In vitro effects of glycyrrhetinic acid and hyaluronic acid on the growth of vulvovaginal Candida albicans and other yeasts

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Summary

Aims. The present study aimed to test the *in vitro* activity against *Candida albicans* and non-*albicans* strains of 18-β glycyrrhetinic acid (18-β GA) and hyaluronic acid (HA), both alone and in combination. This antimicrobial activity was assessed using the National Committee for Clinical Laboratory Standards (NCCLS) method on *Candida* strains that were isolated from patients with recurrent vulvovaginal candidiasis (RVVC).

Results. Our results demonstrate that the anti-*Candida* activity is independent from antifungal susceptibility level and the fact that the growth inhibition is stronger at acidic pH level makes the two drugs a promising biological alternative for the topical treatment of vulvovaginal candidiasis (VVC) and RVVC.

Conclusions. Furthermore, the reduction of both budding cells formation and germ tube elongation, on mammalian cell monolayers, may explain the observed growth inhibition and suggest a decreased virulence, respectively.

Introduction

Mucocutaneous candidiasis can be divided into nongenital disease and genitourinary disease (1). Clinical manifestations of genitourinary candidiasis are mainly represented by vulvovaginal candidiasis (VVC) in women, balanitis and balanoposthitis in men, and candiduria in both sexes (1). Among the many causes of vaginitis, VVC is the second most common after bacterial vaginosis and more than 40% are diagnosed in primary care settings (2). A recent survey conducted in five European countries and in the United States reported a prevalence of VVC between 29% and 49%, and a prevalence of recurring VVC (RVVC, defined as a 12-month period with 4 or more infections) of 9% (25). Among *Candida* spp. recovered from vaginal swabs, *Candida albicans* is the most common (1, 12), nevertheless *Candida* non-*albicans* have increasingly been identified as the cause of vulvovaginitis, differing considerably with regard to virulence and antimycotic drug susceptibility (12). Indeed, treatment for *Candida glabrata* or *Candida krusei* VVC is generally unsuccessful with typical vaginal and oral drugs, and alternative treatments have been proposed, such as boric acid, amphotericin B, and echinocandins, but nevertheless, failures are becoming more common (16). Therefore, there is the need for new therapeutic strategies against non-*albicans* VVC, exhibiting distinct mechanisms of action and/or evasion of resistance (6, 20).

Glycyrrhizin, a compound derived from *Glycyrrhiza glabra,* and its hydrolytic derivative 18-β glycyrrhetinic acid (18-β GA) have been shown to possess antiviral and antimicrobial activity (7, 9, 13, 15, 17), and it has also been reported an *in vitro* activity against *C. albicans* strains (19). Similarly, hyaluronic acid (HA) too has been described as having a fungistatic activity in some studies (11, 23), and *C. glabrata* and *C. parapsilopsis,* in particular, displayed a HA dose-dependent growth inhibition (3).

The aim of the study was to investigate the antifungal susceptibility of *C. albicans* and non-a*lbicans* strains isolated from RVVC patients, and the activity of 18-βGA and HA, both alone and in combination, against the same strains, in the light of a possible future use of these molecules for the treatment of mucocutaneous candidiasis.

Materials and Methods

Candida yeast isolates

Seven *C. albicans* and 24 non-*albicans* strains (13 *C. glabrata*, 6 *Saccharomyces cerevisiae*, 2 *C. krusei*, 1 *Candida guilliermondii*, 1 *Candida lusitaniae* and 1 *Zygosaccharomyces* spp.) isolated from vaginal exudates of women suffering from RVVC were included in the study. The isolates were cultured on Sabouraud agar with Chloramphenicol (DIFCO USA), and identified by the API 20C system (bioMerieux, Marcy-l'Etoile, France). The antifungal susceptibility testing of the non-*albicans* and non-*Candida* strains was performed, against (amphotericin, fluconazole, itraconazole, ketoconazole, 5-fluorocytosine and voriconazole), with Sensititre Yeast One panels (Trek Diagnostic Systems, UK), and interpretation of results was performed using Clinical Laboratory Standards Institute (CLSI) guidelines.

