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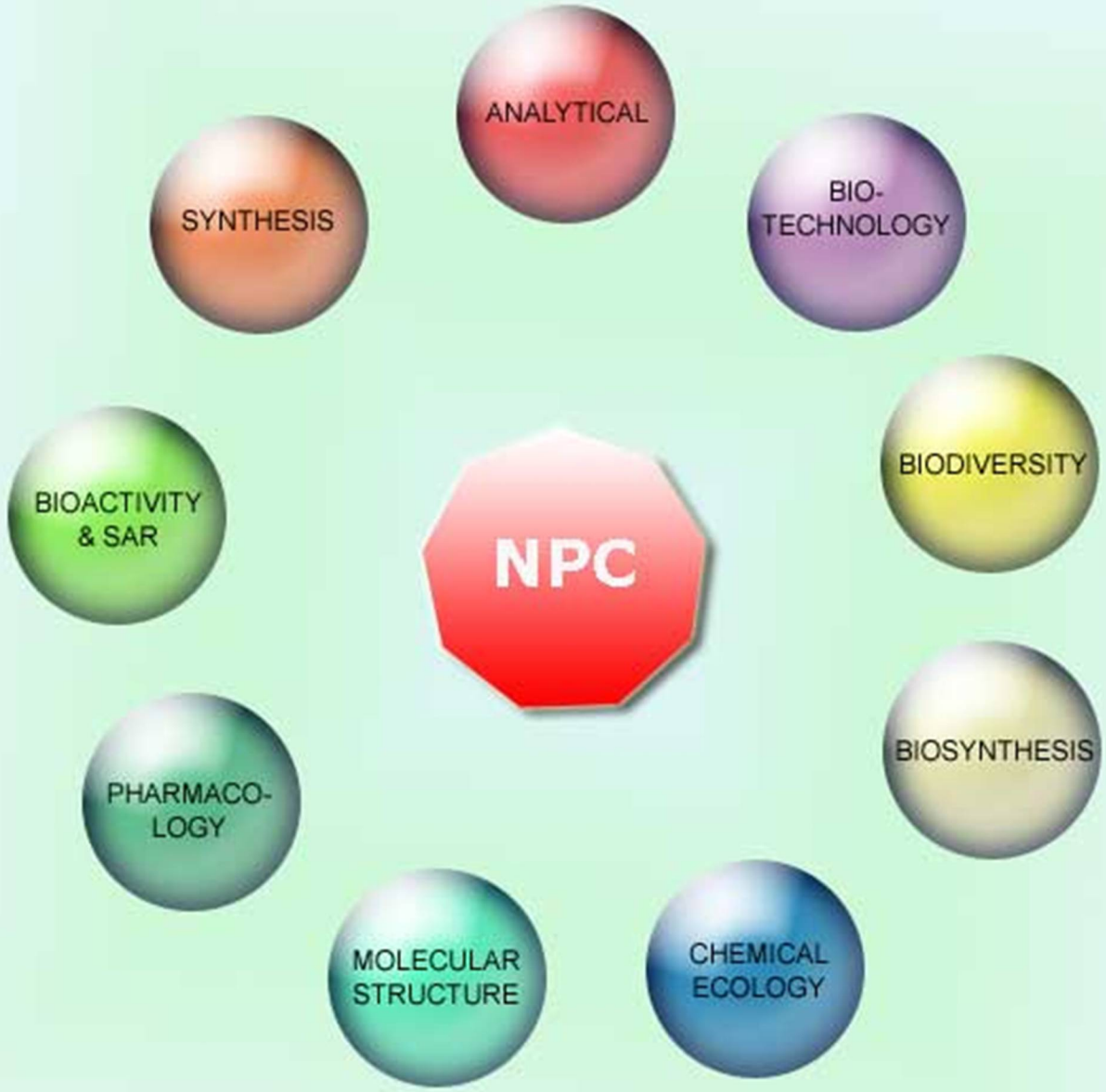

