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Phytochemistry 66 (2005) 649–652
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

A furanocoumarin and polymethoxylated flavonoids from the Yucatec Mayan plant *Casimiroa tetrameria*

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Received 6 December 2004; accepted 8 December 2004
Available online 30 January 2005

Abstract

As part of an ongoing study of the medicinal plants of the Yucatec Maya, *Casimiroa tetrameria* was investigated for its phytochemistry. From an ethyl acetate partition of an ethanol extract of the leaves, eight flavonoids and a furanocoumarin were isolated and characterised as 5,6,2′,3′,5′,6′-hexamethoxyflavone, 5,6,2′,3′,6′-pentamethoxyflavone and 5-methoxy-8-(3″-hydroxymethyl-but-2″-enyloxy)-psoralen using a combination of ^{1}H, ^{13}C NMR and NOESY spectroscopy.

Keywords: Polymethoxyflavones; Furanocoumarin; Yucatec Maya; Rutaceae

1. Introduction

The Yucatec Maya (Mexico) use *Casimiroa tetrameria* Millsp. (Rutaceae), which is commonly known as *Yuy,* for treating gastrointestinal problems, especially diarrhoea, dysentery and gastrointestinal cramps. The usage of this plant as well as those of numerous other species was documented in a detailed ethnobotanical study (Ankli et al., 1999). The closely related *C. edulis* yields an economically important fruit known as white sapote or Mexican apple. Currently six species are recognised in the genus, but a systematic re-evaluation would be desirable and therefore, a detailed comparative phytochemical analysis of these species may be of relevance for such an evaluation. *C. tetrameria* is relatively well circumscribed, with its characteristic five lobed leaves in combination with a fruit which contains only one 13–14 mm long seed (Martínez, 1951). This study is part of an ongoing project on the indigenous use of medicinal plants in the Lowlands of México (Heinrich, 1998, 2003; Leonti et al., 2003).

2. Results and discussion

The phytochemical investigation focused on the ethyl acetate partition of an ethanolic extract of the dried leaves of *Casimiroa tetrameria* (Heneka, 2002; Heinrich et al., 2005). Further fractionation of this extract resulted in the isolation of 13 compounds, two new and six known polymethoxylated flavonoids (**1**–**8**), four flavonoid glycosides (**9**–**12**) and one new furanocoumarin (**13**).

The eight polymethoxylated flavonoids were not all obtained in pure form, but two of them could be identified from a mixture. The structures of the two new compounds 5,6,2′,3′,5′,6′-hexamethoxyflavone (**1**, 9 mg) and

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0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.12.005

5,6,2′,3′,6′-pentamethoxyflavone (**2**, 13 mg obtained as a mixture together with **6**, zapotin, 5,6,2′,6′-tetramethoxyflavone) were elucidated using ^{1}H NMR, ^{1}H–^{1}H NOESY, ^{13}C NMR and EI–MS.

EI–MS indicated a molecular ion of *m/z* 402 ($C_{21}H_{22}O_8$) for compound **1**. Fragments at *m/z* 165 and 137 support the dimethoxy substitution of ring A (cf. Meyer et al., 1985). The ^{1}H NMR spectrum exhibited only four signals in the aromatic region, and four signals corresponding to six –OCH_3 groups. These data suggested a hexamethoxylated flavonoid. The base peak at *m/z* 387 indicated the elimination of a methyl group (–CH_3) and the facile loss of this group in the mass spectrum has been suggested by Dreyer and Bertelli (1967) to indicate a methoxyl group at position C-5 of the flavonoid nucleus. This methoxyl group (δ_H 3.98) gave an NOE correlation to a further methoxyl (at C-6, 3.91, s) which had a further NOE correlation to an aromatic proton at δ_H 7.26 (d) placed at C-7 of the A-ring of the flavonoid. In the COSY spectrum this proton had an *ortho* coupling to a further aromatic proton (δ_H 7.17, d, J = 9 Hz) which was assigned as H-8 and therefore completed the A-ring of the flavonoid. In addition, the data for the A ring of compound **1** were nearly identical with those for zapotin (5,6,2′,6′-tetramethoxyflavone, **6**).

