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Antifungal activity of low molecular weight chitosan against clinical isolates of *Candida* spp.

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Chitosan is a natural polymer derived from chitin, a structural component of fungi, insects and shrimp, which exerts antimicrobial effects against bacteria and fungi. The aim of this study was to investigate the *in vitro* antifungal activity of low molecular weight chitosan (LMWC), and the potential synergy between chitosan and a currently used antifungal drug, fluconazole. The *in vitro* minimal inhibitory concentrations (MICs) of chitosan and fluconazole against 105 clinical *Candida* isolates were measured by the broth microdilution method. LMWC exhibited a significant antifungal activity, inhibiting over 89.9% of the clinical isolates examined (68.6% of which was completely inhibited). The species included several fluconazole-resistant strains and less susceptible species such as *C. glabrata*, which was inhibited at a concentration of 4.8 mg/l LMWC. Although some strains were susceptible at pH 7.0, a greater antifungal activity of LMWC was observed at pH 4.0. There was no evidence of a synergistic effect of the combination of LMWC and fluconazole at pH 7.0. This is the first report in which the antifungal activity of LMWC was investigated with clinical *Candida* strains. The use of LMWC as an antifungal compound opens new therapeutic perspectives, as the low toxicity of LMWC in humans supports its use in new applications in an environment of pH 4.0–4.5, such as a topical agent for vulvovaginal candidiasis.

Keywords chitosan, antifungal susceptibility testing, fluconazole, *Candida*, LMWC

Introduction

Chitosan is a β-1, 4-D-glucosamine polymer derived from the alkaline N-deacetylation of chitin. The latter is a structural component of fungi, insects and crustaceans that is insoluble in most solvents, with the exception of organic acids, e.g., acetic acid [1,2]. Chitosan is a nontoxic, biocompatible and biodegradable product that displays diverse biological and therapeutic properties, including antimicrobial activity [1–7], antitumor activity [2], wound healing [4,8–11] and cholesterol-lowering effects [12–14]. The antimicrobial activity of chitosan has been demonstrated against bacteria (*Bacillus cereus*, *Escherichia coli*, *Staphlyococcus aureus*, *Salmonella enterica*) [1,15] molds (*Botrytis cinerea*, *Fusarium oxysporum*) [1,16], and yeast (*Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata* and *Candida krusei*) [1,17], and has been shown to be dependent on the type of chitosan and the degree of its polymerization [1]. Based on the latter characteristic, chitosan can be classified as low molecular weight (LMWC) or high molecular weight (HMWC). Some reports suggest that shorter oligomers of chitosan, such as LMWC, have greater antifungal activity [5,17]. However, chitosan's mechanism of action has not been completely elucidated. There is some evidence that it acts on the plasma membrane and fungal wall cell, chelating

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trace metals and inhibiting mRNA synthesis [1,17]. Potential synergistic effects between LMWC and conventional drugs, including fluconazole, have yet to be explored.

This is the first time that the antifungal activity of LMWC against 105 clinical isolates of *Candida* spp. is evaluated. Previous studies have only measured its activity against a few strains of *Candida* spp. [3] or environmental fungi [1,5,17]. LMWC exhibited antifungal activity inhibiting over 90% of the clinical strains at pH 4.0. There was no evidence of synergism between LMWC and fluconazole at pH 7.0, since higher pH levels affect the *in vitro* activity of the drug.

Materials and methods

Strains

We analyzed 105 *Candida* strains from the collection of Clinica Dávila Laboratory, Santiago de Chile including several *Candida* species recovered from clinical specimens. The test isolates were stored at −80°C. In addition, the following eight strains were included in the studies as controls; *Candida krusei* ATCC 62258, *Candida albicans* ATCC 64548, *Candida albicans* ATCC 64550, *Candida tropicalis* Rex MY1012, *Candida glabrata* ATCC 90030, *Saccharomyces cerevisiae* ATCC 9763, *Candida lusitaniae* Rex CL2819 and *Candida parapsilosis* ATCC 22019. Strains were identified by the use of the germ tube test [18], microcultivation [19], ChromAgar Candida [20] and API AUX 32C [21].

Antifungal fluconazole susceptibility testing of clinical strains

In vitro antifungal susceptibility tests of the isolates (AST) to fluconazole was performed with a broth microdilution method, in accord with the standards of the European Committee on Antifungal Susceptibility Testing (EUCAST-ASFT) [22], employing *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 as controls. Resistance to fluconazole (Pfizer) was assessed according EUCAST-AFST standard [22–24].

