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ORIGINAL ARTICLE

Effect of new methionine-based ionic liquid on the *CDR1* and *CDR2* gene expression on sensitive and resistant strains of *Candida albicans*

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ABSTRACT

Background and Objectives: Previous researchers showed the antimicrobial ability of ionic liquids (ILs) on different infective agents. ILs can dissolve organic components, especially DNA molecules. Among synthesized eight binary ILs mixtures, we have chosen ([Met-HCl] [PyS]) IL for determining the antifungal ability of IL against *Candida albicans* cells.
Materials and Methods: Well diffusion assay, chrome agar and Germ tube tests were used to detect the *Candida* samples. PCR, real-time-PCR, and flow cytometry tests were performed to determine the IL's rate of toxic ability.
Results: Well diffusion assay revealed the diameters of the growth inhibition zones were the largest in IL with methionine and Proline amino acids. Minimum inhibitory concentration (MIC) and the Minimum fungicidal concentration (MFC) tests showed that they inhibited the growth of the *C. albicans* at a range from 250 µg/ml for sensitivity and 400 µg/ml for resistance, MIC average of all samples were 341.62 ± 4.153 µg/ml. IL reduced the expression of *CDR1* and *CDR2* the genes encoded by the major protein of ABC system transporter by 2.1 (P= 0.009) and 1.2 fold (P= 0.693), revealed by PCR and real time-PCR. In the flow cytometry test, there were increasing dead cells after treating with the ([Met-HCl] [PyS]) even in the most resistant strain.
Conclusion: The novel IL was effective against the most clinical and standard *C. albicans*.

Keywords: Ionic liquid; *Candida albicans*; Candidiasis; Antifungal agents; Methionine

INTRODUCTION

This study aims to research and produce a healthy ionic liquid for the body that is effective on *Candida albicans* strains and prevents its growth. Ionic Liquids (ILs), a deceptive material, are composed of cations and anions. Firstly, ILs were applied in 1914 as non-volatile electrolytes for batteries (1). ILs have many interesting properties, including powerful conductivity of cations or anions (2), low flammability (3), adequate thermal stability (4), adjustable polarity, being solvent in various biochemical substances (5), little moisture pressure, and low melting point (6). Due to their favorite effects, ILs were operated in

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biochemical processes, organic molecular synthesis (7), absorption of CO_2, SO_2 (8), and extraction from nanoparticles (9). Two common ionic liquids, ammonium or phosphonium-based, could produce proper bioactive anticancer, antimicrobial, and antiparasitic drugs (10, 11). Selection and modification of the anion and cation of the quaternary ammonium-based ILs with positive nitrogen could form tune physical properties, such as good melting, water-solubility, rate of conduction, and viscosity (12). Antimicrobial activity of the alkyl group on the different infective agents has been previously shown (13, 14). ILs can dissolve organic components, especially DNA molecules. ILs have low vapor potency leads to reuse repeatedly, unlike volatile components. A report showed that the 3rd descendant ILs could increase the ability to deliver active drug ingredients through increased solubility of its absorption. ILs carrier proteins and amino acids by developing acceptors with strong hydrogen bonds. Recent technology of ILs assessed on the ammonium-based salts is hydrophobic and can induce antimicrobial behavior. An aniline based ionic liquid could kill microorganisms (15). *Candida albicans* is a commensal microorganism in human and animal mucosal flora. They can induce candidiasis, especially in immunosuppressed patients. After immunosuppressing, antibacterial therapy and venous injection by polluted catheter are the other causes of *Candida* spp. overgrowth (16). Changing ions in ionic liquids may alter their chemical and physical properties and create a specific function. One study showed that the northern hybridization technique could detect the expression of two types of genes that encode pumps with efflux ability named the *CDR* and MDR in *C. albicans.* These genes are related to efflux pumps during biofilm growth. Mutation in the genes related to the efflux pumps could prepare some conditions that lead to the susceptibility of *C. albicans* to antifungal drugs. The mutants harboring a deletion of *CDR1*, *CDR2*, or *MDR1* increase susceptibility to fluconazole in planktonic form. *Candida* biofilm development and resistance could be with various mechanisms that may involve different molecular factors (17).

