al., 1978).The structures of all compounds were proposed from their spectral data (^1H, ^{13}C, HMQC, HMBC, IR and MS) and comparison with those reported in the literature.

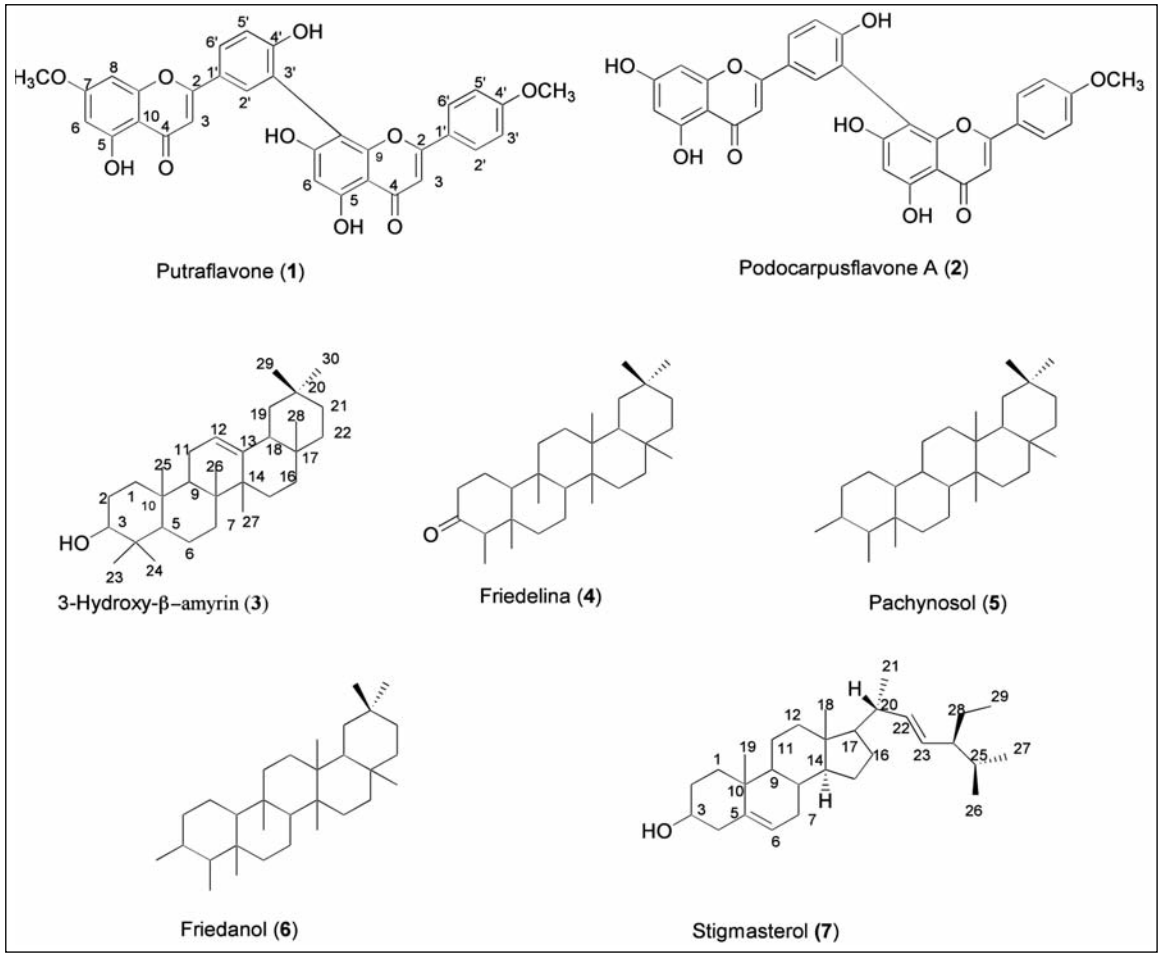

Figura 1
Chemical structures of isolated compounds from *Podocalyx loranthoides*

GENERAL PROCEDURES

The IR spectra were recorded on a SHIDMAZU 470 spectrometer. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. NMR spectra were recorded with a JEOL Eclipse 270 spectrometer operating at 270 MHz and a Bruker DRX-500 with $CDCl_3$, DMSO, and acetone-d_6 as solvent and TMS as internal standard. Low-resolution mass spectra were measured in a VARIAN Saturn 2000 and high resolution mass spectra (HRMS-CI) were measured on a JEOL JMS-AX505WA. Optical rotations were measured in a Lynos Photonies Type SR6, Spannung. TLC analyses were carried out on precoated silica gel G_{254} (Merck) plates and the spots were visualized by UV (254 nm) irradiation and reaction with p-anisaldehyde/H_2SO_4/HOAc reagent. For column chromatography, silica gel 60 (Merk 100-200 mesh) and RP-18 silica gel were used.

