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ANTIFUNGAL ACTIVITY OF FIVE SPECIES OF *POLYGALA*

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ABSTRACT

Crude extracts and fractions of five species of *Polygala* - *P. campestris*, *P. cyparissias, P. paniculat*a, *P. pulchella* and *P. sabulo*sa - were investigated for their *in vitro* antifungal activity against opportunistic *Candida* species, *Cryptococcus gattii* and *Sporothrix schenckii* with bioautographic and microdilution assays. In the bioautographic assays, the major extracts were active against the fungi tested. In the minimal concentration inhibitory (MIC) assay, the hexane extract of *P. paniculata* and EtOAc fraction of *P. sabulosa* showed the best antifungal activity, with MIC values of 60 and 30 µg/mL, respectively, against *C. tropicalis, C. gattii* and *S. schenckii*. The compounds isolated from *P. sabulosa* prenyloxycoumarin and 1,2,3,4,5,6-hexanehexol displayed antifungal activity against *S. schenckii* (with MICs of 125 µg/mL and 250 µg/mL, respectively) and *C. gattii* (both with MICs of 250 µg/mL). Rutin and aurapten isolated from *P. paniculata* showed antifungal activity against *C. gattii* with MIC values of 60 and 250 µg/mL, respectively. In the antifungal screening, few of the isolated compounds showed good antifungal inhibition. The compound α-spinasterol showed broad activity against the species tested, while rutin had the best activity with the lowest MIC values for the microorganisms tested. These two compounds may be chemically modified by the introduction of a substitute group that would alter several physico-chemical properties of the molecule, such as hydrophobicity, electronic density and steric strain.

Key words: Polygalaceae, *Polygala* species, antifungal activity, rutin, α-spinasterol

INTRODUCTION

In recent years, fungal infections have emerged as a major cause of disease and mortality, largely because of the growing population of immunocompromised patients. In patients affected by AIDS, opportunistic yeasts of the genus *Candida*, such as *C. albicans*, have accounted for approximately 60% of clinical isolates. The appearance of other species of this genus as agents of candidosis may reflect the selection of species that are less susceptible to the action of antifungal agents (39). Approximately 90% of AIDS patients present with oropharyngeal candidosis at least once in the course of the disease, and two-thirds of these patients display the same characteristic at the onset of the symptoms (4).

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On the other hand, *Cryptococcus neoformans*, the major cause of meningitis in AIDS patients, has been identified as the fourth most common cause of life-threatening infection in AIDS patients (36), affecting a more limited percentage (6 - 8%) (4). *Cryptococcus gattii* primarily infects otherwise healthy individuals, but serotype C isolates of *C. gattii* were found to be implicated in HIV-associated infections in California, Botswana and Malawi (6). *Sporothrix schenckii* is the etiological agent of sporotrichosis, a subcutaneous mycosis with a worldwide distribution that can affect humans and other animals (37).

Human mycoses are often not successfully treated because the available antifungal drugs are either ineffective, produce many adverse effects, fail to prevent recurrence of infection, or lead to the development of drug-resistant strains (11). Therefore, there is a distinct need for the discovery of new, safer and more-effective antifungal agents. Natural products from higher plants may provide a new source of antimicrobial agents with possibly novel mechanisms of action (27). In recent years, antimicrobial properties of medicinal plants have been increasingly reported from different parts of the world (17, 18, 24).

The *Polygala* genus belongs to the family Polygalaceae, which contains 600 species (11), 19 of which are found in the state of Santa Catarina, Brazil. A chemical investigation of the genus showed the occurrence of a variety of secondary metabolites, such as xanthones (5, 6, 22, 30), saponins (16, 25, 45), oligosaccharides (15, 21), flavonoids (35, 38), coumarins (12, 13, 32, 33) and styrylpyrones (31, 32).

Various pharmacological activities have been reported for some species of *Polygala*, such as anti-inflammatory activity in *P. japonica* and *P. cyparissias* (10, 19), anxiolytic-like effects in *P. sabulosa* (8, 9) trypanocidal activity in *P. sabulosa* and *P. cyparissias* (34, 35), antinociceptive effects in *P. sabulosa* (40) and *P. cyparissias* (34, 35), antibacterial activities in *P. myrtifolia* (20) and so on. Although the biological activities of various *Polygala* species have been investigated, their antifungal properties have not. Few studies have reported the antifungal activities of species belonging to the genus *Polygala*, such as *P. myrtifolia* (26), *P. gazensis* (3) and *P. nyikensis* (23).

In this paper, we report an evaluation of the antifungal activities of extracts, fractions and isolated compounds from different species of *Polygala*, all of them found in Santa Catarina State, Brazil. Extracts and fractions of *Polygala cyparissias* A. St. – Hil. & Moq., *P. campestris* Gardner., *P. paniculata* Lam., *P. pulchella* A. St. – Hil. & Moq. and *P. sabulosa* A.W. Bennett were tested against human opportunistic pathogenic fungi using a bioautographic assay. The minimal inhibitory concentrations (MICs) of the most active extracts and isolated compounds were then evaluated with the broth dilution method.

