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ORIGINAL ARTICLE

Synthesis of new imidazole-based ionic liquids with antifungal activity against *Candida albicans*

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ABSTRACT

Background and Objectives: *Candida albicans* cause a problematic condition in immunocompromised patients that could not be treated quickly due to the resistant behavior of microorganisms. This study aimed to investigate the effect of a novel ionic liquid (IL) as a new drug on *C. albicans* strains.
Materials and Methods: Seven newly binary ionic liquids mixtures were synthesized, and among them, ([prollinium chloride] [1-methylimidazolium 3-sulfonate] ([pro-HCl][MImS]) was selected and characterized by 1HNMR, 13C NMR, and FT-IR methods. Samples from patients (n=50) with candidiasis were collected and identified through culture media. *ERG11* gene overexpression was related to resistance against azole-bearing drugs. The antibiogram, well diffusion assay, MICs, and MFCs tests were operated. PCR and Real-time evaluated the expression of the *ERG11* gene, and the rate of cell death was detected using Flow Cytometry.
Results: Our data manifested that this novel IL (Ionic Liquid) can inhibit *C. albican*'s growth, reduce the expression of ERG11 and increase dead cells.
Conclusion: The newly synthesized IL had an inhibiting effect on the growth of the *C. albicans* strains and may be used as an alternative candidate for novel drug design.

Keywords: Ionic liquid; Imidazole; Proline; *ERG11*; *Candida albicans*

INTRODUCTION

Ionic Liquids (ILs), a deceptive material, are composed of cations and anions. Firstly, ILs were applied in 1914 as a non-volatile electrolyte for batteries. Due to their favorite effects, ILs were operated in biochemical processes and organic molecular synthesis (1). Some ionic liquids have proper bioactive anticancer, antimicrobial, and antiparasitic properties (2-4). ILs can dissolve organic components, especially DNA molecules, and have low vapor potency leading to reuse repeatedly, unlike volatile components. A report showed that the third descendant ILs could increase the ability to deliver active drug ingredients through increased solubility. ILs could carry proteins and amino acids by binding with hydrogen

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bonds. Ammonium-based ILs are hydrophobic antimicrobials. An aniline base with cation ammonium ion could kill microorganisms (5). The aim of this study was that due to the strong antimicrobial properties of ionic liquids and the resistance of all types of fungi, especially *Candida*, we found it better to measure the antibiotic properties of these liquids. On the other hand, studies show that these liquids are very toxic to body cells. Efforts to reduce the toxicity of ionic liquids led us to combine various amino acids with imidazole ionic liquid to make it non-toxic to body cells while being toxic to *Candida* spp. Therefore, a compound of imidazolium and six separate amino acids was made. Among all ionic liquids and amino acids, the proline-containing IL was more powerful than others in killing *Candida* spp. (6-8). Therapy and venous injection by polluted catheter are the other causes of *Candida* spp. overgrowth (9). Changing ions in ionic liquids may alter their chemical and physical properties and create a specific function. Housekeeping genes are the regions in a genome that are conserved stably more than other genes due to their roles in maintaining essential cellular functions. One of these genes in the yeast is *ACT1*, which helps to identify their phylogeny (10). Various studies showed that molecular techniques such as PCR and the northern hybridization technique could detect the expression of *CDR* and *MDR*, as well as *ERG11* genes in *C. albicans*. The *C. albicans* could resist azoles using several mechanisms, including increased expression of the genes related to the drug efflux pumps. These include *CDR1, CDR2*, and *MDR1* genes. *ERG11*, or sterol 14-demethylase, is a fungal cytochrome P450 enzyme that cooperates in the zymosterol biosynthesis and into ergosterol. *ERG11* gene overexpression is related to resistance to azole-bearing drugs (11). Nuclear Magnetic Resonance (NMR) is a spectroscopy technique to analyze an organic compound's chemical purity and structures. It determines what atoms cooperate in a particular organic compound by two different NMR, ^{1}H, and ^{13}C. The 1H NMR could define the number of hydrogen atoms of an organic molecule. The ^{13}C NMR could determine the number of carbon atoms. This study was designed to synthesize imidazolium-based ionic liquids with six amino acids separately. Their inhibitory effects on the growth of *Candida* species were evaluated. Finally, proline showed the best anti-*Candida* result in the standard strain, sensitive and even resistant to antifungal drugs.

MATERIALS AND METHODS

Synthesis of ionic liquids: providing pre materials. Modifying the cations and anions in ionic liquids lead to a proper specific function (5). All chemical reagents and solvents were commercially available and used without further purification. The 1H and 13C NMR spectra were recorded on an INOVA 500 MHz using DMSO-d6, CDCl3, or D2O as a solvent and calibrated with tetramethylsilane (TMS) as the internal reference. IR spectra were on a JASCO FT-IR410 spectrometer with KBr plates, as shown in Fig. 1. This study showed the effect of the novel IL ([prolinium chloride] [1-methylimidazolium 3-sulfonate] ([pro-HCl] [MImS]) on the overexpression of *ERG11* using a real-time reverse transcription-polymerase reaction (Real-time PCR). It measured the viability of candida cells after treatment with IL.

