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# Synergistic effect between taurine-induced silver ion and itraconazole against azole-resistant *Candida* species and *Candida auris*

Yu-Cin Deng [a], Chi-Jen Shih [b], Shang-Yi Lin [c,d,e], Liang-Chun Wang [f], Tsung-Ying Yang [a,g,h,**], Sung-Pin Tseng [a,f,i,j,*]

[a] Department of Medical Laboratory Science and Biotechnology, College of Health Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Division of Infectious Diseases, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[e] Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[f] Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan
[g] Department of Medical Laboratory and Regenerative Medicine, MacKay Medical College, New Taipei City, Taiwan
[h] Research Institute for Science and Engineering, Waseda University, Japan
[i] Graduate Institute of Animal Vaccine Technology, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan
[j] Center for Tropical Medicine and Infectious Disease Research, Kaohsiung Medical University, Kaohsiung, Taiwan

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ABSTRACT

*Background:* Azole antifungals are the first-line choice for treating candidiasis within a limited antifungal option. However, azole-resistant *Candida* species have increased rapidly, causing severe clinical threats, especially multidrug-resistant (MDR) isolates. The emergence of *Candida auris* has also caused global concerns recently.
*Methods:* Herein, we evaluated the antifungal activity of taurine-induced silver ions (Tau-Ag), prepared by the induction from silver-incorporated mesoporous bioactive glass to address this issue.
*Results:* Our data demonstrated that minimum inhibitory concentrations (MICs) of Tau-Ag ranged from 0.020 to 0.078 mg/mL in 24h and from 0.039 to 0.156 mg/mL in 48h. No hemolysis and cytotoxicity were observed at the MICs. Furthermore, no *in vivo* toxicity related to Tau-Ag was observed in a *Caenorhabditis elegans* model.
In the investigation of antifungal mechanisms, we observed that the reactive oxygen species (ROS) level significantly increased when *Candida* spp. treated with Tau-Ag. Biofilm formation inhibition assays found that Tau-Ag may penetrate the biofilm and eliminate biofilm-forming cells. In the time-kill method, Tau-Ag showed a long-lasting fungistatic effect and superior antifungal effect compared to itraconazole alone. Furthermore, Tau-Ag showed synergistic antifungal effects in combination with itraconazole, effectively restoring its activity.
*Conclusion:* Our results confirmed the potential of Tau-Ag and its combination use with itraconazole to serve as a novel antifungal agent to combat the plight of administration on azole-resistant and MDR *Candida* spp. and *C. auris*.

## 1. Introduction

*Candida* spp. has been documented as an opportunistic pathogen frequently causing superficial mucosal lesions or bloodstream infections[1,2] with a mortality rate of 63.6 %,[3] particularly affecting hospitalized patients and immunocompromised individuals.[4] Vaginal candidiasis is one of the most common fungal infections, which causes about 75 % of women to develop vulvovaginal candidiasis, and 40%–45 % suffer from chronic and recurrent infections.[5] *C. albicans* was the most frequently isolated *Candida* spp. from clinical specimens, but the prevalence of azole-resistant non-albicans *Candida* (NAC) spp. has arisen in recent years, particularly *C. tropicalis* and *C. glabrata*.[6,7] The emerging

* Corresponding author. Department of Medical Laboratory Science and Biotechnology, College of Health Sciences, Kaohsiung Medical University, 100, Shih-Chuan 1st Road, Kaohsiung, 80708, Taiwan.
** Corresponding author. Department of Medical Laboratory and Regenerative Medicine, MacKay Medical College, No.46, Sec. 3, Zhongzheng Rd., Sanzhi Dist., New Taipei City, 252005, Taiwan.
*E-mail addresses:* zegmayang@mmc.edu.tw (T.-Y. Yang), tsengsp@kmu.edu.tw (S.-P. Tseng).

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pathogen *C. auris* has recently caused severe global health threats and nosocomial outbreaks of invasive infections.[8,9]

Considerable research has focused on investigating the antibacterial properties of metals in the biomedical field. In this study, silver-containing mesoporous bioactive glass (MBG-Ag) was utilized as a source of silver ions, and 2 % taurine was employed as a matrix to induce the release of silver ions to prepare Tau-Ag. The synthesis of Tau-Ag provided a high concentration and purity of silver, with a silver content of 272.9 ppm.[10] Without toxic impurities, nearly 100 % cell viability was observed for Tau-Ag at concentrations of 0.08, 0.16, and 0.32 mg/mL, indicating its biocompatibility and feasibility for clinical application.[10]

Currently, only four classes of antifungal agents are available for treating candidiasis. Azoles are commonly the first-line treatment for severe or chronic oral candidiasis and chronic mucocutaneous candidiasis due to their effectiveness and lower toxicity.[11] Azole-resistant *Candida* spp. has increased and posed a threat to candidiasis treatment. To combat the rising threat, exploring combination therapies with existing antimicrobial agents is critical. Itraconazole exhibited a broad spectrum of antifungal activities and applications[12] and is widely used in treating superficial mycoses, subcutaneous, and vaginal candidiasis.[13] We evaluated Tau-Ag's efficacy and synergism with itraconazole to resolve the administration problem of azole-resistant *Candida* spp. and emerging pathogen *C. auris*.

## 2. Materials and methods

### 2.1. Collection of Candida spp.

A total of 32 clinical *Candida* spp. isolates and 1 standard strain were included in our study. 30 azole-resistant *Candida* spp. isolates from blood samples in Kaohsiung Medical University Chung-Ho Memorial Hospital (KMUH) were chosen for the experiments, including 8 *C. albicans*, 20 *C. tropicalis*, and 2 *C. glabrata*. 2 *C. auris* which were associated with the first cases of C. *auris* infection and candidemia in Taiwan were identified from Chi Mei Medical Center (CMMH) in 2018[14] and from KMUH in 2021,[15] separately. The standard strain ATCC 10231 *C. albicans*, which had previously been identified as a MDR strain in the report by the American Type Culture Collection, was selected as the control strain for this study.[16]

### 2.2. Preparation of Tau-Ag

The synthesis of Tau-Ag has been well-established in the previous study.[10] Mesoporous Bioactive Glass (MBG-Ag) was utilized as the source of silver ions, and taurine was employed as the matrix to induce the release of silver ions. In brief, 0.2 g of MBG-Ag powder was immersed with 10 mL of 2 % taurine solution at 160 rpm and 37 °C for 24 h, resulting in a concentration of 20 mg/mL (solid-liquid ratio) of matrix-induced silver ions. Subsequently, the solution was filtered using a 0.22 μm filter to eliminate any solid material. The silver ions induced by the taurine solution were designated as Tau-Ag.