Putraflavone (**1**). Yellow solid, mp 240-243 ºC; IR (KBr) $\gamma_{max}/_{cm}{}^{-1}$ 3300 (OH), 1660, 1657; RMN ^{1}H (270 MHz, DMSO-d_6) δ ppm: 3,74 (s, 1H, **4´-OCH$_3$**); 3.81 (s, 1H, **7-OCH$_3$**); 6.34 (1H, d, *J* = 2,2 Hz, **H-6**); 6.42 (1H, s, **H-6**); 6.76 (1H, d, *J* = 2,2 Hz, **H-8**); 6.88 (1H, s, **H-3**); 6.89 (1H, s, **H-3**); 6.92 (2H, d, *J* = 8,6 Hz, **H-3´**, **H-5´**); 7.16 (1H, d, *J* = 8,4 Hz, **H-5**); 7.66 (2H, d, *J* = 8,9 Hz, **H-2´, H-6**); 7.99 (1H, da, **H-6´**); 8.05 (, 1H, da, **H-2´**); 12.97 (1H, s, **5-OH**); 13.07 (1H, s, **5-OH**).RMN ^{13}C (75 MHz, DMSO–d_6) δ ppm: 56.1 (**4´-OCH$_3$**); 56.6 (**7-OCH$_3$**); 93.2 (**C-8**); 98.6 (**C-6**); 99.2 (**C-6**); 103.7 (**C-3**); 103.8 (**C-3**); 104.2 (**C-8**); 104.5 (**C-10**); 105.2 (**C-10**); 115.1 (**C-3´, C-5´**); 116.7 (**C-5´**); 120.5 (**C-3´**); 121.4 (**C-1´**); 123.5 (**C-1´**); 128.5 (**C-6´**, **C-2´**, **C-6´**); 131.9 (**C-2´**); 155.1 (**C-9**); 157.8 (**C-9**); 160.2 (**C-4´**); 160.8 (**C-5**); 161.1 (**C-5**); 162.4 (**C-7**); 162.8 (**C-4´**); 163.8 (**C-2**); 164.6 (**C-2**); 165.6 (**C-7**); 182.5 (**C-4**); 182.7 (**C-4**).

Podocarpusflavona A (**2**) Yellow solid, mp 228-230 ºC; IR (KBr) $\gamma_{max}/_{cm}{}^{-1}$ 3500 (OH), 1660, 1659; RMN ^{1}H (270 MHz, DMSO-d_6) δ ppm: 3.75 (1H, s, **4´-OCH$_3$**); 6.18 (1H, d, *J* = 2,2 Hz, **H-6**); 6.42 (1H, s, **H-6**); 6.46 (1H, d, *J* = 2,1 Hz, **H-8**); 6.82 (1H, s, **H-3**); 6.83 (1H, s, **H-3**); 6.93 (2H, d, *J* = 8,6 Hz, **H-3´, H-5´**); 7.15 (1H, d, *J* = 8,4 Hz, **H-5´**); 7.67 (2H, d, *J* = 8,9 Hz, **H-2´, H-6´**); 7.97 (1H, dd, *J* = 8,6 Hz; 2,4 Hz, **H-6´**); 8.01 (1H, d, **H-2´);** 12.95 (1H, s, **5-OH**); 13.05 (1H, s, **5-OH**). RMN ^{13}C (75 MHz, DMSO–d_6) δ ppm: 56.1 (**4´-OCH$_3$**); 94.6 (**C-8**); 99.2 (**C-6**); 99.3 (**C-6**); 103.5 (**C-3**); 103.8 (**C-10**); 104.2 (**C-3**, **C-10**); 104.4 (**C-8**); 115.0 (**C-3´**, **C-5´**); 116.3 (**C-5´**); 120.5 (**C-1´**, **C-3´**); 123.5 (**C-1´**); 128.5 (**C-2´**, **C-2´, C-6´**); 131.9 (**C-6´**); 155.0 (**C-9**), 158.0 (**C-9**); 160.0 (**C-4´**); 161.9 (**C-5**); 162.5 (**C-4´**); 162.8 (**C-5**); 163.0 (**C-2**); 164.6 (**C-2**); 165.0 (**C-7**); 166.0 (**C-7**); 182.2 (**C-4**); 182.7 (**C-4**).

