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In Vitro Antifungal Activity of Crude Hydro-Alcoholic Extract of *Petiveria alliacea* L on Clinical *Candida* Isolates

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Abstract

Background: The global burden of infections due to *Candida* and the emerging resistance to antifungals has led to the search for new therapeutic alternatives. The aim of the present work it was to evaluate the *in vitro* antifungal activity of crude hydro-alcoholic extract of *Petiveria alliacea* L (HAEPAL) versus fluconazole against *Candida* isolates.

Methods: *In vitro* antifungal activity was evaluated by broth microdilution method in front of 125 *Candida* isolates (60 *C. albicans*). Inoculum of 1.5×106 CFU/mL in sterile saline solution were incubated with five dilutions of the extract (128, 64, 32, 16 and 8 µg/mL). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of HAEPAL showing ≤ 50% growth compared with the extract-free growth control estimated by counting the CFU/mL. Fluconazole susceptibility was assessed by ATB™ Fungus 3 and E-test according to manufacturer's instructions.

Results: HAEPAL showed higher antifungal activity compared to fluconazole. Only four isolates (one *C. albicans*, one *C. gl*abrata and two *C. krusei*) exhibited high MICs (≥ 64 µg/mL) compared to 34 (19 *C. albicans* and 15 *Candida non-albicans*) which showed resistance to fluconazole.

Conclusion: These results show the antifungal potentiality of HAEPAL, which could become a potential alternative for *Candida* treatment.

Key words:

Plant extract; *Petiveria alliacea* L, *Candida*; Fluconazole; Antifungal activity

Abbreviations:

HAEPAL: Hydro-Alcoholic Extract of *Petiveria Alliacea L*; CLSI: Clinical and Laboratory Standards Institute; SSS: Sterile Saline Solution; MIC: Minimum Inhibitory Concentration; MIC^{50}: Lowest Extract Concentration able to Inhibit at Least 50% of Growth Compared with Culture Medium without HAEPAL; SDB: Sabouraud Dextrose Broth; SDA: Sabouraud Dextrose Agar; GM: Geometric Mean; SDD: Susceptible Dose Dependent

Introduction

The incidence of fungal infections has drastically increased over the past three decades and has become a major cause of morbidity and mortality [1]. Antifungal therapy failure depends on multiple factors including microbiological resistance. The use of fluconazole as standard prophylaxis in aids patients and the hematopoietic cell transplantation setting was simultaneously accompanied by increased acquired and innate resistance to antifungal drugs. Azole resistance was first noted in *Candida* species in patients with acquired immunodeficiency syndrome (of 348 isolates tested against fluconazole, and 33% were found to be resistant). With the advent of new azole and echinocandin agents, we have seen the emergence of more azole-resistant and echinocandin-resistant fungi [1,2]. This has stimulated a search for safe and more potent antifungal products. In this sense the plant kingdom has been an important source of large numbers of natural drugs over the years [1,3].

There are approximately 8000 species of Cuban medicinal plants and nearly half are endemic species. This is a rich source for the development of potential therapeutic agents [4]. *Petiveria alliacea* L, popularly known as anamú, is a native plant from Latin America that belongs to the family Phytolaccaceae. Because fresh leafs contain the similar chemical compounds also present in the rest of the plant, it is considered the richest vegetative organ responsible for different pharmacological activities [5].

There are few reports of antifungal activity of *P. alliacea* against fungal species [6-8]. However, literature on the evaluation of various preparations from this plant against yeasts does not provide conclusive results due to the scarce number of studied isolates [9,10]. Given the presence of substances with proven anti-fungal activity in this plant, this work aimed to determine the *in vitro* activity of a crude hydro-alcoholic extract of *P. alliacea* against Cuban clinical isolates of

Candida and compared it with that of fluconazole, by far the most common antifungal used in clinical practice.

Materials and Methods

Microorganisms: A total of 125 *Candida* isolates (60 *C. albicans*, 26 *C. parapsilosis*, 10 *C. tropicalis*, 9 *C. glabrata*, 8 *C. krusei*, 5 *C. lusitaneae*, 3 *C. guillermondii*, 3 *C. kefyr*, 1 *C. inconspicua* and *1 C. ciferri*) from vagina (43 %), hearing canal, blood stream (20 % each one) and oral cavity (17 %) were studied. These were previously identified by conventional methods (colony and yeast morphology on Sabouraud dextrose agar, germinative tube formation, morphology on cornmeal agar with tween 80, standard assimilation and fermentation reactions, urease production and growth at 37°C) [11].

