BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e19897

Article

Synergistic interaction of fluconazole/ sodium bicarbonate on the inhibition of *Candida glabrata* phospholipase gene

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Candida glabrata infections are responsible for deaths of people globally. Fluconazole is known to be less effective against *C. glabrata*, which developed many strategies to evade being destroyed by fluconazole. To achieve enhanced efficacy of fluconazole against *C. glabrata*, the interaction of fluconazole with sodium bicarbonate was investigated using the CLSI guidelines. The efficacy of fluconazole alone and in combination with sodium bicarbonate was evaluated using the time-kill and phospholipase production assays. Eventually, the expression of *PLB* was assessed using semi-quantitative RT-PCR to investigate the inhibitory properties of fluconazole alone and in combination with sodium bicarbonate against *C. glabrata.* The fluconazole/sodium bicarbonate combination displayed synergistic and antagonistic effects (FICI= 0.375-4.25). In *C. glabrata* ATCC, SN 152, and SN 164, the fluconazole/sodium bicarbonate combination exhibited a significant fungicidal activity ($p<0.05$) but antagonistic effect in the case of SN 283. With exception of SN 283, a significant reduction was noted in phospholipase production in clinical isolates of *C. glabrata* treated with fluconazole/sodium bicarbonate combination. The *PLB* was down-regulated significantly by 0.168-0.515 fold in *C. glabrata* treated with fluconazole/sodium bicarbonate. The results suggested fluconazole/sodium bicarbonate to have a potential synergistic interaction in *C. glabrata*, and the underlying mechanism may be associated with phospholipase gene.

Keywords: *Candida glabrata.* Fluconazole. *PLB.* Sodium bicarbonate.

INTRODUCTION

Similar to *Candida albicans, C. glabrata* is an opportunistic fungal pathogen that commonly exists in the gastrointestinal, genitourinary, and respiratory tracts (Silva *et al*., 2012; Charlet *et al*., 2018; Chen *et al*., 2019). *C. glabrata* is the most common cause of non-*albicans Candida* vulvovaginitis, which is associated with high morbidity and mortality rates (Kalaiarasan *et al*., 2018; Makanjuola *et al*., 2018; Kiasat *et al*., 2019; Mikdachi, Spann, 2019; Rodríguez-Cerdeira *et al*., 2019). Importantly, several risk factors such as severe immunosuppression, diabetes mellitus, prematurity, older age, broad spectrum antibiotics, prior usage of antifungal agents, and low socioeconomic status are associated with higher likelihood of non-*albicans* candidiasis (Deorukhkar *et al*., 2014; Makanjuola *et al*., 2018; Rodrigues *et al*., 2019). The potent pathogenic mechanisms of *Candida* species is contributed to various virulence factors, including adherence to the surface, ability to evade host defence, resistance to hydrogen peroxide and derivatives, phenotypic switching, biofilm production, rapid response to changes in the microenvironment, and secretion of extracellular hydrolytic enzymes (Fidel *et al*., 1999; Figueiredo-Carvalho *et al*., 2017; Kalaiarasan *et al*., 2018; Makanjuola *et al*., 2018; Rodríguez-Cerdeira *et al*., 2019; Treviño-Rangel *et al*., 2019). In contrast to the aggressive

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process adopted by other fungal pathogens, *C. glabrata* uses a combination of immune escape and persistence to invade and colonize target cells (Ho, Haynes, 2015; Kasper *et al*., 2015).

Destruction of host tissues by *C. glabrata* may be facilitated by the release of extracellular hydrolytic enzymes, such as phospholipase, proteinase, and esterase, into the local environment. Indeed, phospholipase hydrolyses phospholipids into fatty acids, which can expose receptors to facilitate adherence by disruption of the host cell membrane. Phospholipases are classified into four types of A, B, C or D, depending on the cleavage site of the of the ester linkage within a phospholipid. Phospholipase B represents major hydrolysis and lysophospholipase/transacylase activities in *Candida* species (Yang, 2003; Silva *et al*., 2012; Barman *et al*., 2018).

