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Antifungal Activity of Aqueous Extracts and of Berberine Isolated from *Berberis heterophylla*

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SUMMARY. The *in-vitro* antifungal activity of aqueous extracts of *Berberis heterophylla* was evaluated, as well as the *in vitro/in vivo* antifungal activity of berberine isolated from *Berberis heterophylla.* In addition acute toxicity on fish and toxicity of berberine to embryo-larval stages of *Bufo arenarum* were tested. Berberine displayed a moderate but significant antifungal activity against dermatophytes fungi. Thus, the *in vivo/ in vitro* antifungal activity of this compound, combined with their lower toxic effect in comparison with the reference compounds, indicate that the potential of this alkaloid as a novel class of antifungal agent should be investigated more fully.

RESUMEN. Se evaluó la actividad antifúngica *in-vitro* de extractos acuosos de *Berberis heterophylla* como así también la actividad antifúngica *in vitro* e *in vivo* de berberina aislada de *Berberis heterophylla.* Además, se determinó la toxicidad aguda en peces y en estadíos larvarios de *Bufo arenarum.* Berberina presentó una moderada pero significativa actividad antifúngica frente a dermatofitos. La actividad antifúngica *in vivo/ in vitro* de este compuesto, combinada con su baja toxicidad en comparación con los compuestos de referencia, indica el potencial de este alcaloide para ser estudiado más profundamente como una novedosa clase de agente antifúngico.

INTRODUCTION

Berberis heterophylla Jus., commonly know in Argentina as "calafate", is a common shrub that grows wild and abundantly in open fields in the South region of Argentina (Patagonia Austral). Although some reports [1-4] indicate that other species of *Berberis* have been used as a remedy for different diseases, it appears that, at least in the south of Argentina, these plants had not been therapeutically used. First-hand interviews with local Indian communities in the state of Chubut (Argentina) indicate that the Indian people do not use "calafate" as remedy, but they use the roots only as a tincture [5].

Practically nothing is known about the pharmacological effects of *B. heterophylla*, however our suspects on potential activities of this plant were based on the presence of berberine (Fig. 1), an alkaloid present in abundant form in the extracts of *Berberis* [6,7]. Protoberberines and their

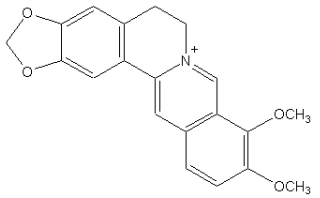

Figure 1. Structure of berberine.

relatives exhibit several types of biological activities [8]. However, to date berberine alone was found to be of clinical value and is being used in the treatment of gastrointestinal disorders. Berberine may act as a insect deterrent and insecticide [9,10]; it has also been reported as an interesting potential antifungal agent [11,12].

Recently we reported[13] an interesting antifungal activity for berberine isolated from *Berberis heterophylla* against *Candida albicans*, which is the leading primary agent causing su-

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PALABRAS CLAVE: Actividad antifúngica *in-vitro/in-vivo, Berberis heterophylla*, Berberina, Dermatofitos, Toxicidad.

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perficial and often fatal disseminated infections in immunocompromised patients [14]. Although candidiasis usually respond readily to treatments, it is difficult to completely eradicate it and patients must receive the antifungal drugs over a long period of time which leads to the development of resistance [15]. It is interesting to note that berberine displayed antifungal activity not only against standardized strains, but against clinical isolated of *Candidas*. We tested it against 8 clinical strains from different body humors from different immunocompromised patients; MIC values ranged from >128 to 16 µg/ml were obtained for berberine indicating a moderate but significant activity against *C. glabrata, C. albicans, C. lusitaniae, C. krusei* and *C. parapsilosis* [13].

On the basis of the above results we were intrigued to know if the infusions of leaves, stem and roots of *Berberis heterophylla* have or have not antifungal effect against dermatophytes fungi. We are also interested to know if berberine isolated from *Berberis heterophylla* possess any antifungal activity against a panel of standardized dermatophytes. On the other hand, in the past it has been shown that many exiting new "lead compounds" found to be highly active on the level of the targeted enzyme (biochemical activity) and on the level of fungal cell (in vitro activity) were either excessively toxic or inactive when tested *in vivo*. Therefore, it is essential that the potential effectiveness of the new compound is also evaluated in animal models. Thus, we wish to report here the in *vitro/in vivo* antifungal activities and toxicity of aqueous extracts and of berberine isolated from *Berberis heterophylla*.

