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Antifungal activity of Curcumin Encapsulated in Polymersome Nanoparticles against Fluconazole Resistant Isolates of *Candida albicans* and its Effect on the Expression level of Efflux Pump *MDR1* gene

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Abstract

Curcumin, active component from *Curcuma longa*, 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 the identification of *C.albicans* in clinical samples, the isolates, resistance to fluconazole was assessed using disc diffusion and broth dilution methods. Six fluconazole-resistant isolates of *C. albicans* were treated with curcumin encapsulated in polymersomes (400 µg/ml) in combination with fluconazole (½ MIC) compared to cells treated with fluconazole (½ MIC) alone. 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% afetr 24 hours. In treated cells, qRT-PCR analysis revealed a decrease in *MDR1* gene expression compared to untreated cells. Curcumin encapsulated in nanoparticles appears to enhance the effectiveness of fluconazole in fluconazole-resistant isolates by reducing *MDR1* gene expression.

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

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1. INTRODUCTION

Fungal infections specially Candida species, 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 estrogen 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 including *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 such as 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 effects has generated the attention of researchers as the third generation of treatments (Awanish Kumar et al., 2014). Given that curcumin, the primary and active component in turmeric (Curcuma longa), possesses antiinflammatory, antitumor, antifungal, and antibacterial properties, it has emerged as a potential therapeutic strategy (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 cancer cells compared to curcumin alone (Pakizehkar, Ranji, Sohi, & Sadeghizadeh, 2020b). 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 study 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 city (in north of Iran). Screening of *C. albicans* isolates was performed on samples of vaginal and cervical secretions from 50 female patients. After a 24-hour incubation period at 35°C, yeast-like colonies on Sabouraud Dextrose Agar (SDA, Quelab, Canada).) were used to evaluate colony morphology, germ-tube tests, and CHROMagar Candida (Charles, Kali, & Joseph, 2015).

2.2. Disk diffusion assay

Disk diffusion assay was conducted strictly according to CLSI 2021. Paper disks containing 10 µg of fluconazole were obtained from HiMedia (India). Following 24 hours incubation period at 35°C, the inhibition 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, Quelab, Canada). *C. albicans* isolates (1.5 x 10^8 cfu/ml) were cultured in SDB in a 96-well microplate with fluconazole concentrations ranging from

2048 µg/ml to 2 µg/ml for a duration of 24 hours at 35°C (Maenchantrarath, Khumdee, Samosornsuk, Mungkornkaew, & Samosornsuk, 2022). The MIC was taken as the lowest concentration of fluconazole which no visible growth was observed by naked eyes (Yuan, Zhou, Su, & Zhang, 2019).

2.4. Curcumin encapsulation in PEG_{400}-OA nanoparticles

Curcumin (Cur) was purchased from Nano Alborz Daru (Tehran, Iran). Oleoyl chloride (OA), polyethylene glycol (PEG, MW 400 KD), and chloroform (trichloromethane) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triethylamine was provided from EMD Millipore (Billerica, MA, USA). Oleoyl chloride (0.01 moles) and PEG_{400} (0.01 moles) were estrificated to synthesize PEG_{400}-OA nanoparticles in the presence of triethylamine (0.012 moles) and chloroform as the solvent at 25°C for 4h. Triethylamine hydrochloride salt was filtered from its organic phase and chloroform was evaporated from PEG_{400}-OA nanoparticles in vacuum oven as described by Pakizehkar *et al* (Pakizehkar, Ranji, Sohi, & Sadeghizadeh, 2020a). In order to load the curcumin into nanoparticles, curcumin was mixed in acetone solution with PEG_{400}-OA nanoparticles to prepare weight/weight ratio of curcumin/PEG_{400}-OA mixture (1:6). After acetone evaporation, 1 mg/ml of this mixture was dissolved in PBS. The experiment was carried out at 25°C, in light-protected condition

2.5 Treatment of C. albicans isolates with Curcumin encapsulated in nanoparticles

To investigate the antifungal effect of Cur: PEG_{400}-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: PEG_{400}-OA (0, 100, 200, and 400 µg/ml). The plates were then incubated at 35°C for 24 hours. After incubation, 20 microliters from each treated sample were cultured on Sabouraud Dextrose Agar (SDA) to evaluate fungal growth inhibition. 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: PEG_{400}-OA and ½ MIC fluconazole, in compared the control group receiving only ½ MIC fluconazole. Total RNA from both study and control groups was extracted using the RNX-Plus™ kit (Sinaclon, 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 including 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'		

