Larvicidal, Antimycobacterial and Antifungal Compounds from the Bark of the Peruvian Plant *Swartzia polyphylla* DC

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The 95% ethanol extract of the bark of *Swartzia polyphylla* DC (Fabaceae) possesses important larvicidal, antimycobacterial and antifungal activity *in vitro*. Bioassay-guided studies performed on the crude ethanol extract afforded T-cadinol as the larvicidal and anti-*Mycobacterium tuberculosis* principle, while the antifungal activity of the extract is due to the presence of the flavonoids biochanin A and dihydrobiochanin A.

Key words antimycobacterial; antifungal; biochanin A; dihydrobiochanin A; larvicidal; *Swartzia polyphylla*

Perú is a country with a large number of medicinal plants, many of which are used for the treatment of infectious diseases,[1)] although only few studies have been conducted to prove their efficacy and safety.[2,3)] Due to the emergence of micro-organism resistance to the common antibiotics[4)] and its worldwide impact on health, our research aims to identify new natural products that may lead to the discovery of new antibacterial agents with higher efficiency and lower toxicity.

As part of our continuing work on bioactive compounds from Peruvian medicinal plants,[5—7)] the *in vitro* antimycobacterial activity of 102 ethanol extracts from 84 plants—used traditionally in Perú for the treatment of inflammatory or infectious disorders—was screened using a tetrazolium microplate assay (TEMA).[8)] In a separate screening, the antifungal and larvicidal activities of over 100 plants, including those previously tested for antimycobacterial activity, were bio-assayed.

As a result of these screenings, *Swartzia polyphylla* DC (Fabaceae) was found to exhibit powerful antimycobacterial action against the sensitive $H_{37}Rv$ and multidrug-resistant *Mycobacterium tuberculosis*. It also inhibited the *in vitro* growth of the dermatophyte *Trichophyton mentagrophytes*, and was active against the larvae of the mosquito *Culex quinquefasciatus*. We are now pleased to report the isolation of the larvicidal, antimycobacterial and antifungal principles present in the crude extract of *S. polyphylla*.

A solvent-partition of the 95% ethanol extract showed that the larvicidal and antimycobacterial activities were concentrated in the hexane fraction, while the 90% methanol fraction was active only in the antifungal assay. The hexane fraction (28 g) was chromatographed on a silica gel column using a hexane–chloroform–methanol gradient. Each fraction (F1—F7) was evaluated for larvicidal and antimycobacterial activity *in vitro*. The most active fraction (F6, 8.7 g) was purified by column chromatography using a hexane–dichloromethane–ethanol gradient and then by MPLC (Lobar Lichroprep silica gel RP-8, 40—63 μm, 310×25 mm, Merck) with acetonitrile–methanol–water (3 : 2 : 2) yielding the most active fraction F-6-4-2 (51.4 mg). This fraction was finally purified by HPLC (Waters Nova-pak H R silica 6 mm, 3.9×300 mm, Waters Model 600E with Waters 2996 PDA detector) using hexane–chloroform gradient (0 to 70%) to obtain T-cadinol (**1**, 9 mg) (Fig. 1).[9,10)]

The 90% methanol fraction (25.5 g) was subjected to column chromatography (silica gel, 0.063—0.200 mm) using a hexane–chloroform–methanol gradient. Each fraction (F1—F9) was tested for antifungal activity *in vitro*. The most active fraction (F3) yielded fraction F3-7-5 (756 mg) after repeated column chromatography using hexane–ethylacetate–methanol as eluent. This fraction was finally purified by MPLC with methanol–water (6 : 4) to afford biochanin A (**2**, 15 mg) and dihydrobiochanin A (**3**, 59 mg) (Fig. 1).[11)]

The bark of *S. polyphylla* contains various flavonoids and isoflavones, some of them with strong activity against carcinogenic bacteria.[11—13)] A bioassay-guided isolation of the 95% ethanol extract of *S. polyphylla* afforded the compound T-cadinol, which showed a moderate anti-*Mycobacterium tuberculosis* activity (MIC=50 μg/ml for the sensitive and multidrug-resistant strains) and strong larvicidal activity. T-cadinol, at a concentration of 300 μg/ml, produces 100% mortality of the larvae of *C. quinquefasciatus* after 1 h exposure. Biochanin A (**2**) and dihydrobiochanin A (**3**) are responsible for the antifungal activity present in the ethanol extract of *S. polyphylla* (Table 1). Both compounds are very active especially against filamentous fungi. The remaining fractions obtained through the bioassay-guided isolation studies were devoid of larvicidal, antimycobacterial or antifungal activities.

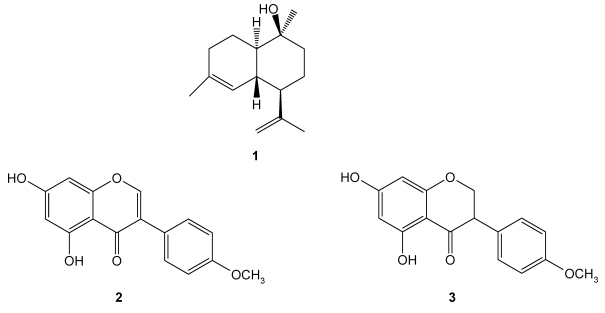

Fig. 1. Structures of Compounds **1**—**3**

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Table 1. Antifungal Activity of Biochanin A (**2**) and Dihydrobiochanin A (**3**)

Compound (μg per well)	Growth inhibition zone diameter (mm)		
	C. albicans	*T. mentagrophytes*	*M. gypseum*
Biochanin A (**2**) (100 μg)	0	31	33
Dihydrobiochanin A (**3**) (100 μg)	0	41	47
Amphotericin B (160 μg)[a)]	31	37	35
Itraconazol (160 μg)[a)]	25	19	27

a) Positive controls.