Evaluation of LMWC antifungal activity

Low molecular weight samples of chitosan (Mw = 70 KDa, >75% deacetylation) from Fluka were washed with acetone and methanol and dried to a constant weight [25]. Optimal test conditions were established prior to determining the minimum inhibitory concentrations (MICs) of isolates by the broth microdilution method. Solubility assays of LMWC were performed with RPMI 1640 (Gibco) containing 2% glucose at pH 7.0 and 4.0, plus acetic acid at varying concentrations (0.01%, 0.1% and 1%). To evaluate the minimum acetic acid concentration that had no effect on the viability of the yeasts, growth curves were determined with the control strain *C. albicans* ATCC 64550 in RPMI 1640 plus 2% glucose at pH 7.0, 4.5 and 4.0, and in varying concentrations of acetic acid (as above). The highest amount of LMWC dissolved by 0.1% acetic acid was 7,400 mg/l. To estimate the antifungal activity of LMWC, growth curves of *C. albicans* ATCC 64550 were generated in the presence of LMWC, i.e., 5, 000, 1, 000 and 100 mg/l in RPMI 1640 plus 2% glucose and in 0.1% acetic acid. Yeast viability was evaluated by colony counts.

To determine the effect of pH on the antifungal activity of LMWC, we tested control and 30 clinical strains. The MICs of chitosan were measured according to EUCAST methodology [22], with some modifications, i.e., the acetic acid used to dissolve LMWC reached a final concentration of 0.1% in the wells of the microplates. MIC measurements were performed using a spectrophotometer at pH 7.0 and 4.0, and LMWC concentrations in the range of 0.5–128 mg/l.

Once the experimental conditions were optimized, MICs of LMWC against all clinical strains and controls were measured using a concentration range of 4.8–2500 mg/l at pH 4.0.

Analysis of synergism between LMWC and fluconazole

Analysis of potential synergy between LMWC and fluconazole was carried out by the chequerboard method [26] at pH 7.0 due to the fact that the antifungal activity of fluconazole is negatively affected at acidic pH [27]. A previous study in our laboratory demonstrated antifungal activity of chitosan against 2 ATCC strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 62258) at pH 7.0 [28]. The fractional inhibitory concentrations of chitosan and fluconazole were calculated for 9 clinical isolates of *Candida* spp. and for control strains according to the following formula: (A)/(MICA) + (B)/(MICB) = FICA+ FICB = Fractional inhibitory concentration (FIC) index. In this formula (A) is the concentration of drug A in a tube that is the lowest inhibitory concentration in its row, (MICA) is the MIC to drug A alone, and FICA the fractional inhibitory concentration of drug A. The formula components (B), (MICB) and FICB are defined in the same fashion but for drug B [26]. Fractional inhibitory concentration index (FICI) values were interpreted as follows; a FICI value of less than 0.5 was considered synergistic, 0.5 to 4 was indifferent, and greater than 4 were considered antagonistic [26]. We also generated death curves using as the control strain *C. tropicalis* Rex MY1012, which was inhibited by low concentrations of LMWC, for which the MIC of fuconazole was 32 mg/l.

