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Evaluating the effect of a novel ionic liquid ([prolinium chloride] [1-methylimidazolium 3-sulfonate]) on a *candida albicans* standard strain and its cytotoxicity to mammalian cells

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ABSTRACT

Candida albicans (C. albicans) stands responsible for most invasive fungal diseases and colonized on the skin and mucosal membranes. The fungus is a part of human natural microflora. Recently, increasing the resistant strains of *C. albicans* led the researchers to search for new drugs in the treatment of candidiasis. This paper aimed to investigate the antifungal activity of ([prolinium chloride] [1-methylimidazolium 3-sulfonate]) on a *candida albicans* standard strain. To reach this purpose, minimum inhibitory concentration (MIC) was performed via microdilution methods. Then to understand the toxicity of the IL, an MTT test was done. The obtained data showed that the IL can inhibit *C. albicans* growth at the 1690 mg/ml concentration ($P<0.0001$). Also, the IL showed low toxicity to mammalian cells at the same concentration ($P< 0.005$). Our results showed that the IL [prolinium chloride] [1-methylimidazolium 3-sulfonate] has a good antifungal activity with low cytotoxicity that may be a good candidate for new drugs design.

1. Introduction

Candida albicans (C. albicans) is a commensal microorganisms and a part of our natural microflora (Li et al., 2002). It commonly lives in our mouth and vagina. When an imbalance occurred in the microflora population, this opportunistic fungus invades its host and make infection. Evidence shows that *C. albicans* is responsible for 60% of mucosal infections and 40% of candidemia cases (Pappas et al., 2018).

Diagnosis of *Candida* infection can be possible through conventional and molecular methods. Three classes of drugs may be used for the treatment of candidiasis including polyene antibiotics, azoles, and echinocandins. Appropriate treatment of fungal infections is difficult due to the similar structure and metabolism of *C. albicans* to eukaryotic hosts. The appearance of high multidrug-resistant (Henriques & Silva, 2021) clinical isolates of *C. albicans* has amplified the need for novel antifungal drugs (Brown et al., 2014).

Ionic liquids (ILs) are a novel class of antimicrobial drugs against many clinically important microorganisms. ILs are composed of cations (mostly organic cations) and organic or inorganic anions with a melting point below 100°C (Welton, 2018). The unique properties of

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ILs such as solubility, thermal stability, hydrophobicity, low vapor pressure (Clare et al., 2019), made them important as researchers are interested in studying them. By choosing various ions it can be possible to obtain an IL with the desired chemical and physical properties. Currently, ILs are considered a promising asset for fighting against many microorganisms (Hartmann et al., 2016).

Some studies highlighted the characterization of ILs. Researchers manifested that ILs based on imidazole and pyridine have a good effect on reducing organisms. ILs based on Imidazole has been reported activity against many fungi and their pathogenicity (Petkovic et al; 2009). The side chain of ILs an effect on their properties as if in a study, it was reported that imidazolium-based Ils with hexadecyl side can inhibit Candida species growth (Bergamo et al., 2014). The component of Imidazolium-based ILs has antifungal and anti-biofilm and may affect the cellular process of fungi. Also, it was mentioned that imidazolium ionic liquids showed potential activity in reducing the content of ergosterol (Schrekker et al., 2013).

Yang et al. (2021) reported that the length of the alkyl chain of the IL molecule is proportional to antimicrobial activity and can limit its cytotoxicity. So by altering the anions and length of ILs, it can be possible to produce more effective and safe drugs that can be replaced with common antifungal drugs (Yang et al., 2021).

Due to increasing azoles resistant strains of *C. albicans* and limited understanding of mechanisms of ILs, this study aimed to investigate the effect of [prolinium chloride] [1-methylimidazolium 3-sulfonate] IL on *C. albicans* standard strain and measure its ability in reducing *C. albicans* cells and its cytotoxicity to human cells.

2. Materials and Methods

Candida strain: The standard *C. albicans* strain was received kindly from the Dr. Amini laboratory and was used in all experiments in this study. The strain was re-cultured on Sabouraud Dextrose Agar (SDA) containing chloramphenicol, incubated at 37℃ for 24 h to obtain a fresh strain.