Experimental procedure. This experiment synthesized six new binary ionic liquids using 1-sulfopyridinium chloride as a cation base. Proline, isoleucine, leucine, glycine, alanine, phenylalanine, and serine were added as anion units separately. All numbered amino acids were used to synthesize six new binary ionic liquids.

([pro-HCl] [MImS]) synthesis. The base, 1-methyl imidazoles (5 mmol), was added with 50 ml dichloromethane under stirring at 0°C temperature by ice bath around the round 250 ml bottle flux. At first, 5 ml of chlorosulfuric acid (5mmol) was added slowly dropwise for about 10 minutes, maintaining the temperature. Then the mixture was stirred for 20 minutes at room temperature until milky color appeared. After that, vacuum filtration was performed at 40 for two h to separate solid salt from the IL. 5 mmol of the synthesized 1-methylimidazolium 3-sulfonate [MImS] dissolved in 100 mL of deionized water and transferred in two necks round-boat bottom flask. The container was placed into bath water at a temperature of 70c. Then five mmol of Proline amino acid was added within one h and stirred heavily for 72 h. The white precipitate was collected. The novel synthesized IL was washed with 30 mL of dichloromethane / vacuumed, and dried at ambient temperature to dehydrate.

Antifungal susceptibility tests: *Candida* samples. In this research, seventy *Candida* samples were taken

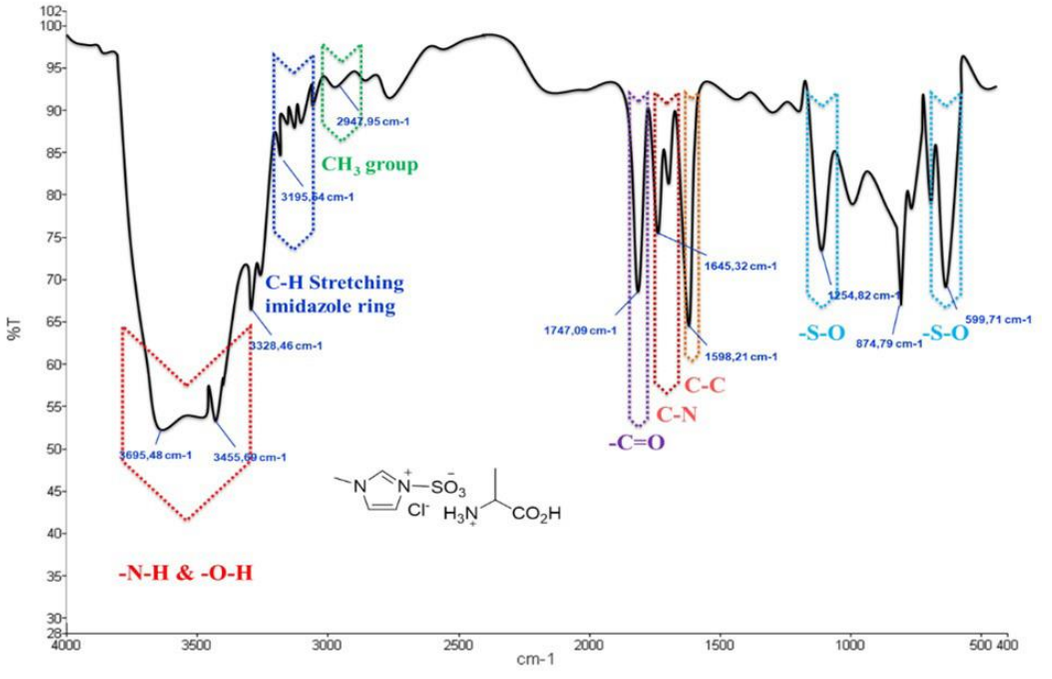

Fig. 1. FT-IR spectrum of [pro-HCl][MImS]

from the oral cavity, skin, and nails of immunocompromised patients with lupus erythematous, diabetic, etc, then were cultured on Sabouraud dextrose agar containing chloramphenicol (40mg/lit) and incubated at 37°C for 24-48 h aerobically (12). To differentiate between *Candida albicans* and other candidates, colony morphology, germ tube formation, and culture in CHROMagar medium of *Candida* were investigated. *Candida albicans* PTCC 5027 was used as the standard and control strain.