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful technique to analyze the chemical purity and structures of organic compound. This technique, which includes ^1H and ^{13}C NMR, can be used to picture the structure of the molecules. The ^1H NMR could define the number and types of hydrogen atoms of an organic molecule. The ^{13}C NMR could determine the number of carbon atoms. Flow cytometry is an automated technique for defining and evaluating the dimensions of single cells and their chemical aspects within a cell population. This technology can detect different fungi species. Flow cytometry detects various fungi cells and micro-colonies. Live, fixed, or non-fixed dead fungi cells stained with fluorescent (18). This study aimed to synthesize methionine-based ionic liquids and their preventing effects on the growth of *Candida* spp. collected from immunocompromised patients and a standard strain. This study aimed to investigate the effect of a new ionic liquid with methionine chloride and pyridinium sulfonate ([Met-HCl] [PyS]) on *C. albicans* growth. All chemical reagents and solvents were commercially available and used without further purification. The ^1H and ^{13}C NMR spectra were recorded on an INOVA 500 MHz using DMSO-d_6, $CDCl_3$, or D_2O as a solvent and calibrated with tetramethylsilane (TMS) as the internal reference. IR spectra were on a JASCO FT-IR410 spectrometer with KBr, shown in Fig. 1.

MATERIALS AND METHODS

Providing pre materials. Modifying the cations and anions in ionic liquids lead to a proper specific function (19).

Experimental procedure: ([Met-HCl] [PyS]) synthesis. Pyridine-N-sulfonic acid was synthetized by the method reported in the previous literature. Then, methionine (9.5 mmol) was added in small portions to a mixture of Pyridine-N-sulfonic acid (0.977g, 5 mmol) in deionized water (20 mL) over 30 min, and the reaction mixture was stirred for 24 h at room temperature. Then the solvent was removed by rotary evaporator under reduced pressure, and the obtained IL was washed with ether (3×20 mL) and dried under vacuum (12, 20).

***Candida* samples, Germ tube forming, and CHROMagar.** We took 70 samples from the oral cavity and skin of immunocompromised patients with candidiasis. Then cultured those on Sabouraud dextrose agar containing chloramphenicol (Armanbiotech, Iran), incubated at 35°C for 24-48 h aerobically. *Candida* isolates were analyzed macroscopically by direct observation, germ tube forming, and

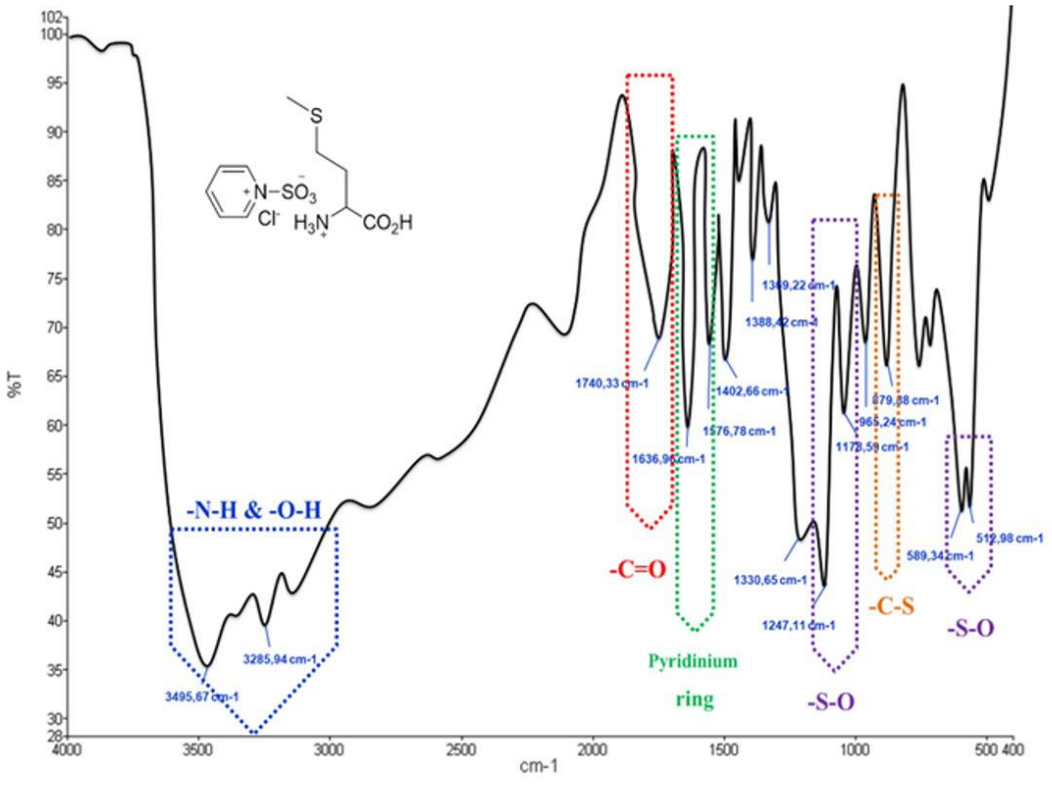

Fig. 1. FT-IR spectrum of ([Met-HCl] [PyS]).