MATERIAL AND METHODS

Plant material

The species *P. cyparissias* (856 g) and *P. paniculata* (3500 g) (deposited at the Herbarium FLOR – UFSC, under number 22744, and Herbarium of the Department of Botany - UFPR, under number 26027, respectively) were collected from Daniela beach, Florianópolis in March 2004. The species *P. pulchella* (1000 g) and *P. sabulosa* (1700 g) (deposited in the Herbarium of the Department of Botany – UFPR, under numbers 28555 and 19640, respectively) were collected in Rancho Queimado in October 2004. *P. campestris* (700 g) (a voucher specimen was deposited in the Herbarium of the Department of Botany - UFPR, under number 7393) was collected in November 2003, also in Rancho Queimado. The plants were identified by Dr. Olavo de Araújo Guimarães, Department of Botany, Universidade Federal do Paraná (UFPR) and Dr. Leila da Graça Amaral, Department of Botany, Universidade Federal de Santa Catarina (UFSC).

Preparation of extracts and fractions

The dried and powdered whole plants were extracted, with

different methods employed for the different species. *P. paniculata* was first extracted three times at room temperature with hexane (the "hexane extract"), then with 96% ethanol (EtOH extract), to avoid the formation of coumarinic artifacts such as those previously described by Pizzolatti *et al*. (33). *P. pulchella* and *P. campestris* were successively extracted three times each with dichloromethane (CH_2Cl_2 extract), ethyl acetate (EtOAc extract) and ethanol (EtOH extract). *P. cyparissias* and *P. sabulosa* were extracted exhaustively at room temperature with 96% ethanol (EtOH extract). The EtOH extract obtained from *P. sabulosa* was then partitioned into *n*-hexane, dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) soluble fractions. Each extract or fraction was filtered, and the solvent was removed under reduced pressure to yield the respective extract.

Isolation of compounds

During evaporation of the ethanolic extract obtained from *P. sabulosa* (456.1 g), formation of a solid compound was observed. This compound was separated and washed with appropriate solvent (CH_2Cl_2 and acetone). The compound was analyzed by IV, ^1H and ^{13}C NMR and identified as 1,2,3,4,5,6-hexanehexol, previously described as occurring in this species by Pizzolatti *et al.* (31). The *n*-hexane fraction of *P. sabulosa* was subjected to column chromatography on silica gel eluted with *n*-hexane/EtOAc gradient. The fractions obtained were then monitored by TLC (Thin-layer chromatography, viewed by spraying with sulfuric vanillin reagent followed by heating at 110 °C) and similar fractions (fr) were combined. The combined fr. 14-26 were recrystallized from acetone to give the sterol α-spinasterol (91 mg).

The CH_2Cl_2 soluble fraction was further subjected to silica gel column chromatography eluted with increasing amounts of ethyl acetate in *n*-hexane to give 95 fr. of 75 mL each. The combined fr. 21-22 were crystallized from hexane/EtOAc (3:1) to yield prenyloxycoumarin (319 g). The combined fr. 25-30 were processed by flash chromatography, yielding dihydrostyryl-2-pyrones 1 (19 mg) and 2 (47 mg). The combined fr. 34-39 were chromatographed on silica gel eluting with a hexane/EtOAc gradient to give the dihydrostyryl-2-pyrone 3 (23 mg) and the styryl-2-pyrone 4 (21 mg). Fraction 49 yielded a yellow powder containing the coumarin scopoletin (17 mg). Fraction 56 was purified by crystallization to yield the styryl-2-pyrone 5 (14 mg).

Detailed nuclear magnetic resonance spectroscopic analysis (^1H and ^{13}C) was performed and the physical and spectroscopic data compared to the confirmed structures of 6-methoxy-7-hydroxycoumarin (scopoletin), 6-methoxy-7-prenyloxycoumarin (prenyloxycoumarin), 4-methoxy-6-(11,12-methylenedioxydihydrostyryl)-2-pyrone (DST 1), 4-methoxy-6-(11,12-methylenedioxy-14-methoxy-dihydrostyryl)-2-pyrone (DST 2), 4-methoxy-6-(11,12-methylenedioxy-10,14-dimethoxy-dihydrostyryl)-2-pyrone (DST 3), 4-methoxy-6-(11,12-methylenedioxystyryl)-2-pyrone (STY 4) and 4-methoxy-6-(11,12-methylenedioxy-14-methoxystyryl)-2-pyrone (STY 5). All of these compounds have been previously found in the five *Polygala* species studied (31, 32).

Precipitation of an amorphous solid from the hexane extract of *P. paniculata* with partial evaporation of the solvent at controlled temperature was observed. This solid was separated and washed successively with *n*-hexane and yielded the compound phebalosin (2 g), identified by ^1H and ^{13}C NMR (33).

The EtOH extract was chromatographed in a silica gel column using a gradient of *n*-hexane/EtOAc/ethanol to give 120 fr. of 75 mL each. Fractions 13-16 and 18-20 were rechromatographed in a silica gel column eluting with an *n*-hexane/EtOAc gradient of increasing polarity to yield the compounds aurapten (87 mg) and □-spinasterol (55 mg), respectively. Fractions 68-75 precipitated a yellow solid, which was separated and washed with methanol to yield the compound rutin (430 mg). These compounds were previously described by Pizzolatti *et al*. (33) and identified by comparison with physical and spectroscopic data (^1H and ^{13}C NMR).