Antifungal activity assay: A broth microdilution method based on M27A3 document of the Clinical and Laboratory Standards Institute (CLSI) [12] was used to determine yeasts susceptibility. Recommended CLSI reference strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included.

P. alliacea hydro-alcoholic extract (HAEPAL) (200 g/L) was prepared by the method described in Public Health Branch Standard 311 (NRSP 311; MINSAP, 1992). Briefly, plant leaves were kept at 40°C until dried and powdered using an appropriate mill; the material was macerated with ethanol 70% (1:5) and stirred 24 h at room temperature; finally it was stored in amber flasks with hermetic caps at 4°C until use. The extract was sterilized by filtration (0.45 and 0.22 µm membranes) and diluted in Sabouraud dextrose broth (SDB) to obtain 256, 128, 64, 32 and 16 µg/mL. Each solution was dispensed (100µL/well) in lines C to G from column 2 to 11. Row B column 2 to 11 was coated with SDB + ethanol 10%. Once the inoculum was added, HAEPAL concentration was diluted to 8, 16, 32, 64 and 128µg/mL (within the range of concentration as set by CLSI for fluconazole) and ethanol decreased to 5% as final concentration.

Candida isolates were transferred to plates with Sabouraud dextrose agar (SDA) and incubated 24-48 h at 37°C. Each culture was suspended in sterile saline solution (SSS). Turbidity was adjusted to McFarland 0.5 scale, equivalent to 1.5×10^6 colony forming units per milliliter (CFU/mL). Suspensions were dispensed (100 µL/well) in column 3 to 11. A reference strain was randomly incorporated in column 2 of each plate as internal control assay. Rows A and H and columns 1 and 12 were coated with SSS in order to reduce the evaporation effects during incubation at 37°C for 24 h.

For reading and interpretation the content of each well was vigorously pipetted and diluted 1:1000 and 1:2000 in SSS. Each dilution (10 µL) was inoculated on SDA and incubated at 37°C for 48-72 h. The CFU/mL was determined for every strain vs each dilution of the extract using a magnifying viewer (Gallenkamp, England) (Figure 1). Minimum inhibitory concentration (MIC) was defined as the lowest extract concentration able to inhibit at least 50% of growth compared with culture medium without HAEPAL. Each procedure was performed three times.

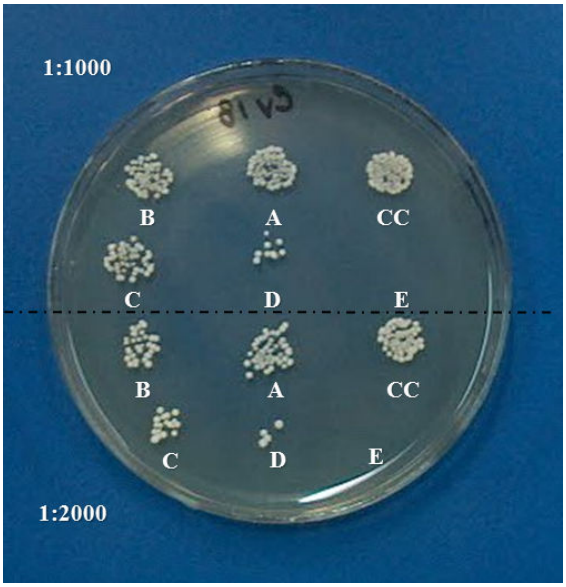

Figure 1: Sabouraud agar plate inoculated with dilutions 1:1000 (upper half of the plate) and 1:2000 (lower half of the plate) with *C. albicans* after 48 h incubation with HAEPAL 8 µg/mL (A), 16 µg/mL (B), 32 µg/mL (C), 64 µg/mL (D), 128 µg/mL (E) and control growth without extract (CC).

For fluconazole susceptibility ATB™ Fungus 3 (bioMérieux, France) was used. When susceptibility interpretation was inconclusive Etest ® strips impregnated with fluconazole (0.016 to 256 µg/mL) (AB BIODISK, Sweden) were used as an alternative method. Both methods were carried out following manufacturer's instructions.

Statistical analysis: Data analysis was performed with 6.07 XLSTAT Excel. Frequencies and percentages were calculated to compare susceptibility values among isolates according to the species. In addition, the MIC ranges and geometric mean (GM) values were determined. The MIC50 was calculated considering the cumulative percentages of isolates inhibited at different concentrations of HAEPAL and fluconazole respectively.

