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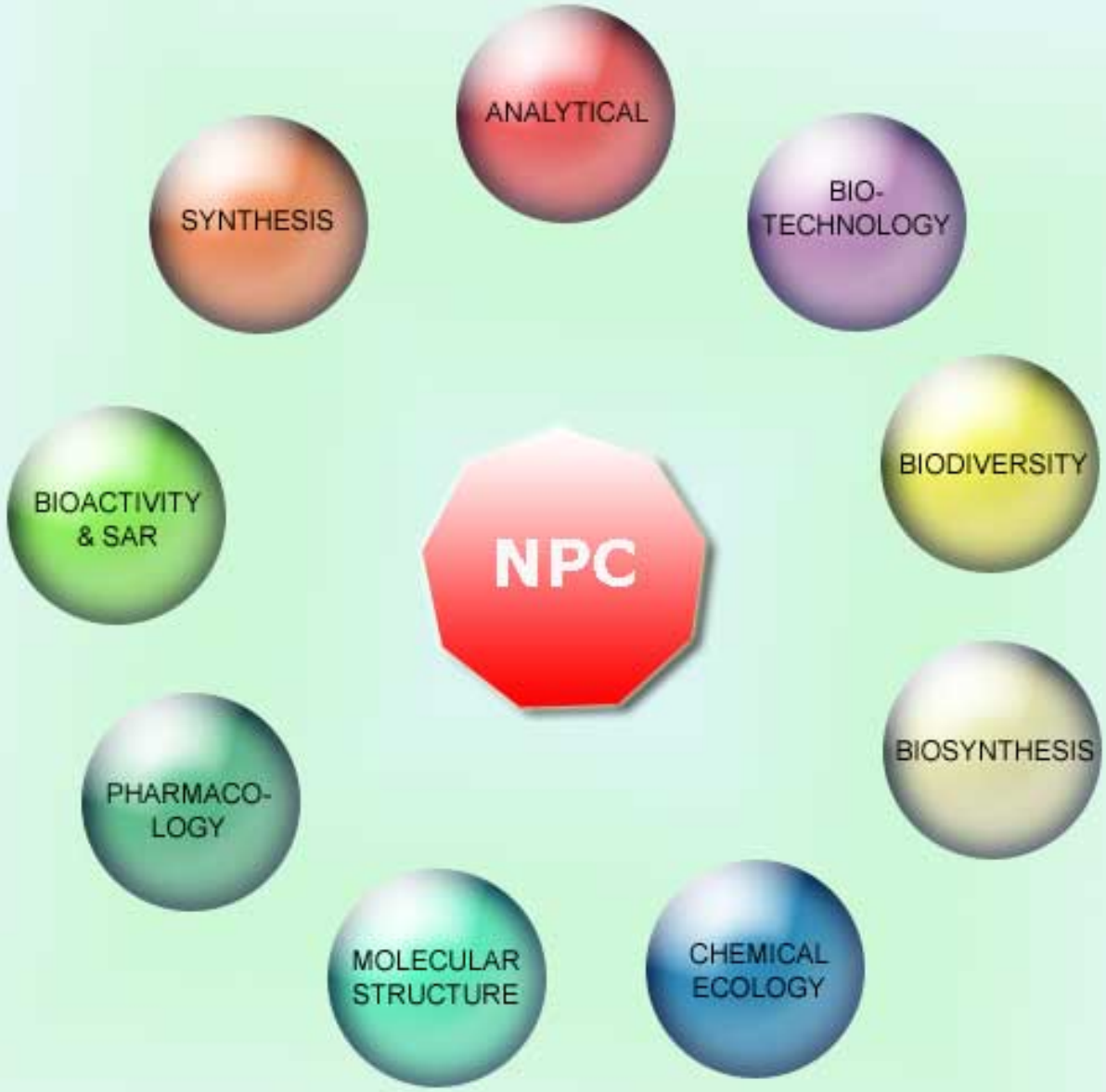


**This Issue is Dedicated to**
**Professor Pedro Joseph-Nathan**
**on the Occasion of his 65th Birthday**

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# Seasonal Phytochemical Variation and Antifungal Evaluation of Different Parts of *Epidendrum mosenii*

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***This paper is dedicated to Professor P. Joseph-Nathan for his 65th birthday.***

