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ORIGINAL ARTICLE

In vitro antifungal potency of the moronecidin-like peptide against *Candida albicans, Candida glabrata,* and *Candida tropicalis*

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ABSTRACT

Background and Objectives: The aim of this study was to investigate the *in vitro* antifungal potency of the moronecidin-like peptide against *Candida albicans*, *Candida glabrata*, and *Candida tropicalis.*
Materials and Methods: To evaluate the antifungal effect of moronecidin-like peptide, the protocol presented in CLSI M27-A3 and CLSI M27-S4 was used and the minimum inhibitory concentration was determined.
Results: The minimum inhibitory effect of moronecidin-like peptide composition was 8 µg/ml for *Candida tropicalis* and *Candida albicans* and 32 µg/ml for *Candida glabrata*. The MIC of nystatin was determined to be 1.25 µg/ml for *Candida glabrata* and *Candida albicans* and 0.625 µg/ml for *Candida tropicalis* strains. The MFC composition of the moronecidin-like peptide was determined for *Candida tropicalis* and *Candida albicans* strains 8 µg/ml and for *Candida glabrata* strain 64 µg/ml. The results of cytotoxicity and hemolysis of the moronecidin peptide test on macrophage showed that moronecidin peptide has no cytotoxicity and toxicity properties.
Conclusion: According to the results of the present study, the moronecidin-like peptide could be a new strategy in the treatment of infections caused by *Candida* strains. The discovery of the exact mechanism of which requires extensive clinical studies in this field.

Keywords: *In vitro*; Fungi; Antimicrobial peptides; Cytotoxicity

INTRODUCTION

Fungal infections (FIs) rapidly increase worldwide and kill more than 1.7 million people yearly (1). The increased use of broad-spectrum antimicrobials, hospitalization in surgical and intensive care units, and the immune-deficient may be implicated in increasing the population at risk of infections (2-4).

In recent years, reports of opportunistic fungal infections have increased, essentially, caused by the *Candida* species (5, 6). *Candida* species are commensal microorganisms in the human respiratory, gastrointestinal, and vaginal tract and are one of the most invasive human fungal pathogens (5, 7-9). More than 15 different *Candida* species can induce human infections; however, most of these infections

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are caused by *Candida albicans, Candida glabrata, Candida tropicalis, Candida krusei,* and *Candida parapsilosis* (5, 10, 11).

Among *Candida* species, *C. albicans* is the most important yeast that can cause life-threatening infections in humans (5). *C. albicans* is a yeast that usually colonizes the intestinal tract, urogenital, and oral mucosa of ~30 to 50%, and skin of ~30-70% of the healthy population (12). Despite that, when the immune system is compromised, *C. albicans* may become an opportunistic pathogen causing life-threatening infections in patients (5). Moreover, the second most pathogenic *Candida* species is *C. tropicalis* which is detected in systemic infections (13). Another Non-*albicans Candida* species is *Candida glabrata,* which has been associated with approximately 15% of all *Candida* bloodstream infections (14, 15). Resistance to antifungals and limited alternatives to antimycotic drugs may be implicated in treatment failure in patients with an invasive *Candida* infection (16). Recently, several studies have indicated the emergence of *C. albicans, C. tropicalis,* and *C. glabrata* resistant to the antifungal agents clinically available (e.g., the azole, amphotericin B, and echinocandins) in different geographic regions (17-19). The development of multidrug-resistant *candida* strains has led to the further complicated therapeutic management of these infections (6, 20, 21). Thus, identifying new antifungal therapeutic options is needed for the treatment of drug-resistant infections.