Results and Discussion

A main concern in medical mycology is the emergence of resistant isolates. This is especially relevant when studying azoles, the most used among the antifungal drugs [13,14]. Fluconazole is one of the widest used for treatment and prophylaxis of fungal infections particularly candidiasis [13,15]. This situation has stimulated a search for new alternatives from natural products of which the herbarium is highly attractive [1,16]. *P. alliacea*, flourishes in Cuba and has several traditional applications such as anti-inflammatory, analgesic, anti-rheumatic, antitumor, hypoglycemic, antipyretic, antispasmodic, abortifacient, diuretic, and sedative among others. It has been also proven its antimicrobial activity against virus, bacteria, protozoa and fungi [17-23]. Most of these activities have been shown for hydro-alcoholic extract from root, stem, and/or leaves maybe because the main active substance are not soluble in water, saline solution or phosphate buffer. Nevertheless, the inclusion of ethanol on the growth control at the highest concentration present on the tested dilution of

HAEPAL, allows conclude that the observed antifungal activity was due the plant extract itself.

In order to avoid contaminations during the *in vitro* antifungal activity of the extract, it was filtrated by using two different porosities. Although the chemical composition of HEAPAL was not monitored during this procedure we assume this did not suffer major change since 0.22 µm (the smallest pore size used) is only able to retain particles larger than such diameter what is far outsized than chemical compounds.

Previous work showed that broth dilution method provides better results than agar diffusion for the evaluated extract [9]. Because there are no standards for determining the susceptibility to natural products, a method was developed based on CLSI standards [12]. The adapted method allowed the evaluation of HAEPAL antifungal activity even though it was used SDB where the extract was previously proved [9] in stated of RPMI media. Table 1 shows the ranges, GM and MIC_{50} of HAEPAL and fluconazole against the studied *Candida* species.

In general, the MICs were higher for HAEPAL. The analysis according to the species indicates that *C. albicans* was the most represented among fluconazole-resistant strains (13 isolates) followed by *C. tropicalis* (3 isolates) and *C. parapsilosis* (2 isolates) while only 2 *C. krusei*, 1 *C. glabrata*, and 1 *C. albicans* required concentrations above 64 µg/mL of HAEPAL to inhibit their growth (data not shown). Fluconazole displayed broad ranges, especially against *C. albicans* and *C. tropicalis* whereas GM of the extract also had wider ranges against those species and *C. glabrata*.

Species	HAEPAL			Fluconazole		
	Range	GM	MIC_{50}	Range	GM	MIC_{50}
C. albicans	08-128	24.33	16	0.75-128	7.01	8
C. parapsilosis	08-32	23.3	16	1-256	5.25	1
C. tropicalis	16-32	24.55	16	0.75-128	11.2	1
C. glabrata	16-128	23.3	16	16	6.98	16
C. krusei	16-128	24,07	32	16-64	7.31	16
C. lusitaneae	08-16	23.3	8	0.25-2	9.58	0.5
C. guillermondii	16-32	25.99	16	1	1.56	1
C. kefyr	16-32	2421	16	0.75-1	5.71	0.75
C. inconspicua	-	-	64	-	-	16
C. ciferri	-	-	32	-	-	1
Total	08-128	24.5	32	0.25-256	6.9	8

Table 1: Range, geometric mean (GM) and MIC_{50} (µg/mL) values of the studied *P. alliacea* L extract and fluconazole against *Candida* isolates.

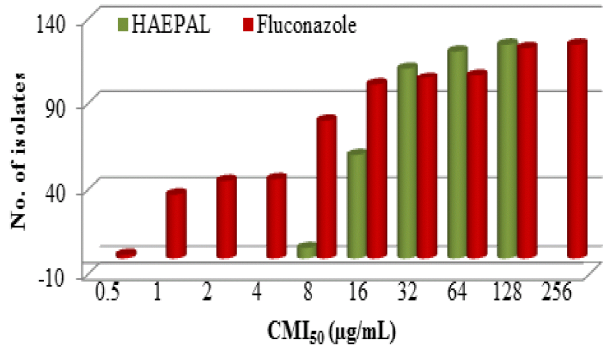

Figure 2: Distribution of accumulated MIC_{50} percentages of hydro-alcoholic extract of *P. alliacea* L and fluconazole against 125 *Candida* spp. isolates.

