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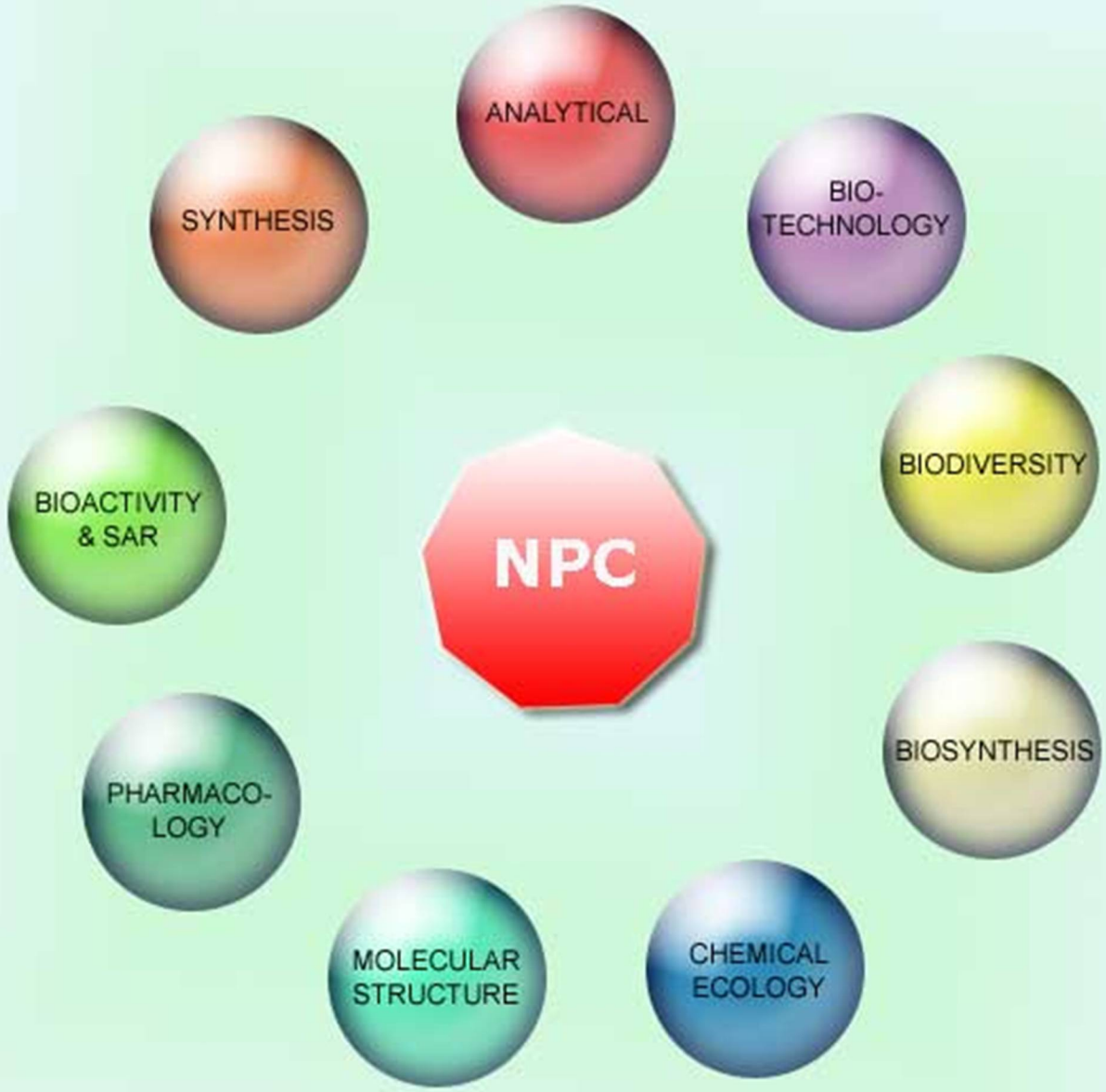