Measurement of non-*albicans* and non-*Candida* growth rate in the presence of 18-βGA

18-βGA was dissolved in ethanol to a final concentration of 10 mg mL^{-1} (stock solution) and stored at 4°C. Doubling dilutions of 18-βGA ranging from 100 μg mL^{-1} to 0.09 μg mL^{-1} were prepared in RPMI 1640 broth (Sigma, St. Louis, Mo.) containing L-glutamine 0.3% (w/v) and glucose 2% (w/v), buffered with 3-(n-morpholino) propanesulfonic acid (MOPS) 0.165 M, at different pH (ranging from 4.0 to 7.0) in 96-well microtiter plates according to the National Committee for Clinical Laboratory Standards (18). After adding the inoculum, the plates were incubated at 35°C and the absorbance was determined spectrophotometrically at 620 nm (OD$_{620}$) after 0, 2, 21, 24 and 48 h of incubation, using a GENios plate-reader (Tecan) and Magellan version 4.0 software. An inoculum with a concentration of 1–2.5×10^3 cells mL^{-1} was standardized spectrophotometrically and validated by quantitative plate counts. All analyses were conducted in duplicate and, for each strain, values were given as the mean of three measurements.

Measurement of *Candida albicans* growth rate in the presence of 18-βGA and/or HA

18-βGA stock solution and HA dissolved in RPMI 1640 broth and were added to the 96 well microtiter plates to obtain the following final concentrations: 18-βGA 0.01% (w/v), 18-βGA 0.02% (w/v), HA 0.2% (w/v), HA 0.4% (w/v), 18-βGA 0.01% (w/v) + HA 0.2% (w/v), 18-βGA 0.02% (w/v) + HA 0.4% (w/v). *Candida albicans* cells were inoculated in every well at a concentration of 1-2.5×10^3 cells mL^{-1} for a final volume of 200 μL. The plates were incubated at 35°C and the OD$_{620}$ was read after 0, 24 and 48 hours, using GENios plate-reader (Tecan) and Magellan version 4.0 software. All analyses were performed in duplicate and, for each strain, values were given as the mean of three measurements. Aliquots from broth cultures in RPMI 1640 broth, set up in the same experimental conditions, were collected at 0, 24 and 48 h of incubation, and were used for: i) determination of colony forming units (cfu mL^{-1}) by plating on Sabouraud agar plates; ii) evaluation of percentage of budding cells by analysis with light microscopy at 40X; iii) inoculation on mammalian cells monolayers VERO and MRC5 (tumoral and normal cells, respectively) grown on cover slip at pH 7. After further 3 hours of incubation at 37°C the cover slips were collected, the monolayers were fixed in a solution consisting of 1 part of methanol and two parts of acetone and were stained with eosin, before microscopic examination. The budding cells were evaluated by phase-contrast light microscopy and hyphal length was measured with a reticle (also called *eyepiece micrometers*) measuring 100 cells for each concentration.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between groups were analyzed using the Student's t test. All data were analyzed by SPSS version 18.0 (SPSS Inc., USA), and P values <0.05 were considered to be statistically significant.

Results and Discussion

Antifungal susceptibility testing

The results obtained by antifungal susceptibility testing, carried out on non-*albicans* and non-*Candida* strains, are summarized in Table 1.

Among non-*albicans* strains, high fluconazole minimum inhibitory concentrations (MICs), of 316 μg mL^{-1}, were observed only in *C. glabrata* (1 strain resistant and 5 strains with dose-dependent susceptibility). On the other hand, all other strains showed susceptibility to almost all antifungal tested. This result is in line with other reports that found a resistance rate of 21.1% to fluconazole (4), more frequently isolated from women suffering from RVVC (18, 21). Recurrent episodes of VVC are in fact more often caused by non-*albicans* species, for which azole antifungal agents are less likely to be effective (21), in particular for *C. glabrata* (22).

Non-*albicans* growth rate in the presence of 18-βGA

18-βGA has been previously demonstrated to have inhibitory activity against *C.albicans* strains (4), isolated from patients with RVVC. Therefore, we tested the antifungal activity of 18-βGA on *C. albicans* and non-*albicans* strains.

Table 1. Antifungal susceptibility of 24 non-*albicans* strains (13 *Candida glabrata* and 11 other species).

	Non-*albicans* and non-*Candida*				*Candida glabrata*			
	MIC$_{50}$	MIC$_{90}$	Range	Mean	MIC$_{50}$	MIC$_{90}$	Range	Mean
AB	0.25	0.5	0.03-05	0.24	0.25	0.5	0.06-0.5	0.25
FZ	4	16	0.25-64	9.01	8	16	0.25-64	13.86
IZ	0.5	0.5	0.016-4	0.64	0.5	0.5	0.016-4	0.96
KZ	0.125	0.5	0.03-2	0.27	0.25	0.5	0.03-2	0.41
FC	<0.03	0.03	<0.03-0.125	0.03	0.03	0.03	0.03	0.03
VOR	0.03	0.25	0.008-1	0.12	0.125	0.25	0.008-1	0.19

AB, amphotericin; FZ, fluconazole; IZ, itraconazole; KZ, ketoconazole; FC, 5-fluorocytosine; VOR, voriconazole.