Assigning the signals for rings B and C was again possible with the help of a ^{1}H NOESY experiment. The two signals at 6.29 (1H, *s*) and 6.67 ppm (1H, *s*) and especially the two signals of methoxyl groups at δ_H 3.75 (6H, *s*) and δ_H 3.88 (6H, *s*), each of them representing a total of six protons, pointed to a symmetrical structure of ring B with a single proton at C-4′. The signal at 6.67 ppm (H-4′) showed an intense NOESY coupling with the singlet signal at δ_H 3.88 (methoxyls at C-3′ and C-5′), which in turn showed an interaction to the methoxyl signal at 3.75 ppm (methoxyls at C-2′/C-6′). This methoxyl resonance gave a NOESY coupling to the remaining resonance at 6.29 (s) indicating that this is H-3. Consequently compound **1** was identified as 5,6,2′,3′,5′,6′-hexamethoxyflavone, which to the best of our knowledge has not been identified in nature.

The NMR data of **2** were similar to those of **1** although only five methoxyl groups were present and this was isolated as a mixture with approximately 17% of zapotin (**6**). The A and C-ring resonances were comparable with those of **1** whereas the presence of two protons at 6.65 (1H, *d*) and 6.98 ppm (1H, *d*) showing an *ortho* coupling to each other (J = 9 Hz) in the ^{1}H spectrum suggested a different B-ring substitution pattern. Again the NOESY spectrum was highly informative with cross-peaks between the doublet at 6.98 ppm and the signal of a methoxyl group at δ_H 3.85 (3H, *s*). Additionally, a cross-peak was observed between the proton signal at 6.65 ppm and an OCH_3-signal at 3.73 ppm (s), which was at the highest field. This pattern established that the two protons were positioned at C-4′ and C-5′ and that there were OCH_3 groups at C-2′, C-3′, and C-6′. Calculating the chemical shift with the increment rule resulted in assigning the signal at 6.65 ppm to H-5′. The remaining methoxyl signal at 3.83 ppm with no interaction with other protons was assigned to a methoxyl group at C-2′. Zapotin (**6**), which is also present in the fraction, was identified by comparing the ^{13}C and ^{1}H NMR data with an authentic sample and by comparison with the literature (Dreyer and Bertelli, 1967). **2** is therefore assigned as 5,6,2′,3′,6′-pentamethoxyflavone and is reported here for the first time.

The main constituent 5,6,3′,4′,5′-pentamethoxyflavone (cerrosilin B, **4**, 162 mg), and 5,6,2′,3′,4′-pentamethoxyflavone (**5**, 8 mg) had identical spectra to previously isolated material from *Sargentia greggii* (Domínguez and Villegas, 1976) and *Ardisia floribunda* (Myrsinaceae, Parveen and Khan, 1987), respectively. Three tetramethoxyflavones (**6** as a mixture with **2**, **7** and **8**) were identified by comparison with already published data. The spectral data of 5,6,3′,4′-tetramethoxyflavone (**7**, 14 mg) and 5,6,3′,5′-tetramethoxyflavone (cerrosillin, **8**, 8 mg) are identical to those from the scientific literature (Dreyer, 1968; Parveen and Khan, 1987, respectively).

The four flavonoid glycosides [quercetin-3-*O*-glucoside (**9**, 8 mg), quercetin-3-*O*-rutinoside (**10**, 11 mg), kaempferol-3-*O*-glucoside (**11**, 15 mg) and kaempferol-3-*O*-rutinoside (**12**, 12 mg)] were also isolated from the ethyl acetate partition and identified by comparing the ^{1}H NMR data with that from the literature (Strack et al., 1989; Parker and Bohm, 1975).

Compound **13** was isolated from the ethyl acetate partition using Sephadex LH 20 eluting with MeOH (100%) and a two-step RP_{18}-HPLC (MeOH–H_2O 50–100 and ACN/MeOH/H_2O – 42.6/5.3/52.1) separation. Four proton signals in the aromatic regions formed a pair of two AB systems (H-3, δ_H 6.27, *d* and H-4, 8.11, *d*) and (H-3′, δ_H 6.98, *d* and H-2′, δ_H 7.61, *d*) confirmed the typical pattern of a linear furanocoumarin (Stavri et al., 2003). The strong downfield shift of one of these protons (H-4) indicated the presence of O-substitution at C-5 (Razdan et al., 1987) and this was confirmed by a signal of an OCH_3-group at 4.16 ppm shown to be in close proximity to H-4 by a correlation in the NOESY spectrum. The remaining four ^{1}H NMR signals [δ_H 1.85 (3H, methyl, *s*), δ_H 4.24 (2H, hydroxymethyl, *s*), δ_H 4.86 (2H, oxymethylene, *d*), δ_H 5.71(1H, olefin, *t*)] were characteristic of a prenyloxy group, in which one of the methyl groups had been oxidised to an hydroxymethyl. This hydroxymethyl group was *cis* with respect to the oxymethylene group of the prenyl substituent on the basis of a NOESY correlation between the two moieties. As C-5, C-6 and C-7 of the coumarin nucleus are all substituted, the prenyloxy group must be placed at position C-8. The base beak of *m/z* 316 suggested a molecular formula of $C_{17}H_{16}O_6$ and compound **13** is therefore identified as the new furanocoumarin