### 2.3. Antifungal susceptibility testing

MIC values were determined using the broth microdilution method according to the CLSI M27-A2 guideline. Interpretations of antimicrobial susceptibility for *C. albicans*, *C. tropicalis*, and *C. glabrata* were based on CLSI guideline M27M44s. For *C. auris*, we adopted the recent tentative breakpoints proposed by the U.S. Centers for Disease Control and Prevention (CDC)[17] since the CLSI guidelines did not provide established MIC breakpoints. Epidemiological cutoff values (ECVs) for the susceptibility testing of *Candida* spp. with no breakpoints were also introduced according to CLSI guideline M57S. Only azoles and echinocandins have MIC breakpoints provided by the CLSI guideline. MDR was defined as an isolate resistant to ≥1 agent in ≥2 drug class according to a previous study.[18] The standard strain *C. albicans* ATCC 10231 has been documented to be resistant to anidulafungin, fluconazole, itraconazole, and voriconazole.[16]

### 2.4. Time-kill method

Fungal suspensions were diluted to approximately $10^4$ CFU/mL, and the antifungal solutions were prepared at concentrations that matched the MIC multiples. Fungal suspensions and antifungal solutions in single or combination were subsequently combined in 96-well microplates. The samples were well-pipetted and collected at specific time points, serially diluted, and plated onto Sabouraud Dextrose Agar (SDA). Colony counts were determined after 24 h of incubation, and time-kill curves were constructed. Synergy was defined as a combination treatment resulting in a ≥ 2-log10 decrease in colony count after 24 h compared to the most active single agent, while an additive effect was indicated by a < 2-log10 but > 1-log10 decrease.[19]

### 2.5. Reactive oxygen species levels measurement

Cellular levels of ROS were analyzed using 2′, 7′-dichloro-dihydro fluorescein diacetate (DCFH-DA) as previously described with some modifications.[20] Fungal suspensions were pre-incubated with 100 μM of DCFH-DA in RPMI 1640 medium for 2 h at 35 °C. Subsequently, the DCFH-DA-treated cells were washed with phosphate-buffered saline (PBS) and resuspended to a density of 0.5 McFarland. The cells were then mixed with Tau-Ag solution in a microplate and incubated for 4 h. Fluorescence intensities were measured using a spectrofluorometric reader at excitation and emission wavelengths of 480 and 530 nm, respectively. The obtained results were normalized using viable colony counts in suspensions.

### 2.6. Biofilm cell viability assay

The viability of biofilm cells was obtained using the MBEC Assay® system as previously described with little modifications.[21,22] The overnight fungal cultures were adjusted to $10^5$ CFU/mL, and 150 μL of the fungal suspension was transferred into a biofilm inoculator [Biofilm Inoculator with 96 Well Base & Cellulose Coated Pegs, MBEC Assay®, Innovotech Inc., Canada.]. The inoculated plates were incubated at 35 °C with 110 rpm for 24 h. Subsequently, the peg lid was rinsed with PBS and transferred to a 96-well plate containing 200 μL of antifungal in each well, followed by a 24-h incubation at 35 °C. 45 min of water sonication were conducted to recover fungal biofilm. The recovered suspension was serially diluted and plated onto SDA plates, with fungal cell counts determined after 24 h of incubation.

### 2.7. Biofilm formation assay

The procedure for biofilm formation and antifungal treatment followed that of the viability assay. The peg lid was rinsed with PBS and transferred to a 96-well plate with 0.05 % crystal violet for 30 min. Following two PBS rinses, the peg lid was immersed in a 96-well plate containing ethanol for 15 min. The suspension was serially diluted, and the biofilm biomass was quantified ($OD_{595}$) using a microplate spectrophotometer.

### 2.8. Hemolysis assay

The hemolytic activity of Tau-Ag was determined with some modifications according to a previous study.[23] The erythrocytes were washed twice with PBS, suspended in 5 % glucose solution to obtain 2 % hematocrit, and treated with 100 μL Tau-Ag solutions. Samples were incubated at 37 °C, 120 rpm for 1 h, and then centrifuged at 700 g for 10 min. The supernatant was transferred to a microplate to measure the optical density of released hemoglobin at 540 nm. The percentage of

hemolysis was calculated as follows: Hemolysis (%) = (OD of test sample – OD of negative control)/(OD of positive control-OD of negative control) × 100 %

### 2.9. Checkerboard microdilution assay

Checkerboard microdilution assays were performed for further analysis of the synergistic efficiency of Tau-Ag in combination with an antifungal agent. The fractional inhibitory concentration indices (FICIs) were calculated by the following equation: FICI = $FIC_{Tau\text{-}Ag}$ + $FIC_{Antifungal}$ = Tau-Ag in combo/Tau-Ag MIC + Antifungal in combo/Antifungal MIC. Tau-Ag in combo and Antifungal in combo represented the concentration of Tau-Ag and itraconazole when used in combination in a specific well, respectively. Tau-Ag MIC and Antifungal MIC represented the MICs of Tau-Ag and the antifungal agent when used alone. The numerators corresponded to the concentrations of the drugs in combination in all wells corresponding to an MIC. The FICIs were calculated as mentioned to evaluate the interaction effects. FICIs ≤ 0.5 were classified as synergistic, 0.5 < FICI ≤ 1 as additive, 1 < FICI ≤ 4 as indifferent, and FICI > 4 as antagonistic.[24,25]

### 2.10. In vivo toxicity study

Toxicity of Tau-Ag on nematode was performed utilizing L4-stage *Caenorhabditis elegans* (*C. elegans*) strain N2 worms for *in vivo* studies. The survival assay was performed as described previously.[26] Briefly, *Escherichia coli* OP50 was initially cultured overnight on nematode growth medium (NGM) agar plates supplemented with Tau-Ag. Subsequently, 40 nematodes were plated on each plate and the alive and dead worms were scored every 24 h, while the surviving ones were transferred to new plates under the same conditions. The viability percentage of survival *C. elegans* was then calculated.

## 3. Results

### 3.1. Antifungal susceptibility testing

In this study, MICs of 9 clinically used antifungal agents were determined for 30 azole-resistant *Candida* spp. including 3 multidrug-resistant isolates, and 2 *C. auris* clinical isolates. The results (Table 1) illustrated that 91 % of the clinical isolates that met the MIC breakpoint criteria were resistant to fluconazole (29/32) and 93 % of the isolates