CHROMagar test. CHROMagar was used for the isolation and differentiation of *Candida* species. The *C. albicans* appeared green color colonies (13). All seventy strains were cultured on CHROMagar, among them, 50 strains were created green colonies as *Candida albicans.*

Germ tube test. To make a complete diagnosis, performed a germ tube test on the strains that were positive in CHROMagar. Horse serum was used to perform this test. As many as 500 microliters were poured into the microtube. Then, from each of the 50 samples, we removed a green colony in CHROMagar and cultured it inside a microtube. The microtubes were incubated for 3 hours at 37°C. Then, the fungus was placed on each microtube on the slide and was looked carefully under the microscope to observe the growth of the germ tube (14). All 50 strains had germ tubes, and as a result, the *C. albicans* diagnosis was confirmed.

Antibiogram test. The resistance and sensitivity of *Candida albicans* strains to amphotericin B, fluconazole, itraconazole, and ketoconazole were investigated by the Kirby- Bauer method based on standard CLSI M100-2018(Performance Standards for Antimicrobial Susceptibility Testing). Antibiogram disks of HiMedia company were used to determine the pattern of drug sensitivity (15).

Disc diffusion assay. To investigate the antifungal effect of six synthesized ionic liquids, the disc diffusion method was used based on standard CLSI M44-A2 (Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts) (16). After evaluating the ILs antifungal ability, the powerful IL against candida growth was selected for continued experimental.

Determination of minimum inhibitory concentration (MIC). CLSI M27-A3 (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts) was used to check the Minimum inhibitory concentration of synthesized ionic liquids. The microdilution method was used to determine the MICs of all six ionic liquids (17).

Determination minimum fungicidal concentration (MFC). To check the fungicidal ability of synthesized ionic liquids, the concentration determined as MIC, and the concentration before and after MIC were cultured on SDA medium, and the concentration which no growth was MFC (18).

Flow cytometry test. Flow cytometry was used to check the ability of the synthesized ionic liquids to kill fungal cells. For this purpose fresh cultures of *candida*, strains were prepared and suspension (0.5 McFarland) was prepared and treated with MIC concentration of ILs. After incubation for 12 hours, the cells were washed with PBS and fixed with 70% ethanol overnight at 4°C. The cells were treated with 200 µg/ml of RNase A and the mixture and incubated for 2 hours at 37°C. For DNA staining, 50 µg/ml of propidium iodide (PI) was added and incubated for 1 hour at 4°C in the dark place, then was studied with flow cytometric analyzer (19).

Evaluation of the *ERG11* gene expression. *ERG11* gene is one of the most important genes in synthesizing proteins involved in the synthesis of ergosterol in the membrane structure of fungi. By inhibiting this gene, it is possible to stop the production of ergosterol and destroy the fungal membrane, followed by the death of the fungus. Therefore, in this research, first, the presence of this gene in the strains was checked and then the effect of synthesized ionic liquids on the expression of this gene was investigated (20).

Detection of *ERG11* via PCR method. To prove the presence of gene *ERG11* in strains of *C. albicans*, DNA extracted was used a SINACLON DNA Extraction kit. Absolute 20 µl volume including 2 µl water, 2 µl forward primer, 2 µl reverse primers, 4 µl DNA, and 10 µl Master mix 2× were used to perform the PCR. Pre-denaturation at 95°C for 3 min started the reaction, followed by 35 cycles. The process was denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 30 sec, and the last step of reaction at 72°C for 5 min. The primers were forwarding 5′-TGCCTGGTTCTTGTTGCATTT-3′ and reverse 5′-AATCGTTCAAGTCACCACCCT-3 (12).

Evaluation of the *ERG11* gene expression rate via Real Time RT PCR method. According to the results, the proline amino acid had the best antifungal ability compared to the other ILs, so, the final evaluation was preventing the effect of the newly synthesized ([Pro-HCl] [MImS]) IL, on the rate of the *ERG11* gene expression by Real-Time RT PCR (21).

RNA extraction and purification. Total RNA samples from strains of *C. albicans* were GTP RNA extracted (DENAzist's RNA Isolation Kit-S-1010-1). In a 2µl tube, 1×10^6 fungal cells collected by centrifugation for 2 min at 10000 rpm were placed, and the supernatant was removed by pipetting. The pellet was resuspended in freshly prepared lysozyme. The tube was incubated at 37°C for 5 min, 400µl of lysis solution was added, following the laboratory protocol. 300 µl of the precipitating solution was added and inverted for 10 min. Then the solution was transferred into a spin column by pipetting and centrifuging the tube at 13000 rpm for 1 min. The spin column was washed three times with 400 µl washing buffer (22).

cDNA synthesis. Materials 2 µl buffer 10×, 2µl M- MLV reverse transcriptase (100unit), and nuclease-free water were poured into the wells. The protocol was initiated by mixing the agents well and centrifuging briefly before pipetting. Then we added the 10 µg total RNA1, Oligo (dT) 18 primer, 1µl dNTP 10mM, and nuclease-free water (top up to 10 µl). 10µl synthesized mix cDNA was incubated at 42°C for 60 min and then incubated for 5 min at 85°C to stop the reaction (21, 22).