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Validation of the Ethnopharmacological Use of *Polygonum persicaria* for its Antifungal Properties

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Polygonum L. genus (Polygonaceae) is represented in Argentina by 21 species and some of them have been used in the traditional medicine of our country to treat affections related with fungal infections, such as skin ailments and vaginal diseases. With the aim of contributing to the correct ethnopharmacological use of this genus, in the present work we describe the antifungal properties of *P. persicaria* (species not studied up to now) and the bio-guided isolation of the main active compounds. Results showed that dichloromethane extracts was the most active with MICs (Minimun Inhibitory Concentrations) between 31.2 – 1000 μg/mL, validating the ethnopharmacological use of *P. persicaria* to treat affections related with fungal infections in the Argentinean traditional medicine.

Keywords: Validation, Ethnopharmacological use, *Polygonum*, Antifungal activity.

Since the early 1980s, fungal infections have emerged as major causes of morbi-mortality, mainly among immunocompromised patients. The majority of deaths were associated with species of *Candida*, *Aspergillus* and *Cryptococcus* [1]. Instead, dermatophytes such as *Trichophyton* and *Microsporum* spp. produce superficial infections (tineas) which are usually not threatening but dramatically diminish the quality of life of human beings [2]. Although it appears to be an array of antifungal agents (polyenes, azoles, allylamines and the recent echinocandins) there are, in fact, few therapeutic options. Decreased susceptibilities of yeasts to the currently available antifungal agents [3] added to the increase in the number of reported cases of resistance [4], have led to a general consensus that new efforts for detecting novel antifungal entities remain a priority. In this context, the study of plants with history of ethnopharmacological use for ailments related to fungal infections, can serve two goals: validation of the use of traditional medicines and finding new leads [5].

Polygonum L. genus (Polygonaceae) is represented in Argentina by 21 species and some of them have been used to treat affections related with fungal infections, such as skin ailments and vaginal diseases [6]. Previous studies of this genus reported that *P. punctatum* possessed antifungal properties against yeasts and dermatophytes [7]. With the aim of contributing to the correct ethnopharmacological use of this genus, in a previous work we described the antifungal properties of *P. acuminatum* [8] and in this work we describe those of *P. persicaria* (species not studied up to now) and the bio-guided isolation

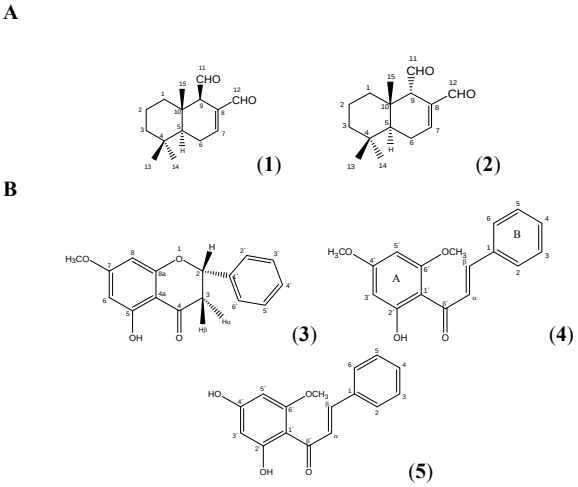

Figure 1: **A**) Sesquiterpene [polygodial (**1**) and isopolygodial (**2**)] and **B**) flavonoids [pinostrobin (**3**), flavokawin B (**4**) and cardamonin (**5**)] isolated from *P. persicaria* DCM extract.

of two sesquiterpene dialdehydes: polygodial (**1**), isopolygodial (**2**), and three flavonoids: pinostrobin (**3**), flavokawin B (**4**) and cardamonin (**5**) (Figure 1). Compounds **1** and **2** were previously isolated from *Drymis spp*. [9,10], *P. punctatum* [7] and *P. acuminatum* [8], while compounds **3**-**5** were previously isolated from *Boesenbergia pandurata*, *Myrica pensilvanica*, *P. ferrugineum* and *Piper spp* [11-13].

