**Objectives**: Novel antifungal treatment options are required for fungi with intrinsic and acquired azole resistance. A series of piperazine-bearing azole derivatives were synthesized and their *in vitro* antifungal activities were confirmed against reference *Candida* strains previously. In this study, their activities against a collection of clinical *Candida* isolates were tested to determine their species-based spectrum and degree of antifungal effect and to identify potential preclinical candidates for antifungal therapy. Additionally, cytotoxicity of the compounds was tested to evaluate their safety.

**Materials & Methods:** Four compounds were synthesized according to the methods validated in previously published reports and their structures and purity were confirmed using NMR and mass spectroscopy. Molecular docking was performed using Glide (Schrödinger LLC, NY) with the X-ray structure of *Candida albicans* CYP51 (PDB ID: 5TZ1) and molecular modeling was performed to predict potential inhibitory effects of the compounds against fungal ergosterol biosynthesis. Minimum inhibitory concentration (MIC, mg/L) values of the compounds were determined *in vitro* against 100 clinical isolates of various *Candida* species, ﻿using the reference microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST Definitive Document E.DEF. 7.4). Isolates with high fluconazole MICs were included in the study (Fluconazole MICs of 32->64 mg/L: *C. albicans* n=1, *Candida auris* n=10, *Candida glabrata* n=6, *Candida guilliermondii* n=2, *Candida krusei* n=8, *Candida parapsilosis* n=4; Fluconazole MICs of 4-16 mg/L: *C. albicans* n=3, *C. glabrata* n=2, *C. guilliermondii* n=6, *Candida inconspicua* n=2, *C. krusei* n=1, *C. parapsilosis* n=1). ﻿ Fluconazole was tested as a comparative control and the experiments were performed in triplicate. Cytotoxicity tests were performed using the MTT (﻿3‐(4,5‐dimethylthiazol‐2‐yl)−2,5‐diphenyltetrazolium bromide) assay.

**Results**: Molecular docking of the compounds predicted high-affinity binding to fungal CYP51 and strong electrostatic engagement with the iron of heme co-factor. Active site for Compound 4 was depicted in Figure 1.

The tested compounds showed highly potent *in vitro* anti-*Candida* activities as compared to fluconazole. Of note, low MICs could be obtained against species with reduced susceptibility/intrinsic resistance/acquired resistance for fluconazole, such as *C. auris, C. inconspicua* and *C. krusei*, remaining > 1 mg/L in general for *C. glabrata* (Table 1).

Compounds 1, 2, 3 and 4 geometric mean (GM) MICs for isolates with fluconazole MICs of ≥32 mg/L were 0.40 mg/L, 1.08 mg/L, 0.91 mg/L and 1.63 mg/L, respectively. For isolates with fluconazole MICs of 4-16 mg/L, GM MICs were 0.37, 0.55, 0.50 and 0.87, respectively.

Cytotoxicity tests revealed high viability for the cells treated with the compounds at their MIC ranges.

**Conclusions**: The tested azoles proved effective against various *Candida* species *in vitro*. Some derivatives were promising against species with reduced fluconazole susceptibility. Based on the cytotoxicity experiments, they appeared to be safe for host tissues at their effective concentrations. Their mechanism of action is believed to be the same as that of azole antifungals i.e. inhibition of CYP51 and thus biosynthesis of ergosterol. Further *in vitro* and *in vivo* assays are warranted to develop these derivatives as preclinical candidates.