5-methoxy-8-(3″-hydroxymethyl-but-2″-enyloxy)-psoralen (21 mg), which is described here for the first time.

3. Experimental

3.1. General experimental procedures

All solvents with the exception of those used in HPLC were of laboratory grade and purchased from Merck (Darmstadt, DE) or Roth (Karlsruhe, DE). Several TLC-systems were developed. For the flavonoids EtOAc/MeOH/H_2O (8:2:1) on silica gel 60F_{254} (Merck) was used. For chromatographic separations the following adsorbents were used: Sephadex LH 20 (Pharmacia, Uppsala), MCI-gel (Mitsubishi Chem. Ind.), RP-18 and silica gel (Merck, Darmstadt).

GC–MS: All experiments were conducted on a Finnigan GC/MS 4000 using an Optima-1 column with helium (8 psi) (detector and injector: 160 °C, split ratio 24:1, detection flame ionisation detector (FID)). NMR experiments were performed using a Bruker AM-400 (^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz)) and a Bruker Avance (^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz)), respectively.

3.2. Plant material

C. tetrameria was collected from the villages and surroundings of Chikindzonot, Ekpeds and Xcocmil, Yucatán, México (1994–1995). Authenticated voucher specimens were deposited at the Herbarium of the National Herbarium of México (MEXU), the Centro de Investigaciones Científicas de Yucatán (CICY) in Mérida, the Instituto Nacional Indigenista (INI) in Valladolid, Yucatán, the ETH Zurich and the Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, London. The plant was identified by A.A. in collaboration with researchers at CICY.

3.3. Extraction and isolation

The air dried and powdered leaves (530 g) were extracted under reflux once with EtOH, 96% (5300 ml, 30 min) and twice with EtOH 70% (5300 ml, 30 ml). The filtrates were combined and the organic solvent was removed under reduced pressure with a rotary evaporator. The resulting residue was freeze-dried yielding 122 g crude extract. Of this extract 110 g were redissolved in H_2O (1500 ml). For further separation this suspension was first extracted with equal amounts (1500 ml) of petrol ether for several times. Afterwards the extraction procedure was repeated in the same way with ethyl acetate. The liquid–liquid separation resulted in an H_2O-fraction (80 g), an ethyl acetate fraction (20 g) and a petroleum ether fraction (10 g, respectively. The ethyl acetate fraction was further separated using various systems of column chromatography: (a) Sephadex LH 20 (MeOH, 100%), (b) RP_{18} (MeOH, 50–100%), (c) RP_{18} ACN/MeOH/H_2O, 42.6/5.3/52.1, 44.6/6.7/48.7 and 51.7/9.5/38.8, respectively), (d), Silica gel_{60} (EtOAc/toluene, 45/55).

3.4. 5,6,2′,3′,5′,6′-Hexamethoxyflavone (*1*)

Yellow amorphous solid; UV (MeOH) λ_{max} nm: 229/328; ^{1}H NMR ($CDCl_3$): δ 7.26 and 7.17 (1H, *d*, J = 9 Hz, H-7 and H-8), 6.67 (1H, *s*, H-4′), 6.29 (1H, *s*, H-3), 3.98 (3H, *s*, OCH_3-5), 3.91 (3H, *s*, OCH_3-6), 3.88 (6H, *s*, OCH_3-3′ and OCH_3-5′), 3.75 (6H, *s*, OCH_3-2′ and OCH_3-6′); ^{13}C NMR ($CDCl_3$): δ 177.8 (C-4), 158.6 (C-2), 152.4 (C-8a), 149.9 (C-6), 149.2 (C-3′ and C-5′), 148.1 (C-5), 140.9 (C-2′ and C-6′), 119.5 (C-4a), 119.2 (C-8), 114.5 (C-3 and C-4′), 113.6 (C-7), 101.7 (C-1′), 62.0 (OCH_3-5), 61.8 (OCH_3-2′ and OCH_3-6′), 57.3 (OCH_3-6), 56.7 (OCH_3-3′ and OCH_3-5′) [C-1′-absent]; EI–MS: *m/z* (intensity%) 402 $[M]^+$ (96), 387 $[M - Me]^+$ (100), 372 $[M\text{-}2Me]^+$ (17), 357 (74), 342 (6), 327 (7), 313 (9), 186 (17), 165 (15), 149 (20), 137 (15), 97 (13), 85 (13), 83 (22), 71 (21), 69 (19), 57 (32), 55 (15), 43 (20).

