Bioguided Identification of Antifungal and Antiproliferative Compounds from the Brazilian Orchid *Miltonia flavescens* Lindl.

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The Orchidaceae family is appreciated worldwide for the beauty of its flowers, and hundreds of species of this family occur in Brazil. Yet little is known about the potential of orchids for therapeutic application. We have investigated bioactive compounds produced by the South Brazilian orchid *Miltonia flavescens* Lindl. Bioguided studies with the fungus *Cladosporium herbarum* allowed the identification of hydrocinnamic acid as the active antifungal compound. In addition, the chloroform fraction exhibited an interesting activity against human cancer cells, and 5,7-dihydroxy-6,4′-dimethoxyflavone isolated from this fraction was found to be active against seven human cancer cell lines, including NCI/ADR-RES ovary sarcoma, with an IC_{50} value of 2.6 μg/mL. This is the first report on the cytostatic activity of this flavone against human ovary sarcoma.

Key words: Miltonia flavescens, Flavone, Human Ovary Sarcoma

Introduction

The Orchidaceae are the largest and an evolutionarily young family among flowering plants. There are over 25,000 known native species, belonging to about 750 genera, besides hundreds of thousands of commercial hybrids. No other family of plants is provided with such a wide variety of shapes, colours, and floral aromas like orchids (Kaiser, 1993; Suttleworth *et al.*, 1991).

Yet the usefulness of orchids is not limited to the fascination caused by its beauty. For centuries, *Vanilla planifolia* has been grown mainly in Central America for the production of vanilla aroma. This makes *Vanilla planifolia* the only orchid species of commercial interest, besides its ornamental use (Suttleworth *et al.*, 1991). There are only few reports on the use of orchids as medicinal plants.

Thus, few groups have worked in recent years on the phytoconstituents of orchids with the aim of discovering bioactive compounds. The most successful case is that of the alkaloid dendrobine isolated from *Dendrobium nobile*. This plant is used in traditional Chinese medicine in the composition of the "Chin-Shih-Hu" medicinal mixture. This alkaloid exhibits antipyretic, hypotensive, and convulsive activities, respectively (Roush, 1980).

In a pioneering work, a survey was carried out to determine the level of alkaloids found in more than 525 orchid species (Lüning, 1964) from several parts of the world, including South America. At that time, small amounts of alkaloids were detected in extracts

from *Miltonia flavescens* Lindl. This is the only data found for this plant in the chemical literature. In this context, the present work initiated the first bioguided chemical study of *M. flavescens*. This interesting orchid is native to northern Paraná State (South Brazil) as an epiphyte on large trees. The plant is characterized by an elongated pseudobulb, topped by two sheets of thin leathery texture. The flowers are found in inflorescences and are characterized by the presence of a prominent sheath. They are pale yellow and have stellate shape (Suttleworth *et al.*, 1991). This plant is used in the region by some residents as garden ornament.

A major current interest is the chemical study of plants aiming to discover compounds against various diseases, such as fungal and cancer ones. Considering that the conventional treatment of ovarian cancer includes surgical techniques, chemotherapy, and radiotherapy, and that these exhibit variable degrees of efficiency, the development of new compounds with chemotherapeutic potential is of fundamental importance. In our work, we have focused on ovarian cancer lines, because this type of cancer is one of the most resilient to treatments currently available, with high mortality rates (du Bois *et al.*, 2004). Therefore, there is a great demand for new drugs that can serve as new therapeutic options exhibiting lower toxicity and requiring a shorter duration of treatment.

Using the aerial parts of the orchid *M. flavescens*, the objective of this work was to isolate and chemically characterize its antifungal and antiproliferative compounds, employing bioguided purification procedures, together with spectroscopic techniques for structural elucidation.

Materials and Methods

Chemical analyses

NMR spectra were recorded on a Varian (Palo Alto, CA, USA) Mercury Plus BB spectrometer, operating at 300.06 MHz for ^{1}H and 75.02 MHz for ^{13}C NMR. Chemical shifts were recorded in ppm with reference to internal tetramethylsilane (TMS) ($\delta = 0.0$ ppm) or to the solvent signal. The solvents used were CD_3OD and $CDCl_3$ (Sigma-Aldrich, Milwaukee, WI, USA). 1D and 2D NMR experiments were carried out (^{1}H NMR, ^{13}C NMR, gCOSY, HMBC, DEPT, HSQC, and NOESY) for the characterization of the isolated compounds.