Compounds **1**-**5** were evaluated for their antifungal activities with the microbroth dilution assay recommended by the Clinical and Laboratory Standards Institutes (CLSI)

Table 1: Antifungal activity (MICs in µg/mL) of *P. persicaria* extracts.

Species	Extract	Antifungal activity (MICs in µg/mL)								
		Ca	*Sc*	*Cn*	*Afu*	*Afl*	*An*	*Mg*	*Tr*	*Tm*
P. persicaria	Hex	I	I	1000	I	I	I	1000	500	1000
	DCM	1000	500	500	1000	1000	1000	125	62.5	31.2
	EtOAc	1000	1000	1000	I	I	I	1000	500	1000
	MeOH	I	I	I	I	I	I	I	I	I
Standards drugs	Ketoconazole	0.50	0.50	0.25	0.12	0.50	0.25	0.04	0.02	0.02
	Amphotericin	1.00	0.50	0.25	0.50	0.50	0.50	0.12	0.07	0.07
	Terbinafine	-	-	-	-	-	-	0.04	0.01	0.04

Ca: *Candida albicans* ATCC 10231; *Sc*: *Saccharomyces cerevisiae* ATCC 9763; *Cn*: *Cryptococcus neoformans* ATCC 32264; *Afu*: *Aspergillus fumigatus* ATCC 26934; *Afl*: *Aspergillus flavus* ATCC 9170; *An*: *Aspergillus niger* ATCC 9029; *Mg*: *Microsporum gypseum* C 115; *Tr*: *Trichophyton rubrum* C 113; *Tm*: *Trichophyton mentagrophytes* ATCC 9972. I: Inactive = MIC > 1000 µg/mL.

Table 2: Antifungal activity (MICs in µg/mL) of compounds isolated from *P. persicaria*.

Compounds		Antifungal activity (MICs in µg/mL)								
		Ca	*Sc*	*Cn*	*Afu*	*Afl*	*An*	*Mg*	*Tr*	*Tm*
1		3.90	15.6	7.8	250	250	250	62.5	7.8	7.8
2		250	125	125	250	250	250	62.5	62.5	31.2
3		250	250	250	125	125	250	62.5	62.5	62.5
4		I	I	250	250	125	250	125	125	125
5		250	250	250	250	250	250	62.5	15.6	15.6
Standards drugs	Ketoconazole	0.50	0.50	0.25	0.12	0.50	0.25	0.04	0.02	0.02
	Amphotericin	1.00	0.50	0.25	0.50	0.50	0.50	0.12	0.07	0.07
	Terbinafine	-	-	-	-	-	-	0.04	0.01	0.04

Ca: *Candida albicans* ATCC 10231; *Sc*: *Saccharomyces cerevisiae* ATCC 9763; *Cn*: *Cryptococcus neoformans* ATCC 32264; *Afu*: *Aspergillus fumigatus* ATCC 26934; *Afl*: *Aspergillus flavus* ATCC 9170; *An*: *Aspergillus niger* ATCC 9029; *Mg*: *Microsporum gypseum* C 115; *Tr*: *Trichophyton rubrum* C 113; *Tm*: *Trichophyton mentagrophytes* ATCC 9972. I: inactive = MIC > 250 µg/mL).

[14] and results are shown in Table 2. They were active against yeasts, *Aspergillus spp*. and dermatophytes with MICs between 3.90 - 250 µg/mL

As it can be observed in Table 2, the five compounds isolated from *P. persicaria*, drimanes as well as flavonoids, all showed antifungal activity. Among them, polygodial (**1**) showed the best activity against yeasts and dermatophytes with MICs between 3.9 to 62.5 µg/mL and it was almost inactive against species of *Aspergillus* genus. Its epimer, isopolygodial (**2**), showed a lower antifungal activity (MICs between 31.2 to 250 µg/mL), suggesting that the C-9 configuration plays an important role in the antifungal activity, as we have been found in a previous paper [8].