*Epidendrum mosenii* is a Brazilian medicinal plant, traditionally used to treat infections and pains. This study reports on the chemical composition and microbiological properties of different parts and in different seasons of this plant. Results demonstrate that 4,3',5'-trihydroxy-3-methoxy-dihydrostilbene (**1**), 4,5-dihydroxy-3,3-dimethoxy-dihydrostilbene (**2**) and pholidotin (**3**) were mainly present in the roots in all seasons, and the yield of mass extract increased their recovery in other parts, such as the stem and leaves for compounds (**2**) and (**3**), in summer and winter, respectively. The antifungal results indicate that compounds (**1**) and (**2**) have interesting activity against *Cryptococcus neoformans, Microsporum gypseum, Trychophyton rubrum* and *Trychophyton mentagrophytes* with MIC values between 62.5 and 125 μg/mL. Taken together, these results strongly suggest that the antifungal properties of *E. mosenii* are related, at least in part, to the presence of dihydrostilbenes **1** and **2**, and this is useful for quality control of phytopreparations based on this plant, justifying the popular use of this plant to treat infections.

**Keywords:** *Epidendrum mosenii*, antifungal activity, seasonal variation, dihydrostilbenes.

*Epidendrum mosenii* Rchb (Orchidaceae), known as "orquidea-da-praia", occurs frequently in the south of Brazil, where it is used for ornamental purposes and also in folk medicine, to treat a variety of disorders, including infections and pain [1]. Phytochemical and biological investigations of the *Epidendrum* genus are rare. However, studies conducted with the chloroform-methanol extract of *E. rigidum* inhibited radicle growth of *Amaranthus hypochondriacus*. Bioassay-guided fractionation of this extract furnished three phytotoxins, the stilbenes gigantol, batatasin III and 2,3-dimethoxy-9,10-dihydrophenanthrene-4,7-diol, together with some known flavonoids and triterpenoids [2]. Previous studies carried out in our laboratories with *E. mosenii* have demonstrated interesting biological properties, particularly antinociceptive effects in mice, related to the presence of steroids and triterpenes [3-5].

We have also previously reported that the methanolic extract of this plant significantly lowered the blood glucose of aloxan-induced diabetic rats, but it was not effective in inhibiting the contractile response elicited by acetylcholine on Guinea-pig ileum and rat duodenum [6,7]. The importance of this plant from both the phytochemical and medicinal points of view led us to deepen the study of its chemical composition and its biological properties.

In a previous preliminary study, some extracts and a fraction containing a mixture of dihydrostilbenes from *E. mosenii* exhibited antifungal activity [5], and we report here the antifungal properties of these

compounds against a panel of human opportunistic pathogenic fungi and the seasonal variation of the active compounds in the different parts of *E. mosenii* using HPLC analyses.

DCM extracts of different parts of *E. mosenii* were analyzed by HPLC in order to quantify the amount of 4,3',5'-trihydroxy-3-methoxy-dihydrostilbene (**1**), 4,5-dihydroxy-3,3-dimethoxy-dihydrostilbene (**2**), and pholidotin (**3**) in each extract, since compounds **1** and **2** exhibited antifungal action and compound **3** is one of the main antinociceptive agents of this plant [4]. A typical chromatogram showed retention times of 28.6, 31.0 and 35.9 (±1) minutes for compounds **1**, **2** and **3**, respectively.

The compound concentrations were determined using the mean area values and the linear regression obtained throughout the calibration curve for each standard compound. Good linearity was obtained for all the compounds, with $r^2$ values of 0.997 (**1**), 0.999 (**2**) and 0.978 (**3**), respectively. The amounts (**1**-**3**) were calculated in different seasons based on the yield (in mg) of extract from 100 g of dried plant part examined (Figures 1-3).

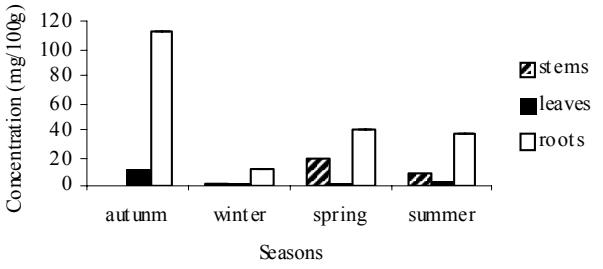


**Figure 1**: Concentrations of **1** in different parts and seasons of *E. mosenii* (mg/100 g dried plant).

The concentration of **1** found in the roots was considerably higher than that of the other parts of the plant and dependent on the season in which it was collected. The roots collected in autumn, exhibited the largest amounts of **1**, followed by those collected in spring and summer (Figure 1).

In relation to compound **2**, the concentrations in the roots and stems were considerably higher than those found in the other parts, regardless of the season in which they were collected. The stems collected in winter, and the roots collected in autumn exhibited a high amount of **2** (Figure 2).

