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Pyranochromones from *Dictyoloma vandellianum* A. Juss and Their Cytotoxic Evaluation

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One new chromone 3,3-dimethylallylspatheliachromene methyl ether (**1**), as well as five known chromones, 6-(3-methylbut-2-enyl) allopteroxylin methyl ether (**2**), 6-(3-methylbut-2-enyl) allopteroxylin (**3**), 3,3-dimethylallylspatheliachromene (**4**), 5-*O*-methylcneorumchromone K (**5**) and spatheliabischromene (**6**), two alkaloids, 8-methoxy-*N*-methylflindersine (**7**) and 8-methoxyflindersine (**8**), and two limonoids, limonin diosphenol (**9**) and rutaevin (**10**), were isolated from *Dictyoloma vandellianum* A. Juss (Rutaceae). Cytotoxic activities towards tumor cell lines B16-F10, HepG2, K562 and HL60 and non-tumor cells PBMC were evaluated for compounds **1** – **6**. Compound **1** was the most active showing IC_{50} values ranging from 6.26 to 14.82 μg/ml in B16-F10 and K562 cell lines, respectively, and presented IC_{50} value of 11.65 μg/ml in PBMC cell line.

Keywords: Chromones, Limonoids, Alkaloids, Cytotoxic activities, *Dictyoloma*, Rutaceae.

Introduction

The search for new anticancer agents from natural resources has led to the discovery of several drugs used in the treatment of tumors, *e.g.* paclitaxel or camptothecin.[1] The main secondary metabolites produced by plants belonging to the Rutaceae family include phenylpropanoids such as coumarin, lignans and flavonoids, terpene limonoids and alkaloids.[2] The genus *Dictyoloma* contains two species, *Dictyoloma peruvianum* Planch., which occurs in Peru and Bolivia, and *Dictyoloma vandellianum* A. Juss., in Brazil.[3] Previous studies have demonstrated the presence of quinolone alkaloids, limonoids and pyranochromones in the leaves,[4] roots[5] and bark[6] of the Brazilian species. Although chromones are found in numerous plant families, their occurrence in the Rutaceae family is restricted to few genus such as *Dictyoloma*, *Spatelia*, *Cneorum* and *Ptaeroxilon*. These oxygenated heterocycles exhibit a wide range of biological activities,[7] including cytotoxic effects.[7 – 11]

The present study describes the isolation and characterization of six pyranochromones, one of them unprecedented, from the root bark of *D. vandellianum*. Two quinolone alkaloids were identified in the root core, and two limonoids were isolated from the flower. The structure of the new compound was established using spectroscopic methods (IR, MS and NMR). Cytotoxic activities towards tumor cell lines and non-tumor cells were investigated for the six pyranochromones.

Results and Discussion

The CH_2Cl_2 extract from root barks yielded one new chromone, 3,3-dimethylallylspatheliachromene methyl ether (**1**), as well as five kown chromones: 6-(3-methylbut-2-enyl) allopteroxylin methyl ether (**2**), 6-(3-methylbut-2-enyl) allopteroxylin (**3**), 3,3-dimethylallylspatheliachromene (**4**), 5-*O*-methylcneorumchromone K (**5**) and spatheliabischromene (**6**; *Fig. 1*).

Comparison of the ^{1}H- and ^{13}C-NMR spectra data to those reported in the literature allowed the identification of chromones **2**,[6] **3**,[6] **4**,[12] **5**[13][14] and **6**.[15]

Chromone **1** was isolated as an amorphous yellow solid and was identified as 3,3-dimethylallylspatheliachromene methyl ether. Spectral data (IR and NMR) revealed the presence of a prenylated pyranochromone at C(8) (*Table 1*). The absorption peak