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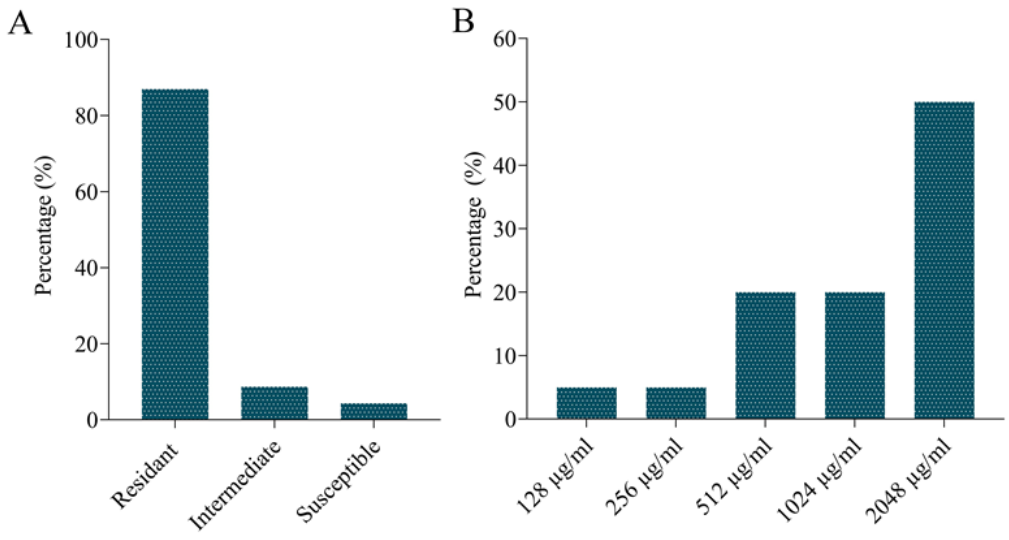

Figure.1. Fluconazole susceptibility profile. A. The percentage of fluconazole susceptibility in *C. albicans* isolates. B. MIC of fluconazole in the resistant isolates in broth microdilution method.

2.7. Statistical analysis

The significance differences between the groups were evaluated using the student's t-test. A 0.05 *P-value* was employed to ascertain statistical significance. The mean ± SD was used to express the results.

3. RESULTS

3.1. Physicochemical properties of Cur: PEG400-OA

In our previous investigation, the ratio of curcumin in Cur: PEG_{400}-OA solvent (PBS) was reported to be 1 mg/ml. 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: PEG_{400}-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., 2020b).

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 susceptible, and two were intermediate (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).

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

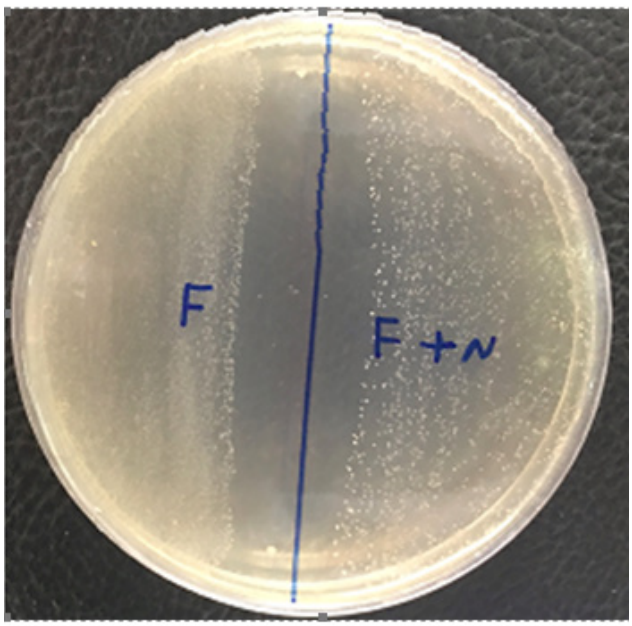

Figure. 2. Growth inhibition in *C. albicans* isolate number 7 treated with group F (fluconazole, ½ MIC) and group F+N (Cur: PEG400-OA nanoparticles (400 µg/ml) in combination with fluconazole (½ MIC))