CHROMagar medium of *Candida*, For isolation and differentiation of major clinical-significant *Candida* species. *Candida albicans* produce a green color in CHROMagar (21, 22).

Antifungal susceptibility test. The resistance and sensitivity of *C. albicans* strains against amphotericin B, fluconazole, itraconazole, and ketoconazole (HiMedia Company) were investigated by the Kirby-Bauer method, based on standard CLSI M100-2018 (Performance Standards for Antimicrobial Susceptibility Testing) (21).

Well diffusion assay. The antifungal activity of IL was determined by the agar well diffusion test based on standard CLSI M44-A2 (Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts). The *C. albicans* strains were cultured 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 ([Met-HCl] [PyS]) IL and some ILs composed of other amino acids, allowed to diffuse at room temperature for 2 h. The plates were incubated at 37°C for 24 h. After incubation, the diameters of the growth inhibition zones were measured (23).

Determination of MICs. According to CLSI M27-A3 (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts) the microdilution method, determine the ionic liquid's minimum inhibitory concentration (MICs). 100 ml of RPMI 1640 add to any wells then the ionic liquid was added, and different concentration of ILs was made by serial dilution method (2, 4, 8, 16, 32, 64, 128, 256, and 1024 mg/L), the strain suspension (1.5×10^8 CFU/ml) was added and incubated at 37°C for 48 h. The *C. albicans* in the culture medium were the negative control. Medium culture in 100 ml of the ionic liquid was a positive control. The phenomena of trailing were clarified as reducing turbidity compared to positive control but regularly attending in all wells; the suspension needed to become optically clear (24-26). The paradoxical growth was characterized as the growth of microorganisms at highly raised concentrations, further the obvious MIC (27-29).

Determination MFC. MFCs were estimated by pouring 0.1ml from MIC (without growth) wells onto

SDA plates. The MFC was the lowest IL concentration, destroying≥ 99.9% of cells. The positive controls were the Fluconazole solution (30).

Detection of *CDR1* and *CDR2* with PCR method. The PCR detected the gene expression of the *CDR1* and *CDR2* in *C. albicans* strains. The SINACLON DNA Extraction kit (Tehran Cavosh Clon- Iran) was used for extracted all the DNA samples from the *C. albicans* strain in Tehran, Iran. Absolute 20 µl volume including 2 µl water, 10pmol/µl forward primer (sequence primers present in Table 1), 10pmol/µl reverse primer, 100 ng DNA, and 10 µl Master mix 2× were used to perform the PCR. Predenaturation at 95℃ for 3 min started the reaction followed by 35 cycles. The process was denaturation at 95℃ for 30 sec, annealing at 57℃ for 30 sec, extension at 72℃ for 30 sec, and the last step of reaction at 72℃ for 5 min. The base design of the primers used for this step to investigate the presence of the gene from clinical samples of *C. albicans* originated from research and synthesized by SINA COLON.CO, Iran (17).

Determination of the expression of the *CDR1* and *CDR2* via Real Time RT PCR method: RNA extraction and purification. Total RNA samples from clinical isolates of *C. albicans* strain and *C. albicans* ATCC 10231 were GTP RNA extracted (RNA Isolation Kit-S-1010-1, Dena Zist Asia, Iran). In a 2 µl tube, 1×10^6 fungal cells collected by centrifugation (2 min at 10000 rpm) were placed, and the supernatant was removed by pipetting. The pellet was re-suspended in freshly prepared lysozyme. The tube was incubated at 37℃ for 5 min. 400 µl 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. After discarding the supernatant, 500 microliters of washing Buffer1 was added and centrifuged. After discarding the supernatant, 750 microliters of washing buffer2 was added. The supernatant was discarded after centrifugation, and the RB column was placed in a new collection tube for complete drying and centrifuged for 3.5 minutes at 12,000 rpm at 4 degrees Celsius. The RB column was placed in the elution tube, and 50 microliters of nuclease-free water were added to the center of the RB column. After one minute, centrifugation was performed for 1.5 minutes with a rotation of 12000 rpm at 4 degrees Celsius, and RNA was kept at minus 70 degrees Celsius. Thereafter 2 µl buffer10×, 2 µl M- MLV reverse transcriptase (100unit) was poured and nuclease-free water was in the wells. The protocol was initiated by mixing the agents well and centrifuging briefly before pipetting. Then we added 10 µg total RNA1, Oligo (dT) 18 Primer (*18-mer* oligonucleotide with 5'- and 3'-hydroxyl ends), 1 µl dNTP 10mM, and nuclease-free water (top up to 10 µl). 10µl synthesized mix cDNA was incubated at 42℃ for 60 min and then incubated for 5 min at 85℃ to stop the reaction.