**Table 1**
Antifungal susceptibility testing of 9 antifungals for 32 *Candida* spp.

| | | MIC[a] (mg/L) | | | | | | | | | MIC[b] (mg/mL) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antifungals | | VOC[c] | IZ[e] | POS[e] | FZ[c] | 5-FC | AMB | AND | MCF | CAS | Tau-Ag | |
| *Spp.* | No. | | | | | | | | | | 24h | 48h |
| ***C. albicans*** | 103–116 | **1 (R)** | >16 | **>8 (NWT)** | 4 | <0.06 | 1 | 0.12* | 0.03 | 0.25 | 0.039 | 0.078 |
| | 105–8 | **>8 (R)** | >16 | **>16 (NWT)** | **>256 (R)** | <0.06 | 0.5 | 0.125 | <0.008 | 0.125 | 0.078 | 0.156 |
| | 105–41 | **>8 (R)** | >16 | **>8 (NWT)** | **256 (R)** | <0.06 | 1 | 0.06 | 0.015 | 0.12 | 0.039 | 0.078 |
| | 107–40 | **>8 (R)** | >16 | **8 (NWT)** | **>256 (R)** | 0.125 | 1 | 0.031 | 0.016 | 0.031 | 0.039 | 0.078 |
| | 107–43 | 0.5 | >16 | **2 (NWT)** | **16 (R)** | 0.12 | 1 | 0.06 | 0.015 | 0.03 | 0.039 | 0.078 |
| | 107–105[c] | **>8 (R)** | >16 | 0.06 | **>256 (R)** | 0.12 | 0.5 | 0.12 | 0.06 | **4 (R)** | 0.020 | 0.039 |
| | 108–14 | **>8 (R)** | >16 | **>8 (NWT)** | **128 (R)** | 0.12 | 0.5 | 0.12 | <0.008 | 0.12 | 0.039 | 0.078 |
| | 108–19 | **>8 (R)** | >16 | **>8 (NWT)** | **32 (R)** | 0.25 | 1 | 0.12 | 0.015 | 0.25 | 0.039 | 0.078 |
| | ATCC 10231 | nd | nd | nd | nd | nd | nd | nd | nd | nd | 0.039 | 0.078 |
| ***C. tropicalis*** | 103–8 | **8 (R)** | **2 (NWT)** | **1 (NWT)** | **128 (R)** | <0.06 | 1 | 0.25 | 0.06 | 0.03 | 0.020 | 0.039 |
| | 103–33 | **1 (R)** | **>64 (NWT)** | **1 (NWT)** | **64 (R)** | >64 | 1 | 0.25 | 0.03 | 0.25 | 0.020 | 0.039 |
| | 103–76 | **0.5 (R)** | **>16 (NWT)** | **1 (NWT)** | **32 (R)** | 0.12 | 0.5 | 0.25 | 0.06 | 0.5 | 0.020 | 0.039 |
| | 103–77 | **8 (R)** | **>16 (NWT)** | **>8 (NWT)** | **32 (R)** | 0.25 | 1 | 0.25 | 0.06 | 0.25 | 0.020 | 0.039 |
| | 103–81 | **1 (R)** | **>16 (NWT)** | **1 (NWT)** | **64 (R)** | 0.25 | 0.5 | 0.25 | 0.03 | 0.25 | 0.020 | 0.039 |
| | 103–89 | **2 (R)** | **>16 (NWT)** | **1 (NWT)** | **64 (R)** | 0.12 | 0.5 | 0.25 | 0.03 | 0.25 | 0.020 | 0.039 |
| | 103–97 | **4 (R)** | **4 (NWT)** | **1 (NWT)** | **64 (R)** | 0.06 | 1 | 0.25 | 0.03 | 0.12 | 0.020 | 0.039 |
| | 103–105[c] | **>8 (R)** | **8 (NWT)** | **1 (NWT)** | **>256 (R)** | <0.06 | 1 | **1 (R)** | 0.12 | 0.25 | 0.020 | 0.039 |
| | 103–115 | 0.5 | **1 (NWT)** | **1 (NWT)** | **16 (R)** | 0.06 | 1 | 0.12 | 0.06 | 0.25 | 0.020 | 0.039 |
| | 103–118 | **>8 (R)** | **>16 (NWT)** | **1 (NWT)** | **8 (R)** | 0.12 | 1 | 0.12 | 0.06 | 0.25 | 0.020 | 0.078 |
| | 105–14 | **>8 (R)** | **>16 (NWT)** | **4 (NWT)** | **8 (R)** | 0.12 | 1 | 0.12 | 0.03 | 0.25 | 0.039 | 0.078 |
| | 105–44 | **8 (R)** | **1 (NWT)** | **1 (NWT)** | **256 (R)** | <0.06 | 1 | 0.25 | 0.03 | 0.25 | 0.039 | 0.078 |
| | 105–59 | **4 (R)** | **>16 (NWT)** | **1 (NWT)** | **8 (R)** | <0.06 | 1 | 0.12 | 0.03 | 0.12 | 0.039 | 0.078 |
| | 106–6 | **>8 (R)** | **1 (NWT)** | **0.5 (NWT)** | **64 (R)** | >64 | 1 | 0.03 | 0.03 | 0.03 | 0.039 | 0.078 |
| | 106–13 | **>8 (R)** | **1 (NWT)** | **0.12 (NWT)** | **>256 (R)** | 0.25 | 1 | 0.063 | 0.016 | 0.016 | 0.039 | 0.078 |
| | 106–14 | **>8 (R)** | **>16 (NWT)** | **1 (NWT)** | **16 (R)** | 0.06 | 1 | 0.25 | 0.03 | 0.06 | 0.039 | 0.078 |
| | 106–41 | **>8 (R)** | **>16 (NWT)** | **>8 (NWT)** | **64 (R)** | <0.06 | 1 | 0.25 | 0.06 | 0.06 | 0.039 | 0.078 |
| | 106–48 | **1 (R)** | **>16 (NWT)** | **1 (NWT)** | **8 (R)** | 0.12 | 1 | 0.12 | 0.06 | 0.12 | 0.039 | 0.078 |
| | 107–15 | **>8 (R)** | **>16 (NWT)** | **>8 (NWT)** | **256 (R)** | <0.06 | 1 | 0.12 | 0.03 | 0.25 | 0.078 | 0.156 |
| | 107–50 | **1 (R)** | **>16 (NWT)** | **1 (NWT)** | **64 (R)** | 0.06 | 1 | 0.12 | 0.03 | 0.25 | 0.039 | 0.078 |
| ***C. glabrata*** | 105–54[c] | **2 (NWT)** | **>16 (NWT)** | **>8 (NWT)** | **64 (R)** | <0.06 | 1 | **0.5 (R)** | 0.12 | **1 (R)** | 0.078 | 0.156 |
| | 107–5 | **4 (NWT)** | **>16 (NWT)** | **>8 (NWT)** | **128 (R)** | <0.06 | 1 | 0.03 | 0.015 | 0.03 | 0.039 | 0.078 |
| ***C. auris*** | **KMUH** | 0.12[d] | 0.125 | 0.12[d] | 8[d] | ≤0.06[d] | 1[d] | 0.12[d] | 0.06[d] | N/P n[d] | 0.039 | 0.078 |
| | **CMMH** | 0.25[d] | 0.25 | 0.25[d] | 16[d] | ≤0.06[e] | 0.5[e] | 0.5–1[e] | 0.25–0.5[e] | N/P nd | 0.078 | 0.156 |

**Abbreviations:** VOC, voriconazole; IZ, itraconazole; POS, posaconazole; FZ, fluconazole; 5-FC, flucytosine; AMB, amphotericin B; AND, anidulafungin; MCF, micafungin; CAS, caspofungin; R, resistant; NWT, non-wild-type; N/P, non-performed. nd, non-detected.

**Notes.**

[a] MIC determined after 24 h of incubation.

[b] MIC determined at 24 and 48 h.

[c] multidrug resistant.

[d] adapted from the reference of[23].

[e] adapted from the reference of[23] and.[22]

All experiments were conducted in triplicate.

were resistant to voriconazole (26/28). ECVs for *in vitro* susceptibility testing of *Candida* spp. with no breakpoints were introduced. Non-wild-type was defined as the MIC of the isolate greater than the ECV. The results showed 100 % of isolates were determined to be itraconazole non-wild-type (22/22) while 97 % of the isolates were posaconazole non-wild-type (29/30), and all the *C. glabrata* (2/2) were voriconazole non-wild-type. The MICs of Tau-Ag were recorded at 24 h and 48 h, ranging from 0.020 to 0.078 mg/mL (solid-liquid ratio) at 24 h and from 0.039 to 0.156 mg/mL at 48 h. Specifically, The MICs for Tau-Ag against MDR *C. albicans* ATCC 10231 were also conducted, measuring 0.039 mg/mL at 24 h and 0.078 mg/mL at 48 h. The results underscored the potential of Tau-Ag as a promising antifungal agent, showing efficacy against MDR isolates, azole-resistant isolates, and the emerging pathogen *C. auris*.