C. glabrata reduced susceptibility or intrinsic resistance to the azole antifungals, resulting in the treatment failure (Amirrajab *et al*., 2016). Antifungal combination therapies are thus urgently needed to fight *C. glabrata* infections. Combination therapies with azole antifungals were reported against *C. glabrata* (Fidel *et al*., 1999; Carrillo-Muñoz *et al*., 2014; Campitelli *et al*., 2017), but thus far, there are no data published on the efficacy of fluconazole/sodium bicarbonate on *C. glabrata*. The present study was, therefore, performed to ascertain the effectiveness of the fluconazole/sodium bicarbonate combination on clinical isolates of *C. glabrata*. The expression profiles of *PLB* gene involved in the *C. glabrata* treated with fluconazole/sodium bicarbonate combination were also investigated in this study.

MATERIAL AND METHODS

Source of *Candida glabrata*

Microbiological and molecular studies were performed on three clinical isolates of *C. glabrata* from recurrent vulvovaginal candidiasis (SN 152, SN 164 and SN 283) obtained from the stock collection of Microbiology Laboratory, Cellular and Molecular Research Centre, Yasuj University of Medical Sciences. Clinical isolates were inoculated onto Sabouraud Dextrose Agar (SDA, Merck, Germany) medium and incubated at 35 °C for 24 h at the microbiology laboratory. *C. glabrata* ATCC 90030 was used as the reference control. The reliability of *C. glabrata* colonies was confirmed by CHROMagar™ *Candida* (CHROMagar Microbiology, Paris, France), germ tube formation, and molecular methods using the universal fungal primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 1990; Alizadeh *et al*., 2017; 2018).

Synergism testing

The synergistic interaction between fluconazole and sodium bicarbonate against clinical isolates of *C. glabrata* was determined via the broth microdilution method (CLSI, M27-A3 and M27-S4). One hundred microliters of a twofold dilution of fluconazole (Merck) ranging 0.03–64 µg/mL and sodium bicarbonate (Merck) ranging 48–50000 µg/mL alone or in combination were dissolved in a standard Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) with 0.2% glucose [buffered to pH 7.0 with 0.165 M of morpholinophos-phonyl sulfate (MOPS)]. Aliquots of the solution were seeded in the wells of 96-well U-bottom microtitre plates (Maxwell, China) in the presence of 100 µL of 0.5-5 × 10^3 colony-forming units (CFU)/mL of *C. glabrata* clinical isolates and incubated at 4 °C for 2 h. The minimum inhibitory concentration (MIC) was assayed at 35 °C after 24 h of incubation. MICs were interpreted with the WHONET software (Alizadeh *et al*., 2017). The synergistic interaction of the fluconazole/sodium bicarbonate combination was assessed on the basis of the fractional inhibitory concentration index (FICI), which represents the sum of the FICIs of each antifungal agent. The FICI was calculated for each antifungal agent through dividing the MIC of each antifungal agent combination by its MIC alone. Indeed, antifungal agent interactions were considered to be synergistic with a FICI ≤ 0.5, partial synergy with a FICI > 0.5 but < 1.0, additive with a FICI of 1.0, indifferent with a FICI of 1.0 but < 4.0, and antagonistic with a FICI of ≥ 4.0 (Khodavandi *et al*., 2010).

Time-kill method

Time-kill experiments were performed with fluconazole and sodium bicarbonate alone and in

combination at 2× MIC, 1× MIC, ½× MIC, and ¼× MIC levels. The mixtures were inoculated with *C. glabrata* and adjusted to give a final concentration of about 10^6 CFU/mL. After 0, 2, 4, 6, 8, 10, 12, 24, and 48 h of incubation at 35 °C, the respective cell suspensions were collected, diluted tenfold serially, and 100 µL of each dilution was spread on SDA. Colonies were counted after 24 h of incubation at 35 °C and the CFU/mL was calculated accordingly (Scheetz *et al*., 2007; Khodavandi *et al*., 2018).

