Antibacterial effect of Curcumin encapsulated in polymersome nanoparticles on the expression of efflux pump *MDR1* gene in fluconazole resistant isolates of *Candida albicans*

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ABSTRACT

Curcumin, a natural product of turmeric, is known for its antibacterial and antifungal properties. *Candida albicans* is a major fungal pathogen with high mortality rate, particularly in immunocompromised patients. This study aimed to evaluate the effect of curcumin encapsulated in nanoparticles (polymersomes) in combination with fluconazole on the expression of the *MDRI* gene in drug-resistant isolates of *C. albicans*. This descriptive cross-sectional study involved obtaining 50 clinical samples, from women with vulvovaginal infections at Al-Zahra hospital (Rasht, Iran). After identifying the strains, resistance to fluconazole was assessed using disc diffusion and broth dilution methods. Six fluconazole-resistant isolates of *C. albicans* were treated with ½ MIC fluconazole (control) alone and in combination with curcumin encapsulated in nanoparticles. After 24 hours, the two cell groups were cultured on Sabouraud dextrose agar (SDA) to estimate the cell death rate. The expression of the *MDR1* gene was quantitatively investigated using the qRT-PCR method in treated and untreated isolates. Our finding indicated that combined therapy with ½ MIC fluconazole and curcumin encapsulated in nanoparticles (at a concentration of 400µg/ml) reduced fungal growth by up to 50% within during 24 hours. In treated cells, qRT-PCR analysis revealed a decrease in *MDR1* gene expression compared to untreated cells. Curcumin appears to enhance the effectiveness of fluconazole in fluconazole-resistant isolates by reducing *MDR1* gene expression.

Key words: *Candida albicans*; Curcumin; Nanoparticles; *MDR1*; qRT-PCR.

1. INTRODUCTION

Candida species, fungal infections, have become more prevalent worldwide over the past few decades. Consequently, *C. albicans* has emerged as the primary cause of fungal infection in humans (Garcia-Gomes, Curvelo, Soares, & Ferreira-Pereira, 2012). Candidiasis, a common fungal infection, poses significant health challenges for individuals with conditions such as HIV infection (Awanish Kumar et al., 2014), organ transplantation, and cancer chemotherapy (Garcia-Gomes et al., 2012). Despite the availability of numerous antifungal medications, the use of azole and non-azole drugs can lead to adverse effects, including liver damage, changes in oestrogen levels, allergic reactions, and the development of drug-resistant (Awanish Kumar et al., 2014).Azole resistance in *C. albicans* arises from various molecular mechanisms, such as alterations in ergosterol biosynthesis pathways, overexpression of genes involved in efflux pump systems like *CDR1*, *CDR2*, and *MDR1*, and overexpression or mutation of *ERG11* genes (Paul et al., 2020). Evidence suggests that reduced intracellular drug accumulation, associated with the upregulation of genes like *MDR* and *CDR*, plays a crucial role in azole resistance in *C. albicans* (Zhang et al., 2019).

Due to the side effects of antifungal drugs and the increasing, resistance to these medications, the use of natural herbal drugs with inhibitory effect has generated the attention of researchers as the third generation of treatments (Awanish Kumar et al., 2014). Given that curcumin, the primary and active ingredient in turmeric, possesses anti-inflammatory, anti-tumor, anti-fungal, and antibacterial properties, it has emerged as a significant herbal components (Awanish Kumar et al., 2014; Rahbar Takrami, Ranji, & Sadeghizadeh, 2019).

