J. Braz. Chem. Soc., Vol. 16, No. 6B, 1454-1457, 2005.
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0103 - 5053 $6.00+0.00

Short Report

Diploflavone, a New Flavonoid from *Diplotropis ferruginea* Benth. (Fabaceae)

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A análise química de *Diplotropis ferruginea* Benth. resultou no isolamento da 3-metoxiflavona, 3-metoxi-6-*O*-prenil-6",6"-dimetilcromeno-(7,8,2",3")-flavona, à qual foi dado o nome trivial de diploflavona **(1)**, bem como da 3,6-dimetoxi-6",6"-dimetilcromeno-(7,8,2",3")-flavona **(2)**. A estrutura do novo composto foi estabelecida por análises espectrais. A atividade citotóxica dos compostos isolados foi testada contra células NCI-H292 (carcinoma de pulmão), HEp-2 (carcinoma de laringe) e KB (carcinoma epidermóide oral). As células HEp-2 foram as mais afetadas pelas substâncias testadas.

The chemical examination of *Diplotropis ferruginea* Benth. resulted in the isolation of a new 3-methoxyflavone, 3-methoxy-6-*O*-prenyl-6",6"-dimethylchromene-(7,8,2",3")-flavone, to which was given the trivial name diploflavone (**1**); as well as the known 3,6-dimethoxy-6",6"-dimethylchromene-(7,8,2",3")-flavone (**2**). The structure of the new compound was established by spectral analyses. Cytotoxic activity of the isolated compounds was tested against the cells NCI-H292 (lung carcinoma), HEp-2 (larynx carcinoma) and KB (oral epidermoid carcinoma). The cells HEp-2 were the most affected by the substances tested.

Keywords: *Diplotropis ferruginea*, Fabaceae, flavonoids, cytotoxicity

Introduction

The Fabaceae have a cosmopolitan distribution, consisting of *ca* 700 genera and more than 17000 species.[1] The genus, *Diplotropis* consists of approximately 22 species, including, *Diplotropis ferruginea* Benth. Investigations of only two species have been reported in the literature: the isolation of quinolizidine alkaloids from *Diplotropis martiusii*,[2] and flavonoids from *Diplotropis purpurea*.[3]

Diplotropis ferruginea is a tree native to Northeastern Brazil, where it is popularly known as "sucupira". It is used in folk medicine for the treatment of rheumatism, arthritis and diabetes.[4] Recently, a chemical investigation of this species resulted in the isolation of lupeol, ethyl 2-hydroxy-4-methoxy-6-propyl benzoate[5] and of the flavonoid 3,4,5,8-tetramethoxy-6,7,2",3"-furanoflavan.[6] Spasmolytic activity was reported for the crude EtOH extract of this plant.[7]

This paper describes the isolation of two more flavonoids, whose structures were established by spectroscopic techniques, mainly EIMS and 1D and 2D NMR.

Experimental

General experimental procedures

Melting points were determined on a REICHERT, model R3279 "Kofler" apparatus, and are uncorrected. IR spectra were obtained in KBr on a BOMEM model MB 100