Phospholipase production assay

Clinical isolates of *C. glabrata* were analysed for phospholipase production assay by growing the isolates on phospholipase agar [10 g peptone, 40 g dextrose, 16 g agar, and 80 mL Egg Yolk Emulsion (Fluka, Chemie AG, Buchs, Switzerland) per 1000 mL of distilled water] (Price *et al.,* 1982; Khodavandi *et al*., 2018). Clinical isolates of *C. glabrata* treated with fluconazole and sodium bicarbonate alone and in combination at 2× MIC, 1× MIC, ½× MIC, and ¼× MIC levels were grown overnight at 37 °C in RPMI 1640. Subsequently, suspensions of approximately 2×10^5 cells/mL of each isolate were inoculated onto the surface of the phospholipase agar plate medium and incubated at 30 °C for 72 h. The phospholipase index (Pz) was defined as the ratio of the colony diameter (mm) to the total colony diameter plus the precipitation zone. Accordingly, a Pz value of $0.82 \leq Pz \leq 0.88$, $0.75 \leq Pz \leq 0.81$, and $Pz \leq 0.74$ denoted negative, weak, moderate activity, and strong phospholipase activities, respectively, by the isolates.

Semi-quantitative analysis of *PLB* gene expression in *C. glabrata*

Expression of *PLB* in *C. glabrata* treated with fluconazole and sodium bicarbonate alone and in combination was analysed by semi-quantitative reverse transcriptase (RT)-PCR. Total cellular RNA was extracted from *C. glabrata* treated with fluconazole and sodium bicarbonate at 2× MIC, 1× MIC, ½× MIC, and ¼× MIC, each alone and in combination following the manufacturer's operating instructions of RNeasy Mini Kit (Qiagen, Hilden, Germany). The extracted RNA was treated with Deoxyribonuclease I (DNase I; SinaColon, Karaj. Iran). Subsequently, RNA was qualified by 1.2% (w/v) formaldehyde denaturing agarose gel electrophoresis and its concentration was measured using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA). Total cellular RNA (0.5 µg) was copied into single-stranded cDNA using M-MuLVRNase H´ reverse transcriptase and random hexamer oligonucleotides using a first strand cDNA synthesis kit (SinaColon, Iran) according to the manufacturer's instructions.

C. glabrata PLB and actin genes were amplified from the synthesized cDNA with primers designed via NCBI/Primer-BLAST and analysed by the OligoAnalyzer tool (https://eu.idtdna.com/pages/tools/oligoanalyzer) (Table I). RT-PCR was performed using the PCR Master Mix (Ampliqon A180306, Odense, Denmark) with 12.5 µL of *Taq* 2x Master Mix, 1 µL of (10 pmol/µL) each of forward and reverse primers, 3 µL of (7 ng/µL) template cDNA, and 7.5 µL of PCR grade H_2O on a Techne thermocycler system (Bibby Scientific, USA). Amplification conditions were as follows: 5 min at 95 °C, 25 cycles of 3-step cycling, denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were visualised under UV light using a gel documentation system (Bio-Rad, USA) to verify the amplicon quantity prior to the gene expression or sequence analysis.

TABLE I - Primers and their specifications used in this study

Primer	Orientation	Sequence	Length (bp)	NCBI GenBank accession number
PLB	Forward	5' ATGGCTGGTCTTTCTGGTGG 3'	839	AF498582
	Reverse	5' AGGTCACGCTCTTGCTTCAA 3'		
Actin	Forward	5' GTTGACCGAGGCTCCAATGA 3'	384	FN394020
	Reverse	5' TGAGCAGCGGTTTGCATTTC 3'		

Relative expression level of *PLB* gene was calculated based on known concentrations of DNA standard molecules. Relative transcript abundances were examined by volume-based analyses using the standard volumes and a regression curve with logistic regression model. The intensity of PCR products, amplified with *PLB* and actin genes in the agarose gel, was calculated based on the comparison with a standard concentration (MassRuler Low Range DNA Ladder, Ready-to- Use, Fermentas) using the Rotor-Gene Q - Pure Detection software (version 2.3.1, Qiagen). The fold change of target gene expression level was calculated as the target/reference ratio in a treated sample relative to target/reference ratio in an untreated control sample. Differentially expressed gene with a statistical significance and a fold change of $\geq$ 2- fold or $\leq$ 0.5 was considered as significantly up- or down-regulated, respectively (Alizadeh *et al*., 2017). The PCR products were analysed by a sequencing service (Macrogen Seoul, South Korea). The sequence of the nucleotide obtained for each gene was analysed using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequences were deposited to GenBank: BankIt - NCBI - NIH (https://www.ncbi.nlm.nih.gov/WebSub/).