Operative process of the Real-Time RT PCR. 0.8 micromole designed primers used in this presentation were according to the previous study (31). The content of the 20 µl provided solution was 50 ng of cDNA, 12.5 µl of 2× SYBR Green, 1 µl of each primer, and 10 µl of ddH_2O, followed by putting the mixed solution in the 95℃ for 60 sec, 95℃-15 sec for denaturation, 55℃-15 sec for annealing, 72℃-45 sec for extension, and the final extension for 72℃-45 sec. ACT1 (F:ATTATATGTTTAGAGGTTGCTGCTTTGG

R: CAATTCGTTGTAGAAGGTATGATGCC), housekeeping, was designed as an internal reference gene. Calculation of the relative target-gene expression was a fold change of 2-ΔΔCT value.

Then, we performed the final evaluation to clear the preventing effect of the newly synthesized ([Met-HCl] [PyS]) IL on the *CDR1* and *CDR2* genes expression by Real Time RT PCR. The primer sequences

Table 1. GenBank accession to primer sequences of *CDR1* and *CDR2*

Gene	GenBank accession number	Product length (base pairs)	Primer sequences
CDR1	X77589	93	Forward: 5'-TGTGTACTATCCATCAACCATCAGC-3'
			Revers: 5'- CACCAAAATAAGCCGTTCTACCA-3'
CDR2	U63812	125	Forward: 5'-TGGCAAACAATCCAACAATACA-3'
			Revers: 5'-AATCAAGGGAATAGATGGGTCA-3'

used for this evaluation was shown in Table 1. The reaction was performed in an EcoTM Real-Time model device (Illumina biotech company, USA). For amplification in each well (48-well plate), a 20-microliter mixture of nuclease-free water (10 µl), Cybergreen reaction mixture (6 µl), template DNA (2 µl), and specific primers (1 µl), for each gene was prepared. The time and temperature program of the reaction was carried out in three stages: the first stage in one cycle to activate the hot start monopolymerase enzyme and initialize the template DNA at a temperature of 95°C for 3 minutes; the second step alternately during 40 cycles of annealing at 95°C for 5 seconds and joining and extension at 60°C for 20 seconds; The third step is applying a cycle with a temperature of 95°C for 15 seconds, 60°C for 30 seconds and 95°C for 15 seconds to draw a curve.

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 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 mixtured then 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, then studied with Flow cytometric analyzer.

Statistical analysis. The data analysis using One-way ANOVA and the Tukey test, in which $p \leq 0.05$ were considered statistically significant. All experiments 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 8.

RESULTS

Characterization of the new synthesized IL. This item was confirmed by FT-IR, ^{1}H NMR, and ^{13}C NMR. The IR spectrum of the IL is shown in Fig. 1. The broad band at 2500-3400 cm^{-1} arises the overlaid NH_3^+ stretching band and the O-H stretching. The vibrational band at 1740 cm^{-1} can be assigned to the C=O bond. The absorptions at 1636 and 1576 cm^{-1} are characteristic of carbon-carbon aromatic stretching vibrations. The bands at 1178 and 589 cm^{-1} can be assigned to the vibrational mode of the S-O band of the SO_3- group.

The ^{1}HNMR of the IL gave signals in D2O. The IL contained five aromatic hydrogens that belonged to the pyridine ring that appeared at 8.0 ppm (t, 2H), 8.5 ppm (t, ^{1}H), and 8.7 ppm (d, 2H). The CH_3 group of amino acid gives a singlet at 2.02 ppm. Also, CH_2 of the *c*-Carbone varied between 2.0 and ~2.20 ppm. The methylene hydrogens of *b*-Carbone and methine hydrogen of *a*-Carbone appear as triplets in 2.60 and 4.1 ppm. The ^{13}C NMR displays the signals for the eight carbon atoms in the IL. The chemical shifts at 143.7, 148.6, and 151.9 ppm were assigned to the pyridine ring. Also, the chemical shift at 185.1 ppm can be attributed to the carboxyl group of the amino acid moiety of the IL. The chemical shifts that appeared at 24.0, 28.3, 53.4, and 59.2 ppm can be related to the carbons of the other carbon atoms of the amino acid moiety of the IL.