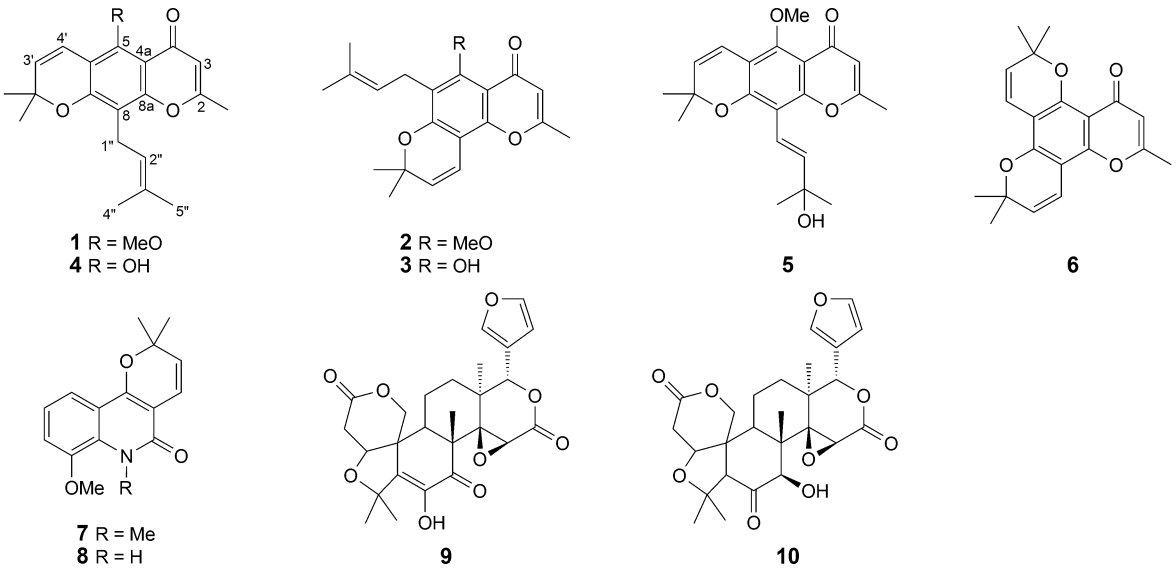


Figure 1. Chemical structures identified in *Dictyoloma vandellianum*.

observed in the IR spectra at ν_{max} 1656 cm^{-1}, characteristic deformation of the α,β unsaturated carbonyl group, along with signals at δ(H) 2.28, 5.96 and δ(C) 162.9, 19.6, 110.8 and 177.1, confirmed the presence of the heterocycle 2-methyl-2,3-dihydro-4-pyrone. Signals of the prenylated group were detected at δ(H) 1.66, 1.79, 3.41, 5.19- 5.14 and at δ(C) 21.6, 121.3, 131.3, 17.6 and 25.4. NMR spectra also showed signals of a fused 2,2-dimethylpyrane at δ(H) 1.43, 5.67, 6.70 and at δ(C) 27.8, 76.3, 116.1 and 130.0. The presence of a methoxy substituent was supported by the signals at δ(H) 3.82 and δ(C) 62.2. The HMBC spectrum revealed correlations between the MeO group (δ(H) 3.82) and C(5) (δ(C) 152.7). The *doublet* at δ(H) 3.41 (H–C(1″)) was correlated with C(8a), C(7) and C(8) (δ(C) 156.0, 154.7, and 112.8, respectively), supporting a linear structure for compound **1**. The other correlations observed in HMBC and HMQC allowed the unambiguous assignment of the H-atoms and carbons of compound **1**. The electron impact mass spectrum showed a molecular ion peak at *m/z* 340 $M^{+\cdot}$, corresponding to the molecular formula $C_{21}H_{24}O_4$, and a base peak at *m/z* 325, produced by the loss of a methyl group (*Scheme 1*).

8-Methoxy-*N*-methylflindersine (**7**) and 8-methoxyflindersine (**8**) were purified from the root core MeOH extract. The two alkaloids were identified based on the comparison of their ^{1}H- and ^{13}C-NMR spectra with the literature values.[4] Alkaloid **8** has been previously isolated from the *D. vandellianum* leaves; the presence of alkaloid **7** in this species is reported in the present work for the first time.

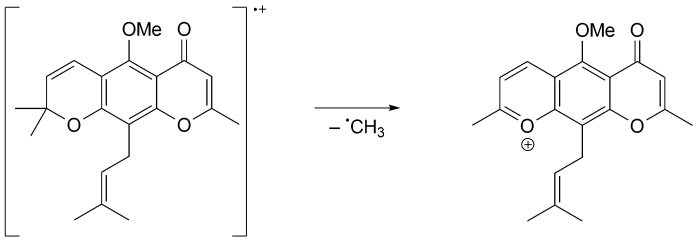
Limonin diosphenol (**9**) and rutaevin (**10**) were isolated from the inflorescences and were characterized by comparison with literature data.[15][16] Although highly oxygenated limonoids carrying a furan ring attached to the *D*-ring are dominant in the Rutaceae family, to the best of our knowledge this is the first report of the isolation of compounds **9** and **10** in *D. vandellianum*.