### 3.2. Time-kill curves

Time-kill assays were conducted on four *Candida* spp. (Fig. 1), including MDR *C. albicans* ATCC 10231 (Fig. 1A), MDR *C. glabrata* 105-54 (Fig. 1B), *C. tropicalis* 106-6 (Fig. 1C) and *C. auris* KMUH (Fig. 1D). Tau-Ag exhibited antifungal activity against all *Candida* spp. within 2 h at concentrations corresponding to 1, 2, and 4 times the MIC. Fungal cell counts were significantly reduced in the Tau-Ag treated groups compared to the control group after 8 h. This visible reduction continued from 8 to 12 h and showed a significant fungistatic effect at 24 h. Minimal or no regrowth of fungal cells was observed at 48 h, with no fungal cells detected at Tau-Ag concentrations of 0.156 and 0.313 mg/mL. Furthermore, time-kill curves of itraconazole against the four *Candida* spp. were also included to compare the antifungal effect. Our results indicated that Tau-Ag at the MIC demonstrated superior fungistatic activity after 4 h compared to itraconazole at the MIC or the highest final concentration of itraconazole. Unlike Tau-Ag-treated groups, the itraconazole-treated groups showed an increase in cell counts after 24 h, nearly reaching the levels of the control group at 48 h. These findings strongly support the potential of Tau-Ag as an effective and long-lasting antifungal agent.

### 3.3. ROS levels

We investigated the potential antifungal activity of Tau-Ag and the combination with itraconazole by analyzing ROS levels in the 4 *Candida* spp. included in the time-kill assay based on the previous study with some modifications.[20] Briefly, these *Candida* spp. were treated with Tau-Ag concentration corresponding to 1, 2, and 4 times the 24-h MIC and also treated with itraconazole or the combination of 1xMIC Tau-Ag and itraconazole for 4 h based on the results from the time-kill curves (Fig. 1). Fig. 2 indicated ROS generation in all *Candida* spp. when treated with Tau-Ag, ROS levels increased significantly ($p < 0.01$) in the Tau-Ag treated groups compared to the control groups. Additionally, we observed and a dose-dependent effect of ROS generation under Tau-Ag treatment. Additionally, the ROS levels under the combination treatment of 1×MIC Tau-Ag and itraconazole increased significantly ($p < 0.001$) compared to the control groups and also showed significant increases compared to the single antifungal treatments ($p < 0.0001$ compared to the itraconazole group; $p < 0.05$ compared to the 1×MIC Tau-Ag group) These findings provided insights into the antifungal activity of Tau-Ag and its underlying mechanism against azole-resistant *Candida* spp.

### 3.4. Inhibition of biofilm formation

Two methods were employed to evaluate the efficacy of Tau-Ag in inhibiting biofilm formation. The biofilm cell viability test evaluated its ability to eradicate biofilm-forming cells, while the crystal violet method

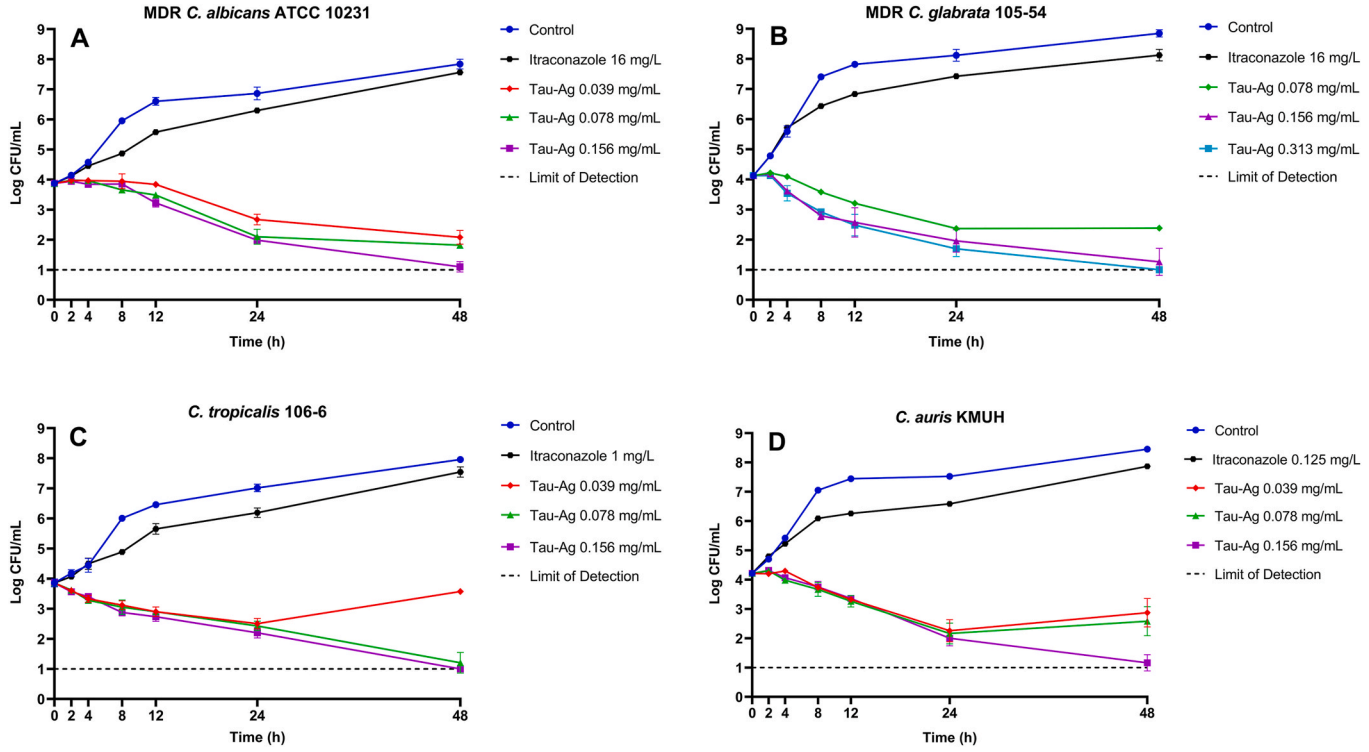


**Fig. 1.** Time-kill curves of Tau-Ag and itraconazole against *Candida* spp. Time-kill curves of MDR *C. albicans* ATCC 10231 (A), MDR *C. glabrata* 105-54 (B), *C. tropicalis* 106-6 (C), and *C. auris* KMUH (D) were determined. The Tau-Ag concentration corresponded to 1, 2, and 4 times the MICs were 0.039, 0.078, and 0.156 mg/mL for (A), (C), (D), and 0.078, 0.156, and 0.313 mg/mL for (B). The itraconazole concentrations were 16 mg/L for (A) and (B), 1 mg/L for (C), and 0.125 mg/L for (D). Cell counts were determined at specific time intervals (0, 2, 4, 8, 12, 24, and 48 h). Dashed horizontal lines (—) represent the limit of detection (LOD) for the assay. The experiments were repeated in triplicate.