There is mounting evidence supporting the hypothesis that curcumin, when combined with antibacterial agents, can have a significant impact on microorganisms (Khalil et al., 2012). Although the role of curcumin in the diets of many countries and its antifungal properties have been recognized since ancient times (Ranji, 2014; Sharma et al., 2011), its poor solubility in aqueous environments results in low bioavailability in both culture environments and in vivo studies (Ranji, 2014). In recent years, there has been widespread interest in using carriers, such as liposome, micelle, chitosan, nano capsule and polymersome to deliver curcumin into cells. Studies have shown that curcumin loaded in polymersome nanoparticles exhibits greater release and efficacy on target cells compared to curcumin alone (Pakizehkar, Ranji, Sohi, & Sadeghizadeh, 2020). Given the increasing resistance of C. *albicans* isolates to azoles, particularly fluconazole, it is imperative to explore approaches to mitigate this resistance. The objective of this research was to investigate the antifungal effects of polymersome nanoparticles containing curcumin in reducing *MDR1* gene expression in fluconazole-resistant *C. albicans* isolates.

2. MATERIALS AND METHODS

2.1. Collection of *C. albicans* isolates

This study was conducted at Al-Zahra Hospital in Rasht, Iran. Screening for the presence of *C. albicans* was performed on samples of vaginal and cervical secretions from 50 female

patients. After a 24-hour incubation period at 37°C, yeast-like colonies on Sabouraud Dextrose Agar (SDA) were evaluated using colony morphology, germ-tube tests, and CHROMagar Candida (Charles, Kali, & Joseph, 2015).

2.2. Disk diffusion testing

Disk diffusion testing was conducted strictly according to CLSI standard M44-A2 (Balouiri, Sadiki, & Ibnsouda, 2016). Paper disks containing 10 mcg of fluconazole were obtained from HiMedia, India. Following 24 hours incubation period at 37°C, the zone diameter was measured, and fluconazole-resistant isolates were identified and selected.

2.3. Determination of minimum inhibitory concentrations

In accordance with CLSI M27A2 guidelines, the MIC test for fluconazole was conducted using Sabouraud Dextrose Broth (SDB). *C. albicans* isolates were cultured in SDB in a 96-well microplate with fluconazole concentrations ranging from 2048 µg/ml to 4 µg/ml for a duration of 48 hours at 37°C.

2.4. Preparation of curcumin entrapped in polymersome nanoparticles

Polymersome nanoparticles, comprising a blend of oleic acid (OA) and polyethylene glycol (PEG400) were procured from Nano Daro Alborz Company (Tehran, Iran). Curcumin (E, E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene3,5-dione) (Chemical formula: C21H20O6) with a purity of ≥94% (MW: 368,38 g/mol) was obtained from Sigma-Aldrich (Germany). Triethylamine (Billerica, USA) and chloroform (Sigma Aldrich, America) served as solvents during the four-hour synthesis of oleoyl chloride (Sigma Aldrich, USA) and polyethylene glycol (Sigma Aldrich, USA), with the reaction conducted at 24°C. Subsequently, the organic phase underwent filtration to eliminate trimethylaniline hydrochloride, and a vacuum device was utilized to evaporate chloroform at 40°C.

The chemical structure of the polymersome nanoparticles was analyzed using FTIR. Next, curcumin and polymersome nanoparticles (PEG400-OA) were mixed (Pakizehkar et al., 2020). The efficiency of curcumin entrapment in nanoparticles and the size of the curcumin entrapped in polymersome nanoparticles (Cur: PEG400-OA) were assessed through various physico-chemical analysis. The final product was stored in a light-protected environment at 4°C.

2.5 Treatment of fluconazole-resistant *C. albicans* isolates with Cur: PEG400-OA

To investigate the antifungal effect of Cur: PEG400-OA, 60 µl of microbial suspension containing C. *albicans* isolates (1.5 x 10^8 cfu/ml) was added to the wells, along with ½ MIC fluconazole and varying concentrations of Cur: PEG400-OA (0, 100, 200, and 400 µg/ml). The plates were then incubated at 37°C for 24 hours. After incubation, 20 microliters from each treated sample were cultured on Sabouraud Dextrose Agar (SDA) to determine the Minimum Fungicidal Concentration (MFC). Each test was carried out at least twice (Alalwan et al., 2017).