Operative process of the real-time RT PCR. The designed primers used in this presentation were according to the previous study (22). The content of the 25 µl provided solution was 0.5 µl of cDNA, 12.5 µl of 2×SYBR Green, 1µl of each primer, and 10µl of ddH_2O, followed by putting the mixed solution in the 95°C for 60 sec, 95°C-15 sec for denaturation, 55°C-15 sec for annealing, 72°C-45 sec for extension, and the final extension for 72°C-45 sec. ACT1, housekeeping, was designed as an internal reference gene. Calculation of the relative target-gene expression was a fold change of 2-Δct value.

Statistical analysis. The data analysis using One-way ANOVA and Tukey test, in which $p\leq 0.05$ were considered statistically significant. All experiment results were reported as mean ± standard deviation (n=3), and Spss 26 was used. The difference in gene expression was calculated using GENE6 software, and the graphs were drawn by Graph Pad Prism 9.

RESULTS

Characterization of the new synthesized ILs. This item was confirmed by FT-IR, H NMR, and C NMR. The IR spectrum of the IL is evaluated in Figs. 1-3. The broadband at 3695-3455 cm^{-1} arises from the overlaid NH^{+} stretching band and the O-H stretching. The strong intensity bands attributed to the imidazole ring's stretching modes are located in the 3111-3195 cm^{-1} spectral range. The absorption bands of the va(CH_3) and vs(CH_3) groups in the IL are observed at a frequency of about 2947 cm^{-1}. The vibrations of C–N and C–C bonds of the imidazole ring generally appear coupled and can be observed in the 1645-1598 cm^{-1} range. The presence of carbonyl moiety is proved due to the sharp absorption band in 1747 cm^{-1}. The sharp peak at 874 is assigned to N-S bonds. The FT-IR vibrations at 1245 and 599 cm^{-1} are consigned to the S–O bond of sulfonic acid and SO3- group (asymmetric stretching and blending modes, respectively).

The H NMR of the IL is shown in Fig. 2. The signal assigned to the methyl group of amino acid moiety and imidazole ring of the IL appeared at 1.49 ppm (d) and 3.85 ppm (s), respectively. The spectrum shows the chemical shifts of the CH and CH_3 protons of the amino acid (4.06 ppm (t) and 1.49 ppm (d), respectively). The CH signal is splitted into a quartet (four lines), and the CH_3 signal is splitted into a doublet (two lines) because of the J-coupling with the neighboring protons. Other signals that appeared at 8.60 ppm, 7.37 ppm, and 7.10 ppm were related to the hydrogen of the imidazole ring.

In Fig. 3, ^{13}C NMR of [pro-HCl] [MImS] showed three sharp peaks at 143, 148, and 151 ppm, which were related to the imidazole ring. Also, the peak at 185 ppm was assigned to the carbonyl group of –COOH. The signal related to the carbon of the CH_3 group belonging to amino acid and imidazole rings was located at 24 and 28 ppm, respectively. The peak at 59 was related to the methylene group of amino acids.

CHROMagar test. CHROMagar was processed for seventy *Candida* samples. As shown in Fig. 4, fifty samples exposed a green color detected as *C. albicans.*

Germ tube test. All seventy samples were under germ tube test, and the fifty ones, which had been positive in CHROMagar, were demonstrated germ tubes (Fig. 5).

Well diffusion assay. The effect of the novel ILs on the *C. albicans* strains growth were determined,