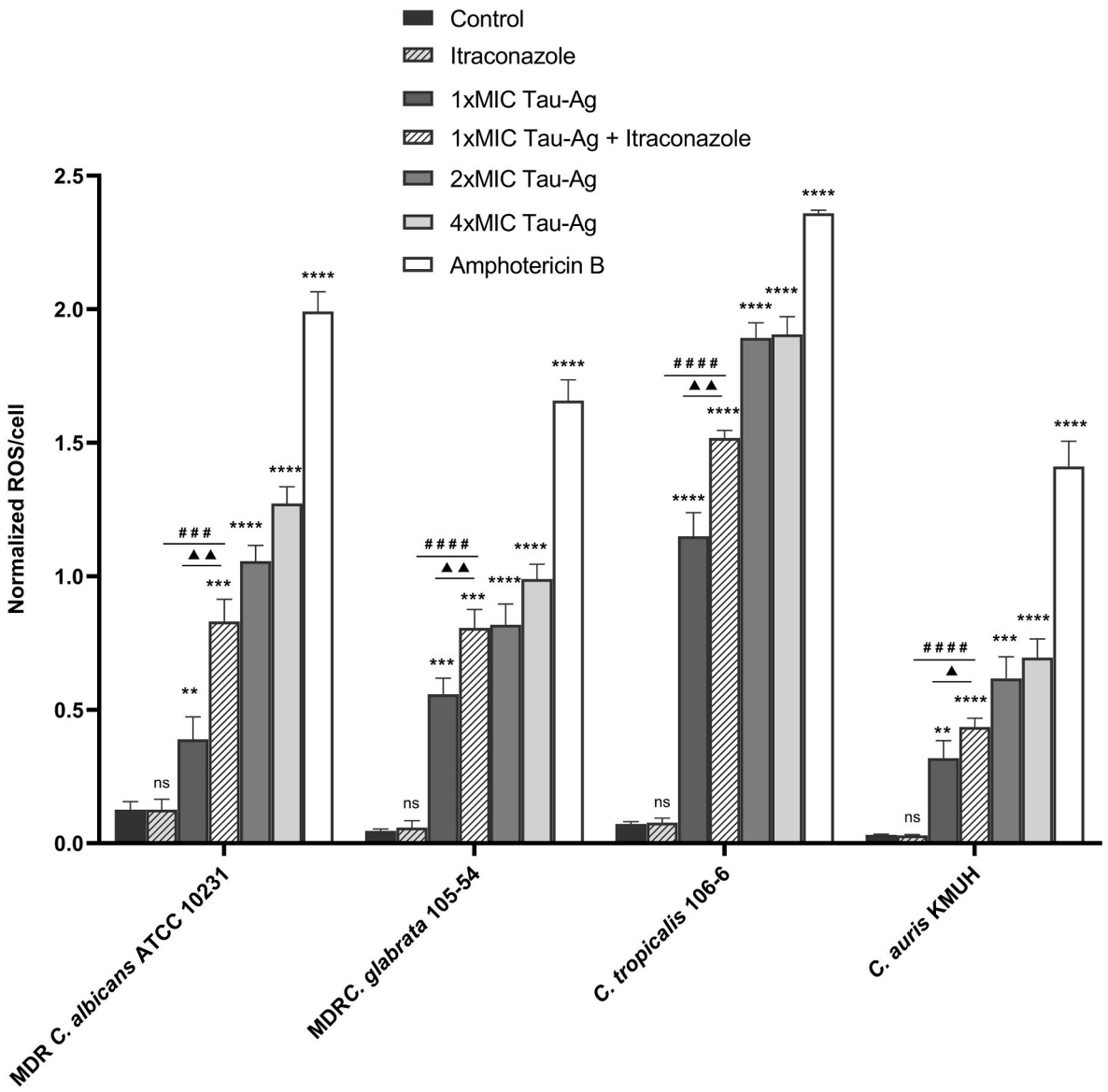


**Fig. 2.** Effect of Tau-Ag on ROS generation in *Candida* spp. The effect of Tau-Ag on ROS generation in MDR *C. albicans* ATCC 10231, MDR *C. glabrata* 105-54, *C. tropicalis* 106-6, and *C. auris* KMUH were determined. Tau-Ag was administered at 1,2,4 times the 24-h MIC concentrations of (0.039, 0.078, and 0.156 mg/mL for MDR *C. albicans* ATCC 10231, *C. tropicalis* 106-6, and *C. auris* KMUH, and 0.078, 0.156, 0.313 mg/mL for MDR *C. glabrata* 105-54), representing 1, 2, and 4 times the 24-h MIC, respectively. Itraconazole at the concentration of 16 mg/L for *C. albicans* and *C. glabrata*; 1 mg/L for *C. tropicalis*; 0.125 mg/L for *C. auris* was treated and the combination treatment of 1xMIC Tau-Ag and Itraconazole utilized the same concentration to the Itraconazole group. Amphotericin B at the concentration of 0.125 μg/mL against the four *Candida* spp. was utilized for positive control. The experiments were repeated in triplicate. The ROS levels were analyzed by an independent *t*-test using GraphPad Prism 8.0.1 Software, with ns (non-significant); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$) indicating statistical significance compared to the control group. # # # ($p < 0.001$); # # # # ($p < 0.0001$) compared to the 1xMIC Tau-Ag group; ▲ ($p < 0.05$); ▲▲ ($p < 0.01$) compared to the Itraconazole group.

assessed Tau-Ag's capacity to disrupt the extracellular matrix. Fig. 3A demonstrated a notable reduction in MDR *C. albicans* ATCC 10231 biofilm-forming cell count with Tau-Ag at concentrations from 0.039 ($p < 0.001$) to 1.25 mg/mL ($p < 0.0001$), showing superior antibiofilm activity compared to itraconazole. Similar trends were observed for *C. tropicalis* 106-6 (Fig. 3B). However, the crystal violet method showed less significant differences between Tau-Ag-treated and untreated groups for both isolates (Fig. 3C and D). Absorbance values only differed significantly at Tau-Ag concentrations of 0.625 mg/mL ($p < 0.05$) and 1.25 mg/mL ($p < 0.01$) for MDR *C. albicans* ATCC 10231, and at 0.313, 0.625 mg/mL ($p < 0.05$), and 1.25 mg/mL ($p < 0.01$) for *C. tropicalis* 106-6. This suggests that Tau-Ag may not directly disrupt the extracellular matrix, so we inferred that Tau-Ag exerted its antibiofilm effects by penetrating the biofilm and eliminating biofilm-forming cells.

### 3.5. Cytotoxicity of Tau-Ag on erythrocytes

Hemolytic assays were conducted to assess the potential toxicological effects of Tau-Ag on erythrocytes. Tau-Ag was tested at concentrations ranging from 0.020 to 20 mg/mL. Fig. 4 showed no hemoglobin release at concentrations of 0.020, 0.039, 0.078, and 0.156 mg/mL. Furthermore, Tau-Ag resulted in 80 % non-hemolyzed red blood cells ($CC_{80}$) at a concentration of 5 mg/mL, 32–250 times higher than the 24-h MICs (0.02–0.078 mg/mL). Nearly 100 % cell viabilities were noticed for Tau-Ag in concentrations of 0.08, 0.16, and 0.32 mg/mL as previously determined.[10] These findings provided essential insights into the safety of Tau-Ag regarding erythrocyte integrity and further supported its potential as a safe and effective antimicrobial agent.