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spectrophotometer. ^{1}H and ^{13}C NMR spectra were run on a Jeol Eclipse+ 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, using $CDCl_3$ as solvent (approximately 10 mg of sample were dissolved in 0.5 mL of solvent and transferred into a 5 mm NMR tube) and solvent signals were used as internal reference for the chemical shifts δ_H 7.26 ($CHCl_3$) and δ_C 77.00 ($CDCl_3$). The one-dimensional (1D) ^{1}H and ^{13}C NMR spectra were acquired under standard conditions (5 mm multinuclear probe). The two-dimensional (2D) experiments were acquired and processed with the Delta software provided by Jeol. Standard pulse sequences were used for all experiments. ^{1}H-^{1}H-COSY spectra were obtained with X-points 512/Y-points 256, X-resolution 11.7 Hz/Y-resolution 23.4 Hz, X-acquisition time 85.4 ms/Y-acquisition time 42.7 ms, pulse 90°, relaxation delay 1.5 s, zerofill: 4. For homonuclear 2D ^{1}H-^{1}H-NOESY experiments were used mixing time 0.5 s, X-points 512/Y-points 256, X-resolution 11. 7Hz/Y-resolution 23.4 Hz, X-acquisition time 85.4 ms/Y-acquisition time 42.7 ms, pulse 90° relaxation delay 1.5 s, zerofill: 4. Two-dimensional inverse hydrogen detected heteronuclear shift correlation ^{1}H-^{13}C-HMQC-$^{1}J_{CH}$ spectra were obtained with $^{1}J_{CH}$ = 140 Hz, X-points 1024/Y-points 128, X-resolution 5.86 Hz/Y-resolution 196 Hz, X-pulse 90°/Y-pulse 90°, X-acquisition time 0.17 s/Y-acquisition time 5.09 ms, pulse 90°, relaxation delay 2.0 s, gradient 1/3 1 ms square, zerofill: 4. Two-dimensional inverse hydrogen detected heteronuclear long-range correlation ^{1}H-^{13}C-HMBC-$^{n}J_{CH}$ (n=2 and 3) experiments were carried out by using $^{n}J_{CH}$ = J constant 140 Hz/J long range 8 Hz, X-points 1024/Y-points 128, X-resolution 5.86 Hz/Y-resolution 196 Hz, X-pulse 90°/Y-pulse 90°, X-acquisition time 0.17 s/Y-acquisition time 5.09 ms, pulse 90°, relaxation delay 2.0 s, gradient 1/3 1 ms square, zerofill: 4. EIMS were measured at 70 eV on a GC/MS System Shimadzu QP-5050.

Plant material

The stem bark of *Diplotropis ferruginea* was collected in the municipality of Caraúbas, State of Rio Grande do Norte, Northeastern Brazil in May 2002. Botanic material was identified by Prof. Maria de Fátima Agra, of the Laboratório de Tecnologia Farmacêutica. A voucher specimen (AGRA & D. ALMEIDA 5559) is deposited at the Herbario Prof. Lauro Pires Xavier (JPB), of the Universidade Federal da Paraíba.

Extraction and isolation

The dried and powdered stem bark of *D. ferruginea* (3 kg) was exhaustively extracted with 95% EtOH at room temperature. The extract was concentrated under vacuum yielding 95 g of the crude product. This was suspended in a MeOH:H_2O (3:7 v/v) mixture and partitioned with hexane, $CHCl_3$ and EtOAc. The hexane fraction was then subjected to silica gel column chromatography and eluted with hexane, $CHCl_3$ and MeOH in an increasing polarity gradient to give 152 fractions. The fractions were monitored by TLC and classified into 25 groups. Fraction 97-102 was purified by preparative TLC over silica gel using $CHCl_3$:MeOH (9:1) to afford flavonoid **1** (61 mg) and the fraction 89-96 was purified in the same way using hexane:EtOAc (2:1) to afford flavonoid **2** (123 mg).

Biological assay

The cytotoxic activity assays were based on the methylazoetetrazolium (MTT) method or the 3-(4,5-dimethylazol-2-yl)-3,5-diphenyltetrazolium bromide method.[8] For the evaluation of cytotoxity the cellular strain HEp2 (larynx carcinoma) NCIH-292 (lung carcinoma) KB (mouth carcinoma)[9] with proven viability were used. The cells were grown in MEM- Minimal Essential Medium[10] with 10% bovine fetal serum containing 1% antibiotics solution (penicillin 1000 UI mL^{-1} + streptomycin 250 mg mL^{-1}) and 1% glutamine (200 μM). A cellular suspension of 5×10^4 cells mL^{-1} was used and distributed in plates of 96 wells. The test samples of 0.15 mL were added into each well. The plates were incubated for 72 h at 37 °C in a humid atmosphere enriched with 5% CO_2. After incubation 15 mL MTT in phosphate buffered saline (BPS) solution at (5 mg mL^{-1}) was added into each well. After 2h the culture medium was removed and 100 μL of DMSO were added in each well for quantitation of blue formazan. The readings were performed with the aid of a Multskan ELX 800 cell reader (Bio-Tec Instruments – USA) at 540 nm.