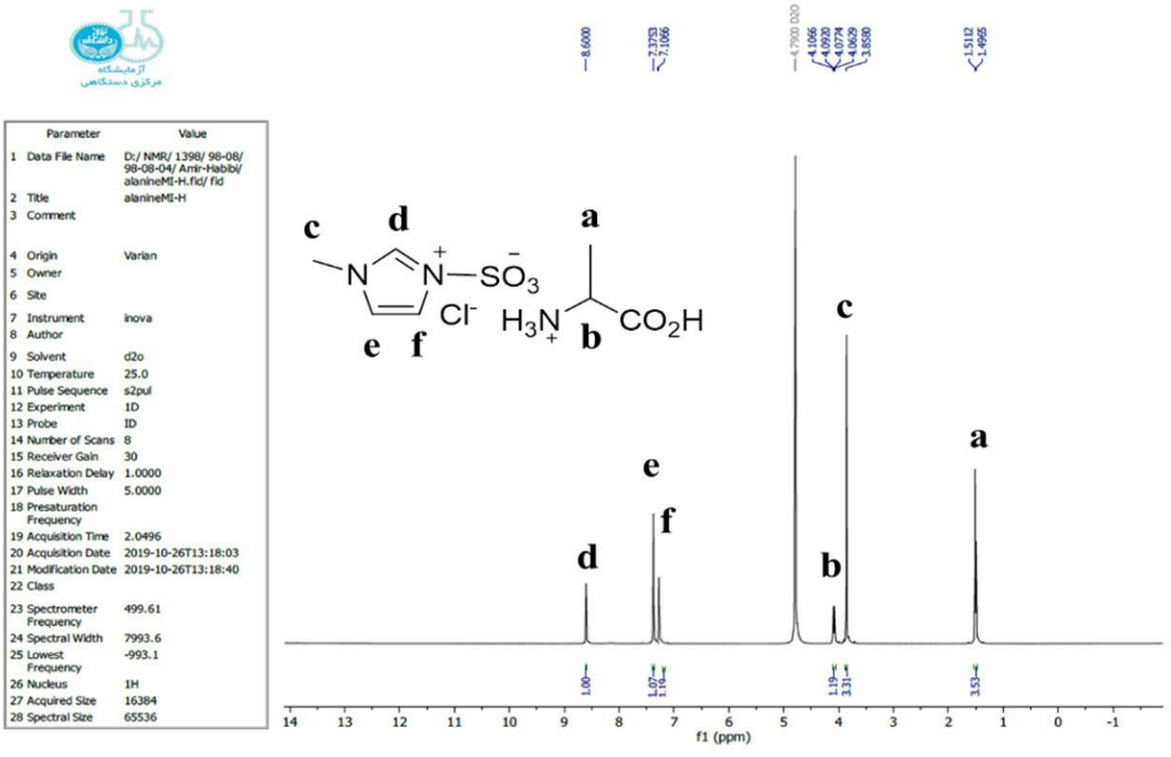

Fig. 2. ^{1}H NMR spectrum of the [pro-HCl][MImS]

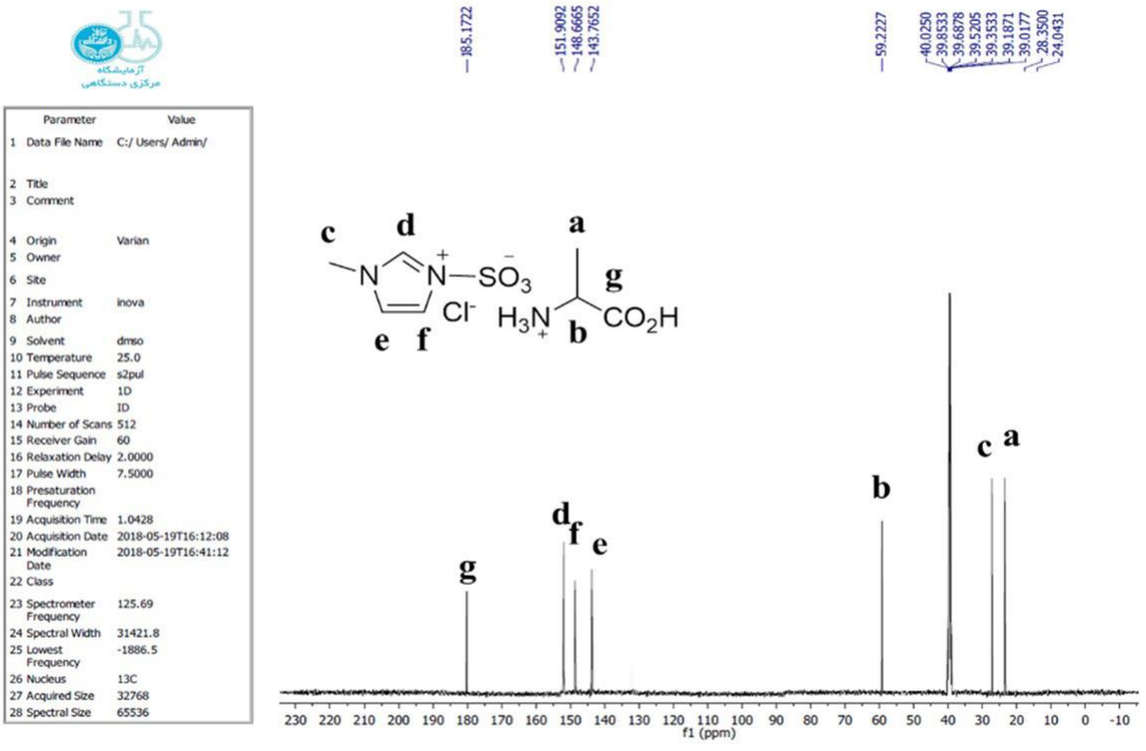

Fig. 3. ^{13}C NMR spectrum of the [pro-HCl][MImS]