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Antifungal Activity of Saponin-rich Extracts of *Phytolacca dioica* and of the Sapogenins Obtained through Hydrolysis

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A saponin-rich extract of *Phytolacca dioica* L. berries, its acid hydrolysate, and its major aglycone, phytolaccagenin, were assayed for antifungal activity against ATCC standard cultures of *Candida albicans* and *Cryptococcus neoformans*, and against clinical isolates of these fungi. The activity of the extract was either low or negligible, but the hydrolysate, containing the sapogenins, including phytolaccagenin, and also pure phytolaccagenin, showed promising antifungal potency. Hydrolysis of a natural product extract is shown to be a useful modification leading to improved bioactivity.

Keywords: Saponins, phytolaccagenin, antifungal activity.

Invasive fungal infections are a major cause of morbidity and mortality in immunocompromised patients [1,2]. Although there appears to be a plentiful armamentarium of antifungal drugs in clinical use, in fact only a modest number of drugs are available [3]. In addition, they show undesirable toxicity, and either facilitate recurrence or lead to the development of resistance due, in part, to the intensive prophylactic use of antifungal drugs [4]. There is, therefore, a clear and urgent need for the discovery of new alternative compounds for antifungal therapy [5]. Plants provide unlimited opportunities for the isolation of new antifungal products because of their unmatched chemical diversity [6,7]. In fact, numerous antifungal compounds have been isolated from them [8-13], including some natural saponins that have demonstrated either strong or at least modest activity [14-17].

The plant genus *Phytolacca* is well known as a source of triterpenoid saponins containing aglycones with oleanolic acid-derived structures (oleanolic [18], phytolaccagenic [19,20], serjanic [21], jaligonic [22] and esculentagenic [23] acids, hederagenin [24], bayogenin [25] and phytolaccagenin [21,25,26]).Among the species of this genus, *P. dioica* L. has been shown to contain the bidesmosidic saponins esculentoside S and esculentoside L_1 [27], which are glycosides of phytolaccagenic acid and phytolaccagenin, respectively (Figure 1), the major sapogenins of this plant [28].

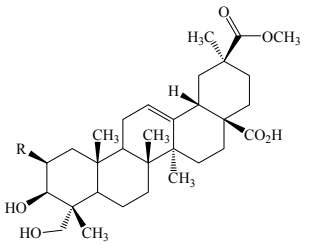

Figure 1: Structures of phytolaccagenic acid (R = H) and phytolaccagenin (R = OH).

Regarding the phytochemistry of the genus *Phytolacca*, both phytolaccoside B {3-*O*-(β–D-xylopyranosyl)-phytolaccagenin = phytolaccasaponin G [29] = esculentoside B [30]} isolated from *P. americana*

[29,31-33], *P. esculenta* [22,31], *P. dodecandra*, *P. acinosa* [29] and *P. tetramera* [26], and phytolaccoside E {3-*O*-(β−D-glucopyranosyl-(1→4)-β−D-xylopyranosyl)-phytolaccagenin = phytolaccasaponin E = esculentoside A} produced by *P. americana* [33,34] and *P. esculenta* [31], showed activity against the plant pathogen *Cladosporium herbarum* [34], and against a panel of human opportunistic yeasts and molds [26]. In addition, recent studies have demonstrated that phytolaccoside B alters the morphology of the fungal cell wall by increasing the amount of chitin and thus causing the death of the fungus [35].

It is worth noting that the antifungal activities exhibited by saponins of the genus *Phytolacca* might be useful for the development of antifungal drugs and agrochemicals. Nevertheless, although it is clear that saponins represent an interesting platform for the development of antifungal compounds, it is also well known that their complex chemical structures demand sophisticated techniques for their isolation and structure elucidation [16]. Since it has been reported recently that the chemical transformation of natural product mixtures to generate chemically engineered extracts can be an entry point to discover bioactive compounds [37,38], the saponin-rich *n*-butanol extract of *P. dioica* berries was subjected to acid hydrolysis This is expected to produce a sapogenin-enriched mixture, reducing the molecular weight and increasing the average lipophilicity of its constituents. Consequently, the generated compounds should have better chances of entering the fungal membrane, as compared with the original natural components, and exert their effects either there or in the cytoplasm.

