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Article

Flavanones from *Vernonia diffusa**

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Isolaram-se do extrato metanólico da madeira de *Vernonia diffusa* duas flavanonas: hesperidina e a nova flavanona, 3'-metilhesperetina (homoesperetina) e sacarose que foi identificada como derivado acetilado. A homoesperetina foi identificada como a aglicona obtida da hidrólise do novo glicosidio natural 7-rutinosilhomoesperetina. Do extrato em diclorometano foi identificada a mistura de sistosterol e estigmasterol. Foi preparado o novo derivado octaacetilhesperidina. As determinações estruturais foram realizadas através da análise dos dados espectrométricos de RMN de ^{1}H e ^{13}C incluindo experiências de DEPT, ^{1}Hx^{1}H-COSY, ^{1}Hx^{13}C-COSY e NOEDIFF.

From the methanolic extract of the wood of *Vernonia diffusa* two flavanones were isolated and identified: hesperidin and a new flavanone, 3'-methylhesperetin (homoesperetin) and sucrose, which was identified as its acetyl derivative. The homoesperetin was identified as the aglicone obtained in the hydrolysis of the new natural flavanone glycoside, homoesperetin-7-O-rutinoside. From the dichloromethane extract, a mixture of sitosterol and stigmasterol was isolated together with a mixture of aliphatic acids. The new octaacetylhesperidin derivative was also prepared. Structural determination was made by ^{1}H and ^{13}C-NMR spectrometric data including DEPT, ^{1}Hx^{1}H-COSY, and ^{1}Hx^{13}C-COSY and NOEDIFF techniques.

Keywords: Vernonia diffusa, *compositae, homoesperetin, flavanones*

Introduction

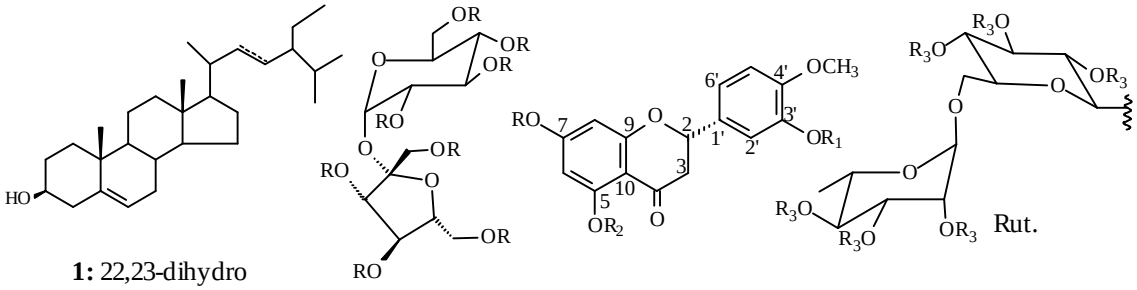
Vernonia diffusa Less belongs to the Vernoneae tribe which has many endemic genera occurring in Brazil. Some of them are used as adornment, medicinal or as pasture's bee. This species is a tree widely distributed in the plains and in the Serra do Mar's forest in São Paulo, Rio de Janeiro, Paraná and Santa Catarina.

A review on the previous phytochemical investigations of this *genus* revealed the study concerned 138 *Vernonia* species among which 38 are from Brazil. The chemical constituents found are triterpenes, steroids, and lignoids, but the more frequent compounds are sesquiterpenoid lactones and flavonoids[1]. These two classes of compounds have been used as taxonomic markers of this genus. The literature has presented many publications of pharmacological activities of sesquiterpene lactones and flavonoids which have been isolated from *Vernonia* species[1,2,3]. In this first chemical investigation of *V. diffusa* we report the isolation and identification of two flavanones besides sitosterol, stigmasterol and sucrose. Eriodictiol is the only flavanone isolated from *Vernonia* species so far (*V. hindei*)[4].

Results and Discussion

Our phytochemical investigation of *V. diffusa* by chromatographic fractionation of the wood's dichloromethane and methanol extracts yielded a mixture of sitosterol (**1**) and stigmasterol (**2**), sucrose (**3**), the known flavanone hesperidin (**4**) and two new flavanones, 3'-methylhesperetin (**7**), named homoesperetin and its 7-O-rutinoside (**5**).