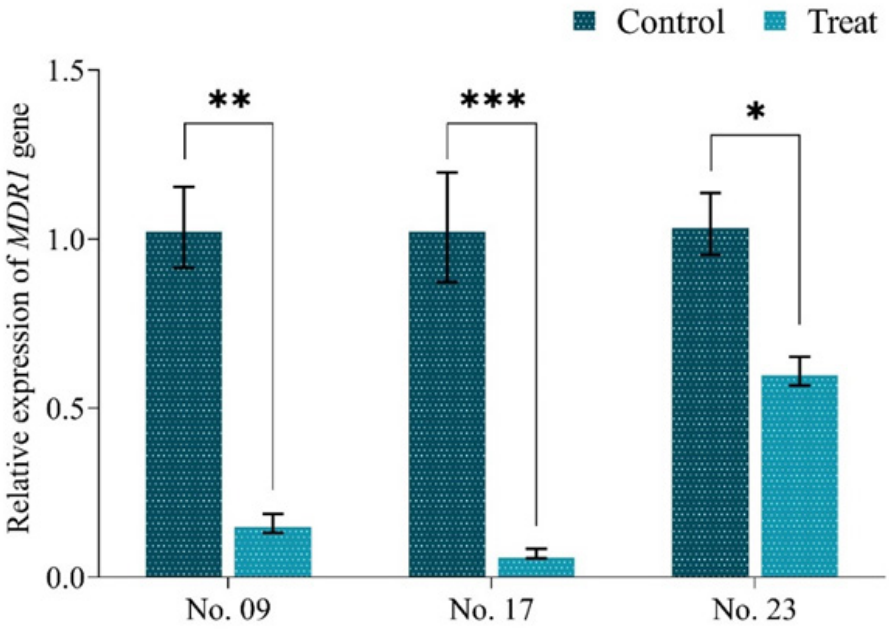

Figure. 3. Quantitative expression of MDR1 gene in *C. albicans* isolate treated with 400 µg/ml of Cur: PEG400-OA in combination with ½MIC fluconazole compared to the control (½MIC fluconazole). The results are represented as mean±SD. Significant difference between treat and control was calculated based on *P>0.05, **P<0.01, and ***P<0.001.

3.3. Downregulation of *MDR1* gene 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 downregulation in *MDR1* gene in all three isolates (Fig. 3).

4. Discussion

Curcumin (diferuloylmethane) as a lipophilic polyphenol is bright yellow and an active compound of turmeric (rhizomes) (Bisht, Wagner, & Bulmer, 2010), has anticancer, antiinflammatory, antioxidant and nontoxic properties (Wilken, Veena, Wang, & Srivatsan, 2011). This therapeutic herbal compound is soluble in oil and insoluble in water (Hanif Mughal, 2019). To improve the bioavailability of fragile and hydrophobic drugs, nanocarriers have been developed to protect the encapsulated drug from enzymatic degradation, provide controlled release, alter their pharmacokinetics, prolong their residence in plasma, and improve their cytoprotective and antioxidant effects (Abboud et al., 2015; Iurciuc-Tincu et al., 2020). The targeted delivery and cellular internalization of curcumin are also improved by its encapsulation in nanoparticles and nano-emulsions (Sahab-Negah et al., 2020). Polyethylene glycol (PEG) is one of the polymers that has caught the interest of numerous researchers to be used in the formulation of liposomes. Both biocompatibility and biodegradability are strong points of this polymer. Pakizehkar et al. showed that curcumin-loaded PEG400-OA nanocarriers (polymersomes) had a spherical shape with an appropriate mean size of 259.5±1.5 nm (Pakizehkar et al., 2020a). In this study, we observed anti-fungal effects of curcumin encapsulated in polymersome nanoparticles on *C. albicans* isolates with under-expression of MDR1 efflux pomp gene.

Approximately 90% of *C. albicans* isolates 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 infec-

tions, 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 that curcumin, as an antifungal compound, can downregulate genes involved in the synthesis of the cell wall of *C. albicans* (Rajasekar et al., 2021; Zhou et al., 2024). 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 (Yean Sheng 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 nanoparticles (polymersomes) was confirmed at 400 µg/ml within 24 hours, a concentration lower than that required in the study by Garcia-Gomes *et al*.

Negi *et al*. revealed that curcumin can be as an Efflux Pump Inhibitor (EPI) in multidrug resistant clinical isolates of *Pseudomonas aeruginosa* (Negi, Prakash, Gupta, & Mohapatra, 2014). Fekri Kohan *et al*. demonstrated that Silybin extracted from Silybum marianum L. had inhibitory effect on the expression of efflux pump genes (acrA, acrB and tolC) in uropathogenic E. coli isolates (Fekri Kohan et al., 2024). Lee et al. reported that the curcumin through decreasing Hsp90 downregulated *CDR1* efflux pomp gene in *C. albicans* (Y. S. Lee et al., 2022). Kaempferol, as a flavonoid, in combination of fluconazole decreased the expression of *CDR1*, *CDR2* and MDR1 efflux pomp genes in *C. albicans* (Shao, Zhang, Wang, Li, & Wang, 2016). In this study, MDR1 gene downregulated in the clinical isolates of *C. albicans* after treatment with curcumin encapsulated in polymersome nanoparticles (400µg/mL) and fluconazole (½MIC) compared to treatment with fluconazole (½MIC) alone. Our quantitative analysis suggests that curcumin encapsulated in polymersome (PEG400-OA) nanoparticles can increase the fluconazole susceptibility in *C. albicans* isolates partially through downregulation of MDR1 efflux pump gene and in result increasing fluconazole entrapment into fungal cells and enhancing cell death induced with fluconazole.

5. CONCLUSION

Our results showed that curcumin encapsulated in polymersome nanoparticles had a synergistic effect with fluconazole on *C. albicans* clinical isolates. Additionally, downregulation of the *MDR1* gene allowed more fluconazole to be taken up and retained in the cell, while decreasing the efflux of the drug from the cell. Consequently, this enhanced the antifungal drug's efficacy in inducing cell death.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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