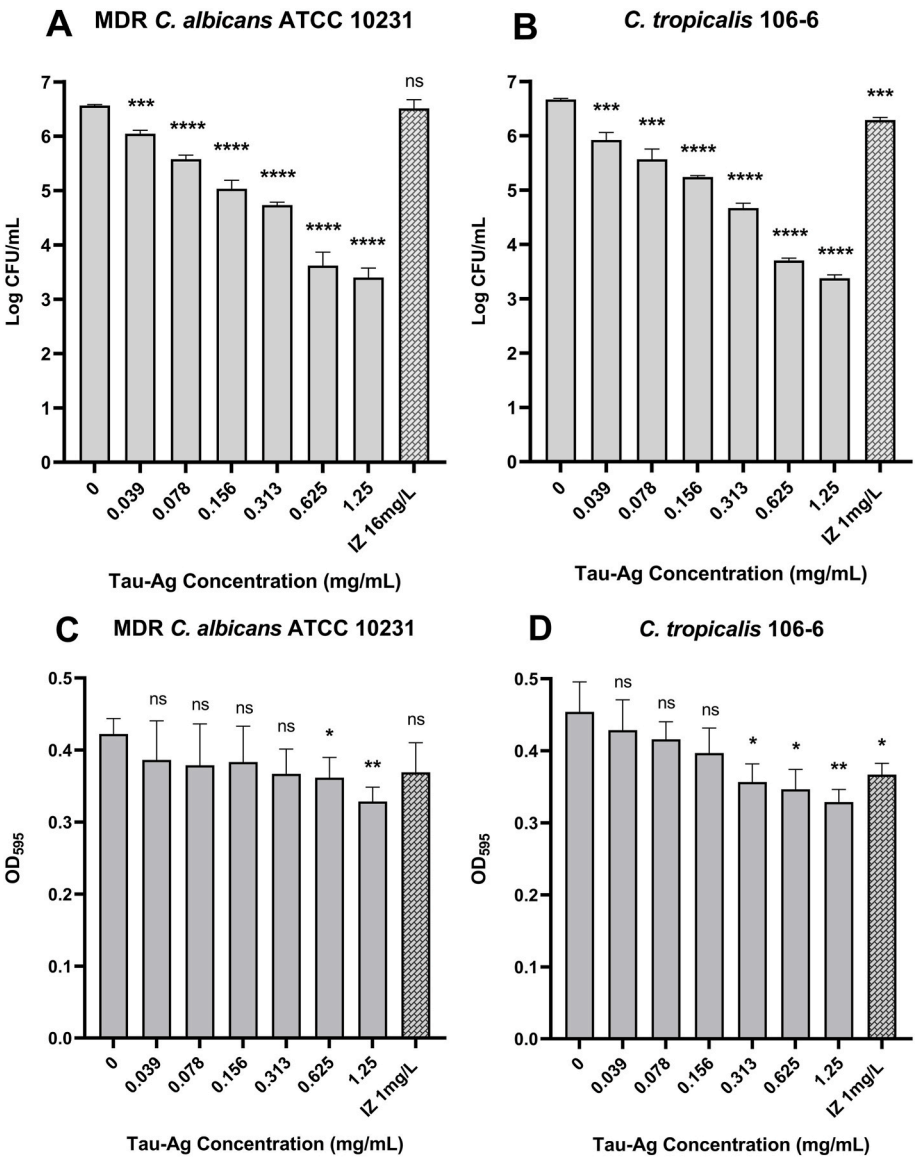


**Fig. 3.** Biofilm inhibition of Tau-Ag by cell viability assay (A), (B) and crystal violet assay (C), (D) against *Candida* spp. Inhibition of biofilm formation by Tau-Ag and itraconazole (antifungal agent control) against MDR *C. albicans* ATCC 10231 and *C. tropicalis* 106-6 was determined. Tau-Ag concentrations ranging from 0.039 to 1.25 mg/mL, corresponding to 0, 1, 2, 4, 8, 16, and 32 times the 24-h MIC, were utilized for evaluation. An independent sample *t*-test was used to indicate statistical significance, with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$), and ns (non-significance). Experiments were conducted in triplicate, and absorbance values were analyzed using GraphPad Prism 8.0.1 Software.

### 3.6. Toxicity of Tau-Ag in Caenorhabditis elegans

*C. elegans* models were employed for *in vivo* studies. No statistically significant difference in *C. elegans* survival rates was observed between the Tau-Ag-treated groups (at 1, 2, and 4 times the 24-h MIC against MDR *C. albicans* ATCC 10231) and the untreated group over the 14-day observation period, indicating the absence of toxicity towards the nematodes (Fig. 6). This data indicated the treatment with Tau-Ag could be potentially safety for further application.

### 3.7. Synergistic effect of Tau-Ag in combination with itraconazole

Tau-Ag exhibited a synergistic antifungal effect with an overall FICI of 0.25 (Table 2) when combined with itraconazole in 58 % (19/33) of the tested isolates while showing an additive effect in 42 % (14/33) of the tested isolates. Furthermore, the combination use of Tau-Ag and itraconazole overcame antifungal resistance in 63.6 % (14/22) of itraconazole non-wild-type isolates. The time-kill curve of Tau-Ag in combination with itraconazole against MDR *C. albicans* ATCC 10231 was also analyzed (Fig. 5). The fungal cell counts of the group treated with the combination of Tau-Ag (0.2 mg/mL) and itraconazole (0.5 mg/L) decreased by approximately 2-log10 within 24 h compared to the single-drug treated groups, indicating a synergistic effect of Tau-Ag with itraconazole and resulted in a more than 32-fold decrease in the itraconazole concentration required for inhibiting fungal cell growth from > 16 mg/L to 0.5 mg/L.

## 4. Discussion

Much research has focused on investigating the antimicrobial activities exhibited by silver nanoparticles (AgNPs).[27,28] However, AgNPs showed instability in solutions, which tend to aggregate, causing the loss of antimicrobial efficacy and treatment failure, with adverse reactions like vascular thrombosis.[29] The toxicity of silver has also been a prominent concern. Most of the studies utilized silver nitrate as the primary source of silver through chemical reduction methods to produce AgNP,

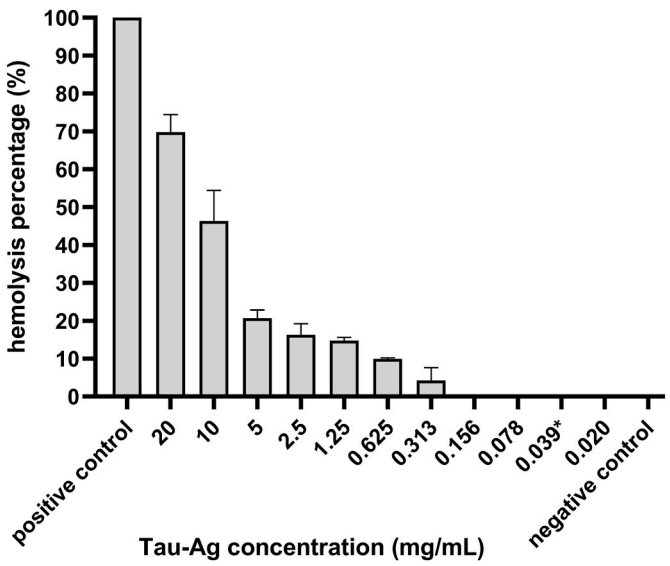


**Fig. 4.** Hemolytic activity of Tau-Ag. Erythrocytes were treated with Tau-Ag solutions at concentrations ranging from 0.020 to 20 mg/mL in 5 % glucose solution. * MIC of Tau-Ag for *Candida* spp. (0.039 mg/mL). Positive and negative controls were conducted using 0.1 mg/mL Triton X-100 solution [0.01 % (vol/vol) Triton X-100] and Tau-Ag-free 5 % glucose solution, respectively. The experiments were repeated in triplicate.