MATERIAL AND METHODS

Plant material

Berberis heterophylla Juss, leaves and stems were collected in Comodoro Rivadavia, Chubut, Argentina in June 2000, and was authenticated by Ing. Agr. Mónica Stronati (Department of Biology, National University of Patagonia). A voucher specimen was deposited in the Herbarium of the Natural Sciences Faculty of the National University of Patagonia "S.J.B."

Extraction and isolation

Dried and powdered roots of *B. heterophylla* (730 gr) were extracted with methanol at room temperature for 96 h (2 L each) by adding fresh solvent every 24 h and eluting. The combined methanol extractable were concentrated to a dark brown residue (33.5 g). The dry extract was dissolved in 1% aqueous HCl and the solution was extracted with Cl_2CH_2. Next, the aqueous phase was basified to pH 8 - 9 with 15% NH_3 and extracted with CH_2Cl_2. The precipitate obtained was filtered and then purified by repetitive column chromatography on silica gel (Cl_2CH_2:MeOH, 9:1; 8:2) to produce Berberine, as yellow needles (701.4 mg g) which was identified by ^{1}H NMR, ^{13}C NMR and compared with the spectral data from literature values [16].

Antifungal in vitro assay

The microorganisms used for the fungistatic evaluation were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). *Trichophyton mentagrophytes* ATCC 9972, *Microsporum canis* C 112, *Trichophyton rubrum* C 113, *Epidermophyton floccosum* C 114 and *Microsporum gypseum* C 115 were maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to reported procedures [18] and adjusted to 106 spores with colony forming ability/ml.

The antifungal activity of berberine was evaluated with the agar dilution method by using Sabouraud-chloramphenicol agar as previously described [19-21]. Stock solutions of compounds (10 mg/mL in DMSO) were diluted to give serial two-fold dilutions that were added to each medium, resulting in concentrations ranging from 0.10 to 250 µg/mL. MIC for each compound was defined as the lowest concentration that produces no visible fungal growth after the incubation time.

Antifungal in vivo assay

Male albino guinea-pig (breeder: Bioterio Central - UNSL) with a body weight of 400-500 g were used, with six animals randomly assigned to each dose group. The animals were kept in fully air-conditioned animals rooms maintained at 20-22 °C and fed with guinea-pig pellets and water *ad libitum*.

Techniques used for the trichophytosis model have been summarized and discussed by Rippon & Ryley [22,23]. We use the method suggested by Polac [24], which has been used previously by us with others compounds obtaining excellent results [25]. Himalayan spotted white guinea pigs weighing 400-500 g were used. For each experiment, *T. mentagrophytes* (initially obtained from *T.m*. ATCC 9972 purchased from the American

type culture collection (Rockville MD, USA)) was freshly isolated from air infected guinea pigs.

The conidia from these heavily sporulating primary cultures on Sabouraud glucose agar were suspended in honey using mort and pestle. Small quantities of this suspension containing approximately 10^5-10^6 conidia were applied to the animals on the shaven skin of one flank, witch had been previously roughened with emery paper. Plucking and shaving of the skin is necessary as this provides some trauma to the area and insures a "take" of the inoculum. The inoculation was performed in subcutaneous way using an hypodermic syringe. This inoculation procedure leads to the development of anti-inflammatory mycotic foci that reach the maximum intensity after two weeks and heal spontaneously after three weeks. Infection was followed by assessing the lesion clinically and ascribing an arbitrary score based on redness, scaling, indurations, erythema, thickening and ulceration. The antifungal treatment begins five days after inoculation.

Each guinea-pig was treated topically with a cream formulation of both berberine and ketoconazole. For this purpose compounds were dissolved with DMSO and then incorporated in a cream (1% concentration). The creams were spread on the infected skin area of the animals with Driglaski spatula. The treatment was applied once daily on the 5, 7 and 9 days during the test.