Microorganisms

For evaluation of antifungal activity, strains from the American Type Culture Collection (ATCC), Rochville, MD, USA were used: *C. albicans* ATCC 18804 *C. krusei* ATCC 20298, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 2001, *S. schenckii* ATCC 20679 and *C. gattii* ATCC 32608. All fungal strains were maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK).

Culture media and inoculum

Saboraud dextrose agar was used for the bioautographic tests. The synthetic medium RPMI (Sigma, St. Louis, MO, USA) with L-glutamine was buffered to pH 7.0 with 0.165 m morpholine propanesulfonic acid (MOPS; Sigma). The test was performed according to the CLSI M27-A standard (28) for determination of the Minimal Inhibitory Concentration (MIC). Inoculum of the fungal cultures of *Candida* spp. and *C. gattii* were prepared by picking five colonies of 21 mm in diameter from 48 h-old cultures grown at 35ºC. The chosen colonies were suspended in 5 ml of sterile 0.85% saline. For the susceptibility test, the resulting suspension was vortexed for 15 s and the cell density adjusted to $1.5 \pm 1.0 \times 10^3$ cells/mL by spectrophotometric methods at 530 nm (29). To obtain Y cells of *S. schenckii,* the fungal culture was cultured on brain heart infusion agar containing 1% glucose (BHID) at 37°C and maintained by continuous weekly passages. The cells were harvested 4 to 6 days after the last passage and packed into a small volume of sterile saline using a sterile cotton swab. The cells were then suspended in saline and thoroughly vortexed. If large aggregates existed, they were allowed to settle for several minutes, and the supernatant was collected. The suspension was then diluted in RPMI medium to obtain a final inoculum size of approximately a $1x10^5$ - $5x10^5$ cells/mL. For bioautographic tests, the inoculum suspension was appropriately diluted in Saboraud broth to a final concentration of approximately 10^5 cells/mL.

Antifungal assays

Bioautographic assay: Extracts and fractions were dissolved in dimethylsulfoxide (DMSO) at 100 µg/mL. 20 µL of the solution was applied to TLC plates (plates of silica gel of $60F_{254}$, Merck, Darmstadt, Germany) with graduated micropipettes. The plates were then submerged twice for five minutes in fungal suspensions and incubated at 35°C in a hermetically sealed bell-jar for 48 h for the *Candida* species and 72 h for *C. gattii* and S. *schenckii*.

Subsequently, plates were sprayed with *p*-iodonitrotetrazolium violet (Sigma®) (INT) (5 mg/mL) and incubated again for 4 hours at 36 ± 1°C. Inhibition zones were observed, and the diameter of inhibition in millimeters was measured. Solvents and DMSO were used as negative controls, while Amphotericin B (Sigma) was used as the positive control. Samples of the culture medium plus microorganisms were used as the growth control. Tests were performed in triplicate.

Susceptibility test

Broth microdilution testing was performed in accordance with the guidelines in CLSI document M27-A (28). The susceptibility was determined with the microbroth dilution method, performed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, MI, USA).

The extracts and fractions were dissolved in DMSO after addition of RPMI. Later, serial dilutions were made with RPMI, maintaining a constant volume of 1000 µL in each tube. In this way, the extracts were tested at eight concentrations that varied from 7.8 to 1000 µg/mL. From each dilution, brackets of 100 µL were transferred to the microplates.

As a control for growth and sterility control, the RPMI was used without the addition of extract or solvent. The culture medium together with the solvent was used as a control for the toxicity of the solvent. Amphotericin B (Sigma) was included at a concentration of 0.03 at 4.0 µg/mL as the positive antifungal control.

After the assembly of the plates, each fungal strain was inoculated and the plates incubated at 35°C for 48 hours for the *Candida* species and 72 hours for *C. gattii* and *S. schenckii*.

Tests were performed in triplicate. The endpoints were determined visually through comparison with the endpoints of the drug-free growth control well. The minimum inhibitory concentration (MIC), expressed in μg/mL, was defined as the lowest extract concentration at which the well was optically clear. Extracts, fractions and isolated compounds with MIC values ≤ 1000 μg/mL were considered active.

RESULTS AND DISCUSSION

Physical and spectroscopic data of the compounds isolated from *P. sabulosa* and *P. paniculata* are presented bellow.

DST 1 (4-methoxy-6-(11,12-methylenedioxydihydrostyryl)-2-pyrone): m.p. 137 - 140°C; IR (KBr, ν cm^{-1}): 3104, 2924, 1710, 1646, 1567, 1489, 1457, 1410, 1242, 1030, 925, 821. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.72 (d, 1H, *J*=7.2 Hz, H-13), 6.65 (d, 1H, *J*=1.8 Hz, H-10), 6.60 (dd, 1H, *J*=7.2 Hz, 1.8 Hz, H-14), 5.92 (s, 2H, O$C\underline{H}_2$O at C-11 and C-12), 5.70 (sl, 1H, H-5), 5.40 (sl, 1H, H-3), 3.77 (s, 3H, 4-OCH_3), 2.87 (t, 2H, H-8), 2.70 (t, 2H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 171.3 (C-2), 165.1 (C-4), 164.4 (C-6), 147.9 (C-11), 146.3 (C-12), 133.8 (C-9), 121.4 (C-14), 108.9 (C-10), 108.5 (C-13), 101.1 (O$C\underline{H}_2$O), 100.5 (C-5), 87.9 (C-3), 56.0 (4-O$C\underline{H}_3$), 35.9 (C-7), 32.8 (C-8).