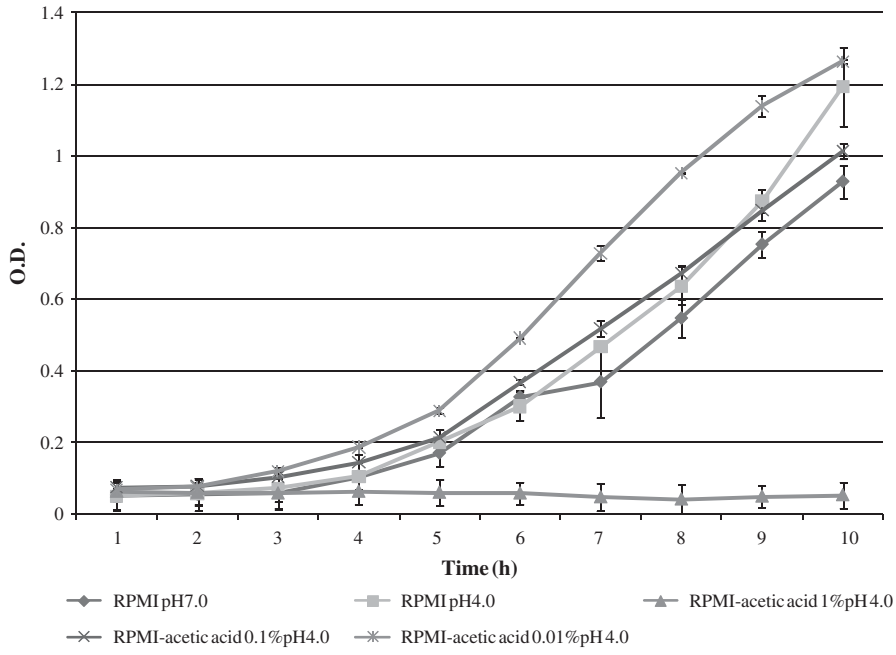

Fig. 1 Effect of acetic acid on the viability (growth curves) of *Candida albicans* ATCC 64550. At a concentration of 1% acetic acid, there was a significant reduction in cell viability.

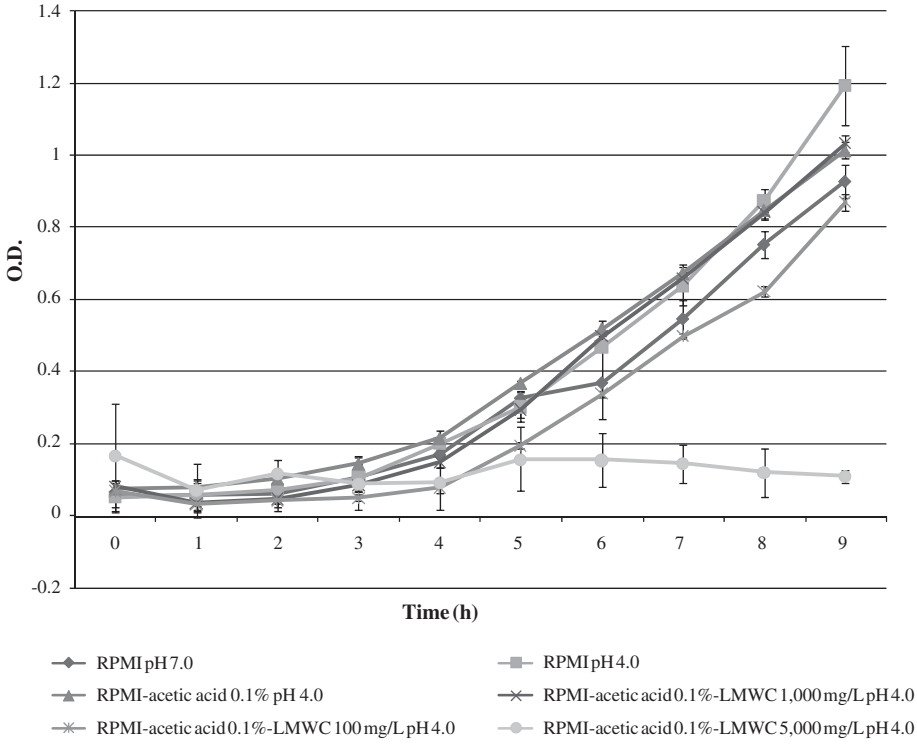

Fig. 2 Antifungal activity of LMWC against *Candida albicans* ATCC 64550. The growth curves indicate an important inhibitory effect at 5,000 mg/l of LMWC.

Results and discussion

We evaluated different solvents for LMWC and found that 4,900–7,400 mg/l of chitosan was soluble in 0.01–0.1% acetic acid, respectively, whereas chitosan was insoluble in DMSO. The minimum concentration of acetic acid that had no effect on the viability of the yeast strains was 0.1% (Fig. 1). Thus, we used 0.1% acetic acid as the solvent in our studies. It is important to note that acetic acid inhibits the growth of *C. albicans* at concentrations of 1% and higher. In previous investigations in which the antimicrobial activity of different molecular weight chitosan compounds or its derivatives were evaluated, acetic acid was used as a solvent but its effect on cell viability was not studied [1,5]. Although dimethyl sulfoxide is commonly used to dissolve drugs that are water insoluble, we found that LMWC was insoluble in this solvent (data not shown). Dimethyl sulfoxide has been used in prior studies to dissolve hydrophobic chitosan derivates [29].