with the toxic impurity of the nitrate group involved causing damage to human health and the environment.[29–34] Green synthesis of AgNPs has become popular recently to prevent the generation of toxic impurities in silver products. Still, biosynthesis is hard to control and possesses a lower yield of silver.[35] Inspired by these studies, we selected different silver sources and synthesis methods to address the problems. Therefore, we utilized taurine which can prevent inflammation and slow down oxidative stress-mediated injuries[36] as the matrix to induce silver ions from silver-incorporated bioactive glass. Tau-Ag demonstrated a high

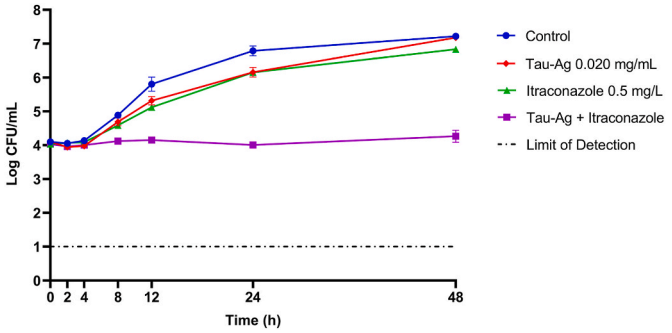


**Fig. 5.** Time-kill curves of Tau-Ag in combination with itraconazole against MDR *C. albicans* ATCC 10231. MDR *C. albicans* ATCC 10231 was selected to evaluate the antifungal efficacy of Tau-Ag combined with itraconazole. Tau-Ag at 0.020 mg/mL (0.5 times the 24-h MIC) and itraconazole at 0.5 mg/L (0.5 times the MIC breakpoint) were used. Dashed horizontal line (—) represents the limit of detection (LOD) for the assay. The experiments were repeated in triplicate.

**Table 2**
FICIs of Tau-Ag in combination with itraconazole and their interaction classifications against the *Candida* spp.

| *Spp.* | No. | Tau-Ag MIC (mg/mL) | | IZ MIC (mg/L) | | FICI | Interpretation |
|---|---|---|---|---|---|---|---|
| | | Alone | Combo | Alone | Combo | | |
| ***C. albicans*** | 103–116 | 0.039 | 0.020 | >16 | 0.5 | 0.531 | ADD |
| | 105–8 | 0.078 | 0.010 | >16 | 0.016 | 0.126 | **SYN** |
| | 105–41 | 0.039 | 0.010 | >16 | 0.25 | 0.266 | **SYN** |
| | 107–40 | 0.039 | 0.020 | >16 | 0.016 | 0.501 | ADD |
| | 107–43 | 0.039 | 0.010 | >16 | 0.125 | 0.258 | **SYN** |
| | 107–105 | 0.020 | 0.005 | >16 | 0.5 | 0.281 | **SYN** |
| | 108–14 | 0.039 | 0.005 | >16 | 0.5 | 0.156 | **SYN** |
| | 108–19 | 0.039 | 0.005 | >16 | 0.5 | 0.156 | **SYN** |
| | ATCC 10231 | 0.039 | 0.010 | >16 | 0.5 | 0.281 | **SYN** |
| ***C. tropicalis*** | 103–8 | 0.039 | 0.002 | 2 | 0.25 | 0.625 | ADD |
| | 103–33 | 0.020 | 0.010 | >16 | 0.125 | 0.516 | ADD |
| | 103–76 | 0.020 | 0.010 | >16 | 0.25 | 0.516 | ADD |
| | 103–77 | 0.020 | 0.010 | >16 | 0.25 | 0.516 | ADD |
| | 103–81 | 0.020 | 0.010 | >16 | 0.25 | 0.516 | ADD |
| | 103–89 | 0.020 | 0.005 | >16 | 1 | 0.313 | **SYN** |
| | 103–97 | 0.020 | 0.002 | 4 | 1 | 0.375 | **SYN** |
| | 103–105 | 0.020 | 0.002 | 8 | 1 | 0.25 | **SYN** |
| | 103–115 | 0.020 | 0.005 | 1 | 0.25 | 0.5 | ADD |
| | 103–118 | 0.020 | 0.01 | >16 | 0.5 | 0.531 | ADD |
| | 105–14 | 0.039 | 0.020 | >16 | 0.5 | 0.531 | ADD |
| | 105–44 | 0.039 | 0.020 | 1 | 0.25 | 0.75 | ADD |
| | 105–59 | 0.039 | 0.010 | >16 | 0.125 | 0.258 | **SYN** |
| | 106–6 | 0.039 | 0.005 | 1 | 0.125 | 0.25 | **SYN** |
| | 106–13 | 0.039 | 0.020 | 1 | 0.016 | 0.516 | ADD |
| | 106–14 | 0.039 | 0.010 | >16 | 0.125 | 0.258 | **SYN** |
| | 106–41 | 0.039 | 0.020 | >16 | 0.125 | 0.516 | ADD |
| | 106–48 | 0.039 | 0.010 | >16 | 1 | 0.313 | **SYN** |
| | 107–15 | 0.078 | 0.020 | >16 | 0.5 | 0.281 | **SYN** |
| | 107–50 | 0.039 | 0.020 | >16 | 0.25 | 0.516 | ADD |
| ***C. glabrata*** | 105–54 | 0.078 | 0.002 | >16 | 0.5 | 0.057 | **SYN** |
| | 107–5 | 0.039 | 0.002 | >16 | 2 | 0.188 | **SYN** |
| ***C. auris*** | KMUH | 0.039 | 0.005 | 0.125 | 0.016 | 0.25 | **SYN** |
| | CMMH | 0.078 | 0.010 | 0.25 | 0.016 | 0.188 | **SYN** |

**Abbreviations:** SYN, synergistic; ADD, additive; IZ, itraconazole; FICI, fractional inhibitory concentration indices.

proportion of ionic silver with excellent stability concurrently safeguarding the purity of silver, which avoids containing toxic impurities.

Studies have revealed that the antimicrobial activity of silver ions can be enhanced by up to one thousand-fold compared to AgNPs.[27,28] The MIC of Tau-Ag for *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. auris* was 0.02–0.078 mg/mL, which was 0.25 μg/mL to 1 μg/mL of silver content, separately (Table 1). The MICs of AgNPs against *Candida* spp. from other studies were compared. Khatoon et al. showed the MICs of AgNPs against antifungal susceptible *C. albicans* ATCC 90028, *C. tropicalis* ATCC 750, and *C. glabrata* ATCC 90030 were 60, 30, 30 μg/mL, separately.[30] Mare et al. showed MIC of AgNPs mediated by spruce bark extract against five standard *Candida* spp. ranged from 20 to 1465 μg/mL.[31] Darwish et al. showed that the MIC of polyvinyl alcohol-capped AgNPs against *C. glabrata* was 54 μg/mL.[32] Radhakrishnan et al. showed that the MIC of AgNPs against wild-type *C. albicans* strain was 40 μg/mL.[33] Ahamad et al. showed the MIC of AgNPs against *C. albicans* was 12.5 μg/mL.[34] Our study found that Tau-Ag showed superior antifungal activity against *Candida* spp. with a silver MIC 10 to 3000 times lower than reported in previous studies.