2.6. Quantitative analysis of *MDR1* gene

The transcription levels of the *MDR1* gene were assessed via qRT-PCR in *C. albicans* isolates treated with 400 µg/ml of Cur: PEG400-OA and ½ MIC fluconazole, with the control group receiving only ½ MIC fluconazole. Isolate RNA from both study and control groups was extracted using the RNX-Plus™ kit (Cinaclon, Tehran, Iran). Subsequently, cDNA was synthesized using the cDNA Synthesis Kit (Yekta Tajhiz Azma, Iran). Quantitative RT-PCR was performed with SYBR®Premix Ex Taq™ II (TaKaRa, Japan) on the Rotor-Gene Q instrument (QIAGEN). The PCR protocol involved an initial denaturation step of three minutes at 95°C, followed by forty cycles of denaturation for five seconds at 95°C, annealing for thirty seconds at 60°C, and extension for five seconds at 75°C.The *ACT1* (Actin) gene served as an internal endogenous control. The primers were in the investigation are listed in Table 1. Each reaction was conducted in triplicate, and the $\Delta\Delta C_T$ method was employed for outcome analysis. The fold change for each was computed using the formula $2^{-\Delta\Delta CT}$.

Table. 1 Sequence of primers used for quantitative RT-PCR

Primer name	Primer sequence	Length of production	Reference
MDR1-F	5'-TCAGTCCGATGTCAGAAAATGC-3'	81 bp	(Alalwan et al., 2017)
MDR1-R	5'-GCAGTGGGAATTTGTAGTATGACAA-3'		
ACT1-F	5'-GCTTTTGGTGTTTGACGAGTTTCT-3'	72 bp	(Alalwan et al., 2017)
ACT1-R	5'-GTGAGCCGGGAAATCTGTATAGTC-3'		

2.7. Statistical analysis

The significance differences between the groups were examined using the student's t-test, one-way, and two-way ANOVA. A 0.05 *P-value* was employed to ascertain statistical significance. The mean ± SD was used to express the results.

3. RESULTS

3.1. Physico-chemical properties of Cur: PEG400-OA

In our previous investigation, the ratio of curcumin in Cur: PEG400-OA solvent (PBS) was reported to be 1 mg/ml (Pakizehkar et al., 2020). Furthermore, it was discovered that curcumin loaded into nanoparticles at a density of approximately 16% with an encapsulation efficiency in Cur: PEG400-OA reaching approximately 97%. The average size of Cur: PEG400-OA was determined to be nearly 259 nm based on dynamic light scattering (DLS) results, while it measured close to 300 nm in the scanning electron microscope (SEM) analysis (Pakizehkar et al., 2020).

3.2. Anti-fungal properties of Cur: PEG400-OA

The morphology and color of the colonies on CHROMagar Candida were utilized for the identification of *C. albicans*. The results indicated that among the 23 isolates in the disc diffusion studies, 20 were resistant to fluconazole, one was sensitive, and two were semi-sensitive (Fig.1. A). Moreover, 50% of the isolates exhibited the highest MIC (2048 µg/ml), indicating a significant level of fluconazole resistance among the isolates (Fig.1. B).