Figure 1A shows the growth curves obtained for 24 non-*albicans* strains incubated with different concentrations of 18-βGA, ranging from 0.09 to 100 µg mL^{-1}, at different pH (4.0, 4.5 and 7.0). Low pH values (4.0 and 4.5) were associated to a stronger inhibitory effect of 18-βGA on yeasts growth than a pH of 7.0. These data suggest that 18-βGA has an inhibitory effect also on non-*albicans* strains and that this activity is pH-dependent.

Only doses ranging from 12.5 to 100 µg mL^{-1} of 18-βGA had a significant inhibitory effect, while the growth kinetics observed for *C. glabrata* strains (Figure 1B) suggested a pH-dependent susceptibility to 18-βGA treatment. Indeed, the growth of *C. glabrata* strains is reduced by 18-βGA at concentrations ranging from 0.39 to 100 µg mL^{-1} at pH 4 and at concentrations ranging from 12.5 to 100 µg mL^{-1} at pH 7. The higher susceptibility of *C. glabrata*
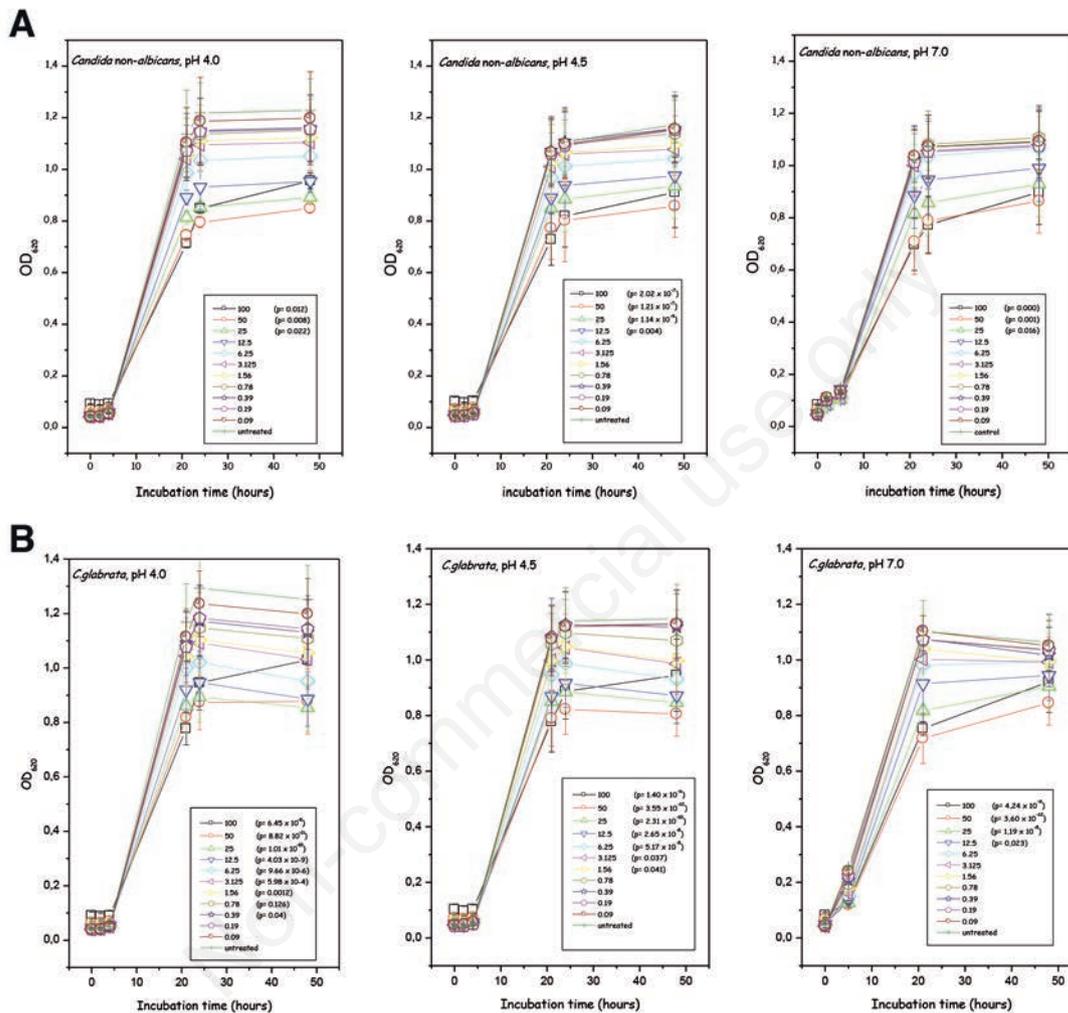

Figure. 1. Growth kinetics of (A) 24 non-*albicans* strains (B) 13 *C. glabrata* cultured at different concentrations of 18-βGA (ranging from 0.09 to 100 µg mL^{-1}). Lines: OD$_{620}$ mean values ±SD: standard deviation.