Piscidins belong to endogenous antimicrobial peptides (AMPs) with broad-spectrum antimicrobial activity, which are potential candidates for antibiotics (22, 23). In 2021, pleurocidin was isolated from *Pleuronectes americanus* and identified as the piscidin family's first member (23). Until now, a variety of AMPs have been reported from different organisms (23-25). Moronecidin is an α-helical AMP, which was first known from hybrid striped bass by Silphaduang et al. (26). Antimicrobial activities of moronecidin have been investigated in several studies, which have suggested high antimicrobial activity (22, 27, 28). Although several studies on the antibacterial effects of moronecidin-like peptides have been carried out (26, 28-30), very few investigations have assessed the antifungal and cytotoxic effects of moronecidin-like peptides (22, 27). For these reasons, the present study focused on the antifungal and cytotoxic activity of the moronecidin-like peptide against standard strains of *C. glabrata, C. albicans,* and *C. tropicalis.*

MATERIALS AND METHODS

Materials and culture media. Sabouraud dextrose agar (SDA; Heidelberg, Germany), chloramphenicol powder (Chol; Sigma-Aldrich, USA), nystatin powder (NYS; Sigma-Aldrich, USA), RPMI-1640 medium pH 7.0, with L-glutamine, without sodium bicarbonate (Sigma Chemical Co.), morpholine propane sulfonic acid (MOPS; Sigma-Aldrich, USA), lyophilized peptide (Pepmic, Suzhou, China), Tris-EDTA buffer (TE Buffer; Sigma-Aldrich, USA), dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), phosphate-buffered saline (PBS) and Triton X-100 and XTT reagent (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxamide-2H-tetrazolium) (Sigma-Aldrich, USA) were purchased.

***Candida* strains.** The *Candida glabrata* PTCC 5297 was purchased from PTCC (Persian Type Culture Collection, Iran), *Candida albicans* strain (GenBank accession no. MT377828), *Candida tropicalis* strain (GenBank accession no. MT377831) used in the present study were obtained from KMU (Kerman University of Medical Sciences, Iran).

Determination of minimum inhibitory concentration (MIC) of nystatin against yeast pathogens. Minimum inhibitory concentration was determined in accordance with the Clinical and Laboratory Standards Institute (CLSI), M27-A3 and M27-S4 recommendations (31-33). *C. glabrata, C. albicans,* and *C. tropicalis* were grown on Sabouraud dextrose agar with chloramphenicol at 30°C for 48-72 hours. The optical density (OD) of yeast cultures was measured at 540 nm for the concentration of 5×10^6 cells/ml. To prepare the stock solution of Nystatin powder, this powder was dissolved in RPMI-1640 medium with pH 7.0, buffered with 165 mM MOPS. A 100 µl of two-fold serial dilutions of nystatin in the concentration range of 0.156 Up to 10 µg/ml was added to each well. Then 100 µl of yeast cell suspensions were added to each well. A 100 µl of culture medium with antibiotic was included as a positive control, whereas 100 µl of culture medium without antibiotic was used as a negative control. Finally, microplates were incubated at 35°C for 48h, and MIC was recorded (34, 35).

Determination of minimum inhibitory concentration (MIC) of moronecidin-like peptide against yeast pathogens. Lyophilized peptide, was diluted

according to the manufacturer's instructions using TE buffer. Serial dilutions (1-128 µg/ml) were prepared from moronecidin-like peptide to determine MIC and minimum fungicidal concentration (MFC). Nystatin stock solution was prepared as a 6.4 mg nystatin powder in 5 ml of DMSO according to clinical laboratory standard institute (CLSI) documents (M27-A3 and M27-S4) (33). A 100 µl of each yeast cell suspension was added to 10 wells of a 96-well microtiter plate. Afterward, 100 µl of the peptide (10 mM sodium phosphate buffer) stock solution with a 512 µg/ml concentration was added to the first test well and mixed. A series of dilutions (10 dilutions) were prepared. The positive control was the well with Luria-Bertani (LB) broth and yeast inoculum but without moronecidin-like peptide; the negative control was the well with LB broth but without inoculum. Microplates were incubated at 37°C for 18 h.

Determination of minimum fungicidal concentration (MFC) of moronecidin-like peptide against yeast pathogens. The minimum fungicidal concentration (MFC) was determined by spotting 50 µL aliquots from wells with no apparent growth (8 to 128 µg/ml for *C. tropicalis* and *C. albicans* and 32 to 128 µg/mL for *C. glabrata*) onto SDA plates and incubating these at 30°C for 96 h. The lowest moronecidin-like peptide concentration producing less than three colonies, which corresponds to killing ≥ 99.9% of the inoculum, was defined as the MFC (36).