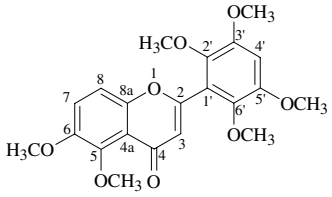
3.5. 5,6,2′,3′,6′-Pentamethoxyflavone (*2*)

Yellow amorphous solid; UV (MeOH) λ_{max} nm: 233/333; ^{1}H NMR ($CDCl_3$): δ 7.26 and 7.18 (each 1H, *d*, J = 9 Hz, H-7 and H-8), 6.98 (1H, *d*, J = 9 Hz, H-4′), 6.65 (1H, *d*, J = 9 Hz, H-5′), 6.27 (1H, *s*, H-3), 3.97 (3H, *s*, OCH_3-5), 3.91 (3H, *s*, OCH_3-6), 3.85 (3H, *s*, OCH_3-3′), 3.83 (3H, *s*, OCH_3-2′), 3.73 (3H, *s*, OCH_3-6′); ^{13}C NMR ($CDCl_3$): δ 178.0 (C-4), 158.5 (C-2), 152.6 (C-8a), 151.8 (C-6′), 149.8 (C-6), 148.6 (C-5), 147.15 (C-2′) 132.1 (C-3′), 119.5 (C-4a), 119.1 (C-8), 115.2 (C-3), 115.0 (C-4′), 113.7 (C-7), 106.3 (C-5′), 62.0 (OCH_3-5), 61.6 (OCH_3-2′), 57.4 (OCH_3-6), 56.7 (OCH_3-3′), 56.3 (OCH_3-6′) [C-1′-absent]; EI–MS: *m/z* (intensity%) 372 $[M]^+$ (81), 357 (100), 342 (11), 327 (35), 312 (3), 297 (3), 177 (13), 165 (19), 150 (14), 149 (97), 137 (19), 85 (28), 83 (39), 71 (21), 57 (34), 44 (21), 43 (19).

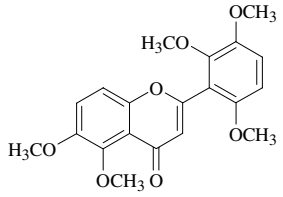
3.6. Methoxy-8-(3″-hydroxymethyl-but-2-enyloxy)-psoralen (*13*)

Colourless amorphous solid; UV (MeCN–H_2O–MeOH, 41.7:52.0:6.3, HPLC-DAD) λ_{max} nm: 224/246/266/314; ^{1}H NMR ($CDCl_3$): δ 6.27 and 8.11 (each 1H, *d*, J = 10 Hz, H-3 and H-4), 7.61 and 6.98 (each 1H, *d*, J = 3 Hz, H-2′ and H-3′), 5.71 (1H, *t*, J = 7.5 Hz, H-2″), 4.86 (2H, *d*, J = 7.5 Hz, CH_2-1″), 4.24 (2H, *s*, CH_2-5″), 4.16 (3H, *s*, OCH_3-5), 1.85 (3H, *s*, CH_3-4″); ^{13}C NMR ($CDCl_3$): δ 160.7 (C-2), 150.7 (C-5), 145.3

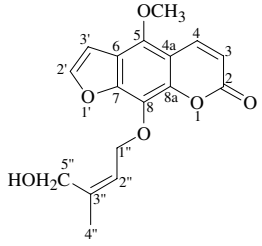
(C-2′), 143.1 (C-8a), 139.7 (C-4), 122.2 (C-2″), 114.6 (C-6), 112.82 (C-3), 107.7 (C-4a), 105.3 (C-3′), 69.3 (C-1″), 61.8 (C-5″), 60.8 (OCH_3-5), 21.5 (C-4″) [C-3′, C-7, C-8′-absent]; EI–MS: *m/z* (intensity%) 233 (85), 232 (100), 217 (98), 189 (32), 161 (20), 133 (10), 105 (11), 95 (9), 77 (14), 63 (9), 53 (8), 43 (28), 41 (13), 39 (13).