Acute toxicity test

We chose the static technique recommended by the U.S. Fish and Wildlife Service [26] which was modified in order to use a lower amount of test compound. The test solutions and test organism were placed in test chambers and kept there during the test. The test began upon initial exposure to the potentially toxic agent and continued for 96 h. The number of dead organisms in each test chambers, was recorded and the dead organisms were removed every 24 h; general observations on the conditions of test organism were also recorded at this time; however the percentage of mortality were recorded at 96 h.

We evaluate the toxic effect of berberine using a toxicity test on fish. Toxicity tests with fish were conducted in 2 liters wide-mouthed jars containing 1 liter of test solution. Fish *Poecilia reticulata sp.* with a size of about 0.7 to 1 cm were tested at each concentration. At least 10 fish were exposed to each concentration for all definitive tests. Five concentrations were used per toxicity test (ranged from 1ppm to 80 ppm).

Toxicity of berberine to embryo-larval stages of Bufo arenarum H.

Tests were performed in temperature regulated environmental laboratory using the static-renewal technique [27]. Embryos of *Bufo arenarum* were obtained *in vitro* [28] and they (n = 20) were put in test chamber of one liter, the first embrionary stages were followed applying the pattern series for this specie described by Del Conte & Sirlim [29]. Berberine was administered in a concentration of 5 ppm using two replicates per test. Larval development of *B. arenarum* was assessed according to De Martin *et al.* [30], and the time of exposure lasted 21 days (larval stage XII). Controls were established and during the experiment embryos and larvae were examined daily observing swimming movements, food consumption and percent survival.

All the bioassays reported here have been performed considering strictly biosafety recommendations and environmental care. Thus, assays using experimental animals were conducted in accordance with the internationally accepted principles for laboratory animals use and care. Laboratory animals were hand led according to the Animal care Guidelines of the National Institute of Health (NIH publication # 85-23 revised in 1985).

RESULTS AND DISCUSSION

Antifungal results

The agar dilution method showed that none of the aqueous extracts tested displayed significant antifungal activity against dermatophytes fungi. These results are in agreement with those previously reported for different aqueous extracts of *B. Heterophylla* tested against *Candida albicans* and other *Candida* species [13]. Thus, our results could account for, at least in part, why the Indian people of Patagonia Austral do not use infusions of *Berberis heterophylla* for the treatment of diseases. In contrast to the above results, berberine isolated from *B. heterophylla* displayed a moderate but significant antifungal effect against all the dermatophytes fungi tested here; being *Ephidermophyton floccosum* the most sensitive specie (Table 1). These results are in a complete agreement with previous papers reporting antifungal [13,31-33] and antibacterial [34] activities for this alkaloid.

In the next step of our study, we evaluated the *in-vivo* antifungal activity of berberine using the trichophytosis model. In this assay ketoconazole was chosen as a reference compound. These results are shown in Table 2.

Our results indicate that berberine display an

Compound	*M.c.*[a]	*M.g.*[b]	*T.m.*[c]	*T.r.*[d]	*E.f.*[e]
Berberine	50	25	25	25	12.5
Amphotericin B	>250	6.21	6.21	25	0.3
Terbinafine	0.01	0.04	0.01	0.04	0.004
Ketoconazole	15	6.25	6.25	15	25

Table 1. MICs values (µg/ml) of berberine against dermatophytes fungi. [a]*Microsporum canis*, [b]*Microsporum gypseum*, [c]*Trichophyton rubrum*, [d]*Trichophyton mentagrophytes*, [e]*Ephidermophyton floccosum*.

Compound	Score test	
	Day 11	Day 16
Berberine	0-1	0
Ketoconazole	0	0
Positive control	2-4	1-2

Table 2. *In vivo* antifungal activity obtained for berberine; ketoconazole was used as reference compound.The following arbitrary score was used: 0 = no findings; 1= few slightly erythematous places on the skin; 2= well-defined redness, swelling with bristling hairs or well-defined redness, bald patches, scaly areas; 3= large areas of marked redness, incrustation, scaling bald patches, ulcerated in places; 4= the same as the positive control, mycotic foci well developed with ulceration in some cases.