Column chromatography (CC) was performed using silica gel 60 (Merck, Darmstadt, Germany) or Sephadex LH-20 (Sigma-Aldrich) as the stationary phase. For thin-layer chromatography (TLC), silica gel 60 G or 60 GF_{254} (Merck) were employed; visualization of spots was carried out by irradiation with ultraviolet light at 254 and 366 nm and/or by spraying with acetic acid/H_2SO_4/anisaldehyde solution (2:1:97, v/v/v) followed by heating.

Polarimetric analyses were performed on a Perkin Elmer (Waltham, MA, USA) 343 polarimeter, using a cuvette with an optical path of 100 mm, a wavelength of 589 nm, and a temperature of 20 °C. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a ThermoFinnigan (Austin, TX, USA) instrument with an oven ramp from 40 to 290 °C at 15 °C/min and a hold for 5 min. Helium was used as carrier gas, at a flow rate of 1.0 mL/min. The injector was maintained at 240 °C, and the split ratio was 1:10. The interface temperature was set at 280 °C. The mass range was m/z 40 – 650 Da, and the detector operated at 70 eV. The samples were diluted at 1 mg/mL in dichloromethane or ethyl acetate.

Tandem atmospheric pressure mass spectrometry (API-MS-MS) analyses were performed on a Micromass (Milford, MA, USA) Quattro Micro API instrument. Samples were diluted in dimethylsulfoxide (DMSO) (0.5 mg/mL). An aliquot of 20 μL was taken and diluted in 10 mL water/methanol (1:1, v/v). For the analyses, voltages were maintained at: capillary, 4 kV; cone, 50 V; extractor, 3 V; RF lens, 0.1 V. The source temperature was maintained at 120 °C, and the desolvation temperature at 350 °C. The desolvation gas used was high-purity nitrogen at a flow rate of 650 L/h. The cone gas was nitrogen with a flow rate of 30 L/h. The analyses were conducted by electron impact (EI) in the positive mode at 12 eV.

Plant material

The aerial parts (leaves, pseudobulbs, roots, and rhizomes) of the species *M. flavescens* (5.0 kg) were provided by the Orchidary of the State University of Londrina (UEL), Londrina, Paraná, Brazil. A voucher specimen is kept alive there.

Antifungal activity

The antifungal bioassays were performed using the technique of plate biorevelation with spores of the fungus *Cladosporium herbarum* ATCC 6670. The microorganism was maintained on potato dextrose agar medium (PDA) at 5 °C. For the assay, spores were

scraped from the agar, suspended in a liquid medium, and sprayed onto plates containing chromatographic fractions. The plates were incubated in a chamber with circulating air at 28 °C for 48 h and then examined for fungal growth. Regions of growth inhibition appeared as clear halos. The commercial product nipazol (propylparabene) was used as positive control.

Cytotoxic activity

Biological assays were performed with the following human cancer cell lines, acquired from the National Cancer Institute (NCI): U251 (glioma, CNS), MCF-7 (breast), PC-3 (prostate), HT-29 (colorectal), K562 (leukemia), OVCAR-3 (ovary carcinoma), and NCI/ADR-RES (ovary sarcoma). All cells were maintained in RPMI 1640 [heat-inactivated fetal bovine serum (FBS)/penicillin/streptomycin] medium. As control, normal human keratinocyte cells (HaCaT) were used. The assays were performed by the colorimetric method with sulforhodamine B (SRB) for evaluation of cell growth (Monks *et al.*, 1991).

Stock solutions of the crude extract, fractions, and purified compounds were prepared in DMSO at a concentration of 0.1 mg/mL. For the activity test, 100 mL of cell culture medium were plated in 96-wells plates. The plates were incubated for 24 h at 37 °C in a humid environment in air containing 5 % CO_2 for cell adaptation.