DST 2 (4-methoxy-6-(11,12-methylenedioxy-14-methoxy-dihydrostyryl)-2-pyrone): m.p. 148 - 151°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.59 (s, 1H, H-10), 6.49 (s, 1H, H-13), 5.88 (s, 2H, O$C\underline{H}_2$O at C-11 and C-12), 5.71 (sl, 1H, H-5), 5.40 (sl, 1H, H-3), 3.77 (s, 3H, 4-OCH_3), 3.74 (s, 3H, 14-OCH_3), 2.85 (t, 2H, H-8), 2.65 (t, 2H, H-7). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 171.1 (C-2), 164.9 (C-4), 164.1 (C-6), 152.2 (C-14), 146.5 (C-12), 140.6 (C-11), 120.1 (C-9), 109.5 (C-10), 100.9 (O$C\underline{H}_2$O), 99.5 (C-5), 94.5 (C-13), 87.5 (C-3), 56.1 (14-O$C\underline{H}_3$), 55.7 (4-O$C\underline{H}_3$), 34.0 (C-8), 27.6 (C-7).

DST 3 (4-methoxy-6-(11,12-methylenedioxy-10,14-dimethoxy-dihydrostyryl)-2-pyrone): m.p. 165 - 167°C; IR (KBr, ν cm^{-1}): 3089, 2951, 2849, 1727, 1646, 1572, 1508, 1474; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.23 (s, 1H, H-13), 5.86 (s, 2H, O$C\underline{H}_2$O at C-11 and C-12), 5.71 (sl, 1H, H-5), 5.40 (sl, 1H, H-3), 3.96 (s, 3H, 10-OCH_3), 3.78 (s, 3H, 4-OCH_3), 3.71 (s, 3H, 14-OCH_3), 2.90 (t, 2H, *J*=7,6 Hz, H-8), 2.58 (t, 2H, *J*=7,6 Hz, H-7).

STY 4 (4-methoxy-6-(11,12-methylenedioxystyryl)-2-pyrone): m.p. 199 - 202°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.45 (d, 1H, *J*=16 Hz, H-8), 7.03 (s, 1H, H-10), 6.88 (s, 1H, H-14), 6.81 (s, 1H, H-13), 6.45 (d, 1H, *J*=16 Hz, H-7), 5.92 (s, 2H, O$C\underline{H}_2$O at C-11 and C-12), 5.70 (sl, 1H, H-5), 5.48 (sl, 1H, H-3), 3.89 (s, 3H, 4-OCH_3).

STY 5 (4-methoxy-6-(11,12-methylenedioxy-14-methoxystyryl)-2-pyrone): m.p. 190 - 191°C; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 7.77 (d, 1H, *J*=16 Hz, H-8), 6.96 (s, 1H, H-10), 6.52 (s, 1H, H-13), 6.46 (d, 1H, *J*=16 Hz, H-7), 5.95 (s, 2H, O$C\underline{H}_2$O at C-11 at C-12), 5.84 (sl, 1H, H-5), 5.40 (sl, 1H, H-3), 3.87 (s, 3H, OCH_3). 3.72 (s, 3H, OCH_3).

Prenyloxycoumarin (6-methoxy-7-prenyloxycoumarin): m.p. 79 - 81°C; IR (KBr, ν cm^{-1}): 1713, 1612, 1561, 1513, 1381, 1280, 1248, 1140, 971, 812. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 7.63 (d, 1H, *J4,3*=9.4 Hz, H-4), 6.86 (s, 1H, H-8), 6.82 (s, 1H, H-5), 6.26 (d, 1H, *J3,4*=9.4 Hz, H-3), 5.49 (t, 1H, $[CH_3]_2$-C=C$\underline{H}$-CH_2), 4.66 (d, 2H, $[CH_3]_2$-C=CH-C$\underline{H}_2$), 3.90 (s, 3H, 6-OCH_3), 1.79 (s, 6H, $[C\underline{H}_3]_2$-C=CH-CH_2). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 161.7 (C=O), 152.0 (C-7), 149.8 (C-10), 146.6 (C-6), 143.3 (C-4), 138.9 (C-3'), 118.6 (C-2'), 113.2 (C-3), 111.2 (C-9), 108.0 (C-5), 101.0 (C-8), 66.2 (C-1'), 56.3 (6-O$C\underline{H}_3$), 25.7 (C-4'), 18.3 (C-5').

