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Antifungal activity of raw extract and flavanons isolated from *Piper ecuadorense* from Ecuador

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Abstract: The MeOH extract of *Piper ecuadorense* Sodiro, Piperaceae, was chosen for metabolite isolation and elucidation due to the strong antifungal activity exhibited, measured by means of the broth microdilution method. Two known flavonoids: pinostrobin (**1**) and pinocembrin (**2**) were isolated from 4.16 g. of dichloromethane extract by column chromatography, using a gradient of hexane/EtOAc. A total of 20 mg of **1** were obtained from the fraction eluted with hexane-EtOAc 95:5 v/v, and 100 mg of **2** were obtained from the fraction eluted with hexane-EtOAc 85:15 v/v. The MIC values of the MeOH extract was 31.25 µg/mL for Trichophyton mentagrophytes ATCC® 28185 and 62.5 µg/mL for *Trichophyton rubrum* ATCC® 28188. The MIC value of pinocembrin was 125 µg/mL for Trichophyton mentagrophytes ATCC® 28185 and Trichophyton rubrum ATCC® 28188. Pinostrobin in antifungal test was not active against fungi tested.

Introduction

Piperaceae family comprises about fourteen genera and 1950 species world wide (Mabberley, 1997) being *Piper* and *Peperomia* the most abundant genera, with about 700 and 600 species, respectively (Joly 1991; López et al., 2002; Danelutte et al., 2003). A review of the Ecuadorian flora reports four genera for Piperaceae family, including 215 *Piper* species, 75 of them endemic (Jorgensen & Leon-Yanéz, 1999). *Piper* species are widely used in several ways in traditional medicine for their antibacterial, antifungal, and disinfectant effects; to prevent pains, stomach-ache, and as remedies against parasites, fever, gastritis, flu, rheumatism, cough, headache, skin, prostate problems etc. (Terreaux et al., 1998; Dyer et al., 2004; Tene et al., 2007).

Piper ecuadorense Sodiro is a native shrub of Ecuador and Colombia, widely distributed in Ecuador between 0-2500 m a.s.l. The plant is popularly known as "matico de monte" and it is used as a traditional remedy by different indigenous communities from Loja and Zamora Provinces, Ecuador, where the aqueous infusion from leaves finds applications for the treatment of hangover, as a disinfectant or in wound healing. Moreover, the "herbal healer" (in quechua: Yurak Hampiyachak) from the Saraguros indigenous community, uses the aerial parts with others plants for "mal del aire" treatment (Andrade et al., 2009).

Fungal infections or mycoses are a common public health problem ranging from superficial to deep infections. Superficial mycoses sometimes reach high endemic levels, specially in tropical areas (Roderick, 2006) and dermatophyte fungi are usually the principal cause (Larypoor et al., 2009). Our research group is interested in searching new antidermatophyte substances from natural resources and by this reason the aim of this research was the isolation and identification of the antifungal active compounds of *P. ecuadorense*, plant that does not report previously phytochemical and biological activity studies.

Materials and Methods

Plant material

The leaves of *Piper ecuadorense* Sodiro, Piperaceae, collected in Pituca, region of Zamora Chinchipe Province, Ecuador, in September 2007 located at 179428 E, 9537781 N coordinates, at 1316 m a.s.l. The plant material was identified by Bolivar Merino, curator of the Universidad Nacional de Loja Herbarium and a voucher sample (PPN-pi-007) was deposited in the Universidad Tecnica Particular de Loja Herbarium.

Extraction and isolation

The extract was obtained from 200 g of dried leaves of *P. ecuadorense* using MeOH as a non selective solvent, by dynamic maceration at 400 rpm for 5 h at room temperature. The extraction was carried out by three times and the filtrates were evaporated under a 50mbar absolute pressure at 37 ºC, obtaining 27.98 g of raw extract. The dried extract (20 g) was processed for removal of sugars by a liquid-liquid partition with a solution of MeOH-H_2O (8:2) and dichloromethane, in proportion 1:1. The organic phase was separated and concentrated under reduced pressure. The sugar free extract (4.16 g) was directly separated by a preparative column chromatography (CC), eluting with a *n*-Hex:EtOAc mixture increasing polarity gradient system. The collected fraction in n-Hex:EtOAc 95:5 led to the isolation of 20 mg of pinostrobin (**1**) and 100 mg of pinocembrin (**2**) were obtained from the fraction eluted with *n*-Hex:EtOAc 85:15. Structures of the two compounds were confirmed by 1D and 2D NMR experiments.