2.1. Susceptibility test

To determine the minimum inhibitory concentration (MICs) of new synthesized ionic liquid, the microdilution methods were used according to the Clinical and Laboratory Standard Institute (CLSI) guideline.

The strain was adjusted to 2×10^2 CFU / ml in 100μl RPMI medium. 100 μl of the ionic liquid was added to the suspension and incubated at 37 ℃ for 48 h. The concentration was two-fold diluted serially (2, 4, 8, 16, 32, 64, 128, 256, 1024 $\mu g/ml$) and negative control was considered with culture medium and *C. albicans*. 100 μl of the IL was added to the medium culture and used as the positive control.

2.2. Well diffusion assay

To investigate the effect of [prolinium chloride] [1-methylimidazolium 3-sulfonate] IL on the standard strain of *C. albicans* used in this study, a well diffusion method was performed according to National Committee for Clinical Laboratory Standards (NCCLS). The *C. albicans* cultures were spread on Sabouraud Dextrose Agar plates with a sterile swab moistened with the fungal suspension at 0.5 McFarland. Subsequently, wells of 8 mm diameter were punched into the agar medium and filled with 100 μl (25 mg/ml) of the saturated IL and allowed to diffuse at room temperature for 2 h. Then the plates were incubated at 37° for 24 h and the diameters zones were measured. The Data was expressed as mean± standard deviation.

2.3. MTT test

MTT assay is a colorimetric assay. In this method, NAD (P) H-dependent cellular oxidoreductase enzymes reflect the number of viable cells present. The test is used to measure cytotoxicity or loss of viable cells. The test was based on the protocol that was described previously (Park et al., 1987).

Firstly, 10×10^4 cells suspended in 100μl of the medium and seeded in 96-well plate for 24h at 37℃ (to adhere the cells at the bottom of the plate) before adding the IL at the concentration at 10^{-4}–10^{-6} μl final concentration. After treatment (24-72 h), the cells were washed twice in PBS, then in each well, 100μl of 0.05 mg/ml MTT in serum-free medium was added, incubated for 3h at 37℃ to allow its metabolism.

In the next step, the produced formazan was dissolved in acidified isopropanol ($100\mu l$). By using a Biotec-ELX800 microplate reader the absorbance was measured and the results were expressed at percentage.

2.4. Statistical analysis

The results of all experiments were reported as mean$\pm$ standard deviation (n=3) and Spss 25 was used.

3. Results

Diameter Zone results: to evaluate the effect of the [prolinium chloride] [1-methylimidazolium 3-sulfonate] IL on the growth of the *Candida* strain used in this study, the zone of inhibition was measured. According to the results, the IL inhibited the growth of the strain up to $29\pm1\ mm$(Fig 1).

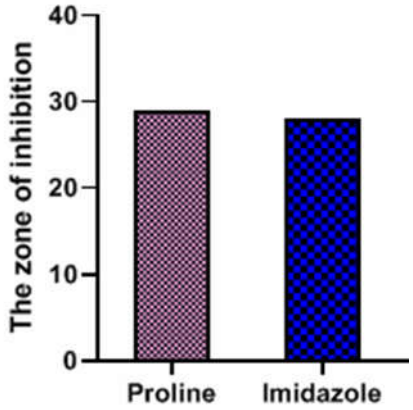

Fig 1. The results of inhibiting growth of C. albicans strain after treatment with [prolinium chloride] [1-methylimidazolium 3-sulfonate] IL

As it was shown in Fig 1, compare to imidazole as control, the IL has a little more effective.

3.1. MIC determination

In this study, the antifungal activity of the IL against *C. albicans* standard strain was investigated. The minimum inhibitory concentration and the diameter zone of inhibition were measured by microdilution and disk diffusion methods respectively. As it was shown in Fig.2, the mean MIC values for the IL with amino acid proline was 1690 mg/ml ($P<0.0001$).