Table 2. Growth kinetics of seven *Candida albicans* strains cultured at different concentration of 18-βGA and of HA, alone and in combination. SD: standard deviation.

Incubation time (hrs)	Control log cfu mL^{-1} (±SD)	18-βGA 0.01% log cfu mL^{-1} (±SD)	% survival	18-βGA 0.02% log cfu mL^{-1} (±SD)	% survival	HA 0.2% log cfu mL^{-1} (±SD)	% survival	HA 0.4% log cfu mL^{-1} (±SD)	% survival	18-βGA 0.01%+ HA 0.2% log cfu mL^{-1} (±SD)	% survival	18-βGA 0.02%+ HA 0.4% log cfu mL^{-1} (±SD)	% survival
0	2.42 (0.13)	2.45 (0.05)		2.43 (0.15)		2.50 (0.3)		2.45 (0.16)		2.50 (0.3)		2.44 (0.2)	
24	5.12 (0.12)	4.59 (0.15)	29.5	4.39 (0.19)	18.6	4.18 (0.19)	11.5	4.21 (0.24)	12.3	4.22 (0.26)	12.6	4.21 (0.24)	12.3
48	5.81 (0.12)	5.45 (0.17)	43.6	5.41 (0.08)	39.8	5.33 (0.1)	33.1	5.24 (0.21)	26.9	5.28 (0.17)	29.5	5.24 (0.15)	26.9

strains to 18-βGA compared to the other non-*albicans* strains suggests that the anti-*Candida* activity of 18-βGA is not influenced by the susceptibility level to azole antifungal drugs.

This finding is of key importance, considering that vaginal environment has an acidic pH.

Indeed, *Candida* spp. is known to cause infections in a number of host niches with markedly different pH levels and the ability of *Candida* spp. to react appropriately to different pH levels is crucial to its pathogenicity (8). Ambient pH influences several physiological functions, including morphogenesis. Under optimal temperature conditions (37°C), filamentation is facilitated by near-neutral ambient pH values, but it is considerably reduced at pH values below 6; virtually only the yeast form exists at a pH of 4 (10).

Candida albicans growth rate in the presence of 18-βGA and/or HA

It has been proven that high molecular weight HA can inhibit the growth of *C. albicans* (23); therefore, the effect of two concentrations of 18-βGA and HA, both alone and in combination, were tested on the growth of seven *C. albicans* strains. The results obtained are reported in Table 2.

The anti-*Candida* activity of both compounds was confirmed, with a survival rates ranging from 11.5% to 29.5% and from 26.9% to 43.6% after 24 and 48 hours of incubation, respectively. In all tests the reduction of growth compared to controls was statistically significant (P<0.0001).

Candida albicans is a dimorphic fungus capable of converting itself between yeast and hyphal forms (26), the former associated with commensal carriage, the latter with disease (10). There is indeed evidence of mutant forms of *C. albicans* that were locked into the yeast form, and were avirulent (10, 24). It has been demonstrated that elongated hyphae evade phagocitic cells, thus representing a pathogenic factor (5, 14). Therefore, the ability of 18-βGA and HA, both alone and in combination, to inhibit or reduce the bud formation in RPMI 1640 broth was tested, and the hyphal elongation on *C. albicans* plated on mammalian cells monolayer after treatment with the two drugs, was measured. The results obtained after two hours of incubation (Figure 2) showed a significant reduction of budding cells (BCs).

In fact, no BCs were detectable in presence of 18-βGA plus HA, and a 3.4% of BCs were detected after incubation in 18-βGA (concentration of 0.01%) against a 7.2% of control BCs. These differences remained statistically significant also after 24 hours of incubation (Figure 2). These data are in agreement with the amount of growth reduction observed in the survival kinetics.