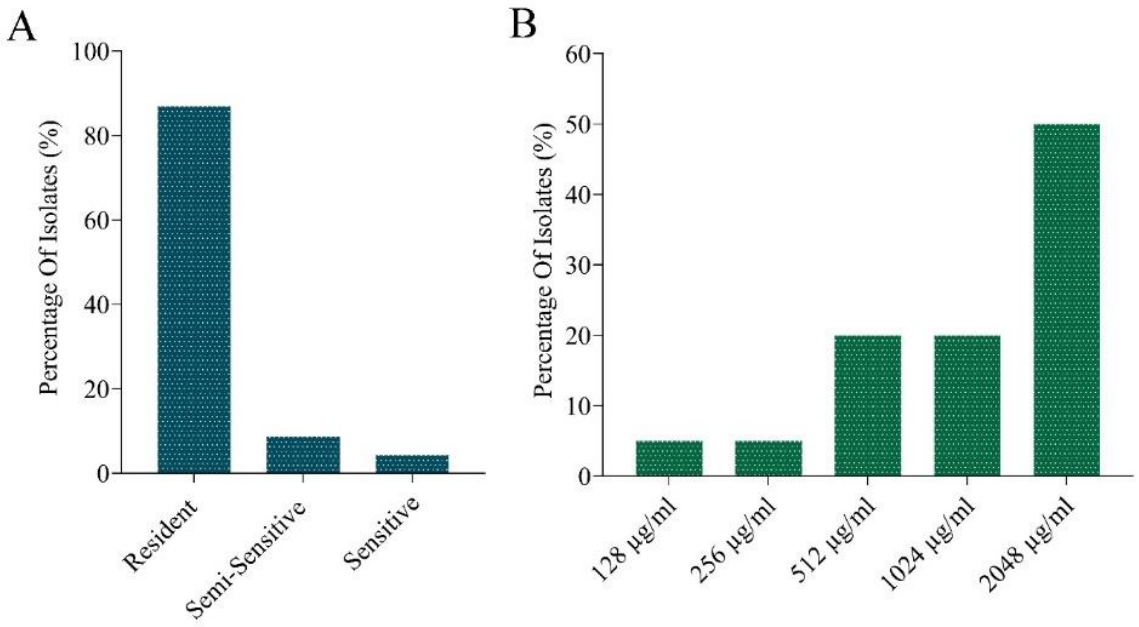

Figure.1. A. Resistance of *C. albicans* isolates to fluconazol via disc diffusion method. B. MIC of fluconazole in isolates

It was determined that the lethal dose to 50% (LD_{50}) of Cur: PEG400-OA in combination with ½ MIC fluconazole was 400 µg/mL. The isolates were cultured on Sabouraud Dextrose Agar (SDA) to validate the MIC results. Based on the Minimum Fungicidal Concentration (MFC) results, 50% of the cells can be annihilated by 400 µg/ml of Cur: PEG400-OA (Fig.2).

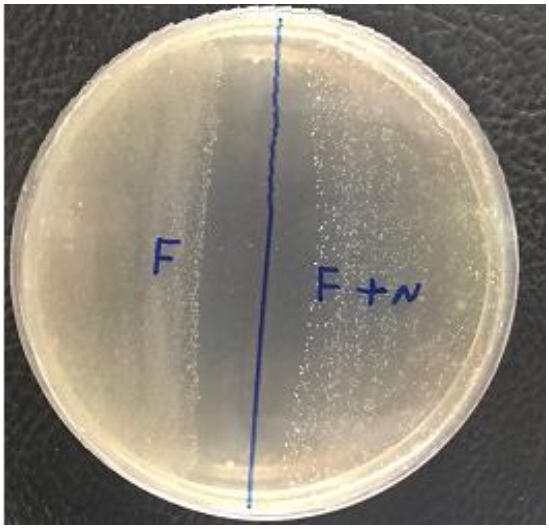

Figure. 2. MFC colony figures in *C. albicans* isolate number 7 treated with ½ MIC fluconazole (F) and ½ MIC fluconazole combined with 400 µg/ml of Cur: PEG400-OA (F+N)

3.3. Reduction of *MDR1* gene expression after treatment with Cur: PEG400-OA

The findings revealed that, compared to the control group (treated with ½ MIC fluconazole alone), the *MDR1* gene expression level in the three isolates under study, which were treated with 400 µg/ml of Cur: PEG400-OA in combination with ½ MIC fluconazole, confirmed a reduction in *MDR1* gene expression in all three isolates (Fig. 3).