Ethical approval

Procedures involving human participants, obtained from Microbiology Laboratory, Cellular and Molecular Research Centre, Yasuj University of Medical Sciences, were in accordance with the ethical standards of the institutional and/or national research committee and with the 2008 Helsinki declaration.

Statistical analysis

Data were analysed statistically using analysis of variance (ANOVA). Means were compared using the Tukey's Post hoc test. A difference with $p < 0.05$ was considered statistically significant. Statistical analysis of time-kill experiments were performed using the SPSS software (version 24; SPSS Inc., Chicago, USA). Relative quantification of gene expression was analysed by the GraphPad Prism software (version 6; GraphPad Software Inc., California, USA).

RESULTS AND DISCUSSION

Clinical isolates of *C. glabrata* were confirmed by microbiological and molecular methods. The ITS sequence of clinical isolate of *C. glabrata* (SN 283) was deposited at DDBJ/EMBL/GenBank under the accession number: MN393005.

Table II shows the results of antibiotic susceptibility of the *C. glabrata* isolates. Our study revealed that one (33%) of the three clinical isolates of *C. glabrata* of recurrent vulvovaginal candidiasis showed resistance to fluconazole. Table III summarizes the MIC and FICI values of fluconazole and sodium bicarbonate alone and in combination against clinical isolates of *C. glabrata*. The MIC of fluconazole alone ranged from 0.5 to 16 µg/mL when fluconazole was used alone, while it decreased to a

range of 0.5 to 2 µg/mL when used in combination with sodium bicarbonate. Compared with sodium bicarbonate used alone (6250–25000 µg/mL), the MICs of sodium bicarbonate reduced 4-fold in concomitant use with fluconazole. Indeed, the fluconazole/sodium bicarbonate combination displayed synergistic (FICI= 0.373-0.499) and antagonist effects (FICI= 4.25) against fluconazole and sodium bicarbonate alone.

TABLE II - Fluconazole susceptibility testing results for clinical isolates of *C. glabrata* analyzed by the WHONET software

Code	Antibiotic name	Antibiotic class	Breakpoints	%R	%I	%S	%R 95%C.I.	Geom. Mean	MIC Range	MICs (%)		
										SN 152	SN 164	SN 283
FLU_NM	Fluconazole	Antifungals	S<=2 R>=8	33	0	67	1.8-87.5	2.52	0.5 - 16	2 (33.3)	16 (33.3)	0.5 (33.3)

SN: Clinical isolates of *C. glabrata.*
S: susceptible; R: resistant; I: intermediate; C.I.: confidence interval

TABLE III - MIC (µg/mL) and FICI values of fluconazole and sodium bicarbonate alone and in combination against clinical isolates of *C. glabrata*

Isolates/ Antifungal	Fluconazole	Sodium bicarbonate	Fluconazole/Sodium bicarbonate		
	MIC	MIC	MIC	FICI	Interpretation
C. glabrata ATCC 90030	2	3152	0.25/782	0.373	Synergism
SN 152	2	6250	0.5/1562	0.499	Synergism
SN 164	16	25000	2/6250	0.375	Synergism
SN 283	0.5	25000	2/6250	4.25	Antagonism

SN: Clinical isolates of *C. glabrata.*

In the time-kill study, the results indicated that the fluconazole/sodium bicarbonate combination exhibited a significant fungicidal activity for ATCC 90030, SN 152, and SN 164 of clinical isolates of *C. glabrata* ($p \leq$ 0.05). Time-kill studies confirmed an antagonistic effect for fluconazole in combination with sodium bicarbonate in the case of SN 283 (Figure 1).