In relation to compound **3**, the results obtained indicate that the production is more pronounced in the roots (Figure 3).

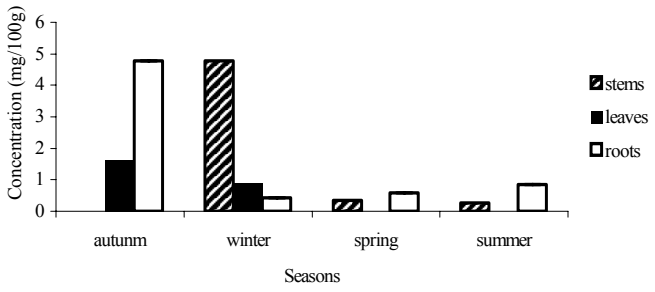


**Figure 2**: Concentrations of **2** in different parts and seasons (mg/100g of dried plant).

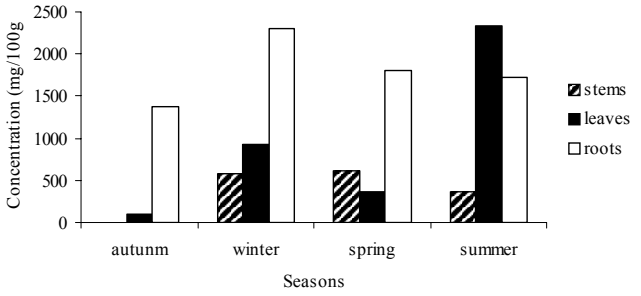


**Figure 3**: Concentrations of **3** in different parts and seasons (mg/100g of dried plant).

We have determined the MIC of compounds **1** and **2**, previously obtained from *E. mosenii* stems, against several opportunistic pathogenic fungi. The antifungal evaluations of these compounds showed that both compounds exert antifungal properties, especially against dermatophytes. As can be seen in Table 2, both compounds were effective against *C. neoformans, M. gypseum, T. rubrum* and *T. mentagrophytes*, with MIC values between 62.5 – 125.0 μg/mL.

In summary, our results add important information regarding the seasonal variation of the most important compounds related to the biological properties of *E. mosenii*. In the case of its antifungal properties, we determined that the roots in autumn possess a high concentration of stilbenes **1** and **2**, both compounds responsible for the antifungal properties. In turn, the stem possesses a good concentration of stilbene **2**, but not of **1,** except in winter. Regarding the steroidal compound **3**, which is responsible for the antinociceptive properties, it is present in the highest amounts in roots in all seasons, but mainly in winter. In contrast, the leaves possess a high concentration of **3**, similar to that observed in the roots, but only in summer. These findings add important data for the rational use of this plant in traditional medicine and open new avenues for continuing its study, which will be of a great profit for the Brazilian population health care.

**Table 2**: Antifungal activity of compounds **1** and **2** from *E. mosenii* against fungi, expressed as minimum inhibitory concentration (μg/mL).

| | Microorganisms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Compounds | *Ca* | *Ct* | *Sc* | *Cn* | *A f* | *Afl* | *An* | *Mg* | *Tr* | *Tm* |
| **1** | >250 | >250 | >250 | 125 | >250 | >250 | >250 | 125 | 62.5 | 125 |
| **2** | >250 | >250 | >250 | 125 | >250 | >250 | >250 | 125 | 125 | 125 |

**1** = 4,3'5'-trihydroxy-3-methoxy-dihydrostilbene; **2** = 4,5-dihydroxy-3,3-dimethoxy-dihydrostilbene; *Ca = Candida albicans*; *Ct = Candida tropicalis*, *Sc = Saccharomyces cerevisiae*, *Cn = Cryptococcus neoformans*, *Af = Aspergillus fumigatus*, *Afl = Aspergillus flavus* and *Aspergillus niger*, *Mg = Microsporum gypseum*, *Tr = Trychophyton rubrum*, *Tm = Trychophyton mentagrophytes*.

## Experimental

***Plant material:*** Different parts (leaves, stems, roots and flowers) of *E. mosenii* were collected at Canto do Morcego, Itajaí, Santa Catarina State, Brazil, during different seasons, from May 2002 to February 2003. The material was authenticated by Prof. Dr Ademir Reis (Herbário Barbosa Rodrigues (HBR), Itajaí, SC) and a voucher specimen was deposited in the same herbarium under number VC Filho 003.