The mixture of compounds **1** and **2** was recognized by analysis of the ^{1}H and ^{13}C-NMR spectral data and comparison with the literature[5]. The relative percentage of **1** (45%) and **2** (55%) was deduced from ^{1}H-nmr integrals of the signals registered for H-22/23 (in **2**) and H-6 (in **1** + **2**).



1: 22,23-dihydro

2: $\Delta^{22,23}$

3: R=H(sucrose)
3a: R=Ac

4: R=Rut, R_1=R_2=R_3=H (hesperidin)
4a: R=Rut, R_1=R_2=R_3=Ac
5: R=Rut, R_2=R_3=H, R_1=Me
6: R=R_1=R_2=H (hesperetin)
7: R=R_2=H, R_1=Me (homoesperetin)

The carbohydrate **3** was identified by comparative analysis with the literature data described for peracetylated sucrose (**3a**)[6,7].

Glycoside **4** was identified by analysis of its spectroscopic data [^{1}H-NMR and ^{13}C-NMR including 2D NMR experiments ^{1}Hx^{1}H-COSY and ^{1}Hx^{13}C-COSY, $^nJ_{CH}$ (n = 1, 2 and 3, COLOC)] and comparison with those previously reported for the hesperidin (hesperetin-7-rutinoside, **4**)[8,9]. The hydrolysis and comparison with authentic samples using the same procedure described in the literature[10] (see experimental) were used to identify the carbohydrates L-rhamnose and D-glucose of the rutinoside unit. The connection (1''' → 6'') of this moiety was confirmed by the chemical shift of the CH_2-6'' (δ_C 66.4 ppm). The presence of a OCH_3 group at 4' position was confirmed by irradiation at δ_H 3.89 (s, 3H) of **4a** which resulted in a 9% NOE at H-5'doublet (δ_H 6.92, d, 8.0 Hz,) and by the cross peak of OCH_3 (δ_H 3.89) and H-5'(δ_H 6.92) with C-4'(δ_C 140.0, $^{2,3}J_{CH}$) in the ^{1}Hx^{13}C-COSY spectra. The same spectrum shows a cross peak of H-2' [δ_H 7.12 (s)] with δ_C 121.0 (C-2', $^1J_{CH}$) and 161.2 (C-3', $^2J_{CH}$) which were used to confirm the location of the hydroxyl group at C-3'. The ^{1}H and ^{13}C-NMR data of the new octaacetyl derivative **4a** are cited in the experimental part.

Spectral and TLC analysis of the hydrolysis products of a crystalline fraction (**VDM-2**) allowed us to identify the aglycone as a flavanone similar to **6** but with two methoxy groups [δ_H 3.89 and 3.81(s, 3H)], 3'-methylhesperetin (**7**). The identification of this flavanone was done by comparison of ^{1}H and ^{13}C-NMR data with those of hesperetin (**6**) and hesperidin (**4**). The presence of the methoxy groups at C-3'and C-4' in **7** was confirmed by an upfield shift of the signals ($\delta_{C\text{-}2'}$ 110.6 and $\delta_{C\text{-}5'}$ 112.6), when compared with that of the same carbon of **4** and **6** ($\delta_{C\text{-}2'}$ 114.4 and $\delta_{C\text{-}5'}$ 112.0). This can be attributed to the γ-effect of the methyl group of the methoxy function at C-3'. Analysis of the aqueous fraction from the hydrolysis by thin layer chromatography (TLC) allowed the identification of the carbohydrates glucose and rhamnose by comparison with authentic samples using the literature methodology[10]. The analysis of ^{1}H-NMR and ^{13}C-NMR spectra data of **VDM-2** led to identification of the signals of **4** and the additional chemical shifts similar to those of **7**. This observation led us to propose the presence of homoesperetin-7-O-rutinoside (**5**), which is the new natural substance that yields **7** upon hydrolysis of VDM-2. The value δ_{CH2} 66.14 for CH_2-OR in the ^{13}C-NMR spectra (PND and DEPT) led us to discard the possibility of two monoglycosylflavanones and to confirm the connection 1''' → 6'' in the rutinoside moiety of **5**.