Table 1. ^{1}H- and ^{13}C-NMR data ($CDCl_3$) for 3,3-dimethylallylspatheliachromene methyl ether (**1**)

Position	δ(H)	δ(C)
2	–	162.9
2-Me	2.28 (*d*, *J* = 0.6)	19.6
3	5.96 (*d*-like, *J* = 0.6)	110.8
4	–	177.1
4a	–	111.5
5	–	152.7
6	–	112.2
7	–	154.7
8	–	112.8
8a	–	156.0
2′	–	76.3
2′-Me	1.43 (*s*)	27.8
3′	5.67 (*d*, *J* = 10.2)	130.0
4′	6.70 (*d*, *J* = 10.2)	116.1
1″	3.41 (*d*, *J* = 7.2)	21.6
2″	5.19 – 5.14 (*m*, *J* = 7.2, 1.2)	121.3
3″		131.3
4″	1.79 (*s*)	17.6
5″	1.66 (*d*, *J* = 1.2)	25.4
MeO	3.82 (*s*)	62.2

δ in ppm, *J* in Hz.

The cytotoxic activity of chromones **1** – **6** was evaluated using the alamar blue assay[17] against B16-F10 (*mouse* melanoma), HepG2 (human hepatocellular



Scheme 1. Formation of base peak.

carcinoma), K562 (human chronic myelocytic leukemia) and HL-60 (human promyelocytic leukemia) tumor cell lines and non-tumor cell PBMC (peripheral blood mononuclear cells; *Table 2*).

All the compounds presented moderate activities against the four tumor cell lines when compared to doxorubicin. The new compound **1** showed the most potent activity towards B16-F10 (IC_{50} = 6.26 μg/ml) and PMBC (IC_{50} = 11.65 μg/ml) and was as efficient as compound **5** against HepG2 (IC_{50} = 9.66 and 9.36 μg/ml, respectively). Compounds **2** and **6** were the most active towards HL-60 (IC_{50} = 9.62 and 9.57 μg/ml, respectively) and compound **5** showed the highest cytotoxic activity against K562 (IC_{50} = 10.01 μg/ml). Compounds **3** and **4** were the less active of the six tested chromones. The cytotoxic activity of compounds **1**, **2**, and **5** was evaluated for the first time in the present work. The cytotoxicity of compounds **4** and **6** was previously assessed in HeLa cells,[8] with moderate activities (ID_{50} = 5 and 10 mg/ml, respectively).

Conclusions

The study of the bark of the roots of *D. vandellianum* allowed the identification of six pyranochromones, one unprecedented. Two alkaloids were isolated from the root core and two limonoids were identified in the inflorescences. The biological activity results showed that the roots of *D. vandellianum* are a source of cytotoxic pyranochromones. The most active compound was 3,3-dimethylallylspatheliachromene methyl ether (**1**), described here for the first time. Although these chromones displayed a moderate cytotoxic activity, they appear as promising leads for anticancer therapy.

Experimental Section

General

All solvents (analytical and high performance liquid chromatography (HPLC) grade) were purchased from *Vetec* (Rio de Janeiro, Brazil), *Quimex* (São Paulo, Brazil), *Qhemis* (São Paulo, Brazil), *Sigma–Aldrich* (São Paulo, Brazil) and *Tedia* (Rio de Janeiro, Brazil) and used without further purification. Thin and preparative layer chromatography (TLC and PLC) were performed on glass plates covered with SiO_2 PF_{254} *Merck 60* and revealed with *Dragendorff* reagent, iodine vapor and/or UV light at 254 and 365 nm. All chromatographic separations were performed on SiO_2 *60* (70 – 230 mesh) from *Merck*, *Sephadex LH-20* (*GE Healthcare*) and functionalized octadecyl (200 – 400 mesh) from *Sigma–Aldrich*. The sizes of the chromatographic columns used were 40 × 4.5 cm, 45 × 5 cm and 15 × 8 cm. IR Spectra: *Bomem* infrared spectrometer mark, *MB100*; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C- (single and two-dimensional) nuclear magnetic resonance (NMR) spectra were obtained on *Varian* spectrometers mark, *Gemini-500* (^1H: 500 MHz and ^{13}C: 125 MHz); *MR-400* (^1H: 400 MHz and ^{13}C: 100 MHz) and *Gemini-300* (^1H: 300 MHz and ^{13}C: 75 MHz) using $CDCl_3$, CD_3OD and