After this period, a control plate was fixed by the addition of 50 μL of 50% trichloroacetic acid (TCA) per well for the determination of cell densities at the time of addition of the formulated samples. To the remaining plates, formulations at concentrations of 0.25, 2.5, 25, and 250 μg/mL were added. Each sample was evaluated in triplicate. After a 48-h incubation, cells were fixed with TCA, and the plates were kept for 1 h at 4 °C. After four washes with distilled water, the samples were maintained at room temperature until they were completely dry.

Fixed cells were stained by adding 50 μL of the protein dye SRB, dissolved to 0.4 % (w/v) in 1 % acetic acid, incubated at 4 °C for 30 min, washed four times with 1 % acetic acid, and then dried at room temperature. The protein-bound dye was solubilized with 10 mM Trizma base (Sigma-Aldrich) at pH 10.5.

Absorbances were measured in a microplate reader at 560 nm and averaged. The mean values were corrected for the absorbance of the respective blank, and the percentage of cell growth relative to the control was calculated and plotted against sample concentration. The standard chemotherapeutic doxorubicin was employed as positive control.

Isolation of chemical constituents

Fresh plant material (5 kg) was ground in a slicer, followed by cold maceration in methanol (25 L) and evaporation of the solvent under reduced pressure at 45 °C. The residue (28 g) was dissolved in water/methanol (1:1, v/v) (120 mL) and partitioned with the solvents *n*-hexane, chloroform, ethyl acetate, and *n*-butanol (5 × 100 mL). This procedure yielded the fractions: *n*-hexane (500 mg), chloroform (2.31 g), ethyl acetate (1.34 g), and *n*-butanol (4.74 g), and the remaining aqueous methanol solution (8.73 g). The *n*-hexane fraction appeared promising for its antifungal activity, while the chloroform fraction showed an interesting antiproliferative profile.

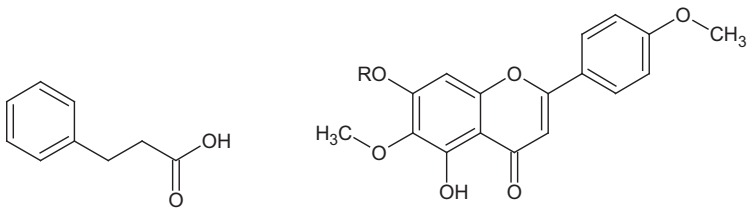
Part of the *n*-hexane fraction (400 mg) was subjected to fractionation by CC on silica gel using as eluents *n*-hexane, ethyl acetate, and methanol in increasing polarity, resulting in 177 fractions (10 mL each) which were grouped according to their TLC profile. The initial non-polar fractions contained a white precipitate, which was then washed twice with *n*-hexane, yielding a mixture of steroids (sitosterol, stigmasterol, and campesterol). All other fractions were complex mixtures.

The chloroform fraction (2.23 g) was subjected to chromatographic purification on silica gel 60 (Merck; 60.0 g, column with 3.0 cm diameter), using *n*-hexane, ethyl acetate, and methanol in mixtures of increasing polarities, collecting 10-mL fractions. The fractions eluting with *n*-hexane/ethyl acetate (17:8) were pooled, and the material re-crystallized from chloroform and *n*-hexane (20 mL), obtaining finally 40.0 mg of yellow crystals of compound **2**.

During crude extract preparation by evaporation under reduced pressure, a brown coloured precipitate was obtained. It had a high sugar content and was further purified by washings with *n*-hexane, chloroform, ethyl acetate, and acetone. Finally, the remaining solid material was re-crystallized from ethanol/distilled water several times to give the pure compound **3** in large quantities.

Hydrocinnamic acid (**1**): Yield: 0.1 mg (85 % pure). – GC-MS (EI, 70 eV): m/z (%) = 150 (56), 104 (63), 91 (100), 77 (19).