Scopoletin (6-methoxy-7-hydroxycoumarin): m.p. 203 - 204°C; IR (KBr, ν cm^{-1}): 3340, 1709, 1267. ^{1}H RMN (400 MHz, $CDCl_3$) δ (ppm): 7.60 (d, 1H, *J3,4*=9.6 Hz, H-3), 6.91 (s, 1H, H-8), 6.85 (s, 1H, H-5), 6.27 (d, 1H, *J4,3*=9,6 Hz, H-4), 6.22 (s, 1H, OH), 3.95 (s, 3H, 6-OCH_3). ^{13}C NMR ($CDCl_3$) δ (ppm): 161.8 (C=O), 150.4 (C-9), 149.9 (C-7), 144.0 (C-6), 143.5 (C-4), 113.6 (C-3), 111.7 (C-10), 107.7 (C-5), 103.4 (C-8), 56.6 (OCH_3).

α-spinasterol (24α-ethyl-5α-cholesta-7,22*E*-dien-3β-ol):

m.p. 153-154°C. IR (KBr, ν cm^{-1}): 3424, 2956, 2937, 2869. ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 5.14 (m, 3H, H-7, H-22 e H-23), 3.60 (m, H-3), 1.03 (d, *J*= 6.6, 21-CH_3), 0.85 (d, *J* = 6.0, 26-CH_3), 0.80 (s, 19-CH_3), 0.80 (d, *J* = 6.3, 27-CH_3), 0.79 (t, *J* = 6.2, 29-CH3), 0.55 (s, 18-CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 140.3 (C-8), 138.9 (C-22), 130.1 (C-23), 118.1 (C-7), 71.8 (C-3), 56.6 (C-17), 55.8 (C-14), 51.9 (C-24), 50.1 (C-9), 43.9 (C-13), 41.5 (C-20), 40.9 (C-5), 40.2 (C-12), 38.7 (C-4), 37.8 (C-1), 34.9 (C-10), 32.6 (C-25), 32.1 (C-2), 30.3 (C-6), 29.2 (C-16), 26.1 (C-28), 23.7 (C-15), 22.2 (C-11), 22.1 (C-21), 21.8 (C-26), 19.7 (C-27), 13.8 (C- 19), 12.9 (C-29), 12.7 (C-18).

1,2,3,4,5,6-hexanehexol: m.p. 166 - 169°C. ^{1}H RMN (400 MHz, $CDCl_3$) δ (ppm): 3.90 (td, 1H, *J*=5.2, *J*=3.4 Hz, H-2 and H-5), 3.80 (dd, 1H, *J*=3.4 Hz, H-3 and H-4), 3.50 (d, 2H, *J*=5.2, H-1 and H-6). All signs showed the 2:1:1 ratio.

Phebalosin (7-methoxy-8-(1',2'-epoxy-3'-methyl-3'-butenyl)-coumarin): m.p. 124°C. IR (KBr, ν cm^{-1}): 3070, 1735, 1608. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.26 (d, *J*=9.6, H-3), 7.62 (d, *J*=9.6, H-4), 7.42 (d, *J*=8.8, H-5), 6.87 (d, *J*=8.8, H-6), 3.99 (d, H-1'), 3.92 (d, *J'*=2.0, H-2'), 5.08 (s, H-4'), 5.30 (s, H-4'), 1.87 (H-5'), 3.97 (s, OCH_3). ^{13}C NMR (400 MHz, CD_3OD) δ (ppm): 162.18 (C-2), 113.05 (C-3), 141.51 (C-4), 129.23 (C-5), 107.81 (C-6), 160.61 (C-7), unobserved (C-8), 154.09 (C-9), 112.76 (C-10), 52.00 (C-1'), 60.92 (C-2'), 113.78 (C-3'), 113.68 (C-4'), 17.68 (C-5'), 56.58 (OCH_3).

Aurapten (7-geraniloxy coumarin): m.p. 65-66 °C. IR (KBr, ν cm^{-1}): 1729, 1592. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.24 (d, *J*=9.5, H-3), 7.64 (d, *J*=9.5, H-4), 7.36 (d, *J*=8.2, H-5), 6.85 (d, *J*=8.2, H-6), 6.82 (s, H-8), 4.60 (d, *J*=6.5, H-1'), 5.47 (t, *J*=6.5, H-2'), 2.11 (H-4'), 2.11 (H-5'), 5.08 (sl, H-6'), 1.76 (s, H-8'), 1.67 (s, H-9'), 1.60 (s, H-10'). ^{13}C NMR (400 MHz, CD_3OD) δ (ppm): 126.12 (C-2), 112.94 (C-3), 143.42 (C-4), 128.64 (C-5), 113.21 (C-6), 161.27 (C-7), 101.57 (C-8), 155.85 (C-9), 112.39 (C-10), 65.46 (C-1'), 118.36 (C-2'), 142.34 (C-3'), 39.49 (C-4'), 26.20 (C-5'), 123.59 (C-6'), 131.94 (C-7'), 16.75 (C-8'), 25.63 (C-9'), 17.69 (C-10').