Table 2. Cytotoxic activity for compounds **1** – **6**

Compounds	IC_{50} [μg/ml] ([μM])				
	B16-F10	HepG2	HL-60	K562	PBMC
1	6.26 (18.41)	9.66 (28.41)	10.14 (29.82)	14.82 (43.59)	11.65 (34.27)
2	11.78 (34.65)	11.70 (34.41)	9.62 (28.29)	15.59 (46.15)	> 25 (73.53)
3	17.58 (53.93)	> 25 (76.69)	13.14 (40.31)	> 25 (76.69)	> 25 (76.69)
4	14.79 (45.37)	19.62 (60.18)	13.13 (40.28)	21.14 (64.85)	16.14 (49.51)
5	12.13 (34.07)	9.36 (26.29)	18.41 (51.71)	10.01 (28.12)	> 25 (70.22)
6	13.37 (41.27)	> 25 (77.16)	9.57 (29.54)	> 25 (77.16)	> 25 (77.16)
Doxorubicin	0.05 (0.09)	0.08 (0.15)	0.14 (0.26)	0.40 (0.74)	0.31 (0.57)

C_5D_5N as solvents and Me_4Si as internal standard. A *Shimadzu*® (Kyoto, Japan) High Performance Liquid Chromatography System, coupled with a *micrOTOF II* (*Bruker Daltonics*, Billerica, MA, USA) with an electrospray ion (ESI) source, was used to perform the ESI-TOF-MS analysis. The LC separation was performed at 32 °C on a *Luna* 5 μm PFP (2) 100Å, 250 × 4.6 mm (*Phenomenex Inc*., Ca, USA) analytical column. Injections (20 μl) were performed using an autosampler (SIL-10AF) with a 100 μl loop. The mobile phase consisted of 1% acetic acid in water (solvent *A*) and MeOH (solvent *B*) using an isocratic mixture of *A* (20%) and *B* (80%) for 30 min. The electrospray ion source was operated in positive and negative ionization mode. The spray voltage was set to 4.5 kV and the capillary temp. to 200 °C. Dry nitrogen (8 l/min) was used as nebulizer gas (4 bar). GC/MS: *Perkin Elmer Clarus 500* gas chromatograph equipped with and *Elite 5MS* capillary column (30 m × 0.25 mm × 0.1 μm). The injections of the samples were made in the split mode (split ratio 25:1) at 250 °C. Hydrogen was used as the carrier gas with a flow rate of 1 ml/min.

Plant Material

The specimens were collected in Piatã, Bahia, Brazil (13°14′43″S, 41°45′28″W) in 26/03/2005. The vouchers specimens were identified by Prof. *M. L. S. G.*, and deposited with the Herbarium Alexandre Leal Costa (ALCB), Institute of Biology, Federal University of Bahia with the registration number 69163. The roots and inflorescences were dried at 45 °C for 72 h and milled, furnishing 384.0 g of the root bark, 1590.0 g of root core and 879.0 g of inflorescences.

Extraction and Isolation

The dried ground root bark (384 g) was macerated three times in CH_2Cl_2 (4 l/7 days) and then three times in MeOH (4 l) for the same period. The CH_2Cl_2 extract of the root bark was submitted to flash chromatography on silica gel, eluting the column with petroleum ether (1 l), hexane (2 l), CH_2Cl_2 (1 l), AcOEt (2 l) and MeOH (1 l). Five solvent fractions were collected, concentrated under reduced pressure and analyzed by TLC (hexane/AcOEt 8:2). The CH_2Cl_2 fraction thus obtained was further separated by column chromatography using an elution gradient hexane/AcOEt (100:0; 98:2, 96:4, 92:8, and 90:10; 100 ml of each mixture). Fractions of 10 ml were collected. The analysis of the ^{1}H-NMR spectrum of fraction 10 (2.48 g) indicated the presence of two major substances. The mixture was subjected to column chromatography (hexane/AcOEt 8:2) allowing the isolation of 3,3-dimethylallylspatheliachromene methyl ether (**1**) (439 mg) and spatheliabischromene (**6**) (148 mg). Chromones **3** (31 mg) and **4** (27 mg) were obtained from *Fr. 6* and compound **2** was purified from *Fr. 8*. The AcOEt fraction (3.0 g) of the CH_2Cl_2 extract of the root bark was chromatographed using a gradient elution of $CHCl_3$/MeOH (100:0, 99:1, 95:5, 90:10, and 0:100, 150 ml of each). Thirteen fractions of 50 ml were collected. 5-*O*-methylcneorumchromone K (**5**) (2.1 g) was obtained from *Fr. 11*.