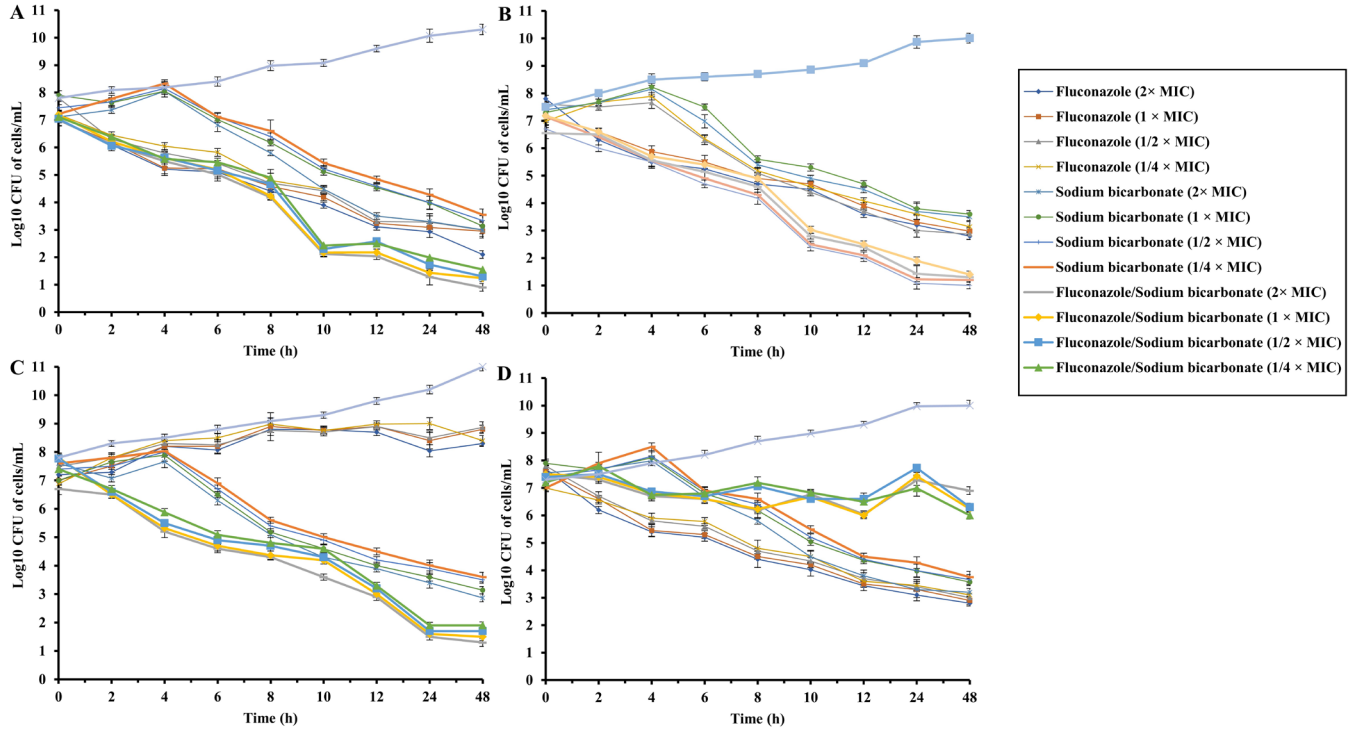

FIGURE 1 - Time-kill curves of fluconazole and sodium bicarbonate alone and in combination against clinical isolates of *C. glabrata* based on MIC. (A) *C. glabrata* ATCC 90030, (B) SN 152, (C) SN 164, and (D) SN 283.

Fluconazole and sodium bicarbonate alone and in combination inhibited the phospholipase activity ($p \leq 0.05$). Table IV shows that the fluconazole/sodium bicarbonate combination manifested strong inhibition of phospholipase activity relative to untreated control in ATCC 90030, SN 152, and SN 164 of clinical isolates of *C. glabrata*. Clearly, the treatment with fluconazole/sodium bicarbonate combination led to 1.60-1.69-fold reduction of phospholipase activity in *C. glabrata* ATCC 90030 compared to the estimated 1.65-1.68-fold and 1.61-1.64-fold reductions of phospholipase activity in SN 152 and SN 164 of clinical isolates of *C. glabrata*. With fluconazole/sodium bicarbonate combination, SN 283 showed no inhibitory effects on phospholipase activity.