Rutin (Quercetin 3-rutinoside): m.p.: 188-190 °C. IR (KBr, ν cm^{-1}): 3415, 1652, 1595, 1496. ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 157.27 (C-2), 134.79 (C-3), 178.33 (C-4), 158.04 (C-5), 97.99 (C-6), 166.21 (C-7), 92.07 (C-8), 167.50 (C-9), 105.37 (C-10), 122.97 (C-1'), 114.85 (C-2'), 145.74 (C-3'), 150.56 (C-4'), 116.23 (C-5'), 122.09 (C-6'), 101.19 (C-1G), 74.52 (C-2G), 76.93 (C-3G), 70.87 (C-4G), 76.02 (C-5G), 67.39 (C-6G), 103.10 (C-1R), 70.26 (C-2R), 71,05 (C-3R), 72.66 (C-4R), 68.54 (C-5R), 16.70 (C-6R). G glucose; R ramnose.

The results of the antifungal screening of *Polygala* spp. are shown in Table 1. The TLC bioassay (bioautography) used in the present study permitted an overview of the antifungal activity of the extracts and fractions of *Polygala* spp. and displayed clear zones on the TLC plates. The hexane fraction of *P. sabulosa* showed a broad spectrum of activity with antifungal properties for all microorganisms used in this study. However, the EtOH extract of *P. sabulosa* was not active against all of the microorganisms tested. All extracts and fractions of *P. paniculata* and *P. pulchella* presented activity against *C. tropicalis.* The other extracts and fractions showed activity for at least one of the fungal species tested.

Table 2 shows the Minimum Inhibitory Concentration (MIC) for the active extracts in bioautography assay. *C. gattii* was the fungal species most sensitive to the tested extracts. The EtOAc fraction of *P. sabulosa* inhibited *C. gattii* and *S. schenckii*, with the lowest active amount being 30 µg/mL. The hexane extract of *P. paniculata* also showed good antifungal activity, with MIC values of 60 µg/mL against *C. tropicalis,* 125 µg/mL against *C. gattii* and 250 µg/mL against *C. glabrata* and *C. parapsilosis.* However, the EtOH extract of *P. paniculata* was active only against *C. tropicalis* (250 µg/mL) and *S. schenckii* (1000 µg/mL).

The hexane and CH_2Cl_2 fractions from *P. sabulosa* had a wide spectrum of action, and the amounts required to inhibit the fungal growth varied between 250 and 1000 µg/mL. However, most of the fungal growth was inhibited at 500 and 1000 µg/mL. Only *C. glabrata* and *S. schenckii* were more susceptible at low concentration to the CH_2Cl_2 fraction of *P. sabulosa*, with an MIC of 250 µg/mL. The EtOH extract of *P. cypariassias* was active only against *C. gattii* (250 µg/mL).

Table 1. Antifungal screening by the bioautographyc method of extracts from selected *Polygala* species.

Plant species	Extract[a]	Bioautography[b]						
		***C. k*[c]**	***C. t***	***C. g***	***C. a***	***C. p***	***Cr. g.***	***S. s***
P. paniculata	Hexane extract	-	+	+	+	+	+	-
	EtOH extract	-	+	-	-	-	-	+
P. sabulosa	EtOH extract	-	-	-	-	-	-	-
	Hexane fraction	+	+	+	+	+	+	+
	CH_2Cl_2 fraction	-	+	+	-	+	-	-
	EtOAc fraction	+	-	-	-	-	+	+
P. campestris	CH_2Cl_2 extract	-	+	+	+	+	-	-
	EtOAc extract	-	-	-	-	+	+	-
	EtOH extract	-	-	-	-	+	-	-
P. pulchella	CH_2Cl_2 extract	+	+	+	-	-	+	-
	EtOAc extract	-	+	-	+	-	+	-
	EtOH extract	-	+	+	-	-	-	-
P. cyparissias	EtOH extract	-	+	+	+	+	+	-

aCH2Cl2 = dichloromethane, EtOAc = ethyl acetate, EtOH = ethanol; b + Present antifungal activity; - No inhibitory activity; cC. k = *C. krusei*; C. t = *C. tropicalis*; C. g = *C. glabrata*; C. a = *C. albicans*; C. p = *C. parapsilosis*; Cr. g = *C. gattii*; S. s = *S. schenckii.*

Table 2. Minimum Inhibitory Concentration (MIC) values (in μg/mL) of five Polygala species in relation to fungal species of medical interest.

Plant species	Extract[a]	MIC value (μg/mL)						
		***C. k*[b]**	***C .t***	***C. g***	***C. a***	***C. p***	***Cr. g.***	***S. s***
P. paniculata	Hexane extract	–	60	250	>1000	250	125	>1000
	EtOH extract	–	250	>1000	–	–	–	1000
P. sabulosa	EtOH extract	–	–	–	–	–	–	1000
	Hexane fraction	500	1000	500	1000	500	1000	>1000
	CH_2Cl_2 fraction	–	500	250	–	>1000	–	250
	EtOAc fraction	>1000	–	–	–	–	30	30
P. campestris	CH_2Cl_2 extract	–	>1000	>1000	>1000	>1000	–	500
	EtOAc extract	–	–	–	–	>1000	>1000	>1000
	EtOH extract	–	> 1000	> 1000	> 1000	> 1000	–	>1000
P. pulchella	CH_2Cl_2 extract	>1000	>1000	>1000	–	–	125	>1000
	EtOAc extract	–	>1000	–	>1000	–	>1000	>1000
	EtOH extract	–	>1000	> 1000	–	–	–	>1000
P. cyparissias	EtOH extract	–	>1000	>1000	>1000	>1000	250	–
Amphotericin B		5	2,5	10	10	5	12	20

aCH2Cl2 = dichloromethane, EtOAc = ethyl acetate, EtOH = ethanol; bC. k = *C. krusei*, C. t = *C. tropicalis*, C. g = *C. glabrata*, C. a = *C. albicans*, C. p = *C. parapsilosis*, Cr. g = *C. gattii*, S. s = *S. schenckii.*