CHROMagar test. 70 samples from people suffering from candidiasis were used, and after culturing on chrome agar medium and separating colonies that were green (as shown in Fig. 2a), and performing germ tube test (as shown in Fig. 2b), 50 samples of *Candida albicans* were identified, and the other tests were performed on these 50 strains.

All positive germ tube tests preserved using the horse serum presented filamentous mycelium, a morphological transition. Both tests revealed fifty of all seventy *Candida* samples were recognized as *C. albicans* strain. Fig. 4b indicates these results.

Antibiogram test. Table 2 shows the effect of four antifungal drugs on *Candida* strains. Results comparing with the standard table of drugs and sensitive and resistant strains were identified. Among these four

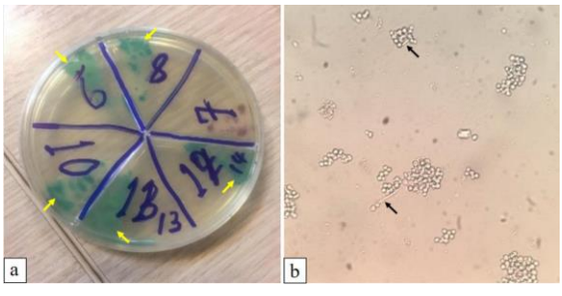

Fig. 2. Chrome agar medium (a) and Germ tube test (b). Yellow arrow: *Candida* pigmentation; Black arrow: germ tube formation.

drugs, fluconazole showed the best fungicidal activity on the *C. albicans* strains (Table 2).

Well diffusion assay of ILs on *C. albicans*. The antifungal activity of the newly synthesized ionic against the *C. albicans* strains and liquids has been shown in Fig. 5. The average inhibition zone of IL by methionine was 32 ± 0.43, IL by proline was 26 ± 1.7, IL by pyridine SO_3H was 25 ± 0.5, IL by serine was 22 ± 1, IL by leucine was 22 ± 1.34, IL by alanine was 20 ± 1.29, IL by valine was 25 ±2.05, IL by glycine was 13 ± 1.5 and IL by phenylalanine was 0.0 ± 0. Comparing the effect of the newly synthesized ILs with some amino acids, the diameters of the growth inhibition zones were the largest in IL with methionine and proline amino acids (Fig. 3).

MIC and MFC test of new synthesized ILs on *C. albicans.* In this research, eight new ionic liquids were synthesized using different amino acids, among them the ([Met-HCl] [PyS]) was shown best and most powerful result to inhibit the growth of the *Candida albicans* strains. MIC range of the ([Met-HCl] [PyS]) was 250 µg/mL for sensitivity and 400 µg/mL for resistant strains (present in Fig. 4). The average MIC was 341.62 ± 4.153 µg/mL. Fig. 4 shows the results of the MFC for all samples. The average of MFC was 587.6 ± 6.59 µg/mL.

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

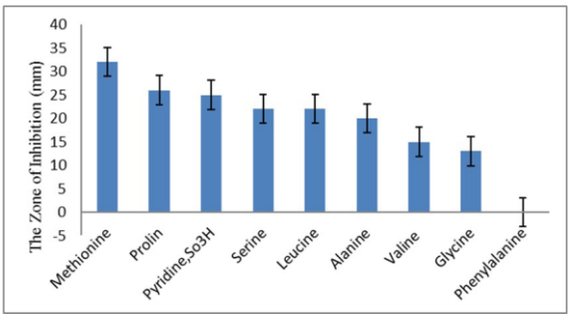

Fig. 3. The growth inhibition zones of the eight new synthesized ILs on *C. albicans* strains.

PCR evaluation of *CDR1* and *CDR2* expression. Before exposure to IL, the fifty strains of *C. albicans* were studied for the expression of *CDR1* and *CDR2* using the PCR method (Fig. 5). Then among fifty clinical isolates of *C. albicans*, 45 numbers (90%) expressed *CDR1* while 44 numbers (88%) expressed the *CDR2* genes. We chose two samples of numbers 4 and 30 to investigate the rate of the ([Met-HCL] [Pys]) on the expression of the *CDR1* and *CDR2* (Fig. 5).

Real-Time PCR evaluation of *CDR1* and *CDR2* rate expression. Evaluating the rate of *CDR1* and *CDR2* expression after the exposure to the ([Met-HCl] [PyS]) IL on the number of 4 and 30 selected isolates by the q Real-Time PCR method. These isolates were selected because of their resistance and sensitive abilities. The results showed a decrease in the expression and fold changing of the proteins (Fig. 6 and Table 3).