TABLE IV - Phospholipase production assay of clinical isolates of *C. glabrata* treated with fluconazole and sodium bicarbonate alone and in combination based on MIC

Antifungals/ Isolates		*C. glabrata* ATCC 90030	SN 152	SN 164	SN 283
Fluconazole	Untreated control	0.55±0.013[a]	0.57±0.015[a]	0.56±0.011[a]	0.58±0.014[a]
	2× MIC	0.87±0.016[e]	0.86±0.014[e]	0.59±0.019[c]	0.88±0.019[e]
	1× MIC	0.86±0.011[d]	0.86±0.011[d]	0.59±0.018[c]	0.87±0.010[d]
	½× MIC	0.85±0.018[c]	0.85±0.013[c]	0.58±0.013[b]	0.85±0.010[c]
	¼× MIC	0.84±0.015[b]	0.84±0.012[b]	0.58±0.015[b]	0.83±0.013[b]

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TABLE IV - Phospholipase production assay of clinical isolates of *C. glabrata* treated with fluconazole and sodium bicarbonate alone and in combination based on MIC

Antifungals/ Isolates		*C. glabrata* ATCC 90030	SN 152	SN 164	SN 283
Sodium bicarbonate	Untreated control	0.55±0.013[a]	0.57±0.015[a]	0.56±0.011[a]	0.58±0.014[a]
	2× MIC	0.84±0.016[d]	0.86±0.010[e]	0.84±0.010[d]	0.85±0.011[d]
	1× MIC	0.83±0.012[c]	0.84±0.018[d]	0.83±0.012[c]	0.83±0.011[c]
	½× MIC	0.82±0.011[b]	0.82±0.010[c]	0.82±0.012[b]	0.82±0.011[b]
	¼× MIC	0.82±0.010[b]	0.81±0.019[b]	0.82±0.010[b]	0.82±0.017[b]
Fluconazole/Sodium bicarbonate	Untreated control	0.55±0.013[a]	0.57±0.015[a]	0.56±0.011[a]	0.58±0.014[a]
	2× MIC	0.93±0.015[d]	0.96±0.010[d]	0.92±0.010[c]	0.59±0.010[b]
	1× MIC	0.91±0.014[c]	0.95±0.014[c]	0.92±0.012[c]	0.59±0.010[b]
	½× MIC	0.88±0.011[b]	0.94±0.010[b]	0.90±0.015[b]	0.58±0.010[a]
	¼× MIC	0.88±0.011[b]	0.94±0.010[b]	0.90±0.010[b]	0.58±0.013[a]

SN: Clinical isolates of *C. glabrata*.
[a–e] Means ± SD in each treatment and column with different superscripts differ significantly ($p < 0.05$).

The expression level of *PLB* was evaluated to investigate the effects of fluconazole and sodium bicarbonate at 2× MIC, 1× MIC, ½× MIC, and ¼× MIC, each alone and in combination on *C. glabrata* ATCC 90030. The expression levels of the *PLB* were significantly down-regulated by fluconazole and sodium bicarbonate alone and in combination ($p \leq 0.0001$). Figure 2 shows the relative quantification of *PLB* treated with fluconazole and sodium bicarbonate alone and in combination using RT-PCR. The fold changes in terms of *PLB* gene expression to untreated control for 2× MIC, 1× MIC, ½× MIC, and ¼× MIC of fluconazole were 0.216 ± 0.02, 0.403 ± 0.008, 0.515 ± 0.02, and 0.659 ± 0.03, respectively ($p \leq 0.0001$). The fold changes regarding *PLB* gene expression for 2× MIC, 1× MIC, ½× MIC, and ¼× MIC of sodium bicarbonate were 0.307 ± 0.02, 0.493 ± 0.03, 0.673 ± 0.009, and 0.817 ± 0.02, respectively ($p \leq 0.0001$). The *PLB* gene was found down-regulated significantly by 0.168 ± 0.04, 0.315 ± 0.01, 0.418 ± 0.02, and 0.515 ± 0.02 after treatment with 2× MIC, 1× MIC, ½× MIC, and ¼× MIC of fluconazole/sodium bicarbonate, respectively ($p \leq 0.0001$). It is important to consider that the effects of fluconazole and sodium bicarbonate alone and in combination are concentration-dependent (Tukey post hoc test, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$). Moreover, there were significant differences among the expression levels of *PLB* in *C. glabrata* ATCC 90030 treated with fluconazole and sodium bicarbonate alone and in combination ($p < 0.05$). The nucleotide and protein sequences of *actin* and *PLB* have been deposited at DDBJ/EMBL/GenBank under the accession numbers MN447432 and MN447433, respectively.