Some species of *Polygala* have previously been shown to have antifungal activity. From the species *P. gazensis*, the chromonocoumarin frutinones A and B and the lignans eudesmin, magnolin, yangambin and kobusin were isolated. Frutinone A showed antifungal activity against the pathogenic plant fungi *Cladosporium cucumerinum* and *C. albicans,* while eudesmin and kobusin were active against *C. cucumerinum* only. Frutinone B, magnolin and yangambin did not present

antifungal activity (3). Two xanthones, 1,7-dihydroxy-4-methoxyxanthone and 1,7-dihydroxy-3,5,6-trimethoxyxan thone, isolated from the roots of *P. nyikensis*, exhibited antifungal activity against *C. cucumerinum* (23). In the *P. myrtifolia* species, the presence of saponins was related to activity against *C. albicans* (26).

Table 3 shows the antifungal activities (MIC values in µg/mL) of compounds isolated from two *Polygala* species, *P. paniculata* and *P. sabulosa,* along with the antifungal activities of the extracts from which they were isolated. The other three plant species did not show good activity; therefore, compounds were not isolated from these species. Scopoletin, DST 1, 2, 3 and STY 4, 5, isolated from the EtOAc extract of *P. sabulosa*, showed weak antifungal activity. In contrast, prenyloxycoumarin and 1,2,3,4,5,6-hexanehexol displayed antifungal activity against *S. schenckii* (with MICs of 125 µg/mL and 250 µg/mL, respectively) and *C. gattii* (both with an MIC of 250 µg/mL). The flavonoid rutin and the coumarin aurapten isolated from *P. paniculata* showed antifungal activity against *C. gattii*, with MIC values of 60 and 250 µg/mL, respectively. Phebalosin, another coumarin isolated from *P. paniculata*, did not show any antifungal activity at concentrations up to 1000 µg/mL. α-spinasterol, isolated from both *Polygala* species, showed antifungal activity between 125 and 500 µg/mL for all the fungal species tested.

Table 3. Antifungal activity of compounds isolated from Polygala sabulosa and P. paniculata (MIC values in µg/mL).

***Polygala* species/Isolated compounds**	**MIC value (µg/mL)**						
	***C. k*[a]**	***C. t***	***C. g***	***C. a***	***C. p***	***Cr. g.***	***S. s***
Polygala sabulosa							
Prenyloxycoumarin	>1000	>1000	>1000	>1000	>1000	250	125
Scopoletin	>1000	>1000	>1000	>1000	>1000	>1000	>1000
DST[b]1	>1000	>1000	>1000	>1000	>1000	>1000	>1000
DST 2	>1000	>1000	>1000	>1000	>1000	>1000	>1000
DST 3	>1000	>1000	>1000	>1000	>1000	500	>1000
STY[c] 4	>1000	>1000	>1000	>1000	>1000	>1000	>1000
STY 5	>1000	500	>1000	>1000	>1000	>1000	>1000
α-spinasterol	500	125	500	500	500	500	250
1,2,3,4,5,6-hexanehexol	>1000	>1000	>1000	>1000	>1000	250	250
Polygala paniculata							
Phebalosin	>1000	>1000	>1000	>1000	>1000	>1000	>1000
α-spinasterol	500	125	500	500	500	500	250
Rutin	500	>1000	>1000	>1000	>1000	60	>1000
Aurapten	>1000	>1000	>1000	>1000	>1000	250	>1000
Amphotericin B	5	2,5	10	10	5	12	20

aC. k = *C. krusei*; C. t = *C. tropicalis*; C. g = *C. glabrata*; C. a = *C. albicans*; C. p = *C. parapsilosis*; Cr. g = *Cr. gattii*; S. s = *S. schenckii*. bDST: dihydrostyrylpyrone. cSTY: styrylpyrone.

The antifungal activity observed against *C. gattii* is significant due to the difficulties associated with the treatment of cryptococcosis and the resistance of this fungus to some antifungal drugs. In our study, the compounds prenyloxycoumarin, aurapten and 1,2,3,4,5,6-hexanehexol showed activity against *C. gatii* with an MIC of 250 µg/mL. The flavonoid rutin exhibited better activity against this fungus, with an MIC of 60 µg/mL.

Previous studies have shown the antifungal activity of some of these compounds. The α-spinasterol isolated from *Solidago microglossa* does not present activity against *Staphylococcus aureus, S. epidermidis, Klebsiella pneumoniae,*

Escherichia coli, Salmonella setubal, Bacillus subtilis, Pseudomonas aeruginosa, Saccharomyces cerevisiae or *C. albicans* (29). In the present work, this compound was active against *C. albicans* with an MIC of 500 µg/mL. Rutin was already shown to be active against *Phytophthora megasperma*, *Cylindrocarpon destructans* (2) and *C. albicans* (MIC of 1000 µg/mL) (14). In the present study, rutin not was active against *C. albicans*, but it showed activity against *C. krusei* and *C. gattii*.