Regarding flavonoids **3**-**5**, there is not a clear difference among the antifungal activities of them against yeasts and *Aspergillus* spp. Nevertheless, chalcone **5** showed a high antifungal activity against *T. rubrum* and *T. mentagrophytes* with MICs = 15.6 µg/mL, eight times higher than the activity showed by chalcone **4** against the same strains. This striking difference in activity against *Trichophyton* spp. could be attributed to the phenolic OH present in compound **5** which is absent in **4**.

These results show that the antifungal activity of *P. persicaria* could be attributed to polygodial but it is clear that the rest of the isolated compounds could contribute to the antifungal behavior of this traditional used species. In addition, these results validate the ethnopharmacological use of *P. persicaria* to treat affections related to fungal infections in the Argentinean traditional medicine and add a new evidence that the ethnopharmacological approach is useful in guiding the discovery of antifungal compounds against dermatophytes, as it was demonstrated in a recent survey among seven Latinaoamerican countries [15].

Experimental

Extracts preparation and compounds isolation: Air-dried aerial parts of each species (100 g) were powdered and successively macerated (3×24 h each) with Hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) with mechanical stirring to obtain the corresponding extracts, after filtration and evaporation. Bioassay-guided fractionation of DCM extract allowed us to isolate the compounds responsible for the antifungal activity. 1.1 g of *P. persicaria* DCM extract were submitted to column chromatography using mixtures of Hex: AcOEt in increasing polarity as elution solvents. We obtained 10 fractions; three of them were actives (fractions 6-8). From 150 mg of fraction 6, by repeated column chromatography, we obtained 55 and 30 mg of compounds **1** and **2** respectively. From 170 mg of fraction 7, by repeated column chromatography, we obtained 50, 46 and 25 mg of compounds **3**, **4** and **5** respectively. Additionally, from 70 mg of fraction 8, we obtained 10 mg of compound **5**. All the compounds were characterized by UV-visible, IR, ^{1}H NMR and ^{13}C NMR spectroscopy.

Antifungal assay: For the antifungal evaluation, strains from the American Type Culture Collection (ATCC, Rockville, MD, USA) and Centro de Referencia en Micología, CEREMIC [C, Faculty of Biochemical and Pharmaceutical Sciences, Suipacha 531 (2000)-Rosario, Argentina] were used: *Candida albicans* (*Ca*) ATCC 10231, *Saccharomyces cerevisiae* (*Sc*) ATCC 9763, *Cryptococcus neoformans* (*Cn*) ATCC 32264, *Aspergillus*

flavus (*Afl*) ATCC 9170, *Aspergillus fumigatus* (*Afu*) ATTC 26934, *Aspergillus niger* (*An*) ATCC 9029, *Trichophyton rubrum* (*Tr*) C 110, *Trichophyton mentagrophytes* (*Tm*) ATCC 9972 and *Microsporum gypseum* (*Mg*) C 115. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 ºC, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained and quantified following reported procedures (CLSI).[14]

Minimum Inhibitory Concentration (MIC) of each extract or compound was determined by using broth microdilution techniques according to the guidelines of CLSI for yeasts: document M27-A2 and for filamentous fungi, M38A. For the assay, stock solutions of extracts or pure compounds (100 µL) were two-fold diluted with the culture medium. A volumen of 100 µL of inoculum suspension [adjusted to $1–5 \times 10^4$ cells/spores as Colony Forming Units (CFU/mL)] was added to each well with the exception of the sterility control where sterile water was added to the well instead. Ketoconazole (Sigma Chem. Co., St. Louis, MO), Terbinafine (Novartis) and Amphotericin B (Sigma) were used as positive controls.

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