*3-Methoxy-6-O-prenyl-6",6"-dimethylchromene-(7,8,2",3")-flavone or diploflavone, (**1**)*

It was obtained as amorphous powder, mp 163-165 °C. IR (KBr) ν_{max}/cm^{-1}: 3062, 2971, 2847, 1620, 1404, 1379, 1300, 1100. EI-MS: *m/z* (%): 418 (8, [M^+]), 364 (19), 349 (100, [M^+ - prenyl]), 335 (38) (Calc. for $C_{26}H_{26}O_5$). ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) (Table 1).

*3,6-Dimethoxy-6",6"-dimethylchromene-(7,8,2",3")-flavone, (**2**)*

It was obtained as amorphous powder, mp 203-204 °C. IR (KBr) ν_{max}/cm^{-1}: 2995, 2844, 1615, 1402, 1382, 1300, 1100. EI-MS: *m/z* (%): 364 (63, [M^+]), 349 (100,

[M^+ - CH_3]), 319 (4) (Calc. for $C_{22}H_{20}O_5$). ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz).

Results and Discussion

Flavonoid **1** was obtained as a colorless amorphous solid. Its molecular formula was deduced as $C_{26}H_{26}O_5$ (14 degrees of unsaturation), supported by the occurrence of the molecular ion at *m/z* 418 in the MS, in combination with ^{1}H and ^{13}C-APT-NMR spectral data. The IR spectrum showed absorptions at 1620 cm^{-1}, attributed to an α-β unsaturated carbonyl group; 3062 cm^{-1} attributed to unsaturated C-H and absorptions in the region 1379-1404 cm^{-1}, suggesting the presence of a *gem*-dimethyl group. ^{1}H NMR of **1** showed signals at δ_H 8.07 (2H, br, d J = 7.7 Hz) and 7.56-7.46 (3H, m) which indicates the possibility of a mono-substituted ring B in a flavonoid. The presence of a 2,2-dimethylchromene moiety was indicated by the characteristic signals of its two vinyl hydrogens forming an AB system[11] at δ_H 5.74 (1H, d, J = 9.9 Hz) and 6.87 (1H, d, J = 9.9 Hz) and a signal at δ_H 1.54 (6H, s) attributed to the two methyl groups. A signal at δ_H 1.79 (6H, s) was also observed and signals at δ_H 4.68 (1H, d, J = 6.2 Hz) and 5.53 (1H, t, J = 6.2 Hz), suggesting the presence of a prenyl group in the molecule. This suggestion is confirmed by the ^{13}C-APT NMR spectra which shows signals at δ_C 18.25 and 25.72, for 2 methyl carbons and a methylene carbon at δ_C 66.29. The chemical shift of the methylene carbon in the ^{13}C NMR indicates that the prenyl group is bound to an oxygen atom. The HMBC experiment showed the location of the *O*-prenyl group at C-6, due to the $^3J_{CH}$ correlation between the signal at δ_H 4.68 (prenyl's methylene hydrogens) with the signal at δ_C 146.06 (C-6). The analysis of all the spectral data for **1** led to the elucidation of its structure as 3-methoxy-6-*O*-prenyl-6",6"-dimethylchromene-(7,8,2",3")-flavone. This substance is described here for the first time and was given the trivial name diploflavone.

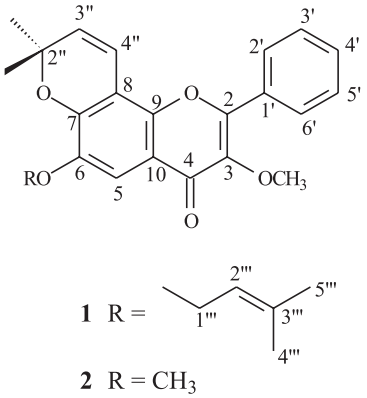

Figure 1. Flavonoids from *Diplotropis ferruginea.*

Flavonoid **2** was isolated as a colorless amorphous solid. Its molecular formula deduced as $C_{22}H_{20}O_5$ (13 degrees of unsaturation), was confirmed by the molecular ion at *m/z* 364 in the MS in combination with ^{1}H-NMR (1D and 2D ^{1}H-^{1}H-COSY) and ^{13}C-APT-NMR spectral data. IR and ^{1}H and ^{13}C-NMR spectra showed the similarity with substance **1**. The only difference between the two substances was the absence of the prenyl moiety in **2**, having a methoxy in the same position. The presence of the methoxy group was indicated by the signal at δ_H 3.97 (3H, s). The substance was thus characterized as the flavonoid 3,6-dimethoxy-6",6"-dimethylchromene-(7,8,2",3")-flavone (**2**), previously isolated from *Bowdichia virgilioides* and the NMR data are in accordance with the literature. [12]