We also evaluated the effect of pH on the viability of fungal cells. The growth of *C. albicans* was not affected at pH 4.0, but was at neutral pH (Fig. 1). At pH 4.0, the antifungal activity of LMWC against *C. albicans* ATCC 64550 was evident at concentrations of 1,000 and 5,000 mg/l of LMWC (Fig. 2).

Using a lower concentration range of LMWC (between 0.5 and 128 mg/l), its antifungal activity against several strains was greater at acidic pH, with MICs between 1- and 4-fold lower at pH 4.0 (Table 1). Some reports have suggested that the polycationic character of chitosan is the most important mechanism of antifungal activity, as the cationic groups would interact with anionic components of the cell wall of the fungus, and destabilize the membrane [1,17]. We observed that the antifungal activity of LMWC increased at acidic pH which might be due to the protonation of the amino group of glucosamine units of chitosan at pH 4.0 as the pKa of LMWC is 6.3 [1,30]. At pH 7.0, we observed a lower antifungal activity, which was restored when the measurements were conducted at acidic pH (4.0) (Table 1). Interestingly, vulvovaginal candidiasis is a frequent infection of the female genital tract. The latter is maintained at pH 4.0 or 4.5 raising the possibility of the use of LMWC as a therapeutic treatment for this type of infection.

Most of the clinical strains were inhibited within the range of 4.8 and 2,500 mg/l LMWC at pH 4.0 (Fig. 3). Antifungal activity of chitosan and its derivatives [3,5, 16,17,28, 30] has previously been demonstrated against members of the genus *Candida*, primarily those species associated with human infections, such as *C. albicans*, *C. krusei* and *C. glabrata* [3]. In this investigation we evaluated a number of important clinical isolates associated with candidiasis ($n = 105$) and found that LMWC had marked antifungal activity. Nearly 90% of the strains were inhibited by LMWC, 68.6% of which were inhibited at a concentration of 4.8 mg/l at pH 4.0 (Fig. 3). When we compared the susceptibility of the strains to LMWC with fluconazole, eight out of nine strains of *C. albicans* and *C. tropicalis* which were resistant to the latter according to EUCAST breakpoints (MIC > 4 mg/l), were inhibited by LMWC of 9.8 mg/l or less (Table 2) [23]. On the other hand, nine strains (six *C. albicans*, one *C. famata* and two *C. krusei*) for which the MIC of fluconazole was 64 mg/l or higher had a LMWC MIC of 9.8 mg/l or less (data not shown). These results suggested that LMWC could be a therapeutic alternative with *Candida* isolates that show

Table 1 Fluconazole MICs of *Candida* spp. and control strains at pH 7.0 and pH 4.0 with concentrations ranging between 0.5 and 128 mg/l.

ID strain	Specie	LMWC MICpH at 7.0 (mg/l)	LMWC MICpH at 4.0 (mg/l)
ATCC 6258	*C. krusei*	32	1
ATCC 64548	*C. albicans*	>128	>128
ATCC 64550	*C. albicans*	>128	>128
Rex MY1012	*C. tropicalis*	2	0.12
ATCC 90030	*C. glabrata*	>128	>128
ATCC 9763	*S. cerevisiae*	>128	>128
Rex Cl 2819	*C. lusitanie*	16	4
ATCC 22019	*C. parapsilosis*	1	0.5
C4	*C. albicans*	>128	>128
C12	*C. albicans*	>128	>128
C14	*C. glabrata*	>128	>128
C15	*C. albicans*	>128	>128
C19	*C. albicans*	>128	>128
C20	*C. albicans*	>128	>128
C21	*C. albicans*	2	0.5
C29	*C. tropicalis*	2	0.5
C34	*C. tropicalis*	>128	>128
C36	*C. tropicalis*	4	0.5
C39	*C. tropicalis*	>128	>128
C41	*C. albicans*	2	1
C42	*C. albicans*	>128	8
C44	*C. albicans*	>128	>128
C55	*C. albicans*	>128	>128
C59	*C. glabrata*	16	2
C60	*C. albicans*	32	2
C62	*C. albicans*	2	1
C90	*C. albicans*	1	0.5
C93	*C. albicans*	32	2
C112	*C. krusei*	>128	>128
C113	*C. famata*	>128	2
C122	*C. glabrata*	>128	>128
C128	*C. krusei*	>128	>128
C151	*C. glabrata*	>128	>128
C152	*C. glabrata*	32	1
C154	*C. krusei*	>128	>128
C157	*C. albicans*	>128	>128
C158	*C. albicans*	>128	>128
C164	*C. albicans*	>128	>128