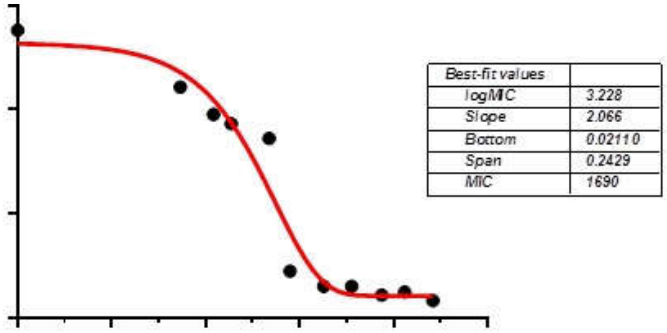

Fig 2. Mean imidazolium- based IL with proline amino acid ([prolinium chloride] [1-methylimidazolium 3-sulfonate] minimum inhibitory concentration (MIC) values for *C. albicans* standard strain

3.2. MTT results

Table 1, shows the viability of mammalian cells after treatment with [prolinium chloride] [1-methylimidazolium 3-sulfonate]. For analyzing the data, One-way ANOVA was used.

Table 1. The effect of each treatment on viability of mammalian cell

	mean	sd	N
Control	100	3.0789	5
2µM	3.6914	0.702	5
4µM	6.2593	0.8025	5
8µM	14.7265	0.9814	5
16µM	52.0516	1.2125	5
32µM	81.2977	2.6143	5

The data manifested that the IL can kill the *candida* standard strain at the concentration of 1690 mg/ml where had low cytotoxicity on mammalian cells at the same concentration ($P < 0.0001$).Fig 3, showed that at the 16 and 32 dilutions, the viability of cells was 50% and 80% respectively. These results showed that treatment with the IL had low toxicity toward the human cells.

Using Tukey's multiple analysis, comparisons between treatment at different dilution was done. As it was shown in table 2, the mean and confidence level confirmed the results at a P-value below 0.005, which is significant.

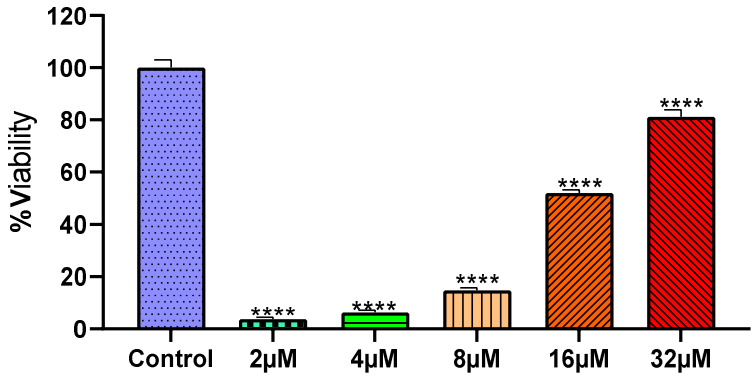

Fig 3. The results of the ([prolinium chloride] [1-methylimidazolium 3-sulfonate]), on human cells viability

Table 2. The mean and *P* value of each dilution of MTT test after treatment

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
2µM vs. Control	-96.31	-99.87 to -92.75	Yes	****	<0.0001
4µM vs. Control	-93.74	-97.30 to -90.18	Yes	****	<0.0001
8µM vs. Control	-85.27	-88.83 to -81.71	Yes	****	<0.0001
16µM vs. Control	-47.95	-51.51 to -44.39	Yes	****	<0.0001
32µM vs. Control	-18.7	-22.26 to -15.14	Yes	****	<0.0001
4µM vs. 2µM	2.568	-0.9920 to 6.128	No	ns	0.2611
8µM vs. 2µM	11.04	7.475 to 14.60	Yes	****	<0.0001
16µM vs. 2µM	48.36	44.80 to 51.92	Yes	****	<0.0001
32µM vs. 2µM	77.61	74.05 to 81.17	Yes	****	<0.0001
8µM vs. 4µM	8.467	4.907 to 12.03	Yes	****	<0.0001
16µM vs. 4µM	45.79	42.23 to 49.35	Yes	****	<0.0001
32µM vs. 4µM	75.04	71.48 to 78.60	Yes	****	<0.0001
16µM vs. 8µM	37.33	33.77 to 40.89	Yes	****	<0.0001
32µM vs. 8µM	66.57	63.01 to 70.13	Yes	****	<0.0001
32µM vs. 16µM	29.25	25.69 to 32.81	Yes	****	<0.0001