According to data presented in Table 2, there is about a 2 (2.1322) fold change reduction in *CDR1* gene expression in IL-treated groups. In contrast, the change of *CDR2* was 1.27 fold nonsignificant (P= 0.693). The decrease in fold change of the *CDR1* gene showed that the novel IL had a significant inhibiting effect on the *CDR1* gene expression (P= 0.009).

Flow cytometry test. Among fifty samples, we selected three numbers of 4, 8, and 7 as sensitive, resistant, and moderate isolates according to the growth inhibition diameter zones (Fig. 7). The ([Met-HCl] [PyS]) IL showed the fungicide ability in all three selected isolates, which means the novel ([Met-HCl] [PyS]) IL showed a superior effect on inhibiting *C. albicans* growth resulting in increasing of dead cells of the fungus. Even in the most resistant isolate (number 8), there was an increase in dead cells compared to the control (P=0.39960). The controls in this test were live *Candida albicance.*

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

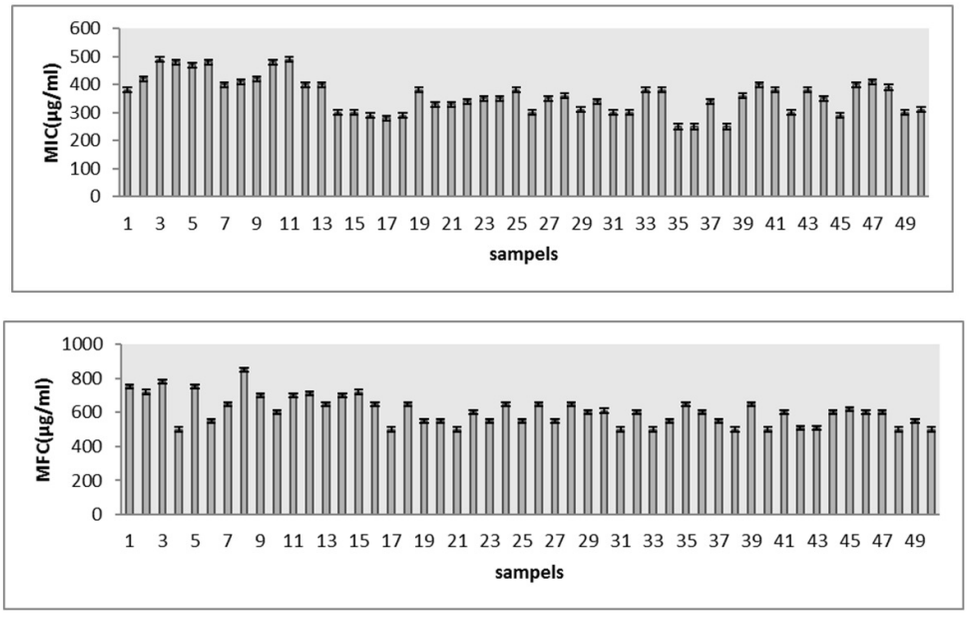

Fig. 4. Diagram of MIC and MFC determination of clinical samples after treatment with ([Met-HCL] [Pys]).