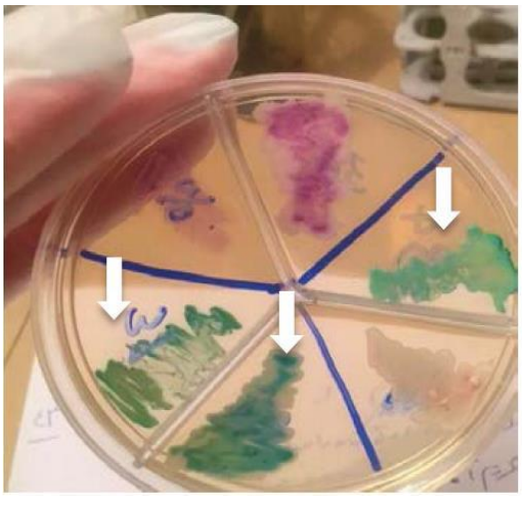

Fig. 4. The CHROMagar plate was operated for the detection of *C. albicans*. The colonies with green colors were *albicans* species.

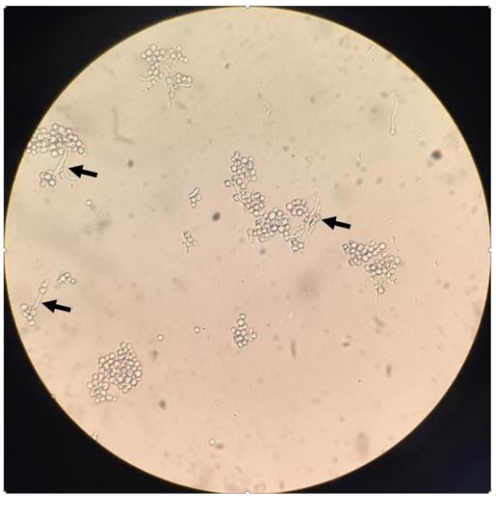

Fig. 5. The germ tube test was operated for the detection of *C. albicans.* The tubes indicated by arrows are pointed to the germ tubes.

and their inhibition zone was measured. The results showed that ILs have antifungal activity (in the range of 13 to 29 mm) except the one with glycine amino acid. Among these novel ILs, [pro-HCl] [MImS], the IL with Proline amino acid significantly showed better antifungal activity (27 ± 1 mm inhibition zone) against *C. albicans* strains (Table 1 and Fig. 6).

Antibiogram test. The Table 2 shows the effect of four antibiotics on cultured samples. Results compared with standard table of these drugs. Among isolates 54% were sensitive and 46% were resistante. Ketoconazole showed minor prevention of the *C. albicans* growth and fluconazole was more effective than other drug (Table 2).

Table 1. The inhibitory of growth effects of six amino acids compared with imidazole as a base in the novel ILs

Amino acids of the novel ILs	**Imidazole	Proline	Alanine	Leucine	Valine	Isoleucine	Glycine
*IZ (mm)	29 ± 1.5	27 ± 1	25 ± 1	22 ± 0.5	19 ± 1.33	13 ± 0.5	14 ± 1

* Inhibition Zone (mm); **Imidazole used as control

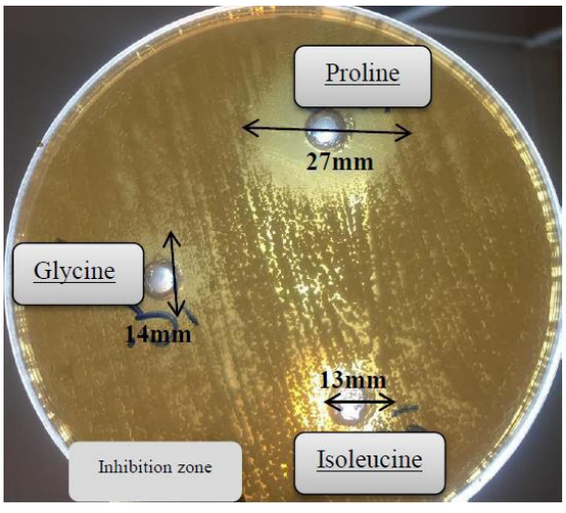

Fig. 6. The IL with proline was the best inhibition zone around of *Candida albicans.*

Table 2. Antibiogram of antifungal drug against the *C. albicans* strains.

Drug	Inhibition Zone (mm) Sensitive	Inhibition Zone (mm) Resistance
Fluconazole	30.2 ± 2.13	10.9 ± 0.943
Amphotericin B	29.4 ± 1.35	10.8 ± 0.87
Itraconazole	18.9 ± 0.83	10.7 ± 1.18
Ketoconazole	16.41 ± 0.57	10.6 ± 0.48

MIC and MFC test of ([pro-HCl] [MImS]) on *C. albicans*. The synthesized ([pro-HCl] [MImS]) inhibited the growth of the *Candida albicans* strains at a range from 192 µg/ml for sensitivity to above 323 µg/ml for resistant strains and an average of all samples 276 ± 4.5 µg/ml. The results of the MCF test for sensitivity were 350 µg/ml and resistance 540 µg/ml, and average of all samples 437 ± 5.25 µg/ml. (Fig. 7) shows the MIC and MFC for all samples.