***Preparation of the samples:*** The different parts of the plant (10 g each) were separately extracted three times, with dichloromethane (200 mL) at room temperature. After evaporation of solvent under reduced pressure, the extracts were dried in vacuum with $P_2O_5$. Pure 4,3',5'-trihydroxy-3-methoxy-dihydrostilbene (**1**), 4,5-dihydroxy-3,3-dimethoxy-dihydrostilbene (**2**) and pholidotin (24-methylene-cycloartanyl-*p*-hydroxy-*cis*-cinnamate) (**3**), were obtained from stems of *E. mosenii*, as previously described [1,8].

***Chromatographic conditions:*** The HPLC system employed consisted of Waters model equipment consisting of a pump 600-F, a 20 μL manual injection loop (Rheodyne 7725i), followed by a line degasser (AF) and equipped with a UV-Vis detector (PDA 2996). A $C_{18}$ column (Phenomenex, 25 cm, 4.6 mm i. d.; 0.5 μm film thickness and 100 A) was used, at room temperature. The solvent system used was a gradient-mixture of acetonitrile/buffer phosphate (*o*-phosphoric acid 0.05%, pH 3.5) from 2:98 v/v until 15:85 v/v, with a flow rate of 0.7 mL/min. The chromatograms were examined at a wavelength range of 200-400 nm. The HPLC grade solvents used were filtered (0.2 μm, Schleicher & Schuell) and degassed by sonication before use. All the extracts were analyzed in triplicate. The sample (10 mg/mL) in methanol/water mixture (20:80) was filtered through a 0.45 μm membrane filter (Millex, Millipore) and directly injected using a 100 μL Hamilton syringe.

***Quantitative analysis:*** The quantification of 4,3',5'-trihydroxy-3-methoxy-dihydrostilbene (**1**), 4,5-dihydroxy-3,3-dimethoxy-dihydrostilbene (**2**) and pholidotin (**3**) was performed throughout the external calibration method. The standard compounds were previously obtained from *E. mosenii* according to Oliveira (1999). The purity (99%) and authenticity were characterized by melting-point determinations and spectroscopic techniques, including, IR, $^1$H NMR and $^{13}$C NMR. The calibration curves were constructed using the conditions described above, with standard samples within the concentration range: 6.6 – 0.12 mg/mL for compound **1**, 4.0 – 0.05 mg / mL for compound **2** and 5.0 - 0.88 mg/mL for compound **3**. The wavelength use for quantification of compounds **1**, **2** and **3** were 223.0, 228.0 and 255.0 nm, respectively. These were diluted in methanol: water (20:80) and then 20 μL was manually injected, in triplicate. The peaks belonging to compounds **1**, **2**, and **3** were identified by comparison with the retention times and absorbance of standard solutions injected under the same conditions. The calibration curves were obtained by linear regression of mean areas integrated using Empower Pro software.

***Antifungal assays:*** For antifungal evaluation, standardized strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, and Centro de Referencia en Micología CEREMIC (C), Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina were used: *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *A. fumigatus* ATTC26934, *A. niger* ATCC 9029, *Trichophyton rubrum* C110, *T. mentagrophytes* ATCC 9972, and *Microsporum gypseum* C115. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30°C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Inoculate of cell or spore

suspensions were obtained according to reported procedures and adjusted to 1-5 x$10^3$ cells/spores with colony forming units (CFU) /mL (NCCLS).

***Antifungal susceptibility testing:*** The Minimum Inhibitory Concentration (MIC) of each extract or compound was determined using broth micro dilution techniques according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory and Standards, NCCLS) for yeasts (M27-A2) and for filamentous fungi (M 38 A). MIC values were determined in RPMI-1640 (Sigma, St Louis, Mo, USA), buffered to pH 7.0 with MOPS. Microtiter trays were incubated at 35$^o$C for yeasts and hialohyphomycetes and at 28-30$^o$C for dermatophyte strains, in a moist, dark chamber. The MICs were recorded visually at 48 h for yeasts, and at an interval according to the control fungal growth, for the rest of the fungi.

For the assay, stock solutions of pure compounds were diluted twice with RPMI from 250 – 0.98 μg/mL (final volume = 100 μL) obtaining a final DMSO concentration of ≤ 1%. A volume of 100 μL of inoculum suspension was added to each well, with the exception of the sterility control, in which sterile water was added instead. Ketoconazole, Terbinafine, and Amphotericin B were used as positive controls. Endpoints were defined as the lowest concentration of drug resulting in total inhibition ($MIC_{100}$) of visual growth, compared with the control wells containing no antifungal growth.

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## ***Review /Account***

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