The incubation for 24 and 48 h in RPMI 1640 broth of *C. albi-*

cans cells, before plating on mammalian cells monolayer (VERO and MRC5), with 18-βGA and HA, both alone and in combination, was associated with a significant reduction of the germ tube elongation (P<0.0001). After 24 h of incubation in RPMI 1640 broth, and subsequent three hours of incubation of yeasts on VERO mammalian cells monolayer, the reduction rate ranged from 27.9 % to 63.6 %. The same procedure on MRC5 mammalian cells monolayer, produced a reduction rate ranging from 17.9% 31.3% to 84.8% 57.3%. Similarly, after 48 h of incubation in RPMI 1640 broth, and subsequent three hours of incubation of yeasts on VERO mam-
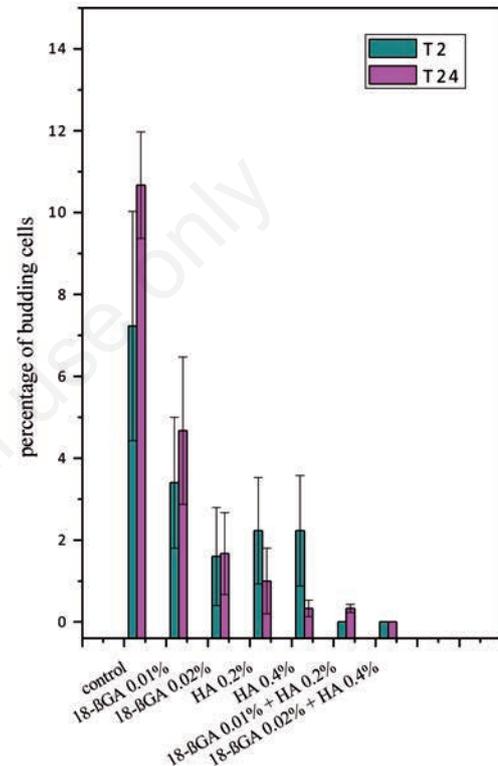

Figure 2. Budding cells of three *Candida albicans* strains incubated, for 2 and 24 hours, in the presence of different concentrations of 18-βGA and of HA alone and in combination. The bars represent standard deviation

Table 3. Effect on *Candida albicans* cells germ tube elongation after incubation for 24 and 48 hours in RPMI 1640 broth containing 18-βGA and/or HA, and after three hours co-incubation with mammalian cells. SD: standard deviation.

Treatment	VERO cells, germinative tube length				MRC5 cells, germinative tube length			
	24 h		48 h		24 h		48 h	
	μm (±SD)	% reduction	μm (±SD)	% reduction	μm (±SD)	% reduction	μm (±SD)	% reduction
Untreated	42.65 (12.5)		48.78 (6.2)		54.20 (9.8)		56.38 (10.1)	
18-βGA 0.01%	30.75 (7.9)	27.90	33.50 (6.5)	31.32	44.50 (5.19)	17.90	39.00 (8.66)	30.83
18-βGA 0.02%	25.44 (9.5)	40.35	26.93 (7.5)	44.79	39.30 (5.60)	27.49	34.63 (9.80)	38.58
HA 0.2%	27.15 (10)	36.34	32.18 (9.6)	34.03	35.20 (6.59)	35.06	34.69 (9.81)	38.39
HA 0.4%	23.25 (11.5)	45.48	27.56 (7.8)	43.50	31.60 (6.83)	41.70	31.00 (8.71)	45.02
18-βGA 0.01% + HA 0.2%	19.43 (9.3)	54.44	24.81 (9.1)	49.14	21.10 (8.64)	61.07	21.69 (7.85)	61.53
18-βGA 0.02% + HA 0.4%	15.5 (11.3)	63.65	20.81 (7.6)	57.34	8.19 (4.57)	84.89	17.44 (5.20)	69.07

malian cells monolayer, the reduction rate ranged from 31.3% 17.9% to 57.3%. Likewise, for MRC5 cells reduction rate ranged from 30.8% to 69.1% (Table 3).

In particular, the combination of 18-βGA plus HA decreased germ tube elongation of more than 49.1%, compared to that obtained with single drug, ranging from 17.9% to 45.5%.

The limitations of this study are represented by the relatively low number of strains evaluated, and the lack of confirmation of the results in animal models. These issues will be addressed by future studies.

Conclusions

In this study we evaluated *in vitro* the (anti-yeasts) anti-*Candida* activity of 18β-GA and HA, alone and in combination, in the light of their possible use as topical treatment for acute VVC. The main results are that 18β-GA and HA anti-*Candida* activity seems independent from antifungal susceptibility, that the growth inhibition seems stronger at acidic pH, and that they reduce both budding cells formation and germ tube elongation on mammalian cell monolayers. Those findings highlight how 18-βGA and HA could be considered a possible alternative to azole antifungal agents for the topical treatment of VVC and RVVC.

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