(**1**)



(**2**)



(**13**)

Acknowledgements

This research would not have been possible without the collaboration of the healers, midwives and other inhabitants of the communities we worked in, who are the traditional keepers of this knowledge. The botanical identification at CICY and MEXU was performed in collaboration with the numerous specialists of this institution.

We are very grateful to Dr. D. Hunkler (formerly School of Chemistry and Pharmacy, University of Freiburg) for recording the NMR spectra and for useful advice, to Prof. Wollenweber (Darmstadt, DE) for a sample of authentic zapotin and to Mrs. M. Weber for performing the GC–MS experiments.

Financial support from the *Graduiertenförderung Baden-Würtemberg* and the *Bernbeck Stiftung* is gratefully acknowledged.

References

Ankli, A., Sticher, O., Heinrich, M., 1999. Medical ethnobotany of the Yucatec Maya: Healers' consensus as a quantitative criterion. Economic Botany 53, 144–160.

Domínguez, X.A., Villegas, D., 1976. Estructura de la Cerrosillina B (5,6,3′,4′,5′-pentamethoxyflavona) aislada de las hojas del Chapote Amarillo (*Sargentia greggii*). Revista Latinoamericana de Quimica 7, 45–46.

Dreyer, D.L., 1968. *Citrus* bitter principles. IX. Extractives of *Casimiroa edulis* Llave et Lex. The structure of zapoterin. Journal of Organic Chemistry 33, 3577–3582.

Dreyer, D.L., Bertelli, D.J., 1967. The structure of zapotin. Tetrahedron 23, 4607–4612.

Heinrich, M., 1998. Plants as antidiarrhoeals in medicine and diet. In: Prendergast, H.D.V., Etkin, N.L., Harris, D.R., Houghton, P.J. (Eds.), Plants for Food and Medicine, Proceedings from a Joint Meeting of the Society for Economic Botany and the International Society for Ethnopharmacology, London 1–6 July 1996. Royal Botanic Gardens, Kew, UK, pp. 17–30.

Heinrich, M., 2003. Ethnobotany and natural products: the search for new molecules, new treatments of old diseases or a better understanding of indigenous cultures?. Current Topics in Medicinal Chemistry 3, 29–42.

Heneka, B., 2002. Isolierung gastrointestinal wirksamer Inhaltsstoffe aus *Casimiroa tetrameria* Millsp., einer yukatekischen Arzneipflanze der Maya (México). PhD thesis. University of Freiburg, Fak. f. Biologie (Available from <http://www.freidoc.uni-freiburg.de/volltexte/442>).

Heinrich, M., Heneka, B., Ankli, A., Rimpler, H., Sticher, O., 2005. Antisecretory and antispasmotic activity of polymethoxylated flavonoids from *Casimiroa tetrameria*. Submitted for publication.

Leonti, M., Sticher, O., Heinrich, M., 2003. Antiquity of medicinal plant usage in two macro-Mayan groups. Journal of Ethnopharmacology 88, 119–124.

Martínez, M., 1951. Las Casimiroas de México. Anales del Instituto de Biología [México, D.F.] 22, 25–81.

Meyer, B.N., Wall, M.E., Wani, M.C., Taylor, H.L., 1985. Plant antitumor agents. 21. Flavones, coumarins, and an alkaloid from *Sargentia greggii*. Journal of Natural Products 48, 952–956.

Parker, W.H., Bohm, B.A., 1975. Flavonol glycosides of *Limnanthes douglasii*. Phytochemistry 14, 553–555.

Parveen, M., Khan, NU-D., 1987. Two new flavones from *Ardisia floribunda* wall in Roxb. Indian Journal of Chemistry 26B, 894–895.

Razdan, T.K., Qadri, B., Harkar, S., Waight, E.S., 1987. Chromones and coumarins from *Skimmia laureola*. Phytochemistry 26, 2063–2069.

Stavri, M., Mathew, K.T., Bucar, F., Gibbons, S., 2003. Pangelin, an antimycobacterial coumarin from *Ducrosia anethifolia*. Planta Medica 69, 956–959.

Strack, D., Heilemann, J., Wray, V., Dirks, H., 1989. Structures and accumulation patterns of soluble and insoluble phenolics from Norway spruce needles. Phytochemistry 28, 2071–2078.