5,7-Dihydroxy-6,4′-dimethoxyflavone (**2**): Yield: 40.0 mg (99 % pure). – GC-MS (EI, 70 eV):



1 **2** R = H

3 R = rutinose

Fig. 1. Chemical structures of the identified bioactive compounds hydrocinnamic acid (**1**), 5,7-dihydroxy-6,4′-dimethoxyflavone (**2**), and 7-*O*-rutinose-5-hydroxy-6,4′-dimethoxyflavone (**3**) from *M. flavescens*.

m/z (%) = 314 (MI – 100), 299 (57), 296 (60), 271 (68), 135 (27), 133 (35), 69 (74). – ^{1}H NMR (300.06 MHz, $CDCl_3$, TMS): $\delta = 3.74$ (3H, s, 4′-OCH_3), 3.84 (3H, s, 7-OCH_3), 6.59 (1H, s, H-3), 6.83 (1H, s, H-9), 7.09 (2H, d, $J = 9$ Hz, H-3′ and H-5′), 8.00 (2H, d, $J = 9$ Hz, H-2′ and H-6′), 13.00 (brd, OH-C-5 and OH-C-7). – ^{13}C NMR (75.02 MHz, $CDCl_3$, TMS): $\delta = 163.3$ (C-2), 103.0 (C-3), 182.1 (C-4), 152.7 (C-5), 131.3 (C-6), 157.3 (C-7), 94.2 (C-8), 152.5 (C-9), 104.1 (C-10), 122.8 (C-1′), 128.2 (C-2′, C-6′), 114.5 (C-3′, C-5′), 162.2 (C-4′), 55.4 (4′-OCH_3), 59.9 (6-OCH_3).

7-O-Rutinose-5-hydroxy-6,4′-dimethoxyflavone (**3**): Yield: 855.0 mg (99% pure). – API-MS-MS (12 eV): m/z (%) = 623.7 ($[M + H]^+$, 100), 645.4 ($[M + Na]^+$, 90). – $[\alpha]_D^{20} = -80°$ (c 0.5 mg/mL, methanol). – ^{1}H NMR (300.06 MHz, DMSO-d_6): $\delta = 12.97$ (1H, s, 5-OH), 8.05 (2H, d, $J = 9$ Hz, H-2′ and 6′), 7.17 (2H, d, $J = 9$ Hz, H-3′ and 5′), 6.94 (1H, s, H-8), 6.93 (1H, s, H-3), 5.13 (1H, d, $J = 7.2$ Hz, H-1″), 4.57 (1H, s, H-1‴), 3.87 (3H, s, OCH_3), 3.77 (3H, s, OCH_3). – ^{13}C NMR (75.02 MHz, DMSO-d_6): $\delta = 164.1$ (C-2), 103.4 (C-3), 182.4 (C-4), 152.2 (C-5), 132.7 (C-6), 156.6 (C-7), 94.4 (C-8), 152.6 (C-9), 105.9 (C-10), 122.7 (C-1′), 128.5 (C-2′), 114.8 (C-3′), 162.4 (C-4′), 114.8 (C-5′), 128.4 (C-6′), 60.4 (6-OCH_3), 55.6 (4′-OCH_3), 100.4 (C-1″), 73.2 (C-2″), 76.5 (C-3″), 69.5 (C-4″), 75.8 (C-5″), 66.0 (C-6″), 100.5 (C-1‴), 70.5 (C-2‴), 70.8 (C-3‴), 72.0 (C-4‴), 68.4 (C-5‴), 17.8 (C-6‴).

Results and Discussion

Antifungal activity

The crude methanol extract of *M. flavescens* and its fractions were tested against the mould *Cladosporium herbarum* (Ascomycota) using the procedure of biorevelation of chromatographic plates. A strong antifungal activity was found for the *n*-hexane fraction, which was therefore investigated for its active compounds. This procedure allowed the isolation of a steroid mixture (sitosterol, stigmasterol, and campesterol), which was identified on the basis of ^{1}H, ^{13}C, and DEPT NMR data. Unfortunately, the amounts obtained for the other fractions were small, which prevented the isolation of their active principles and their analysis by NMR spectroscopy. Thus, biological assays were performed with each fraction. Active fractions were analysed by GC-MS for the identification of the compounds responsible for the biological activity.

This procedure allowed the identification of three bioactive fractions. Two of these were a complex mixture containing phenolic and fatty acid esters, respectively. In general, it is known that the biorevelation procedure presents false positives when lipid samples are tested. The third fraction contained rather pure hydrocinnamic acid (**1**) (85% pure) (Fig. 1); this identified acid is the main antifungal substance present in extracts from this plant. The antifungal activity of this compound is described in the literature (Mao *et al.*, 2006).