The root core was macerated in hexane (7 l) for 7 days (3 times) and then in MeOH (7 l) for 7 days (3 times). The MeOH extract (30.0 g) was fractioned by flash column chromatography using successively petroleum ether (1 l), hexane (1 l), $CHCl_3$ (2 l), Et_2O (2 l), and MeOH (2 l). Five solvent fractions were collected, concentrated under reduced pressure and analyzed by TLC (hexane/AcOEt 8:2 and $CHCl_3$/MeOH 95:5). The $CHCl_3$ sub-fraction was then fractionated by column chromatography using a gradient mixture of hexane, AcOEt, and $CHCl_3$ as eluents: (9:1:1, 85:15:10, 8:2:1, 75:25:10, 7:3:1, 100 ml of each), collecting fractions of 10 ml. The pure alkaloid 8-methoxy-*N*-methylflindersine (**7**) (278 mg) and a mixture containing 8-methoxyflindersine (**8**) were obtained. Compound **8** was purified by isocratic column chromatography using (hexane/AcOEt 8:2) and collecting fractions of 10 ml, allowing the isolation of 40 mg of the pure compound. The inflorescences were macerated three times in MeOH (6 l) for 7 days. The extract was suspended in water (500 ml), filtrated and extracted successively with $CHCl_3$ (5 × 150 ml), Et_2O (5 × 150 ml), and AcOEt (5 × 150 ml). The solvents were removed under vacuum and the $CHCl_3$ fraction (500 mg) was subjected to fractionation on *Sephadex LH-20* using a gradient of MeOH and CH_2Cl_2 as eluent (100:0, 90:10, 80:20, 70:30, 60:40 and 50:50) collecting 24 fractions of 150 ml. Precipitation spontaneously occurred in *Fr. 5*. The solid was filtered and purified by PLC ($CHCl_3$/MeOH 99:1 plus one drop of pyridine), allowing the isolation of limonin diosfenol (**9**) (18 mg) and rutaevin (**10**) (15 mg).

Cells

B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukemia) and HL-60 (human promyelocytic leukemia) tumor cells lines were kindly donated by Hospital A.C. Camargo, São Paulo, Brazil. Cells were maintained in RPMI-1640 medium (*Gibco-BRL*) with 10% fetal

bovine serum (*Cultilab*), 2 mM L-glutamine (*Vetec Química Fina*) and 50 μg/ml gentamycin (*Novafarma*). Adherent cells were collected by treatment with 0.25% trypsin EDTA solution (*Gibco-BRL*). All cell lines were cultured in flasks at 37 °C in 5% CO_2 and subcultured every 3 – 4 days to maintain exponential growth. All cell lines were tested for mycoplasma using a *Mycoplasma Stain Kit* (*Sigma–Aldrich*) to validate the use of cells free from contamination. Heparinized blood from 20 to 35 year-old, non-smoker healthy donors who had not taken any drugs for at least 15 days prior to sampling was collected, and the peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient in a GE Ficoll-Paque Plus. PBMCs were washed and re-suspended at a concentration of 0.3×10^6 cells/ml in RPMI 1640 medium with 20% fetal bovine serum, 2 mM glutamine, and 50 μg/ml gentamycin at 37 °C with 5% CO_2. Concanavalin A (ConA; *Sigma–Aldrich Co.*) was used as a mitogen to trigger cell division in T-lymphocytes. ConA (10 μg/ml) was added at the beginning of culture, and the cells were treated with the test compounds after 24 h. Cell viability was examined using Trypan blue exclusion assay for all experiments. Over 90% of the cells were viable at the beginning of the culture. The Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (#031019/2013).

Cytotoxic Activity

Cell viability was quantified using the alamar blue assay, according to *Ahmed et al.*[17]. Cells were inserted in 96-well plates (0.7×10^5 cells/ml for adherent cells or 0.3×10^6 cells/ml for suspended cells in 100 μl of medium). After 24 h, the compounds (in a range of concentrations varying from 0.19 to 25 μg/ml) were dissolved in DMSO (*Sigma–Aldrich Co.*) and the solution was added to each well and incubated for 72 h. Doxorubicin (purity ≥ 95.0%, doxorubicin hydrochloride, *Laboratory IMA S.A.I.C.*) was used as a positive control (0.08 – 5 μg/ml). Negative controls received the vehicle that was used for diluting the alkaloids (0.5% DMSO). Four (for cell lines) or 24 (for PBMCs) h before the end of incubation, 20 μl of a stock solution (0.312 mg/ml) of the alamar blue (resazurin; *Sigma–Aldrich Co.*) were added to each well. Absorbance at 570 nm and 600 nm was measured using a *SpectraMax 190* Microplate Reader (*Molecular Device*), and the drug effect was quantified as the percentage of control absorbance.

Statistical Analysis

Data are presented as inhibitory concentration of 50% (IC_{50}) values and their 95% confidence intervals obtained by nonlinear regression from at the least three independent experiments performed in duplicate or triplicate. All analyses were carried out using the GRAPHPAD program (Intuitive Software for Science).

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201600276.

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