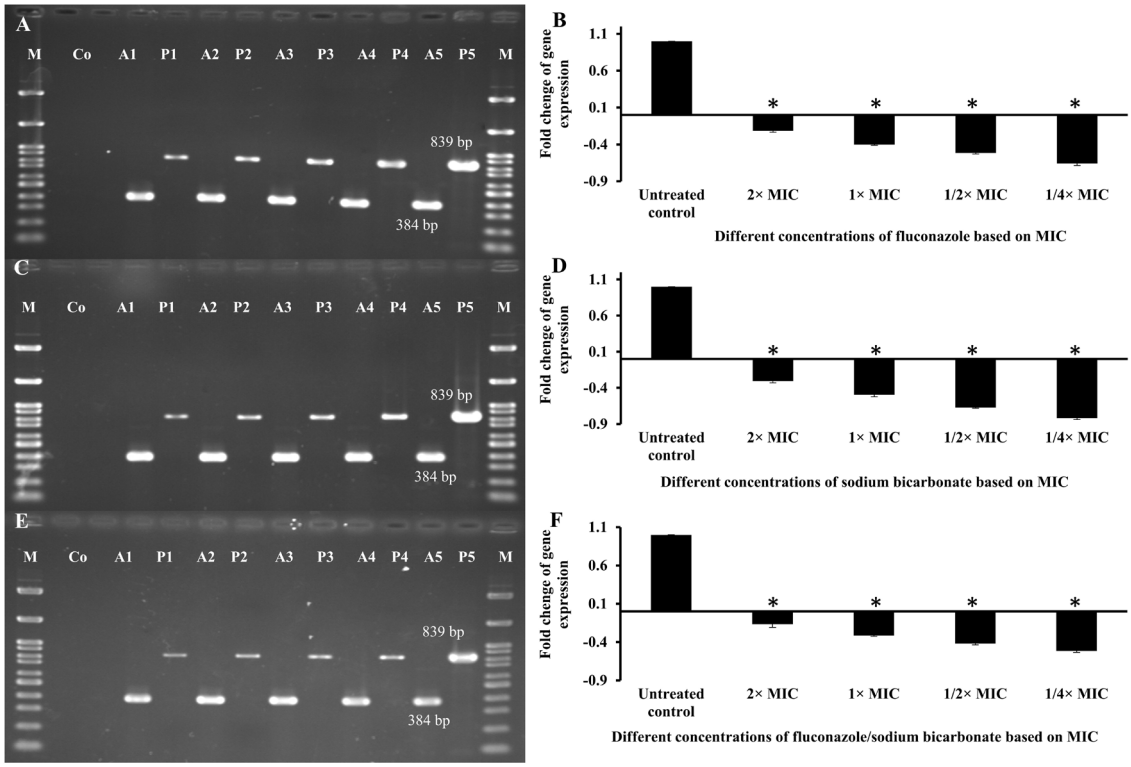

FIGURE 2 - Relative expression level of *PLB* gene at different concentrations of fluconazole (A, B), sodium bicarbonate (C, D), and fluconazole/sodium bicarbonate (E, F) based on MIC in *C. glabrata* ATCC 90030. Co: negative control for PCR, A1: Actin with 2× MIC concentration, P1: *PLB* with 2× MIC concentration, A2: Actin with 1× MIC concentration, P2: *PLB* with 1× MIC concentration, A3: Actin with ½× MIC concentration, P3: *PLB* with ½× MIC concentration, A4: Actin with ¼× MIC concentration, P4: *PLB* ¼× MIC concentration, A5: Actin in untreated control, P5: *PLB* in untreated control, and M: DNA molecular marker. (*) means a significant reduction of gene expression relative to untreated control at $p < 0.0001$. Data are means of fold changes with standard error from three independent experiments amplified in triplicates.