The *n*-butanol extract (BE) of *P. dioica* berries, as well as its acid hydrolysate (BH), were tested against *Candida albicans* ATCC 10231 and *Cryptococcus neoformans* ATCC 32264, using the microbroth dilution assay. The relevance of these fungi to the epidemiology of fungal infections is well known. Candidiasis is now the fourth most prevalent blood-stream infection in immunocompromised hosts, *C. albicans* representing more than 60% of isolates from clinical infections [2]. In turn, *C. neoformans* remains an important life-threatening complication, particularly for patients who have undergone transplantation of solid organs, and, therefore, new compounds acting against this fungus are highly welcome [39].

Results showed that BE did not display any activity at concentrations up to 250 μg/mL (Table 1, line 1), but, in contrast, BH showed promising antifungal activities against the same yeasts.

Table 1: Minimum inhibitory concentrations of the *P. dioica n*-butanol extract (BE) and its acid hydrolysis mixture against standard cultures of *C. albicans* and *C. neoformans*. In addition, MIC values of phytolaccagenin (PG) and two reference antifungals are included.

Sample	MIC *C. albicans* ATCC 10231 (μg/mL)	MIC *C. neoformans* ATCC 32264 (μg/mL)
BE	> 250	> 250
BH	62.5	31.2
PG	62.5	15.6
Amphotericin B	0.25	0.25
Ketoconazole	0.50	0.25

Both mixtures were subjected to TLC (*n*-hexane: ethyl acetate, 30:70), and bioautographed using *C. neoformans* ATCC 32264. This qualitative bioassay showed again that BE did not have any antifungal component and instead, BH inhibited the fungal growth in a zone that could be ascribed to only one compound. The bioassay-guided fractionation of BH led to the isolation and characterization of phytolaccagenin (PG) as the compound responsible for the antifungal activity. In microdilution assays, PG showed similar antifungal behavior to BH (MIC = 62.5 μg/mL) against *C. albicans* and slightly better activity than the hydrolysate against *C. neoformans* (MIC 15.6 and 31.2 μg/mL respectively) (Table 1).

In order to get a closer look at the differences in antifungal properties among BE, BH, and PG, we assayed the three samples in a new panel consisting of six clinical isolates of *C. albicans* and ten of *C. neoformans*, obtained from immunocompromised patients.

The MIC values of the three samples were determined against this extended panel by using three endpoints MIC_{100}, MIC_{80} and MIC_{50} (the minimum concentration of compounds that inhibits 100, 80 and 50 % of growth, respectively) (Table 2). The application of less stringent endpoints, such as MIC_{80} and MIC_{50}, has been shown consistently to represent the *in vitro* activity of compounds [40] and often provides a better correlation with other measurements of antifungal activity [41].

Results showed that BE was almost inactive (MIC ≥ 250 μg/mL) against the whole panel listed in table 2. In contrast, BH and PG showed activity against all strains of the panel. Regarding the behavior of both BH and PG against *C. albicans*, they showed similar MIC_{100} (62.5 μg/mL for both in all strains tested), MIC_{80} (range 31.2 - 62.5 for BH and 62.5 μg/mL for PG) and MIC_{50} values (31.2 - 62.5 μg/mL) for the hydrolysate and the genin respectively. In turn, results of BH and PG against *C. neoformans* showed again that both samples possessed similar activity, but with the difference that this fungal species was more sensitive to BH and PG (MIC range observed: 15.6 - 62.5 μg/mL) than *C. albicans*.