*Mean Diff= mean differences, CL of diff= confidence level of differences, ns= none sense

4. Discussion

Canidida spp are a group of opportunistic fungi that cause candidiasis. In many cases of invasive candidiasis, the most recognized agent is *candida albicans* that is responsible for two-third of these infections (Aashrafi et al., 2020). Normally, the infections caused by this fungus should be treated with common antifungal drugs like azoles, but increasing the resistance species of the fungus makes a serious problem that fails treatment (Luna-Tapia et al., 2018; Henriques & Silva, 2021).

ILs are compounds composed of organic cations and organic or inorganic anions (Busetti et al., 2010). One of the great advantages of ILs

is their variety, which can be achieved by their ions exchange that resulted in obtaining an IL with the desired chemical and physical characteristics. These materials are considered a good agent for fighting against fungi (Hartmann et al; 2016). Synthesizing a series of tert-BuOH-functionalized-imidazolium mesylate salts ([alkyl-t OHim][OMs]) and evaluating their antimicrobial activity on selected pathogenic microorganisms including bacteria (Gram positive and Gram negative), yeast, and fungi showed that the dodecyl substituted ionic liquid [C12-t OHim][OMs] significantly prevented the biofilm formation of S. epidermidis at 100 mM concentration as well as showed noteworthy antimicrobial activity. Again in another study, the results of MIC of dodecyl substituted ionic liquid on *C.albicans* strain NCIM 3628 (MIC $< 2000\ mg/ml$) was reported (Navale et al., 2015). Comparing this results to the results of this study, our novel IL showed better effect on *C. albicans* (MIC $< 1000\ mg/ml$) than the one (dodecyl substituted ionic liquid).

In previous studies toxicity of ILs against many microorganisms was studied. Kulacki &Labertie reported in their work that the [C4 MIM] [Br], [C6 MIM] [Br] and [C8 MIM] [Br] ILs had an antimicrobial effect on *Chlamydomonas reinhardtii* and said that the toxicity of these ILs is more than acetone, benzene, and phenol (Kulacki and Laberti, 2008). In the present study, the novel [prolinium chloride] [1-methylimidazolium 3-sulfonate] showed toxicity against *C. albicans* more than pyridine-based ILs. The imidazolium chloride-based ILs enhanced toxicity toward a subset of microorganisms and can reduce the concentration of antimicrobial compounds necessary to inhibit microbial growth (Yang et al., 2021). Similarly, the results of our study manifested the effect of the [prolinium chloride] [1-methylimidazolium 3-sulfonate] in reducing the concentration that is required for inhibiting microbial growth.

Investigating the effect of the ILs on mammalian cell cytotoxicity is important due to this material toxicity. Cellular damage may arise after treatment with different chemical agents (Amde et al., 2015). In a study, it was demonstrated an increase in antimicrobial efficacy on microbial cultures with specific IL + antimicrobial combinations, while maintaining low cytotoxicity towards mammalian cell (yang et al., 2021). In the present study, the effect of the ([prolinium chloride] [1-methylimidazolium 3-sulfonate] was investigated against mammalian cells. After treatment with the IL, the results showed little increase in toxicity to human cells that is not significant.

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Conflict of interest

There is no conflict of interest

Author contribution

Acquisition of data: Amir Habibi
Analysis and interpretation of data: Dr. Mansour Bayat, Dr. Behin Omidi, Amir Habibi
Drafting of the manuscript: Dr. Pejman Mortazavi, Dr. Mansour Bayat, and Amir Habibi
Critical revision of the manuscript for important intellectual content: Dr. Ali Ezabadi, Dr. Behin Omidi, and Dr. Mansour Bayat.
Statistical analysis: Amir Habibi
Administrative, technical, and material support: Dr. Ali Ezabadi, Amir Habibi

Refereces

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