Experimental

Plant Material *Swartzia polyphylla* DC (Fabaceae) bark was collected in March 2004 in Ramón Castilla, Department of Loreto, Perú, and identified by Irma Fernández. A voucher specimen (IF1569) has been deposited in the Departamento de Ciencias Farmacéuticas of the Universidad Peruana Cayetano Heredia, Lima, Perú.

Bioassays. Antifungal Activity The yeast *Candida albicans* ATCC 90028 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.), while *Trichophyton mentagrophytes* var. interdigitale IHEM 0584 was provided by the Belgian Coordinated Collection of Microorganisms (BCCM/IHEM, Brussels, Belgium). *Microsporum gypseum* IMTAvH 36836 was a clinical isolate obtained from the Laboratorio de Micología of the Instituto de Medicina Tropical "Alexander von Humboldt" (IMTAvH, Lima Perú). *M. gypseum* was isolated from a patient with tinea corporis and was identified by one of us (B.B.) by classical microbiology techniques.[14)]

The antifungal activities of the extracts or pure compounds were evaluated by means of the agar-well diffusion assay. The assay was carried out according to the method of Hufford *et al.*[15)] with some modifications. The media used was Sabouraud Dextrose agar (Difco). Molten agar (20 ml) at 45 °C was aseptically mixed with 1 ml fungal suspension (1×10^4 CFU/ml) and poured into 100 mm×15 mm sterile Petri dishes. For the preparation of the inocula, colonies of fungi were suspended in sterile saline. The suspensions were adjusted turbidimetrically to 0.5 for *C. albicans* and by using a hemacytometer cell counting chamber for *T. mentagrophytes* and *M. gypseum*. The concentration of the suspensions was corroborated by serial dilution plate counts. Once the agar was hardened, 11 mm wells were bored using a sterile cork borer. Aliquots (100 μl) of the ethanolic extracts (25 mg/ml) or pure compounds (1 mg/ml) were placed into the wells and the plates were incubated for 24—72 h at room temperature. Amphotericin B (1.6 mg/ml) (Sigma) and itraconazol (1.6 mg/ml) (Pfizer) were dissolved in DMSO (Sigma) and served as positive controls. Final amounts of isolated compounds and positive controls are shown in Table 1. The tests were carried out in triplicate. The antifungal activity was measured as the diameter (mm) of clear zone of growth inhibition. Solvent controls (95% ethanol and DMSO) were included in every experiment as negative controls.

Larvicidal Activity Batches of 10 third instar larvae of *Culex quinquefasciatus* were put into plastic cups containing 4.8 ml of distilled water. The plant extracts or pure compound were dissolved in 0.2 ml DMSO–ethanol (1 : 1) at different concentrations. Solutions of the extract or pure compound were added to the larvae and their mortality was recorded after 1, 3 and 24 h. The larvae were considered dead if they did not move after prodded with a wooden dowel. Control experiments consisted of adding 0.2 ml of DMSO–ethanol (1 : 1) instead of the extract or pure compound solution.

Anti-*Mycobacterium tuberculosis* Activity Antimycobacterial activity *in vitro* of extracts and pure compounds were evaluated by using the Tetrazolium Microplate Assay (TEMA)[8)] against the sensitive $H_{37}Rv$ ATCC 27294 (American Type Culture Collection, Rockville, Md.) and multidrug-resistant *Mycobacterium tuberculosis* strain (clinical isolate, strain 02 TB DM039EP097).

Bioassay-Guided Isolation of Active Compounds Air-dried powdered plant material (1.38 kg) was extracted by percolation at room temperature with 95% ethanol. The solvent was then evaporated to dryness under reduced pressure at a temperature lower than 40 °C to obtain the crude extract (256 g). This residue was further partitioned between dichloromethane and water. A insoluble solid (165 g) remained between the two phases. The water fraction yield was 2.7 g. The dichloromethane soluble residue was partitioned between hexane and 90% methanol to obtain the hexane (36 g) and 90% methanol (52 g) fractions. The larvicidal and antimycobacterial activities were concentrated in the hexane fraction, while the 90% methanol fraction showed strong antifungal activity.

Structure Elucidation The structures of the isolated compounds were identified by nuclear magnetic resonance (Varian Inova 500) using ^{1}H-NMR (500 MHz), ^{13}C-NMR (126 MHz) and 2D-NMR analysis. $CDCl_3$ was used as solvent for T-cadinol (**1**), whereas CD_3OD was used for the flavonoids biochanin A (**2**) and dihydrobiochanin A (**3**). Electrospray mass spectrometry analysis was conducted at the Mass Spectrometry & Proteomics Facility, Ohio State University, Columbus, OH 43210. The spectral data and our analysis compared satisfactorily with those reported in the literature.[9—11)]

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