Antiproliferative activity

Evaluation of the cytostatic profile of the extract and fractions revealed a potential for the discovery of compounds with antiproliferative activity from the chloroform fraction, as seen in Table I. The chloroform fraction was particularly active against the human ovary sarcoma NCI/ADR-RES line, which is multidrug-resistant.

Chromatography of this fraction led to the isolation of the flavonoid 5,7-dihydroxy-6,4′-dimethoxyflavone

Table I. IC_{50} values (μg/mL) of the crude extract, fractions, and purified compounds **2** and **3** against seven human cancer cell lines and human keratinocytes (HaCaT, normal cells).

Assayed sample	U251	MCF-7	PC-3	HT-29	K562	OVCAR-3	NCI/ADR-RES	HaCaT
Doxorubicin	0.0029	0.043	0.025	0.025	0.40	0.089	0.012	0.028
Crude extract	> 250	> 250	n.e.[a]	> 250	n.e.	> 250	> 250	> 250
n-Hexane fraction	> 250	> 250	> 250	> 250	155.4	> 250	n.e.	69.5
Chloroform fraction	90.4	> 250	250	250	67.2	n.e.	9.3	> 250
Ethyl acetate fraction	> 250	> 250	> 250	> 250	> 250	n.e.	> 250	> 250
n-Butanol fraction	> 250	250	> 250	> 250	250	n.e.	> 250	> 250
Methanol/water fraction	> 250	> 250	> 250	> 250	250	n.e.	> 250	> 250
2[b]	5.6 ± 1.6	6.8 ± 1.4	n.e.	18.5 ± 11.1	n.e.	> 250	2.6 ± 0.7	16.2 ± 7.0
3	> 250	> 250	> 250	> 250	> 250	n.e.	> 250	> 250

[a] n.e., not evaluated.
[b] Mean ± standard error.

(**2**) (Fig. 1) as yellow crystals. The molecular structure was identified on the basis of spectroscopic data, specifically uni- and bidimensional NMR (^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC, and NOESY) and mass spectrometry. MS analyses identified the molecular ion as the base peak at m/z 314. The ^{1}H NMR spectrum showed a characteristic singlet signal at δ_H 6.83 ppm (H-9), typical of the A ring of 5,6,7-substituted flavones, as well as a singlet signal at δ_H 6.59 ppm (s, H-3), characteristic of non-substituted ring C. The positions of the methoxy substituents were confirmed by NOE difference experiments. Spectroscopic measurements revealed data consistent with the literature data of this compound (Horie *et al.*, 1998).

After purification, **2** was subjected to antiproliferative evaluation and found to be active against various cancer cell lines, in particular the ovary sarcoma NCI/ADR-RES line ($IC_{50} = 2.6$ μg/mL). Thus, this compound was responsible for the antiproliferative activity observed for the chloroform fraction.

The compound was also tested against another ovarian cancer cell line (OVCAR-3, ovary carcinoma), but it was little effective up to a concentration of 250 μg/mL. Such specificity is an important factor for better targeting/selection of a medicine in relation to the type of cancer affecting a patient. Moreover, biological activities against specific cell lines may be an important route to discover new peculiar biochemical mechanisms. The IC_{50} value of **2** for inhibition of the growth of non-cancerous human keratinocyte HaCaT cells was (16.2 ± 7.0) μg/mL, *i. e.* (6.2 ± 10.0) times larger than its IC_{50} value against the ovary sarcoma NCI/ADR-RES line.

Furthermore, compound **2** acted differentially on another type of ovarian cancer (OVCAR-3 line), as can be seen in Fig. 2. Frequently, selectivity may provide access to new biochemical pathways in specific cell lines, opening new opportunities for drug development.

In a previous study Liu *et al.* (2007) evaluated the activity of 5,7-dihydroxy-6,4′-dimethoxyflavone in rats carrying tumours of the lines H22 and S180. In both cases, there was a large reduction in tumour growth, thus prolonging the animals' lifetimes. The same compound also showed antimutagenic potential when tested against cells of genetically modified *Salmonella typhimurium*, which indicates its potential for studies against diseases caused by mutant cells (Chulasiri *et al.*, 1992).