Experimental

General experimental procedure

Melting points were determined using a kofler hot stage instrument and are uncorrected; NMR spectra were measured in DMSO-d_6, D_3CCOCD_3, or $CDCl_3$ using TMS as the internal standard, employing a Bruker AC-200 (^{1}H: 200 MHz, ^{13}C: 50.3 MHz); C.C. was run with Silica gel S (Riedel, 0.0032-0.0063 mm); TLC was performed on Silica gel 60 F 254 (Merck).

Plant material

Vernonia diffusa Less, Compositae, was collected in Barra do Piraí, RJ, and authenticated by Dr[a] Marilena de Menezes Silva Conde (IB-UFRRJ). A voucher specimen is available for inspection in the herbarium of Instituto de Biologia, UFRRJ-RJ, Brazil.

Extraction and isolation

The powdered wood (4.0 kg) was extracted by maceration with CH_2Cl_2 and methanol. The solvent was removed

under vacuum to yield 9.1 g of CH_2Cl_2 and 45.02 g of methanolic residue. The CH_2Cl_2 residue (2.0 g) was fractionated in C.C. of Silica gel with dichloromethane as solvent, gradually enriched with MeOH, to afford 200 fractions of 20 mL. The fractions 80-120 were crystallized from dichloromethane/hexane (1:1) to yield a mixture of **1** and **2** (190 mg). The methanolic residue (20.0 g) was precipitated from MeOH/AcOEt (1:1) to yield a solid (400 mg, mp 261°), named **VDM-1**, that was identified as **4**. The soluble part was fractionated in florisil with EtOAc and MeOH. EtOAc fraction yielded KCl (81.0 mg) and a mixture of aliphatic acids. The MeOH fraction was fractionated in a column of silica gel in AcOEt/MeOH (7:3) increasing the polarity to neat MeOH. This procedure afforded 69 fractions. Fractions 5-30 yielded an amorphous material (200 mg), named **VDM-2** whose ^{1}H and ^{13}C-NMR spectra revealed a mixture of glycoside similar to **4**. The analysis of the acid hydrolysis products[10] led us to identify the flavanone homoesperetin (**7**) in the chloroform fraction and the carbohydrates glucose and rhamnose in the aqueous fraction which is in agreement with **5** as natural substance in **VDM-2**. Fractions 35-64 (160.0 mg) yielded impure **4** and fractions 65-69 (45.0 mg) were acetylated with Ac_2O and pyridine. The solution was kept at room temp for 24 h and usual work-up gave a mixture of acetates (45.0 mg). This mixture was fractionated on a silica gel column (AcOEt : MeOH, 7:3) to yield **4a** (10 mg) and **3a** + **4a** (30 mg). The same procedure was done to prepare the acetyl derivatives **4a** (80.0 mg, gum) from **4** (100.0 mg).

*Acid hydrolysis of **VDM-2***

VDM-2 (100 mg) was dissolved in 10 mL of MeOH-H_2O (1:1) with concentrated HCl (1.0 mL) and the solution was kept under reflux for 3 hr. The mixture was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure, filtered in silica gel, to yield **7** (30.0 mg). The H_2O of the solution was evaporated to dryness under reduced pressure after addition of acetone. The sugars were identified as D-glucose and L-rhamnose by comparison with authentic samples by thin layer chromatography (TLC) using silica gel S (Riedel) impregnated with 5% of NaOAc as adsorbent and EtOAc-isoPrOH-H_2O (35:39:26) as eluent. Spots were visualized by spraying with a freshly prepared solution of diphenylamine (4% in EtOH), aniline (4% in EtOH) and concentrated phosphoric acid (5:4:1), after heating for 10 min.