3-Hydroxy-β-amirin (**3**), white solid, 196-198 ºC, ^{1}H NMR (500 MHz, $CDCl_3$);δ:

0.73-1.88 (23H, m, **H-1, H-2, H-5, H-6, H-7, H-9, H-11, H-15, H-16, H-18, H-19, H-18, H-21, H-22**); 0.76 (3H, s, **H-24**); 0.81 (3H, s, **H-25**); 0.92 (3H, s, **H-23**); 0.96 (s, 3H, **H-30**); 1.02 (3H, s, **H-29**); 1.18 (3H, s, **H-28**); 1.20 (3H, s, **H-26**); 1.23 (3H, s, **H-27**); 3.34 (1H, m, **H-3**); 5.15 (1H, sa, **H-12**). RMN ^{13}C (75 MHz, $CDCl_3$) δ ppm: 15.3 (C-**25**); 16.8 (C-**26**); 18.0 (C-**6**); 22.4 (C-**24**); 23.5 (C-**11**); 23.7 (C-**30**); 26.1 (C-**15**); 26.2 (C-**27**); 27.0 (C-**2**); 27,4 (C-**16**); 28.3 (C-**23**); 28.4 (C-**28**); 31.1 (C-**20**); 32.5 (C-**7**, C-**17**); 33.4 (C-29); 34,8 (C-**21**); 37.0 (C-**8**); 37.2 (C-**1**, C-**22**); 37.4 (C-**10**); 40.0 (C-**4**); 41.8 (C-**14**); 46.6 (C-**19**); 47.2 (C-**9**); 47.4 (C-**18**); 48.9 (C-**5**); 76.3 (C-**3**); 121.8 (C-**12**); 145.2 (C-**13**).

Friedelin (**4**), white solid, mp 260-262 ºC, ^{1}H NMR (500 MHz, $CDCl_3$); 1.26-2.40 (m, 25H, **H-1, H-2, H-6, H-7, H-8, H-10, H-11, H-12, H-15, H-16, H-18, H-19, H-21, H-22**); 0,70 (3H, s, **H-24**); 0.84 (3H, s, **H-25**); 0.87 (3H, d, *J* = 6,4 Hz, **H-23**); 0.93 (3H, s, **H-30**); 0.98 (3H, s, **H-29**); 1.03 (3H, s, **H-26**); 1.16 (3H, s, **H-27**); 1.23 (3H, s, **H-28**); 2.20-2.28 (1H, m, **H-4**). 6.9 (C-**23**); 14.7 (C-**24**); 18.0 (C-**25**); 18.3 (C-**7**); 18.7 (C-**27**); 20.3 (C-**26**); 22.3 (C-1); 28.2 (C-**20**); 30.1 (C-**17**); 30.6 (C-**12**); 31.8 (C-**29**); 32.2 (C-**28**); 32.5 (C-**15**); 32.9 (C-**21**); 35.1 (C-**30**); 35.4 (C-**19**); 35.7 (C-**11**); 36.1 (C-**16**); 37.5 (C-**9**); 38.4 (C-**14**); 39.3 (C-**22**); 39.8 (C-**13**); 41.4 (C-**6**); 41.6 (C-**2**); 42.2 (C-5); 42.9 (C-**18**); 53.2 (C-**8**); 58.3 (C-**4**); 59.6 (C-**10**); 213.2 (C-**3**).

Pachynosol (**5**), white solid, 196-198 ºC, ^{1}H NMR (500 MHz, $CDCl_3$); δ: 0.70(3H, s, H-**24**); 0.88 (3H, s, H-**25**); 0.93 (3H, s, H-**30**); 0.95 (3H, s, H-**29**); 0.98(3H, s, H-**26**); 1.01(3H, H-**27**); 1.16 (3H, H- **28**); 3.61 (1H, sa, H-**16**); 1.15 -2.80 (m, H-**1**, H-**2**, H-**6**, H-**7**, H-**18**, H-**19**, H-**21**, H-**22**). ^{13}C NMR (75 MHz, $CDCl_3$): d 22.5 (C-**1**), 41.3 (C-**2**), 55.6 (C-**3**), 42.8 (C-**4**), 41.2 (C-**5**), 18.7 (C-**6**), 53.2 (C-**7**), 37.5 (C-**8**), 37.5 (C-**9**), 56.6 (C-**10**), 35.4 (C-**11**), 29.3 (C-**12**), 39.7 (C-**13**), 39.7 (C-**14**), 43.5 (C-**15**), 75.1 (C-**16**), 32.4 (C-**17**), 42.3 (C-**18**), 35.5 (C-**19**), 28.2 (C-**20**), 33.9 (C-**21**), 36.0 (C-**22**), 6.7 (C-**23**), 14.8 (C-**24**), 19.0 (C-**25**), 20.3 (C-**26**), 32.2 (C-**27**), 28.2 (C-**28**), 31.8 (C-**29**), 35.1 (C-**30**).