Regarding the lack of activity of the coumarins scopoletin and phebalosin, it has been reported that the pharmacological and biochemical properties of simple coumarins may depend on the substitution pattern of the benzene moiety. Sardari *et al.* (41) demonstrated that a free 6-OH is essential and that alkylated derivatives of 7-hydroxycoumarin may show both antifungal and antibacterial properties. Although the coumarins prenyloxycoumarin, phebalosin and aurapten do not possess a free 6-OH, they were not totally devoid of antifungal activity. Nevertheless, the observed lack of activity of scopoletin (MICs > 1000 µg/mL) is in conflict with the results of Sardari *et al.* (41) because this coumarin has a free 6-OH. There are also many studies that describe the antifungal activity of this coumarin against phytopathogens. There is a correlation between scopoletin accumulation and resistance to microbial pathogens in some plants showing important antifungal activity, scopoletin being considered a phytoalexin (42, 1, 43).

As is clear from Table 3, most of the isolated compounds were devoid of antifungal activity up to 1000 µg/mL, and only α-spinasterol, 1,2,3,4,5,6-hexanehexol, prenyloxycoumarin, rutin and aurapten (Figure 1) showed moderate activity toward some fungal species, albeit lower than that displayed by the original extracts or fractions. This interesting and unexpected result could be due to a synergistic action between the compounds of the extracts in the case of *P. sabulosa* and *P. paniculata* or could be ascribed to minor components of the extracts.

Of all the compounds screened, two of them showed antifungal inhibition, whereas eleven did not have significant activity. Rutin and aurapten isolated from *P. paniculata* showed antifungal activity against *C. gattii* with MIC values of 60 and 250 µg/mL, respectively. The compound α-spinasterol showed a wide activity against the species tested and rutin, with the lowest MIC for the microorganisms tested, had the best activity. Chemical modification may improve the activity of these two compounds.

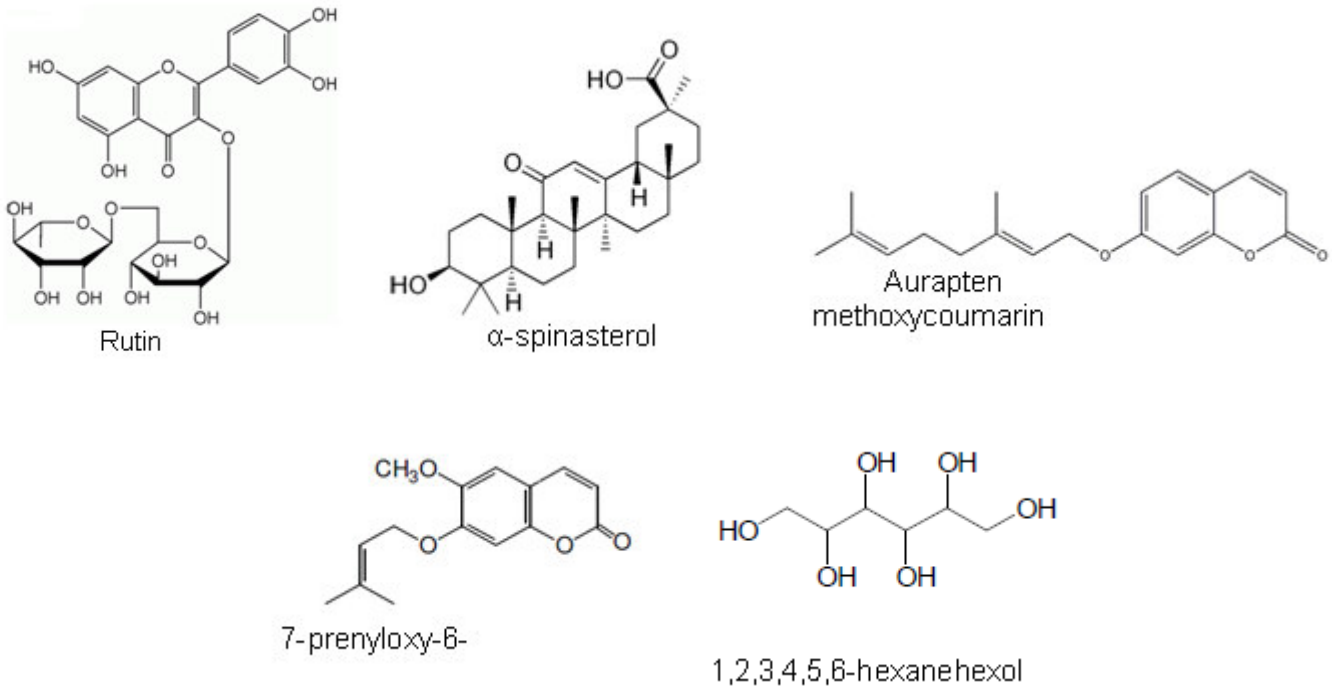

Figure 1. Chemical structure of active compounds.

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