PCR evaluation of *ERG11* gene. The PCR method studied the fifty strains of *C. albicans* for the *ERG11* expression. Among them, 45 numbers (90%) expressed the *ERG11* gene (Fig. 8).

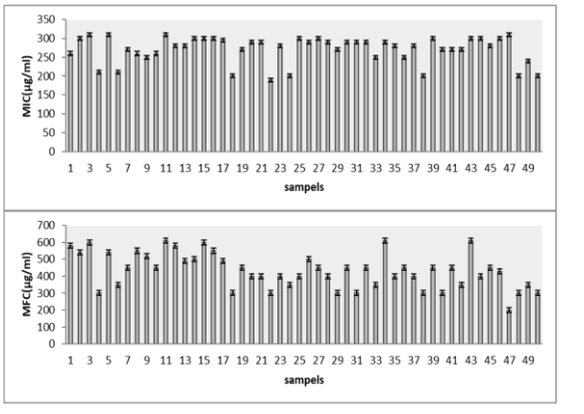

Fig.7. diagram of MIC and MFC determination of *Candida albicans* strains after treatment with ([pro-HCl] [MImS]).

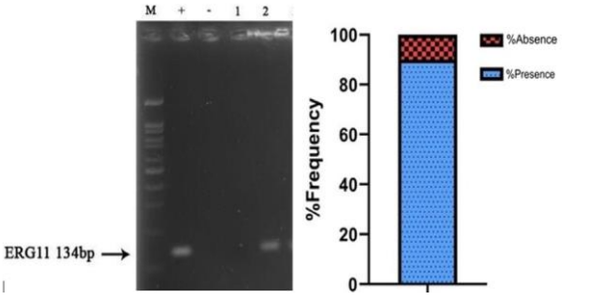

Fig. 8. The expression of the *ERG11* gene was detected by PCR.

Real-Time PCR evaluation of *ERG11* rate expression. Samples 11 and 27, among the most resistant samples, were designated into two groups with and without exposure to ([pro-HCl] [MImS]). As shown, the selected isolates were investigated to determine the decline expression of the *ERG11* after exposure to ([pro-HCl] [MImS]). The gene expression was reduced significantly, and the mRNA fold change of the gene showed a reduction from 1.000 in the untreated strains to 0.618 in the treated one ($\rho<0.05$) (Fig. 9).

Flow cytometry test. The test analysis was performed to determine the number of dead cells after treatment with the new material. The Flow cytometry test results showed an increase in dead cells num-

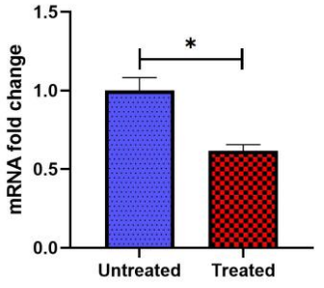

Fig. 9. The RT-PCR; Comparing the fold change of *ERG11* in IL treated group and untreated (control) group. **P= 0.009; ns: Non-significant.

ber of *C. albicans* after treatment with the ([pro-Hcl [MImS]). As was shown in (Fig. 10), by comparing the 3 (isolates 4, 11, and 7) selected isolates, sensitive, resistant and moderate, respectively, with each other and with control samples, it was manifested that dead cells were seen in isolate 4, the sensitive one. In isolate 11, the most resistant ones, the dead cells' was increased significantly (P=0.00272).

DISCUSSION

Candida albicans is the most causative agent of candidiasis, especially in critical and immunocompromised patients. One of the challenges in developing new antifungal drugs is the overexpression of the *ERG11, CDR1* and *CDR2* efflux pumps that cause resistance against the common antifungal medications. Emerging ILs with unique properties of the elongated against the fungal agents, lead the researchers to apply them as new safe administrations against fungi (12, 22). Some of the investigators demonstrated the antimicrobial function of various ionic liquids. imidazolium containing hexadecyl, the ionic liquid, can prevent the growth of microorganisms and *Candida* species. The vast biological activity of ILs depends on thermal stability, charge electronegativity, alkyl agents, and size. ILs can interact between changes in the biomolecules. Biologically active agents such as long alkyl-chain imidazole or 4-benzyl-4-methyl-morpholinium are the most important fields of ILs to have an antimicrobial effect (23). A study reported that fungi are more sensitive to 1- alkyl-3-methylimidazolium ionic liquids than Gram-negative organisms (24). In the present study, six (n=6) novel ILs based on 1-methyl imidazole and various amino acids were synthesized in an unprecedented method and tested their antifungal activities. According to these findings of MIC and vast inhibition zone in the microplates around the *C. albicans* strains, there is the best antifungal ability in the ([pro-HCl] [MImS]) compared to the other ILs synthesized in this study. So the rest of our experiments were performed using ([pro-HCl] [MImS]) IL.