Friedanol (**6**), white solid, 196-198 ºC, ^{1}H NMR (500 MHz, $CDCl_3$); δ: 0.68 (3H, s, H-**24**); 0.84 (3H, s,

H-**23**); 0.86 (3H, s, H-**25**); 0.87 (3H, s, H-**30**); 0.96 (3H, s, H-**29**); 0.98 (3H, H-**26**); 1.02 (3H, H-**27**); 1.26 (3H, H-**28**). ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.0 (C-**1**), 31.8 (C-**2**), 77.5 (C-**3**), 55.0 (C-**4**), 37.5 (C-**5**), 41.0 (C-**6**),22.5 (C-**7**), 52.9 (C-**8**), 35.6 (C-**9**), 61.0 (C-**10**), 35.4 (C-**11**), 29.7 (C-**12**), 37.5 (C-**13**), 38.9 (C-**14**), 30.5 (C-**15**), 35.5 (C-**16**), 30.0 (C-**17**), 42.0 (C-**18**), 35.4 (C-**19**), 28.2 (C-**20**), 32.5 (C-**21**), 40.6 (C-**22**), 11.0 (C-**23**), 14.2 (C-**24**), 18.4 (C-**25**), 18.6 (C-**26**), 20.3 (C-**27**), 31.8 (C-**28**), 35.1 (C-**29**), 32.2 (C-**30**).

Stigmasterol (**7**). White solid, 127-130 ºC, ^{1}H NMR (500 MHz, $CDCl_3$); δ: 0.66 (3H, s, H-**18**); 1.48 (1H, s, H-**2a**); 1.50 (1H, m, H-**24**); 1.51(1H,m, H-**7a**); 1.83 (1H, m, H-**2b**); 1.98 (1H, m, H-**7b**); 2.00 (1H, m, H-**20**); 2.26 (1H, m, H-**4**); 3.50 (1H, m, H-**3**); 4.99 (1H, dd .8.60, 15.20 Hz, H-**23**); 5.13 (1H, dd, 8.60, 15.20 Hz, H-**22**); 5.32 (brs, H-**6**). ^{13}C NMR (75 MHz, $CDCl_3$): d 37.3 (C-**1**), 31.1 (C-**2**), 71.9 (C-**3**), 42.4 (C-**4**), 140.8 (C-**5**), 121.8 (C-**6**), 31.9 (C-**7**), 36.2 (C-**8**), 50.2 (C-**9**), 36.6 (C-**10**), 21.1 (C-**11**), 39.8 (C-**12**), 42.3 (C-**13**), 56.9 (C-**14**), 24.4 (C-**15**), 28.9 (C-**16**), 56.0 (C-**17**), 12.3 (C-**18**), 19.4 (C-**19**), 40.6 (C-**20**), 19.9 (C-**21**), 138.4 (C-**22**), 129.3 (C-**23**), 51.3 (C-**24**), 29.2 (C-**25**), 19.0 (C-**26**), 21.3 (C-**27**), 25.5 (C-**28**), 12.1 (C-**29**).