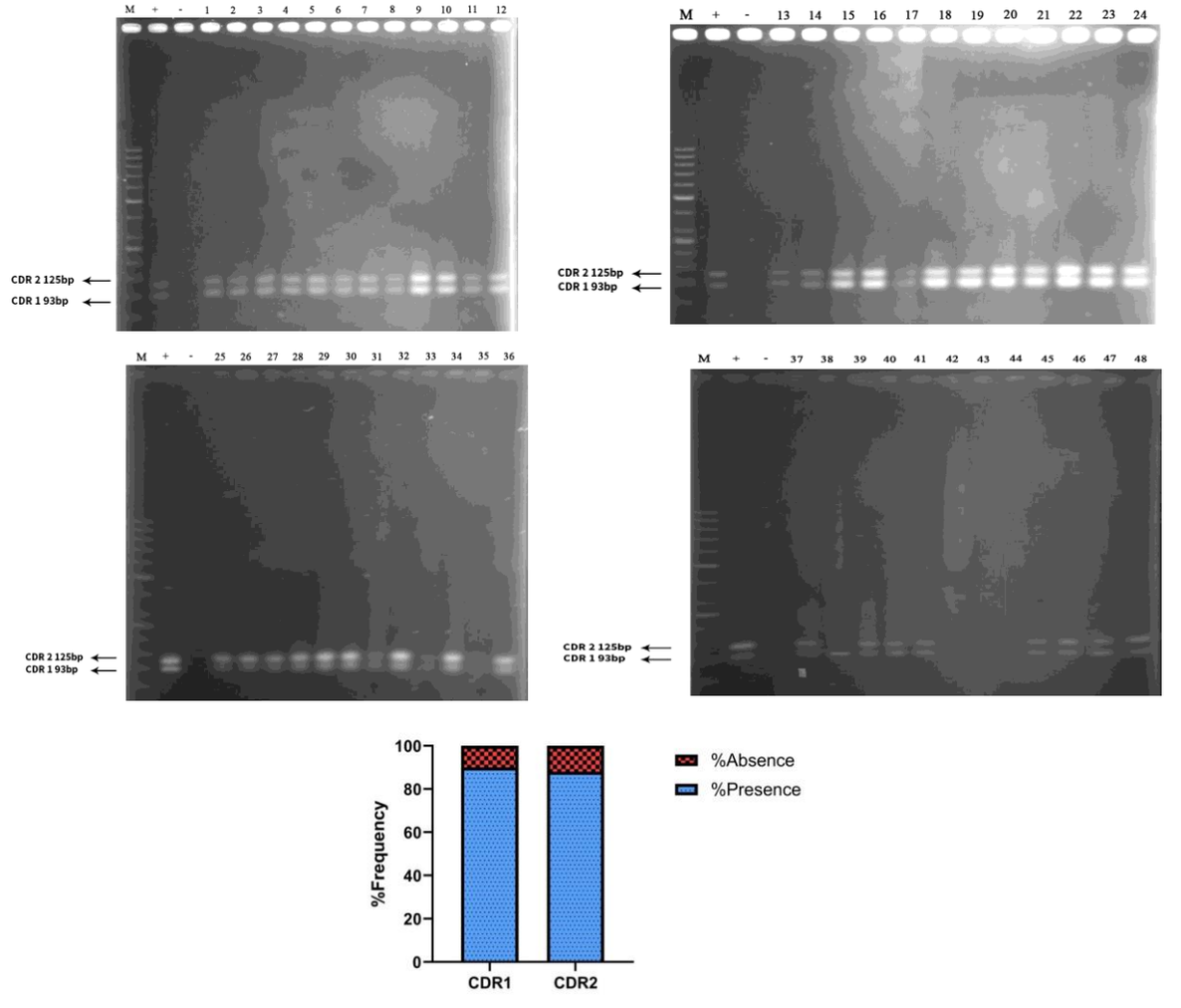

Fig. 5. The percent of expression of the *CDR1* and *CDR2*.

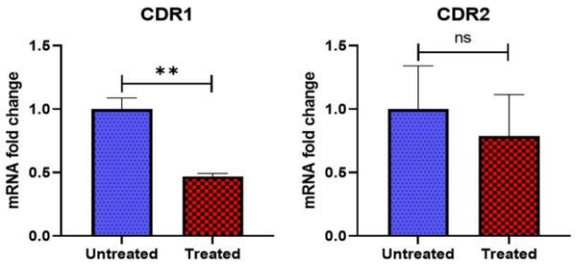

Fig. 6. The q RT-PCR; Comparing the fold change of *CDR1* and *CDR2* in IL treated group and untreated (control) group. **P= 0.009; ns: Non-significant.

Table 3. Fold change of *CDR1* and *CDR2* in treated with ([Met-HCL] [Pys]) and untreated groups.

	brpA **(untreat)**	***brpA*** **(treat)**	**Diff [*brpA* (untreat, treat)**
CDR1	3.02	3.3	-0.28
CDR2	4.37	4.645	-0.275
count	2	2	2
Mean	3.69	3.97	-0.27
STDEV	0.954	0.951	0.0035
P-Value			0.0057
Confidence Level(CI)			94%
Difference (A-B log scale)			-0.277
Fold Change			-1.43

The amount of fold change for the *brpA* gene is equal to -1.43, which indicates that this gene has decreased by 1.43 times in the treated group compared to the untreated group. The Count row indicates the number of examined samples. The Mean row indicates the average of two samples. P≤ 0.05

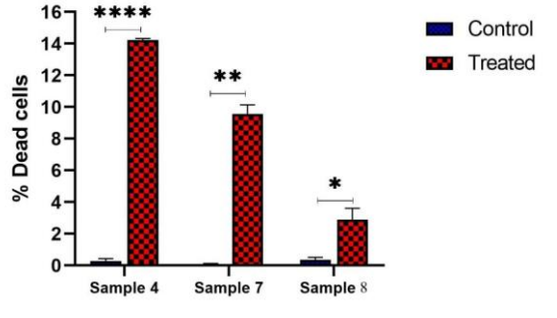

Fig. 7. Flow cytometry revealed the effect of the IL on viability of *C. albicans* isolates from clinical samples.