Some of the most common flavones have been found to have anticancer activity. Luteolin (3′,4′,5,7-

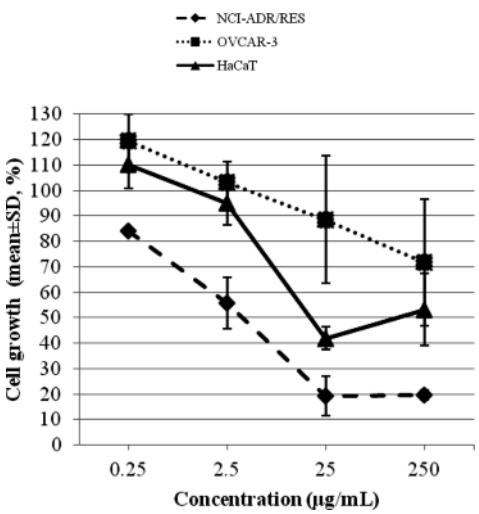


Fig. 2. Concentration-response curve of 5,7-dihydroxy-6,4′-dimethoxyflavone against two lines of human ovarian cancer cells and normal human cells (HaCaT keratinocytes).

tetrahydroxyflavone) arrested the cell cycle and induced apoptosis in many human cancer cells, including prostate cancer cells (PCA) (Ueda *et al.*, 2003; Lim *et al.*, 2007). It reduced the potential metastasis of many cancer cells, and also acted as a preventive agent against cancer invasion (Aalinkeel *et al.*, 2004; Vayalil *et al.*, 2004; Vijayababu *et al.*, 2006). Diosmetin (3′,5,7-trihydroxy-4′-methoxyflavone) selectively inhibited proliferation of the breast adenocarcinoma MDA-MB 468, and was only slightly toxic in normal breast cells (Androutsopoulos *et al.*, 2009). Apigenin (4′,5,7-trihydroxyflavone) has been implicated as chemopreventive agent against prostate and breast cancers (Mak *et al.*, 2006). The antiproliferative activity of the highly oxygenated nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) in human gastric cancer cell lines involves several mechanisms, *i. e.* direct cytotoxicity, induction of apoptosis, and modulation of the cell cycle (Yoshimizu *et al.*, 2004).

Finally, while preparing the crude extract a large quantity of glycosidic material precipitated during solvent evaporation, yielding a large amount of **3** (Fig. 1) after re-crystallization. The mass spectrum obtained by API-MS-MS confirmed the molecular weight of the compound. The ^{1}H NMR spectrum of **3** showed two singlets at δ_H 6.94 and 6.93 ppm, typical of protons of the rings A and C of substituted flavones. The presence of signals in the region between δ_H 3.78 and 3.87 ppm, belonging to methoxy groups, was also noted. The anomeric protons of glucose and rhamnose were observed at δ_H 5.13 and 4.57 ppm, respectively. The position of the disaccharide substituent (*O*-C-7) was determined by a NOE difference experiment, which revealed the anomeric hydrogen signal at δ_H 5.13 ppm and a signal increase in the hydrogen singlet at δ_H 6.94 ppm. The spectroscopic data were consistent with the literature data (Hyun, 2008). The compound inhibited neither the growth of *Cladosporium herbarum* nor that of the human cancer cell lines, most likely due to its high hydrophilicity and difficult permeation through cell membranes. However, this compound may be an interesting source of the aglycone **2** by simple basic hydrolysis.

Conclusion

The bioguided chemical investigation of a *M. flavescens* extract showed that the *n*-hexane fraction has antifungal activity which is due to hydrocinnamic acid. From the chloroform fraction, 5,7-dihydroxy-6,4′-dimethoxyflavone was isolated as the active principle responsible for the antiproliferative activity of this fraction. This compound is selectively active against a human ovary sarcoma cell line, as compared to an ovary carcinoma cell line, and is only slightly toxic to normal cells. This is the first bioguided phytochemical study of a Brazilian orchid with the aim to discover compounds with anticancer potential.

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