Octaacetylhesperidin (**4a**): gum, IR (film, ν_{max}^{NaCl}) cm^{-1}: 2970, 2860, 1740, 1650, 1607, 1500, 1450, 1380; 1240, 1050; ^{1}H-NMR ($CDCL_3$, δ): 7.22 (br d, 8.0 Hz, H-6'), 7.10 (br s, H-2'), 6.92 (d, 8.0Hz, H-5'), 6.40 (d, 2.2Hz, H-8), 6.24 (d, 2.2 Hz, H-6), 5.34 (dd, 10.0; 3.1 Hz, H-2), 5.22 (m, H-3"), 5.18 (m, H-1", 3"', 4"'), 5.12 (m, H-4"), 5.10 (m, H-2"'), 4.95 (t, 9.8Hz, H-2"), 4.59 (br s, H-1"'), 3.9 (s, OCH3), 3.8-3.9 (m, H-5", 5"'), 3.6 (m, H-6"), 2.92 (dd, 18.6; 10.0Hz, H-3ax), 2.6 (dd, 18.6; 3.1Hz, H-3-eq), 1.9-2.1, 2.26, 2.30 (s, $\underline{H}_3C$-CO), 1.1 (d, 6.0Hz, H-6'"); ^{13}C-NMR($CDCl_3$, δ): 188.5(C-4), 179.0-169.0 (O-$\underline{C}$=O), 163.5 (C-7), 161.8 (C-5), 161.2 (C-3'), 151.8 (C-9), 140.0 (C-4'), 130.7 (C-1'), 124.7 (C-6'), 121.0 (C-2'), 109.5 (C-10), 105.8 (C-6), 102.1 (C-8), 97.9 (C-1"'), 97.5 (C-1"), 73.2 (C-5"), 72.3 (C-3"), 70.8 (C-2"'and C-4"'), 69.2 (C-2"0, 68,9 (C-3"'), 68.6 (C-4"), 66.5 (C-5"'), 66.4 (C-6"), 55.8 (OCH_3), 20.1-20.4 ($\underline{C}H_3$-CO), 17.5 (C-6"').

Homoesperetin-7-O-rutinoside (**5**): (mixture with **4**), ^{1}H-NMR (DMSO-d_6, δ): 12.3(HO-5), 6.92 (br s, H-6',5'), 6.95 (s, H-2'), 6.10 (br s, H-6, 8), 5.50 (dd, 10.0; 3.10 Hz, H-2), 3.1-3.6 (m, H-C-O), 4.90 (d, 6.0 Hz, H-1"), 4.60 (br s, H-1"'), 3.82, 3.80 (s, OCH_3), 2.70-3.20 (m, 2xH-3), 1.07 (d, 6.0Hz, H-6'"); ^{13}C-NMR($CDCl_3$, δ): 197.1(C-4), 165.2 (C-7), 162.6 (C-5), 163.1 (C-9), 147.0 (C-3'), 146.0 (C-4'), 130.0(C-1'), 118.0 (C-6'), 112.0(C-5'), 110.5 (C-2'), 96.4 (C-6), 95.7 (C-8), 100.0 (C-1"'), 99.5 (C-1"), 78.6 (C-3"), 76.3 (C-5"), 73.0(C-4"'), 70.4 (C-2"'), 70.3 (C-3"'), 69.6 (C-4"), 69.2 (C-2"), 68.4 (C-5"'), 66.1 (C-6"), 55.8 (2xOCH_3), 17.9 (C-6"').

Homohesperetin (**7**): gum; IR (film, ν_{max}^{NaCl}) cm^{-1}:3476 2917, 1648, 1607, 1521, 1277, 1205;^{1}H-NMR (DMSO-d_6, δ): 12.2 (br s, HO-5), 7.9 (br s, 7-O-H), 6.96 (br d, 8.0 Hz, H-2', H-5', 6'), 5.90 (br s, H-6, 8), 5.40 (br d, 12.0; H-2), 3.81 (s, OCH_3), 3.84 (s, OCH_3), 3.06 (dd, 14.0; 12.0Hz, H-3ax), 2.76 (dd, 14.0; 3.0 Hz, H-3-eq). ^{13}C-NMR (DMSO-d_6, δ): 195.9 (C-4), 165.6 (C-7), 163.0 (C-5), 163.5 (C-9), 146.9 (C-3'), 145.7 (C-4'), 130.0 (C-1'), 118.2 (C-6'), 112.7 (C-5'), 110.6 (C-2'), 103.7 (C-10), 95.6 (C-6), 96.7 (C-8), 55.8 and 55.9 (OCH_3). EIMS *m/z* (rel. Int.): 316(37, M^{+}), 191(25), 164(100), 152(22), 151(80), 137(30), 124(20).

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