CDR1 and *CDR2* efflux pumps that cause resistance against the common antifungal medications (17). Emerging ILs with unique properties of the elongated against the fungal agents, lead the researchers to apply them as new safe administrations against fungi (17). Some of the investigators demonstrated the antimicrobial function of various ionic liquids (12, 32). Imidazolium containing hexadecyl, the ionic liquid, can prevent the growth of microorganisms and *Candida* species (33, 34). The vast biological activity of ILs depends on thermal stability, charge electronegativity, alkyl agents, and size. ILs can interact between changes in the biomolecules (35, 36). Biologically active agents or molecules are the most important fields of ILs to be studied (32, 37). In this study, eight (n= 8) newly designed ILs were designed with 1-sulfopyridinium chloride as the precursor and eight (n=8) various amino acids and properly characterized using spectroscopic techniques. Novelty is the key feature of the protocol to synthesize the ILs used in this study. According to these findings of MIC and vast inhibition zone around the *C. albicans* strains, there are the best antifungal ability in the ([Met-HCl] [PyS]) compared to the other ILs synthesized in this study. So the rest of our experiments were performed using ([Met-HCl] [PyS]) IL. Some researchers synthesized the diethanolamine Lactic IL successfully, by replacement of halogenated ion fluid. They confirmed this IL using the Fourier transform infrared (FT-IR) spectra and nuclear magnetic resonance (NMR) spectroscopy. We synthesized the novel ILs successfully and characterized them using FT-IR and NMR spectra (38). The other researcher applied a series of 1-alkylquinolinium bromide against clinically relevant microbes such as *C. albicans.* They reported that tested ILs had broad-spectrum antimicrobial activity against fungi and Gram-positive bacteria (39). In this presentation, we evaluated the antifungal activity of all eight novel ILs similar to the previous study (20). Comparing the efficiency of the novel ([Met-HCl] [PyS]) MIC (MIC <1 mg/mL) of our study with previously reported by some researchers with 3-Choropropanoic acid base IL on the *C. albicans* strains (ATCC, American Type Culture Collection: MIC >169 µg/mL; ATCC: MIC >338 µg/mL) showed a better antifungal activity of our ([Met-HCl] [PyS]) novel IL (40). The work of Yang et al. (2021) exhibited the 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 ([Met-HCl] [PyS])

against *C. albicans* (41). In the present study, after treatment with the novel ([Met-HCl] [PyS]), the zone inhibition diameter was 32 ± 0.43 mm exhibited a better effect on inhibiting *C. albicans* growth. *CDR1* and *CDR2* are important glycoprotein structures in fluconazole resistance in *Candida* species (42). Fructose administration over than efficient diet could induce fluconazole resistance in *C. albicans* via the activation of *CDR1* and *MDR1* transporters (43). Upregulations of multidrug efflux pumps to belong to the ATP-binding cassette transporters superfamily controlled by *CDR1/CDR2* proteins were involved in most *C. albicans* fluconazole-resistant strains (44). So in the present study, we investigated the role of the novel ([Met-HCl] [PyS]) on the expression level of these two proteins. This study showed a significant reduction of the *CDR1* expression and fold change from 1.000 to 0.469 in non-treated and treated strains (P=0.009). This data showed that the ([Met-HCL] [Pys]) could suppress the expression of the protein *CDR1* significantly. Kaempferol (natural flavonol) can inhibit *CDR1* expression more than *CDR2* in fluconazole-resistant *C. albicans* strains (45). Similarly, our data showed a reduction in the fold change of the *CDR2* expression from 1.000 to 0.787 in non-treated and treated samples, which was not significant (P=0.69). 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 (46). Flow cytometry could detect and evaluate the quantitation of yeast death and viability (47). Its cytotoxicity evaluation was dependent on dose and time (48). 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 (18). The results of the flow cytometry test of the present study confirmed the effect of the novel ([Met-HCl] [PyS]) IL on increasing the dead cells of *C. albicans* after treatment. 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 ([Met-HCl] [PyS]) IL against *C. albicans* strains. ([Met-HCl] [PyS]) can be a candidate for treating candidiasis with effective cytotoxicity to resistant *Candida* cells.

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