Hemolytic activity assay. The ability of moronecidin-like peptide to lysis erythrocytes was assessed according to the protocol of Stark et al. (37). Briefly, freshly collected human erythrocytes were washed and resuspended in sterile phosphate-buffered saline (PBS) to 4% (vol/vol), and 100 µl of peptide solution (at a final concentration of 25, 50, and 100 µg/ml) were inoculated into round-bottom 96-well plates. The 0 and 100% hemolysis were determined with agents of PBS and 0.1% Triton X-100, respectively. The plates were incubated for 1 hour at 37°C and then centrifuged at 1000 rpm for 5 minutes. The supernatant (100 µl) was then transferred to a fresh 96-well flat-bottom plate, and the amount of hemoglobin was measured by absorbance at 540 nm using a spectrophotometer.

Determination of cytotoxicity of moronecidin-like peptide. The toxicity of moronecidin-like peptide at different concentrations (25, 50, and 100 µg/ml) was evaluated using macrophage cells. First, macrophage cells were cultured in a 1640 RPMI medium enriched with 10% heat-inactivated fetal bovine serum (FBS), 6.5% CO_2, and a humid atmosphere at 37°C to form a monolayer; then the supernatant was removed and the monolayer cells were washed with 10 ml of PBS buffer. Trypsin and EDTA mixture (1 ml) were added to the flask and incubated at 37°C for 1-2 minutes to allow the cells to detach from the surface of the flask. The 1640 RPMI medium (5-7 ml) was inoculated to re-suspend the detached cells. The flask contents were gently mixed and transferred to 15 ml falcon tubes and centrifuged at 1000 g for 5 minutes. The supernatant was removed and two ml of 1640 RPMI medium was added into the cell pellet; then, the cells were counted by a Neubauer chamber. The cell suspension was prepared at 1×10^5 cells/ ml concentration. Next, 50 µl of 1640 RPMI medium and 10% inactivated FBS was added to each well. A 50 µl of the cell suspension was transferred to other wells and diluted to the final concentration of 5×10^4 CFU/ml. The flasks were incubated in 6.5% CO_2, 37°C, and humid atmosphere for 24 hours. The formation of cellular monolayers was examined by an inverted microscope. Serial dilutions of the moronecidin-like peptide (25, 50, and 100 µg/ml) were added to each well, and the plates were incubated at 37°C. The positive control was the wells with cell suspension but without moronecidin-like peptide, and the negative control was the wells with sterile cell culture medium but without moronecidin-like peptide. All the measurements were repeated in triplicate in three separate experiments. Changes in the morphological appearance of the cells and mitochondrial activity in the presence of moronecidin-like peptide, were compared with the control (23). Changes in mitochondrial activity were measured by cell viability and proliferation 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxamide-2H-tetrazolium (XTT) assay (38).

RESULTS

The MIC values of moronecidin-like peptide and nystatin are summarized in Table 1, respectively. The MIC values of moronecidin-like peptide were 8 µg/ml against *C. tropicalis* and *C. albicans* and 32 µg/ml against *C. glabrata*. The MIC values of nystatin were 1.25 µg/mL against *C. albicans* and *C. glabrata* and 0.625 µg/ml against *C. tropicalis* (Table 1).

The MFC values of moronecidin-like peptide were 8 µg/mL against *C. tropicalis* and *C. albicans* and 64 µg/mL against *C. glabrata* (Table 1).

The cytotoxic activity of the moronecidin-like peptide did not differ between cells treated with the peptide at concentrations of 25, 50, and 100 µg/ml and cells untreated with the peptide (control). Statistical analysis of the XTT assay´s results with graphpad prism software showed that the absorbance of cells in the present and absence of the Moronecidin-like peptide has no significant difference ($P > 0.05$) (Fig.1).

The hemolytic activity of moronecidin-like peptide was evaluated using human erythrocytes. Almost no effect was observed on the moronecidin-like peptide (25, 50, and 100 µg/ml concentrations) on human erythrocytes compared with the control group.