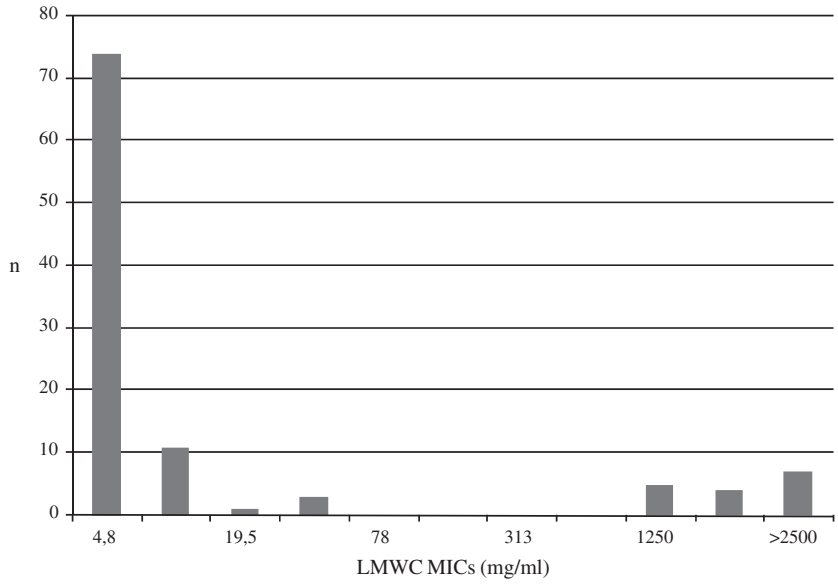

Fig. 3 Distribution of LMWC MICs at pH 4.0 for clinical isolates of *Candida* spp.

some degree of *in vitro* resistance to antifungal drugs such as fluconazole. Seven of 12 strains of *C. glabrata* that presented high MICs of 16 mg/l or greater to fluconazole were inhibited by LMWC concentrations less than 4.8 mg/l. However five strains of this species required concentrations of 2,500 mg/l of LMWC or greater (data not shown). These results were consistent with a previous study in which strains of *C. glabrata* exhibited higher resistance to LMWC [3].

In assays of eight control strains and nine clinical isolates of *Candida* spp., LMWC was not synergistic with fluconazole at pH 7.0, although some strains had FICI values that were closer to synergy at 0.5 (data not shown).

Table 2 LMWC MICs *versus* fluconazole susceptibility of *Candida* spp.

	Fluconazole susceptibility					
	C. albicans		*C. tropicalis*		*C. parapsilosis*	
LMWC (mg/l)	S	R	S	R	S	R
4.9	53	6	3	1	2	0
9.8	5	1	1	0	0	0
19.6	1	0	0	0	0	0
156	0	0	1	0	0	0
312	0	0	1	0	0	0
625	1	0	0	0	0	0
1250	2	0	0	0	0	0
2500	1	0	0	0	0	0
>2500	7	1	0	0	0	0
Total	70	8	2	2	2	0

Susceptible (S) $\leq$ 2 mg/l; resistant (R) $>$ 4 mg/l according EUCAST clinical MIC breakpoints.

It is important to emphasize that antagonism between LMWC and fluconazole was not observed, which lends support to the idea that LMWC could be used for the delivery of conventional antifungal drugs [31–33]. Combination of LMWC and fluconazole at acidic pH was not performed because fluconazole is inactive *in vitro* at a pH near to 4.0 [27], although an *in vivo* synergism cannot be discounted. A previous study evaluated the effect of fluconazole on *Candida albicans* under *in vitro* conditions resembling the vaginal microenvironment. In this study, fluconazole showed a fungicidal synergistic effect at pH 4.2 by its combination with acetic acid [27].

Given that conventional antifungal therapies are currently insufficient to control some mycoses, these results open new therapeutic options for antifungal treatment. The low toxicity of chitosan in humans [2,17] also supports LMWC as a good candidate for new applications. LMWC represents an innovative agent for topical use in the treatment of superficial mycoses, such as vulvovaginal candidiasis, because it is not absorbed by the intestinal tract [2,34,35]. Finally, since LMWC is soluble at pH 4.0, it would be effective in the treatment of candidiasis, which commonly occurs in an environment of pH 4.0–4.5, even in the case of *C. glabrata*.

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Declaration of interest: None.

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