Instruments and materials

^{1}H and ^{13}C NMR spectra were measured on a Varian 600 MHz (600 and 125 MHz) instrument, using methanol-d3 (Aldrich) as a solvent. Chemical shifts were reported in units (ppm) and coupling constants (J) in Hz. Silica gel (Merck 0.015-0.040 mm) was used for column chromatography (CC) and silica gel 60 25EA (Aldrich, 0.50 and 1 mm) for analytical and preparative TLC. Spots on chromatograms were detected under UV light (254 and 365 nm) and by spraying with a vanillin/sulphuric acid solution followed by heating.

Minimum inhibitory concentration (MIC) determination

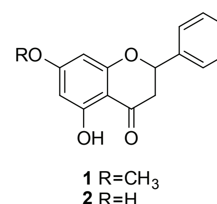
MIC values were determined by the broth microdilution method according to the M 38-A document (CLSI, 2002), using a final concentration of $5x10^4$ spores/mL of *Trichophyton mentagrophytes* ATCC® 28185 and *Trichophyton rubrum* ATCC® 28188, well known dermatophytes causant of skin diseases. The MIC was defined as the lowest concentration of substance that prevented growth, which was determined by the appearance of mycelial growth after 96 h of incubation. Solutions of the test compounds were prepared in DMSO (2 mg/100µL). The assay was carried out in 96-well microtiter plates and the two-fold serial dilution was employed to get concentrations of 1000 µg/mL to 7.81 µg/mLl. Incubation was at 30 ºC for 96 h. Itraconazole was used a positive control with a MIC value of 0.48 µg/mL.

Results and Discussion

The dichloromethane extract (4.16 g) from leaves of *Piper ecuadorense* was submitted to fractionation by column chromatography on silica gel obtaining compounds **1-2**. The flavanones pinostrobin (**1**) (5-hydroxy-7-methoxyflavone) (Thusoo et al., 1981) and pinocembrin (**2**) (5,7-dihydroxyflavanone) (Jung et al., 1990) were identified by comparison of their physical and spectral data with those previously reported in literature (Burke et al., 1986; Usia et al., 2002; Wu et al, 2002; Feld et al., 2003; Grael et al., 2005; Yenjai & Wanich, 2010). The spectral NMR data are shown below.

Pinostrobin (**1**) amorphous powder; ^{1}H NMR (600 MHz, MeOH-d): δ ppm 2.82 (dd, *J*=17.18, 2.64 Hz, 1 H) 3.14 (dd, *J*=17.18, 13.21 Hz, 1 H) 3.82 (s, 3 H) 5.50 (dd, *J*=12.88, 2.97 Hz, 1H) 6.04 - 6.08 (m, 1 H) 6.10 (d, *J*=1.98 Hz, 1 H) 7.38 (d, *J*=7.27 Hz, 1 H) 7.40 - 7.45 (m, 2 H) 7.51 (d, *J*=7.27 Hz, 2 H); HRESIMS: *m/z* 270.0887 [M]+ (calcd. for $C_{16}H_{14}O_4$, 270.0892).

Pinocembrin (**2**) amorphous powder; ^{1}H (600 MHz, MeOH-d): d ppm 2.78 (dd, *J*=17.02, 3.52 Hz, 1 H) 3.10 (dd, *J*=17.02, 12.91 Hz, 1 H) 5.47 (dd, *J*=12.62, 3.23 Hz, 1 H) 5.90 (d, *J*=1.76 Hz, 1 H) 5.94 (d, *J*=2.35 Hz, 1 H) 7.35 - 7.39 (m, 1 H) 7.40 - 7.44 (m, 2 H) 7.50 (d, *J*=7.63 Hz, 2 H); HRESIMS: *m/z* 256.0730 [M]+ (calcd. for $C_{15}H_{12}O_4$, 256.0736).