Table 1. The MICs and MFCs of different concentrations (µg/ml) of Moronecidin-like peptide and Nystatin against tested *candida* strains

***Candida* Strains**	**MICs (Moronecidin-like peptide)**	**Nystatin**	**MFCs (Moronecidin-like peptide)**
Candida tropicalis	8	0.625	8
Candida albicans	8	1.25	8
Candida glabrata	32	1.25	64

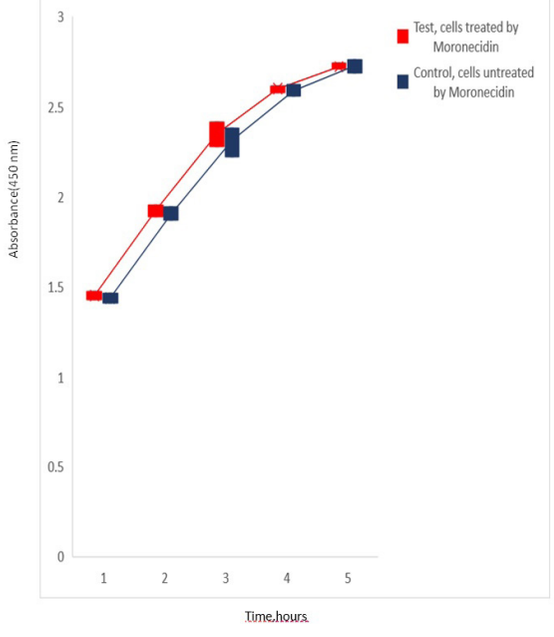

Fig. 1. XTT assay for macrophage cells (10^4) in the present (100 µg/ml) and absence of the Moronecidin-like peptide

DISCUSSION

The purpose of this study was to determine the effect of a moronecidin-like peptide on *Candida* species. Moronecidin-like peptides have been shown to exhibit broad-spectrum antibacterial action against Gram-positive and Gram-negative bacteria (29, 39). We have demonstrated that the composition of moronecidin-like peptides has antifungal efficacy against clinical isolates of *C. glabrata*. This result is in accord with previous studies conducted on *Candida* strains (22, 39). Mohammadi et al. (22), compared the antimicrobial activity of a new moronecidin-like peptide extracted from *Hippocampus comes* with moronecidin from *hybrid striped bass.* They demonstrated that both AMPs have good antifungal activity. Lauth et al. (2002), observed that moronecidin inhibited *Listeria monocytogenes* and *Staphylococcus aureus* growth with a MIC of between 1 and 5 µM (28).

Polybia MPI peptide was studied by Wang et al. in *C. albicans* and *C. glabrata* for its antifungal activity, mechanism of membrane breakdown, and suppression of biofilm formation. They found that this peptide's antifungal properties were linked to its ability to affect fungal membranes and that it could be used as a new antifungal treatment option (40). The antifungal activity of moronecidin-like peptide on the three-standard species of the examined candidates was demonstrated in the current study, which agrees with previous study. Fluconazole-resistant *C. glabrata* was tested in the laboratory with hepcidin-20 peptide, and the results showed that it had antifungal activity in the human vaginal fluid (24). Moreover, additional research has shown that non-amphipathic cationic and cathelicidin peptides have antimicrobial activity against *Candida* species (41-43).

According to our results, the moronecidin-like peptide had no toxicity or hemolytic activity on human cells. Houyvet et al. (2018) demonstrated that the amidated form is highly toxic to all three types of red blood cells (human, sea bass, and lesser-spotted dogfish RBCs) in the hemolytic assay (29). Amidated peptides are much more active on prokaryotic and eukaryotic cells than non-amidated peptides in in vitro tests (29). As with other peptides, the C-terminal amidation of moronecidin-like peptide has been shown to increase the cytotoxic action of the peptide (44, 45). We did not investigate susceptibility to salinity in moronecidin-like peptide, but as proven for moronecidin, salt was not a restriction (28).

We showed that the moronecidin-like peptide had no cytotoxic and hemolytic effect on human cells. Moreover, the current study's findings indicate that the moronecidin-like peptide has acceptable antifungal efficacy against selected standard candida isolates. Despite the fact that further research is required to describe and optimize the peptide's antifungal activity, we suggest that this peptide should be investigated further as a potential therapeutic agent for the treatment of candida infections.

Ethics approval. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC.1398.368.).

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