Table 2: Comparative minimum inhibitory concentrations (MIC_{100}, MIC_{80} and MIC_{50}) of the *Phytolacca dioica* *n*-butanol extract (BE), its hydrolysate (BH), and phytolaccagenin (PG) against clinical isolates of *Candida albicans* (*Ca*) and *Cryptococcus neoformans* (*Cn*). For the sake of comparison, the MIC_{100} of both samples against ATCC standard cultures and those of amphotericin B (Amph B) against the clinical isolates are included.
[a] NA = not active (MIC_{100} > 250 μg/mL).

			MIC_{100}		MIC_{80}		MIC_{50}		MIC_{100}
		BE	BH	PG	BH	PG	BH	PG	Amph B
Ca	ATCC10231	NA[a]	62.5	62.5					
Ca	C 125	NA	62.5	62.5	62.5	62.5	31.2	62.5	0.78
Ca	C 126	NA	62.5	62.5	31.2	62.5	31.2	62.5	1.56
Ca	C 127	250	62.5	62.5	62.5	62.5	31.2	31.2	0.78
Ca	C 128	NA	62.5	62.5	31.2	62.5	31.2	62.5	1.56
Ca	C 129	NA	62.5	62.5	62.5	62.5	62.5	31.2	0.78
Ca	C 130	250	62.5	62.5	31.2	62.5	31.2	31.2	0.39
Cn	ATCC 32264	NA	31.2	15.6					
Cn	IM 983040	NA	62.5	31.2	62.5	31.2	31.2	15.6	0.25
Cn	IM 972724	250	62.5	62.5	62.5	31.2	31.2	15.6	0.13
Cn	IM 042074	NA	62.5	62.5	62.5	31.2	31.2	15.6	0.06
Cn	IM 983036	NA	62.5	62.5	62.5	31.2	15.6	15.6	0.25
Cn	IM 00319	NA	62.5	62.5	62.5	31.2	62.5	31.2	0.13
Cn	IM 972751	NA	62.5	31.2	31.2	31.2	15.6	15.6	0.25
Cn	IM 031631	NA	62.5	31.2	31.2	31.2	15.6	15.6	0.25
Cn	IM 031706	250	62.5	31.2	31.2	31.2	15.6	15.6	0.13
Cn	IM 961951	NA	62.5	62.5	62.5	31.2	31.2	15.6	0.25
Cn	IM 052470	NA	62.5	31.2	62.5	31.2	31.2	15.6	0.06

In conclusion, (a) acid hydrolysis of the either inactive or marginally active *n*-butanol extract of *P. dioica* berries (BE) led to a mixture containing sapogenins (BH, where phytolaccagenin and phytolaccagenic acid are presumably the major constituents [28]), which was not only more active against the standard fungal strains, but also against the clinical isolates of *C. albicans* and *C. neoformans*; (b) phytolaccagenin was responsible for practically all the activity of BH, as the difference observed in the activity of both samples for all isolates was in no case more than one two-fold dilution; (c) *C. neoformans* proved to be more sensitive than *C. albicans* to the *P. dioica* sapogenins and to pure phytolaccagenin.

This work demonstrates that the hydrolysis of a saponin-rich extract led to a new mixture with stronger and promising activities that could open new avenues for the discovery of novel antifungal agents. In addition, the work provides new evidence that the chemical modification of a natural product extract can lead to mixtures with improved properties that can be used as sources of bioactive compounds [37].

Experimental

Plant material and chemistry: *Phytolacca dioica* L. (Phytolaccaceae) berries, collected at the Faculty of Sciences campus of the University of Chile in Santiago in February 2008 (voucher specimen SQF 22461, Herbarium of the Faculty of Chemical and Pharmaceutical Sciences) were dried, ground and extracted sequentially with hexanes, CH_2Cl_2 and MeOH. From the concentrated MeOH extract abundant crystals of sucrose precipitated and were removed Dilution with water, extraction with *n*-BuOH, and concentration to dryness afforded BE. This was heated under reflux for 3 h with 3 M HCl, which on cooling deposited a brown solid (BH). BH was chromatographed on silica gel to obtain phytolaccagenin in sufficient quantity for the biological assays. The purity (> 95%) and identity of phytolaccagenin were established by TLC and HPLC, and by one- and two-dimensional ^1H and ^{13}C NMR spectroscopy.