The 2D experiments HMQC and HMBC were used to confirm the ^{1}H and ^{13}C chemical shifts of **1** (Table 1) and **2**.[12]

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for **1** including results obtained by heteronuclear 2D shift-correlated HMQC and HMBC spectra, in $CDCl_3$ as solvent and TMS as internal reference. Chemical shifts in δ (ppm) and coupling constants (J, in parenthesis) in Hz*

Carbon	^{1}H-^{13}C-COSY-$^1J_{CH}$		^{1}H-^{13}C-COSY-$^nJ_{CH}$	
	δ_C	δ_H	$^2J_{CH}$	$^3J_{CH}$ e $^4J_{CH}$
C				
2	154.31	-		H-2'/H-6'
3	140.96	-		MeO-3
4	174.21	-		H-5
6	146.06	-		2H-1'"
7	147.93	-		H-5; H-4"
8	110.16	-	H-4"	H-3"
9	146.15	-		H-5; H-4"
10	117.19	-	H-5	
1'	131.29	-	H-2'/H-6'	H-3'/H-5'
2"	77.91	-	3H-5"/3H-6"	H-4"
3'"	137.78	-	3H-4'"/3H-5'"	2H-1'"
CH				
5	106.5	7.52 (s)		
2',6'	128.1	8.07 (br, d, 7.7)		
3',5'	128.44	7.56-7.46 (m)		
4'	131.24	7.56-7.46 (m)		H-2'/H-6'
4"	115.32	6.87 (d, 9.9)		
3"	131.27	5.74 (d, 9.9)		3H-5"/3H-6"
2'"	119.48	5.53 (t, 6.2)	2H-1'"	3H-4'"/3H-5'"
CH_2				
1'"	66.29	4.68 (d, 6.2)		
CH_3				
MeO-3	60.02	3.89 (s)		
MeO-6	-	-	-	-
5",6"	27.83	1.54 (s)		H-3"
4'"	18.25	1.79 (s)		H-2'"
5'"	25.72	1.79 (s)		H-2'"

* Homonuclear ^{1}H-^{1}H-COSY spectra were also used for these assignments. Chemical shifts of hydrogen atoms obtained from 1D ^{1}H NMR spectrum. Carbon atoms corresponding to C, CH, CH_2 and CH_3 deduced by comparative analysis of {^{1}H}- and APT-^{13}C spectra.

Table 2. Cytotoxic activity of **1** and **2** against the cells KB, NCI-H 292 and HEp-2

	Cell strain					
	KB		NCI-H - 292		HEp-2	
Compound	Concentration (μg mL^{-1})	% Inhibition	Concentration (μg mL^{-1})	% Inhibition	Concentration (μg mL^{-1})	% Inhibition
1	10.0	26.2	10.0	29.1	10.0	41.0
	5.0	21.2	5.0	26.7	5.0	24.8
	2.5	19.0	2.5	9.8	2.5	3.4
	1.25	18.0	1.25	11.0	1.25	3.4
2	10.5	18%	10.0	10.0	10.0	21.0
	5.0	16%	5.0	9.2	5.0	17.9
	2.5	15%	2.5	3.8	2.5	13.8
	1.25	15%	1.25	3.8	1.25	8.8

KB (oral epidermoid carcinoma), NCI-H - 292 (lung carcinoma), HEp-2 (larynx carcinoma).

The cytotoxic activity of cells NCI-H292 and KB were not affected by flavonoids **1** and **2**, however, cells HEp-2 were affected by diploflavone (**1**). At the concentration of 10 μg mL^{-1} it showed an inhibition of proliferation of 41% (Table 2).

Acknowledgements

The authors are grateful to the Instituto do Milênio do Semi-Árido (IMSEAR/CNPq), CAPES and FAPERJ by financial support. Sincere thanks are also due to NAPRALERT.

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Received: January 13, 2005
Published on the web: October 31, 2005