Some researchers synthesized the Diethanolamine Lactic IL successfully, by replacement of halogenated ion fluid. They confirmed this IL using the fourier transform infrared (FTIR) spectra and the nuclear magnetic resonance (NMR) spectroscopy. We synthesized the novel ILs successfully and characterized them using IF and NMR spectra (25). The other researcher applied a series of 1-alkylquinolinium bromide against clinically relevant microbes such as *C. albicans.* They reported tested ILs had broad-spectrum antimicrobial activity against fungi and Gram-positive bacteria. In this presentation, we evaluated the antifungal activity of all eight novels ILs similar to the previous study (26). Comparing the efficiency of the novel ([pro-HCl] [MImS]) MIC (MIC <1 mg/ml , 276 ± 4.5 µg/ml) of our study with previously reported by some researchers with 3-Choropropanoic acid base IL on the *C. albicans* strains (ATCC: MIC >1.69 mg/ml; ATCC: MIC

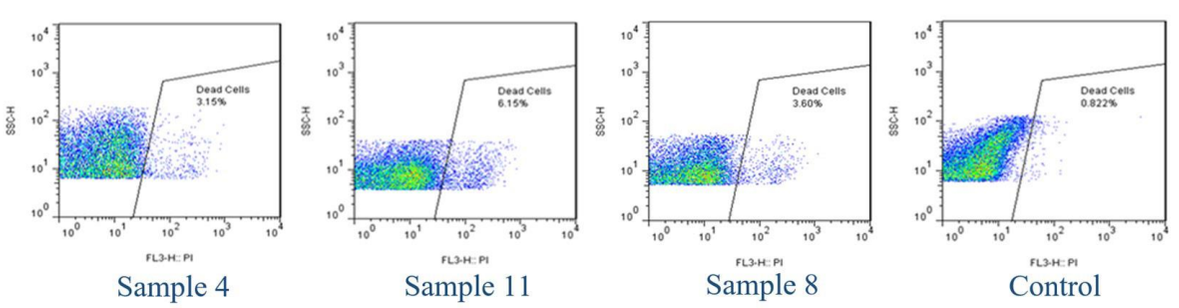

Fig. 10. Flow cytometry dot plots of *C. albicans* cells after treatment with ([pro-Hcl [MImS]). Dead cells of a sensitive (sample 4), moderate (sample 8), resistant (sample 11) strains, and the control one of *C. albicans* after treatment.

>3.38 mg/ml) showed a better antifungal activity of our ([pro-HCl] [MImS]) novel IL (27). The work of Yang et al. (2021) exhibited antimicrobial activity of imidazolium chloride-based ILs + tetracycline/ doxycycline enhanced toxic activity against both bacteria and yeast. These antibiotics inhibit protein synthesis. But they had limited efficacy due to antibiotic resistance. Interestingly we found the complementarity effect and efficient antifungal activity of ([pro-HCl] [MImS]) against *C. albicans* (28). In the present study, after treating with the novel ([pro-HCl] [MImS]), the zone inhibition diameter was 27 ± 1 mm exhibited a better effect on inhibiting *C. albicans* growth (29). ERG11 is important glycoprotein structures in fluconazole resistance in *Candida* species (30). A study on 165 isolates of the *Candida* species revealed that 28 cases were resistant to fluconazole. Expression of the ERG11 gene was confirmed in 10 isolates. Therefore overexpression of the *ERG11* gene may contribute to azoles resistance. The obtained data from Real-time PCR showed a significant reduction in the *ERG11* expression and the mRNA fold change from 1.000 (untreated strains) to 0.618 (treated strains) ($\rho < 0.05$). This reduction results in antifungal activity and killing properties of our novel IL against *C. albicans* strains. Anti-fungal abilities of ILs have earned attention in research because of the different chemical processes. In a paper, flow cytometry was used to demonstrate the cytotoxicity of methyl alkyl imidazolium-based ILs in *Saccharomyces* yeast. Flow cytometry could detect and evaluate the quantitation of yeast death and viability. Its ILs cytotoxicity evaluation was dependent on dose and time (31). The main advantage of flow cytometry over other methods is the synergy of detection speed and accuracy. This means that a large number of cells can be identified in terms of viability or function and morphology (32). The results of the flow cytometry test of the present study confirmed the effect of the novel ([pro-HCl] [MImS]) IL on increasing the dead cells of *C. albicans* cells after treatment (Fig. 10). Each method used in this study is evidence of the antifungal effect of our new synthesized IL on *C. albicans* strains.

CONCLUSION

This investigation revealed the antifungal ability of the ([pro-HCl] [MImS]) IL against *C. albicans* strains. ([pro-HCl] [MImS]) can be a candidate in treating candidiasis with effective cytotoxicity to resistant *candida* cells.

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