Azoles, more specifically triazoles, remain as the main options for the treatment of *Candida* infections (Pierce, Lopez-Ribot, 2013; Nami *et al*., 2019). Azole antifungal agents are cytochrome demethylase system inhibitors with antifungal activity against *Candida* species. Azoles are compounds that prevent the formation of ergosterol, which is a key regulator of membrane fluidity in fungal cell. Fluconazole is known to be less effective against *C. glabrata,* which developed many strategies to evade being destroyed by fluconazole (Chong *et al*., 2018; Madhavan *et al*., 2018). The interaction of fluconazole with sodium bicarbonate was investigated to achieve enhanced efficacy of fluconazole against *C. glabrata*.

The present study demonstrated the effectiveness of fluconazole/sodium bicarbonate combination against clinical isolates of *C. glabrata*. Fluconazole/sodium bicarbonate combination showed 66.67% synergism with clinical isolates of *C. glabrata*. For decades, sodium bicarbonate has wide medical and industrial applications (Letscher-Bru *et al*., 2013; Dobay *et al*., 2018). Sodium bicarbonate inhibits planktonic form of different bacteria and biofilm formation by *Pseudomonas aeruginosa*. Bacterial growth inhibition by sodium bicarbonate is triggered by intracellular cAMP production with pH responsiveness (Xie *et al*., 2010; Dobay *et al*., 2018). Few researchers have documented the antifungal properties of sodium bicarbonate against *Candida* species (Sousa *et al*., 2009; Letscher-Bru *et al*., 2013; Najafi *et al*., 2016). Synergistic interaction of baicalin/sodium bicarbonate in clinical isolates of *C. albicans* has been reported recently (Shao *et al*., 2019). It was observed that the MICs of baicalin and sodium bicarbonate alone were > 2048 µg/mL, and those of baicalin and sodium bicarbonate in combination decreased 16-32 folds with FICI in a range of 0.094-0.375.

Enhanced killing with the fluconazole/sodium bicarbonate combination was observed for ATCC 90030, SN 152, and SN 164 of clinical isolates of *C. glabrata*. Of particular interest was our finding of decreased killing with fluconazole/sodium bicarbonate combination for the SN 283 isolate. We believe that this distinction between the behaviour of the different clinical isolates of *C. glabrata* is the developed strategies to evade being destroyed by fluconazole (Madhavan *et al*., 2018).

This study demonstrated significant, concentration-dependent changes in the expression level of *PLB* after exposure to fluconazole/sodium bicarbonate combination. Our findings reveal that sodium bicarbonate might be able to down regulate the expression level of *PLB* alone and in combination with fluconazole. Exposure to fluconazole/sodium bicarbonate combination simultaneously could further down regulate the expression level of *PLB* significantly ($p < 0.05$). This may be explained by the sodium bicarbonate promoting the effects of fluconazole to fungicidal activity (Marchetti *et al*., 2000). In an important report, Li *et al*. (2016) concluded that *C. albicans* treated with fluconazole/ budesonide combination was able to down regulate the expression of phospholipase-related genes, PLB1-5 and *PLC1*. Similarly, Khodavandi *et al*. (2018) observed a down regulation of gene expression of *PLB* gene in *C. tropicalis* cells treated with fluconazole/ amphotericin B combination. Khodavandi *et al*. (2019) found that *LIP1* and *LIP4* genes in *C. tropicalis* were down regulated significantly by the fluconazole/clotrimazole combination.

CONCLUSION

Taken together, our observations demonstrated that sodium bicarbonate could be a candidate of synergism with fluconazole against clinical isolates of *C. glabrata*. The non-toxic nature of sodium bicarbonate along with the promising results further strengthens its candidature for continued future investigations. The potential for inhibition of *PLB* gene in *C. glabrata* suggests that the antifungal mechanism of fluconazole and sodium bicarbonate alone and in combination is deeply involved with the phospholipase gene.

ACKNOWLEDGEMENTS

The authors are thankful for financial support of this study by the Islamic Azad University of Yasuj. Results of the current study are part of an MSc thesis (10943455).

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Received for publication on 08th November 2019
Accepted for publication on 09th July 2020