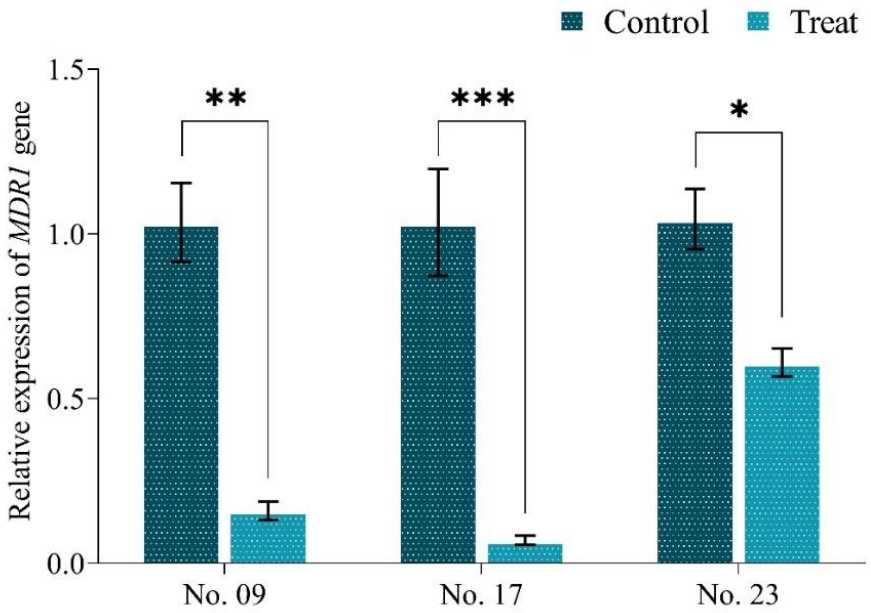

Figure. 3. Quantitative expression of *MDR1* gene in *C. albicans* isolate treated with 1400 µg/ml of Cur: PEG400-OA in combination with ½MIC fluconazole compared to the control (½MIC fluconazole). The results are represented as mean±SD. Significant difference between treat and control was calculated based on **P>0.05*, ***P<0.01*, and ****P<0.001*.

4. DISCUSSION

Researchers often regard curcumin as a potential new adjunctive medication for treating various diseases due to its broad-spectrum qualities and safety profile even at high doses (Rahbar Takrami et al., 2019). In this study, it was found that after 24 hours, Cur: PEG400-OA (400 µg/ml) combined with ½ MIC of fluconazole inhibited the growth of isolates. Moreover, the qRT-PCR method confirmed downregulation of the *MDR1* gene compared to the control group. Approximately 90% of *C. albicans* cases are attributed to Candida *vaginitis*, which constitutes one-third of vulvovaginitis cases and is experienced by at least 70% of women at some point in their lives (Baldesi et al., 2017; Ishikane, Hayakawa, Kutsuna, Takeshita, & Ohmagari, 2016). The prevalence of recurrent Vulvovaginal Candidiasis (VVC) in women is approximately 8% (Jeanmonod, Jeanmonod, Christopherson, & Spivey, 2019). Various researchers are currently exploring different methods of treating infections, including the use of antimicrobial herbal compounds as supplements or alternatives to traditional treatments to expedite patient recovery and reduce hospital mortality rates (Hu, Zhang, Kong, Zhao, & Yang, 2017; Sadiq et al., 2016; Vinodkumar, Nakkeeran, & Renukadevi, 2017). Additionally, due to the increase in resistance to opportunistic infections and their high prevalence in hospitals over the past few decades, researchers are seeking alternative approaches to their treatment (Motahhary Tashi & Ranji, 2017; Rahbar Takrami et al., 2019; Ranji & Rahbar Takrami, 2017).