The studied formulation did not undergo any fractionating and purification process, therefore all the constituents, with and without antifungal activity, were present in the solution. This implied that the concentrations of active ingredients with possible antifungal activity were "diluted" in the extract and for that reason higher concentrations of the crude extract were necessary which could explains that HAEPAL GM ranges and MIC values were higher than those of fluconazole. Despite this, over 95% of the isolates were inhibited at HAEPAL MIC ≤ 128 µg/mL.

Figure 2 shows the accumulated MIC_{50} values obtained with the extract and fluconazole. Most isolates (80%) were inhibited with fluconazole ≤ 8 µg/mL. HAEPAL however, showed activity against fluconazole resistant and susceptible dose dependent (SDD) isolates. Of the total isolates included in this study, 109 (87.2%) vs. 123 (98.4%) were inhibited at 8 and 64 µg/mL of fluconazole and HAEPAL respectively.

Currently there are no established criteria to define the antifungal activity of natural products [3]. However there are several studies that report the association between this activity and MIC values. Holetz et al. suggested that MICs of ≤ 100 µg/mL correspond with good activity, 100 to 500 µg/mL with moderate and >500 µg/mL with weak activity [18]. Duarte et al. proposed that MIC values of <500 µg/mL represent a strong action, 600 – 1500 µg/mL moderate and >1500 µg/mL slight

action [19]. Webster et al. suggest that plant extracts with MICs of ≤ 1000 µg/mL encompass a strong antifungal potency [20]. Regardless of the various criteria for categorizing the antifungal activity of a natural extract, HAEPAL exhibited relatively low effective MIC values.

Previous studies have shown that *P. alliacea* is active against certain bacteria, protozoa and viruses [8,10,21-23]. While some authors were unable to detect antifungal effects of this plant after studying hydro-alcoholic extracts against *C. albicans* strains [10,22] others demonstrated activity against *Colletotrichum gloeosporides*, *Cladosporium cladosporiodes*, *C. sphaerospermum*, dermatophytes, six species of *Candida* and other yeast (*Saccharomyces*, *Rhodotorula*, *Trichosporon* and *Cryptococcus*) [6,7,9]. These discrepancies could be explained by differences in the method for extract preparation, the solvent, the parts of the plant used, the technique applied in susceptibility studies, and the amount of the active ingredients in preparations. The latter condition can vary depending on the region and weather conditions where the plant is harvested [24].

Former phytochemical screening showed that *P. alliacea* contains substances with proven antifungal activity such as triterpenes, derived alkaloids, flavonoids and saponins [25]. These metabolites probably act by inhibiting the biosynthesis of ergosterol or other sterols present in the fungal membrane, damaging and altering its permeability resulting in loss of essential intracellular elements [26,27]. Especially the saponins are able to form pores in the lipid membrane, to increase cell permeability and inhibit biofilm formation, rendering fungal cells more susceptible to osmotic stress [28,29]. Another potential mechanism by which chemical components could have antifungal activity is the inhibition of triglycerides and phospholipids biosynthesis as well as oxidative and peroxidative enzymatic activity; this would lead to the accumulation of toxic concentrations of hydrogen peroxide that contribute to the deterioration of subcellular organelles and cell necrosis [27]. However, it is difficult to define a specific action mechanism since the results of the studies done to date are not conclusive. The high concentration of these compounds in leaves, which were used in the preparation of HAEPAL, could explain the obtained results in this study.

In conclusion, the assessment of the *P. alliacea* L extract against 125 clinical *Candida* isolates showed a prominent antifungal effect. Comparison of its activity with that of fluconazole, a major antifungal used in candidiasis management, demonstrated the therapeutic potential of this plant, which could be useful for treatment. Future studies of the extract fractions could help to define the compound(s) responsible for the antifungal activity. Other methods such as lyophylization should also be evaluated for the active compounds obtained from the plant.

Competing of Interests:

None of the authors of this article have a conflict of interest with any product or company listed in this article.

Author's Contributions

MTIZ: design of the study, interpretation of data, drafted the manuscript; REVM: susceptibility tests with HAEPAL and analysis of primary data; JIF: HAEPAL preparation and intellectual contribution to the study; CMFA and GFMM: analysis and interpretation of data, critical reviewed the draft; MRPL and EXV: susceptibility tests against fluconazole and the analysis of primary data, critical reviewed the draft; JFM: conception of the study, critical reviewed the draft. All authors read and approved the final manuscript.

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