Results and discussion

A total of eleven compounds were isolated from the organic extracts of the stems and leaves of *Podocalyx loranthoides* by repeated chromatographic separation over silica gel. The structures of the isolated compounds were deduced by extensive NMR and mass spectral analyses. The EI mass spectra of compound **4** gave an ion peak at m/z 426 corresponding to a molecular formula $C_{30}H_{50}O$. The ^{13}C NMR spectrum displayed a total of 30 carbons signals. The DEPT NMR experiment, indicated that 7 carbons are quaternary ones, including a ketone group; and the rest of 23 carbons were classified as 8 methyls, 11 methylenes, 4 methines and 7 quaternary carbons. The ^{1}H NMR spectrum of compound **4** showed 7 three proton singlets at δ 0.70, 0.84, 0.87, 0.93, 0.98, 1.03, 1.16 and a doublet (J = 6.4 Hz) centered at δ 0.87. These were attributed to methyl groups present in a triterpene type friedelane. On the basis of the above spectral features, compound **4** was characterized as friedelin, the identity of which was further substantiated by comparison of its spectroscopic data with published values (Gottlieb et al., 1985). This is the first report of occurrence of friedelin from *P. loranthoides*. The compounds **3**, **5**, **6**, **7** were characterized by comparison of their ^{1}H NMR and ^{13}C NMR spectral data with published one as well as by co-TLC with authentic samples. The ^{1}H and ^{13}C NMR of three compounds showed signals due to hydroxyl group and tetracyclic triterpenes; detailed analysis of the NMR spectra with the aid of ^{1}H-^{1}H, ^{1}H-^{13}C, and long range ^{1}H-^{13}C, suggested that they were well known structures identified as: 3-hydroxy-,-amirin (**3**), pachynosol (**5**), and friedanol (**6**).

The known natural steroid **7**, was identified by analysis of their spectral data and comparison with literature values, mainly ^{13}C NMR chemical shifts described for stigmasterol (**7**).

Podocarpusflavone A (**2**) was identified mainly by analysis of ^{1}H and ^{13}C NMR and specially HMQC and HMBC experiments. The ^{1}H NMR spectrum showed signals typical of a flavonoid structure. Two chelated hydroxyl groups were observed at δ 12.95 and 13.05 ppm.

The ^{13}C NMR spectrum of compound **2** showed 31 signals, showing two carbonyl groups at δ 182.2 and 182.7 ppm. Among the aromatic carbons, one methoxyl group was also found at δ 56.1 ppm. The presence of two carbonyls, suggesting a flavonoid dimmer, which contain two flavone units. The analysis of HMBC spectrum data was very important to identify the linkage between the flavonoid units. The cumulated data from the HMBC experiment indicated characteristic correlations which defined the po - sition of each aromatic ring The data of NMR spectra related to splitting pattern and coupling constants also suggested a pentasubstituted aromatic ring with a 5,7-dioxigenation pattern for the A ring of one flavone. Finally carefully comparison of our data with those published for podocarpusflavone A, indicated that the compound **2** has the same structure. Additional the position of the methoxyl group was done by UV spectral analysis using $AlCl_3$ reagent.

The compound **1** was analyzed under the same criteria used for podocarpuflavone A (**2**). The major difference between this compound and the previous described, was the presence of two methoxyl groups. Comparison with the literature data indicated that the compound **1** is the well known biflavone putraflavone (**1**) (Suárez et al., 2003).Unequivocal assignments of NMR signals were obtained by long-range correlations. The location of the OMe on positions 7 and 4′ was established by Difference NOE experiments between H-6/H-8 and OMe-7 (δ 3.82), and between H-3′/H-5′ and OMe-4 (δ 3.76), respectively.

The structure of aliphatic compounds tetradecane, octadecane, nonadecane and hexadecane were established by mass spectroscopic. All the compounds herein described are reported as the principal metabolites found in the first phytochemical analysis of the plant *Podocalyx loranthoides.*

Conclusión

The latest revision of the Euphorbiacea, which reported 320 genera, lists only the species Podocalyx *loranthoides* Klotzsch under the genus *Podocalyx* (Steyermark et al., 1999). Many species under the big family Euphorbiaceae are particularly rich in terpenoids, specially diterpenes and triterpenes (Lima et al., 2003; Merrit and Ley, 1992, Rocha Barbosa et al., 2003, Salatino et al., 2007), several genus are also reported with high concentrations of biflavonoids as principal constituents, making this class of compounds valuable chemotaxonomic markers under the spurge family (Canelón et al., 2005; Del Rayo-Camacho et al., 2000; Hnatyszyn et al., 1987). The results herein reported, showed that the triterpenes and, biflavones, principal constituents founds in the aerials parts of *P. loranthoides,* are compounds which have been previously isolated in the Euphorbiacea family. This finding, the presence of the previously described compounds in *Podocalyx loranthoides,* strongly supports the placement of the genus *Podocalyx* under the Euphorbiaceae. All the constituents are in agreement with the assignment of this genus under the spurge family. However, interestingly, after finish this research we found recently in the literature, a new classification for this species, now *Podocalyx loranthoides* is included under the Malpighiales family (APG II, 2003), but our results, like we describe previously in this paper, strongly support the chemical characterization of the *Podocalyx* under the Euphorbiaceae.

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