Several studies have shown the curcumin, as an antifungal compound, can downregulation genes have involved in the synthesis of the cell wall of *C. albicans* (Rajasekar et al., 2021; Zhou et al., 2024). Kali *et al*. identified a clear relationship between curcumin and antibiotics in several biofilm-producing bacterial isolates (Kali, Bhuvaneshwar, Charles, & Seetha, 2016). Numerous studies (Bezerra, y Araújo, Alves-Júnior, Damasceno, & Oshiro-Junior, 2024; Cheraghipour et al., 2021; Garcia-Gomes et al., 2012; Hajifathali, Lesan, Lotfali, Salimi-Sabour, & Khatibi, 2023; Tsopmene et al., 2024) have demonstrated the antifungal effect of curcumin (at concentrations ranging from 10 to650 µg/ml) alone or in combination with antifungal agents such as MCD (α-methyl cinnamaldehyde) (Narayanan et al., 2020), caspofungina (Alalwan et al., 2017), and fluconazole (Lee et al., 2022) against *C. albicans.* In a previous study by Soliman *et al.*, extracts of *Asparagus tenuifolius*, toothbrush tree (*Salvadora persica*), henna leaf (*Lawsonia inermis*), and purslane (*Portulaca oleracea*) at concentrations between 25 and 100 µm/ml exhibited anti-Candida effects after 24 hours using the disc diffusion method (Soliman et al., 2017). Furthermore, Garcia-Gomes et al. discovered that curcumin had a remarkable ability to prevent a C. *albicans* isolate from developing resistance to fluconazole. At 11 µM, it was able to restore sensitivity to this azole. Notably, an efflux pump may be involved in contributing to the isolate's resistance to fluconazole (Garcia-Gomes et al., 2012). In the present study, the anti-fungal property of Cur: PEG400-OA was confirmed at 400 µg/ml within 24 hours, a concentration lower than that required in the study by Garcia-Gomes *et al.*

The general mode of action of azole antifungals involves inhibiting lanosterol 14-alpha-demethylase, which converts lanosterol in fungal cell membranes into ergosterol (Monk & Keniya, 2021). *MDR1* is typically expressed at low levels, but a significant proportion of

fluconazole-resistant in *C. albicans* clinical isolates permanently overexpress *MDR1*. Additionally, it has been demonstrated that *MDR1* overexpression plays a crucial role in the isolate's resistance; deletion of the *MDR1* gene from overexpressing *C. albicans* strains results in a decrease in the mutants' resistance to fluconazole (Bergin et al., 2023). In the study by Khosravi *et al*., overexpression of *MDR1* and *CDR2* genes was observed in 75% of *C. albicans* isolates (Rad, Falahati, Roudbary, Farahyar, & Nami, 2016).Chen *et al*. observed that all fluconazole-resistant isolates in their study exhibited elevated expression of *CDR1* and *CDR2*, and some of these isolates also showed increased expression of *MDR1* and *ERG11* (Chen et al., 2010). In the present study, a reduction in *MDR1* gene expression was observed in several fluconazole-resistant *C. albicans* isolates. It appears that curcumin, with its inhibitory effect on the expression of this gene, reduces the presence of this transporter of the efflux pump system on the cell surface, leading to less fluconazole being pumped out of the cell. This reduction in the release of fluconazole results in an increase in the drug's cytoplasmic concentration and a decrease in the amount that affects the cell. Considering that various factors play a crucial role in the development of drug resistance, and resistance can vary among different fungal strains due to various mutations or the acquisition of new genes, the limitations of this research include the variability in the response of each strain to the drug at specific dose, which can affect the expression of genes involved in drug resistance.

5. CONCLUSION

According to this study, curcumin trapped in polymersome nanoparticles exhibits a synergistic effect with fluconazole on *C. albicans* isolates. Additionally, reducing the expression of the *MDR1* gene allows more fluconazole to be taken up and retained in the cell, while decreasing the efflux of the drug from the cell. Consequently, this enhances the antifungal drug's efficacy in inducing cell death.

ACKNOWLEDGEMENTS

The authors of this article would like to be grateful to Islamic Azad University, Rasht, for funding the research based on research grant number 19280/1/16/17.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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