The antifungal activity was performed by the broth microdilution technique, the same to determine the growth inhibition of test organisms in the presence of decreasing concentrations of the extracts, which are diluted in the culture medium or broth. The results of antidermatophyte activity of pinocembrin and pinostrobin are for the first time reported in this study and are showed in Table 1.

Table 1. Minimun Inhibitory Concentration (MIC) of *Piper ecuadorense* Sodiro, pinocembrin (1) and pinostrobin (2).

Compounds/extract	Antifungal activity MIC (µg/mL)	
	Trichophyton mentagrophytes	*Trichophyton rubrum*
MeOH extract	31.25	62.5
Pinocembrin	125	125
Pinostrobin	NA	NA

NA: no active

According to Cos et al. (2006) for all anti-infective bioassays the IC50 of pure compounds should be bellow 25 µM and for mixtures below 100 µg/mL. Considering that MIC can be expressed as 90% inhibition or higher, and comparing the MIC values of the extract and for the two compounds (Table 1) we can conclude that the strong anti-fungal activity showed by the MeOH extract is not due to these isolated compounds. An extensive investigation of all possible metabolites compounds present in the MeOH extract should be carried out to determine all the possible active compounds. It is really remarkable that only pinocembrin exhibited antifungal activity wich demonstrates that the hidroxyl group in C-7 is necessary for the biological activity instead of a methoxyl group in C-7 as showed in pinostrobin. A study conducted by Pouget et al. (2001) on a group of flavonoids and their antiproliferative effect in MCF-7 human breast cancer cell line, showed that pinostrobin exhibited a 80% of inhibition, in contrast, pinocembrin exhibited less than 20% of inhibition. This confirms the fact that compounds very similar in structure can act by different mechanisms in different systems.

Earlier bioassays on pinocembrin (**2**) isolated from leaf resin of eastern cottonwood *Populus deltoides* showed against spore germination of four fungal pathogens of cottonwood suggested that it is most active against *Melampsora medusae* (toxic at 16 ppm), least active against *Septoria musiva* (no inhibition at 32 ppm), and intermediate in activity against *Cytospora chrysosperma* (toxic at 32 ppm) and *Marssonina brunnea* (9% of control germination at 32 ppm). Also in a choice assay, the flavanone pinocembrin isolated as the most active principle from *Flourensia* oolepis aerial parts showed strong antifeedant activity against *Epilachna paenulata, Xanthogaleruca luteola* and *Spodoptera frugiperda* with an antifeedant index (AI%) of 90, 94 and 91% ($p<0.01$) respectively, at 50 µg/cm^2 (Shain & Miller, 1982; Diaz et al., 2009)

Moreover, pinostrobin isolated from *Polygonum lapathifolium* ssp. nodosum quickly penetrates through cytoplasm to the cellular nucleus of the cultured cells, and gives intensive apoptotic response in stimulating leukemic cells *in vitro*. The number of apoptotic cells increased with the concentration of pinostrobin: 10 nM, 25 and 60%; 100 nM, 45 and 76%; 1 microm, 70 and 88% for Jurkat and HL60 cell lines, respectively (Smolarz et al., 2006).

Pinostrobin and pinocembrin have been also isolated from the chloroform extract of the red rhizome variety of *Boesenbergia pandurata* (Robx.) Schltr. and the *n*-hexane and ethyl acetate extracts of the roots of *Renealmia nicolaioides* showed significant topical anti-inflammatory activity in the assay of TPA-induced ear edema in rats. (Tuchinda et al., 2002; Gu et al., 2002). Other studies have demonstrated that pinostrobin inhibits voltage-gated sodium channels of mammalian brain (IC50 23 µM) based on the ability of this substance to suppress the depolarizing effects of the sodium channel selective activator veratridine in a synaptoneurosomal preparation from mouse brain (Russell et al., 2010). Moreover pinocembrin has showed anti-inflammatory activity in the sheep red blood cell-induced delayed-type hypersensitivity reaction. (Sala et al., 2003).

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Authors' contributions

JR contributed in collecting plant sample, running the laboratory work, analysis of the data and drafted the paper. LC contributed to biological studies and NMR analysis. VM contributed in collecting plant sample, identification and confection of herbarium. SA contributed to plant collection. OM designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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