Microorganisms and media: For the microbroth dilution method, standard strains (*C. albicans* ATCC 10231, *C. neoformans* ATCC 32264) were obtained from the American Type Culture Collection, Rockville, MD, USA. Six clinical isolates of *C. albicans* and 10 of *C. neoformans* were kindly provided by the Centro de Referencia en Micología (C, CEREMIC, Rosario) and the Malbrán Institute (IM, Av. Vélez Sarsfield 563, Buenos Aires). Their voucher numbers are presented in Table 2. They were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30ºC, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid), and sub-cultured every 15 days to avoid pleomorphic transformations. Inocula were obtained according to reported procedures [40,41] and adjusted to 1-5 × 10^3 cells/spores with colony forming units (CFU/mL).

Antifungal susceptibility tests: Minimum inhibitory concentrations (MIC) of BE, BH, and PG were determined by using broth microdilution techniques according to the guidelines of the CSLI (formerly NCCLS) [40]. MIC values were determined in RPMI-1640 (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with MOPS. Microtiter trays were incubated at 30ºC in

a moist, dark chamber, and MICs were recorded at 48 h. For the assay, stock solutions of either the extract or pure compound were diluted with RPMI from 1000 or 250, respectively, to 0.98 μg/mL (final volume = 100 μL) and final DMSO concentration ≤ 2%. Inoculum suspension (100 μL) was added to each well with the exception of the sterility control, where sterile water was added instead. Ketoconazole and amphotericin B (Sigma Chemical Co., St. Louis, MO, USA) were used as positive controls. Endpoints were defined as the lowest concentration of either extract or compound resulting in total inhibition (MIC_{100}) of visually observed growth compared with the growth in the control wells containing no test compound. To determine the minimal concentrations causing 80 or 50% inhibition of fungal growth (MIC_{80} or MIC_{50}), the percentages of inhibition of the clinical isolates of *C. albicans* and *C. neoformans* were determined at 7 concentrations: 250, 125, 62.5, 31.25, 15.62, 7.8, and 3.9 μg/mL, for BH and PG). The tests were performed in 96-well microplates. Sample test wells (STW) were prepared with stock solutions of BH or PG in DMSO (≤ 2%), diluted with RPMI-1640 to each final concentration (100 μL). An inoculum suspension (100 μL) was added to each well. A growth control well (GCW) containing medium, inoculum, and the same amount of DMSO used in the STW, but sample-free) and a sterility control well (SCW) (sample, medium and sterile water instead of inoculum) were included for each strain tested. Microtiter trays were incubated for 48 h in a moist, dark chamber at 30ºC. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphotericin B was used as a positive control. Tests were performed in triplicate. Reduction of fungal growth due to each sample concentration was calculated as follows:

% inhibition = 100 – (OD_{405} STW – OD_{405} SCW) / OD_{405} GCW – OD_{405} SCW.

From these data, MIC_{80} and MIC_{50} were determined. They were defined as the lowest concentration of either BH or PG that showed 80% or 50% reduction of growth, respectively.

Bioautography: BE, BH and PG were analyzed in duplicate by TLC. One of the plates was sprayed with anisaldehyde/sulfuric acid reagent followed by heating at 100ºC for 5-10 min to visualize the spots [42]. The other plate was bioautographed as follows: warm agar-Sabouraud medium with 0.02% phenol red (1 mL/cm^2) containing a *C. neoformans* ATCC 32264 inoculum of 1-4 × 10^5 cells/mL, quantified in accordance with reported procedures [43,44], was distributed over the developed TLC plate. After solidification of the medium, the plate was put into a sterile Petri dish, covered, and incubated overnight at 28ºC. Subsequently, the bioautogram was sprayed with an aqueous solution (1 mg/mL) of MTT (methylthiazolyltetrazolium chloride) and incubated for 2 h at 28ºC. Yellow inhibition zones appeared against a dark brown background [44].

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