Ionic silver is a form of silver that performs antimicrobial effects. AgNPs exhibited antimicrobial effects by releasing silver ions to generate ROS when exposed to the cells,[37] indicating a similar antifungal mechanism to Tau-Ag. Studies by Mare et al. and Zhou et al. have shown a dose-dependent effect between AgNPs and ROS levels in *Candida* spp., which confirmed the antifungal mechanism of AgNPs.[31] The detailed mechanism of AgNPs-induced ROS generation and fungal cell death was investigated by Hwang et al.[38] Further investigation is necessary to comprehensively investigate the mechanisms of ROS generation induced by Tau-Ag. Partha et al. used a case-control study with a posttest control group design to perform time-kill method of fluconazole, itraconazole and voriconazole against *C. albicans* ATCC 14053.[39] With the same initial inoculum ($10^4$ CFU/mL) and the measured time intervals, time-kill curves of Tau-Ag (Fig. 1) showed superior fungistatic effect since regrowth of fungal cells after 8 h and consistent increase after 24 h–48 h were observed in the time-kill curves of azole antifungals. The comparison of time-kill curves between Tau-Ag and azole antifungals indicated Tau-Ag's potential to address the azole resistance of *Candida* spp. Tau-Ag showed gradual effectiveness in inhibiting biofilm formation (Fig. 3), and we also observed that Tau-Ag may penetrate the biofilm and eliminate the biofilm-forming cell. Through MTT assays, Ahamad et al. also suggested that AgNPs effectively inhibited biofilm formation and disrupted mature biofilm.[34] Further studies on the synergistic effect of Tau-Ag and itraconazole in inhibiting biofilm formation should be conducted. The toxicity of silver ions has been a prominent concern, but no hemolysis was found at 1, 2, or 4 times the MIC of Tau-Ag against azole-resistant *Candida* spp. (Fig. 4). Due to the superior antifungal properties and purity of silver content, Tau-Ag showed no cytotoxicity at the MICs. Previous studies have evaluated the hemolytic activity of AgNPs. Khatoon et al. showed that AgNPs caused 5.6 % lysis in RBCs at MIC, and the hemolytic activity of the inherent antifungal agent, fluconazole, was also performed and showed 26.4 % of hemolysis at MIC.[30] Further evaluation of the hemolytic activity of the combination use of Tau-Ag and itraconazole could be conducted. In the current work, we performed *in vivo* studies through *C. elegans* models to further evaluate its toxicity (Fig. 6). Tau-Ag at 0.039–0.156 mg/mL (e.g., approximately 0.5–2 ppm[10]) showed no toxicity to the nematodes, concurrently performing better antifungal activity than the inherent antifungal agents. Further *in vivo* studies would be performed to confirm the treatment efficiency of Tau-Ag.

Limited treatment options were available for the treatment of candidiasis.[40] Combining first-line antimicrobial agents with other medications is the most effective way to reduce the time and cost to address drug-resistant microbes since it can effectively enhance antimicrobial activity and reduce the dosage.[41] In our study, Tau-Ag showed a synergistic effect with itraconazole against *Candida* spp., with a synergistic percentage against *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. auris* of 89 %, 40 %, 100 %, and 100 %, respectively (Table 2). We confirmed the synergism by further performing the time-kill method (Fig. 5). Yassin et al. characterized the green biosynthesized silver nanoparticles. They found their synergistic efficiency against *C. albicans*, *C. tropicalis*, and *C. glabrata* in combination with itraconazole, recording relative synergism percentages of 57.78 %, and 36.37 %, 74.32 %, respectively. The MIC of the biogenic AgNPs was 2.5 μg/mL against fluconazole-susceptible *C. tropicalis* ATCC 13803, demonstrating the highest susceptibility to the biogenic AgNPs.[42] Our findings of Tau-Ag showed similar synergism percentages with itraconazole but showed superior antifungal effect against both azole-resistant and MDR *Candida* spp., with the silver content of MIC needed only 0.25–1 μg/mL, 2.5 to 5 times lower than the previous study.

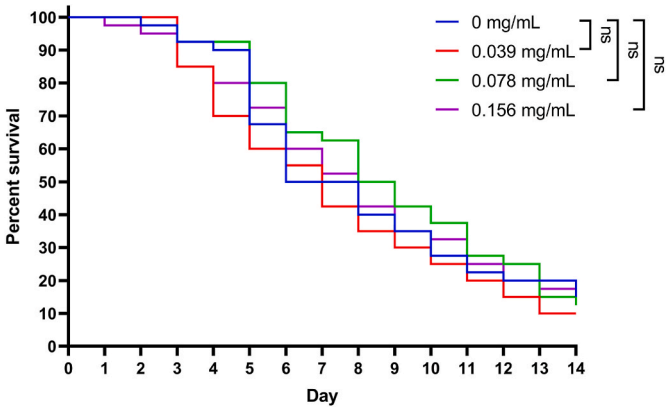


**Fig. 6.** Toxicity of Tau-Ag on *C. elegans* Each group of survival studies consisted of 40 adult worms (L4 stage). Nematode viability was monitored and recorded every 24 h over 14 days or until all worms died. A control group of worms remained untreated for comparison. The viability results were presented as the percentage of survival *C. elegans* utilizing a Kaplan-Mier survival curve generated by GraphPad Prism 8.0.1 Software. ns (non-significance).

Given the synergism results, we sought to apply the synergism properties to treating azole-resistant *Candida* spp. Itraconazole was typically administrated orally in clinical practice or topically via creams, ointment, or pastes to improve local delivery of itraconazole.[43] Treatment of recurrent vulvovaginal candidiasis has attracted attention in recent years. Maciel et al. highlighted the importance of developing and evaluating an anti-*Candida* cream based on silver nanoparticles in treating recurrent vulvovaginal candidiasis.[44] Further research on the murine vulvovaginal infection model could be conducted to evaluate Tau-Ag's potential to complement itraconazole gel, ointment, or cream for treating chronic and recurrent vulvovaginal candidiasis. Further studies should be conducted to evaluate the cytotoxicity of the combined use of Tau-Ag and itraconazole on human vaginal epithelial cells. Regardless of the type of candidiasis, Tau-Ag could effectively address the challenges posed by azole-resistant and MDR *Candida* infection in clinical administration.

## 5. Conclusion

This study evaluated Tau-Ag's antifungal activities, mechanisms, and synergistic effects with itraconazole through *in vitro* and *in vivo* testing while maintaining no cytotoxicity. Tau-Ag exhibited strong potential as a novel antifungal agent effective against azole-resistant *Candida* spp., especially MDR isolates. The antifungal effects were consistent across *C. albicans*, *C. tropicalis*, and *C. glabrata*, as well as *C. auris*, indicating broad-spectrum efficacy against azole-resistant *Candida* spp. Tau-Ag also showed superior efficacy compared to itraconazole, along with synergistic effects. These findings suggested Tau-Ag as a promising candidate for treating azole-resistant and MDR *Candida* infections.

## CRediT authorship contribution statement

**Yu-Cin Deng:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation. **Chi-Jen Shih:** Resources, Data curation. **Shang-Yi Lin:** Writing – review & editing, Resources, Data curation. **Liang-Chun Wang:** Data curation. **Tsung-Ying Yang:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sung-Pin Tseng:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare no conflicts of interest.

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The graphical abstract was created with BioRender.com.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2025.01.001.

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