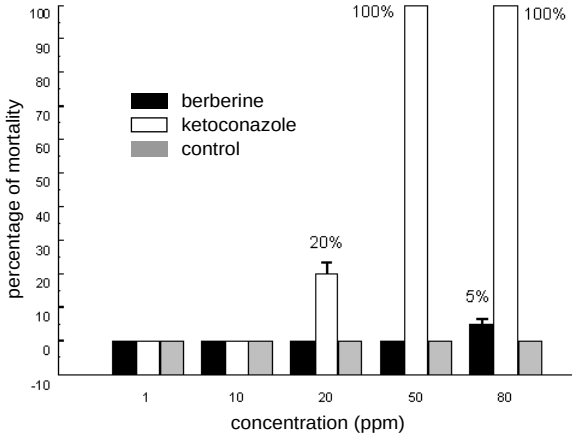

Figure 2. Results of acute toxicity test, showing the percent of mortality at different concentrations. Numerical values at column heads represent the percentage of mortality.

interesting *in vivo* antifungal effect in this animal model. Although the *in vivo* activity obtained for berberine is lower with respect to obtained for ketoconazole; the *in vivo* activity displayed by berberine is still significant.

All tested dermatophytes were inhibited at 50 µg/ml and some of then at lower concentration. The most sensitive species was *Epidermophyton floccosum*. Since dermatophytes are a group of fungi which characteristically infect the keratinized areas of the body and dermatomycoses are very difficult to eradicate, it is very interesting to note that berberine showed *in vitro/ in vivo* activity against dermatophytes.

Acute toxicity assays

It is clear that toxicity of any new potential antifungal agent is a key factor for the future perspectives of this class of compounds, therefore we performed a preliminary study on the potential acute toxicity of berberine. It is crucial to determine the actual capacities and limitations of this alkaloid acting as antifungal agent. In this preliminary study we chose two different test: acute toxicity on fish and toxicity to embryo-larval stages of *Bufo arenarum (H)*.

Acute toxicity tests are generally used to determine the level of toxic agent that produces an adverse effect on a specified percentage of the test organism in a short period of time. Because death is normally an easily detected and obviously important adverse effect, the most common acute toxicity test is the acute mortality test. The most important data obtained from a toxicity test are the percentage of test organisms that are affected in a specified way by each of the treatments. The results derived from these data is a measure of the toxicity of the potentially toxic agent to the test organisms under the conditions of the test or, in other words, a measure of the susceptibility of the test organisms to the potentially toxic agent.

The results of acute toxicity obtained for berberine and ketoconazole are shown in Figure 2.

Both berberine and ketoconazole do not show acute toxicity at low concentrations (1 and 10 ppm). However, ketoconazole displayed 20% and 100 % of mortality at 20 ppm and 50 ppm respectively. It should be noted that at these concentrations berberine is not toxic. Berberine shows only a very low percentage of mortality (5%) from 80 ppm.

Concentration	Number of hatched larvae	Defective developments		Deaths		Motility	Food consumption
		No	%	No	%		
5 ppm	20	0	0	0	0	normal	normal

Table 3. Results obtained for toxicity of embryo-larval stages of *Bufo arenarum (H).*

It is evident that the acute toxicity of ketoconazole is significantly higher with respect to that of berberine. These results obtained for berberine are in agreement with those attained for the toxicity of embryo-larval stages of *Bufo arenarum H.* see (Table 3). Thus to 5 ppm of concentration, berberine do not display embryo toxic activity neither embryo lethal effect.

CONCLUSIONS

The aqueous extracts of *B. Heterophylla* do not possess significant antifungal activity against dermatophytes fungi tested here. These results combined with those recently reported [13] for the antibacterial activity of these extracts, account for why the Indian people of Patagonia Austral do not use this plant as a remedy. On the other hand, berberine isolated from *B. heterophylla* displayed an interesting *in vitro/in vivo* antifungal activity against a panel of dermatophytes fungi. These results are in agreement with the inhibitory effects of protoberberines (including berberine) on sterol and chitin biosyntheses in *Candida albicans* reported by Park *et al.* [34]. It had been reported that berberine would be an interesting candidate acting as antifungal agent. Our results are an additional support for this assumption. The *in vivo/in vitro* antifungal activity of this compound, combined with their lower toxic effect in comparison with ketoconazole, indicate that the potential of this alkaloid as a novel class of antifungal agent should be investigated more fully.

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