

In-Vitro Antifungal Activity of Nano Encapsulated Caprylic Acid and EFG1 Gene Expression Profile in *Candida albicans*

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ABSTRACT

Backgrounds: Due to the emergence of multidrug-resistant *Candida* species, the discovery of new antifungal agents with minimum side effects is essential. The aim of this study was to investigate the antifungal activity of caprylic acid and nano-encapsulated caprylic acid against *C. albicans* as well as their effect on the expression of *EFG1* gene.

Materials & Methods: In this laboratory trial study, the minimum inhibitory concentration (MIC) of caprylic acid and nano-encapsulated caprylic acid against *C. albicans* was evaluated at various concentrations (400-625 and 1.3-50 μ L/mL, respectively). Real time-PCR was performed to assess the expression level of *EFG1* gene. Cytotoxicity effect of caprylic acid and nano-encapsulated caprylic acid was evaluated on SW480 cell line using MTT test.

Findings: Antifungal activity findings displayed that MIC₉₀ and MIC₅₀ values of caprylic acid were 500 and 450 μ g/mL, respectively, whereas MIC₉₀ and MIC₅₀ values of nano-encapsulated caprylic acid were 6.2 and 3.1 μ g/mL, respectively. The expression of *EFG1* gene significantly decreased in the groups treated with caprylic acid and nano-encapsulated caprylic acid compared to the control group. According to the cytotoxicity evaluation findings, the viability of cells treated with caprylic acid was significantly higher than that of cells exposed to nano-encapsulated caprylic acid.

Conclusions: According to the obtained results, nano-encapsulated caprylic acid successfully inhibited *C. albicans* growth at a lower concentration compared to caprylic acid. Overall, it was found that nano-encapsulated caprylic acid is a promising antifungal agent against *Candida* species; however, further studies are needed to be performed about nano-encapsulation of caprylic acid.

Keywords: *Candida albicans*, Caprylic acid, Nano-encapsulation, *EFG1* gene, Antifungal agent

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Introduction

Candida albicans is a polymorphic fungus which causes as opportunistic fungal infection in immunocompromised patients [1]. *Candida* infections are mainly caused by the organism's ability to form biofilm on living and non-living surfaces, respectively. According to some reports from the United States, the mortality rate due to *Candida* infections associated to the use of medical equipment is estimated to be as high as 30%, which imposes high cost to the community for antifungal therapies. Today, biofilm formation is known as the main cause of pathogenesis of *C. albicans*. While this phenomenon could turn into a systemic infection, it is characterized by drug resistance [2-5].

Biofilm formation starts from changes in phenotypic cells, distinct from planktonic cells, to hyphal formation and in mature biofilm, extracellular matrix surrounds all materials [6-7]. Genetic analysis has revealed that network genes navigate biofilm formation. One of the major regulators of biofilm formation in *C. albicans* is enhanced filamentous growth protein I (*EFG1*) [8]. On the other hand, *EFG1* as a transcription factor has several target genes that regulate the expression of hyphal genes including *HWP1*, *HYR1*, and *ALS3* [8-10]. Understanding the gene expression patterns in *Candida* biofilm is of great interest to investigators in order to identify potential antifungal targets. Application of conventional antifungal drugs has been limited due to toxicity, side effects, and the development of multidrug-resistant *Candida* species [11-13]. Recently, the use of natural-based compounds has attracted much attention because of their antifungal characteristics. Previous studies have shown that fatty acids are effective against pathogenic fungi in plants and human [14]. Caprylic acid found in coconut is one of the triglycerides family members, including

caprylic acid (CAP, C8), capric (C10), caproic (C6), and lauric (C12) fatty acids. A previous study reported that medium-chain fatty acids had potent antibacterial, antifungal, and anti-inflammatory properties [15]. Therefore, these properties make caprylic acid as a useful treatment for fungal infections. In addition, unlike surfactants and synthetic peptides, fatty acids have shown no toxicity, hypersensitivity, carcinogenicity, and mutagenic effects on mammalian cells so far [16-17].

Objectives: Nowadays, the design of drug delivery nano systems, such as liposomes, micelles, nanoparticles, antibody conjugates, and polymer conjugate systems, has attracted much attentions. This novel approach represents a significant improvement in pharmaceutical systems due to enhanced targeted and controlled drug delivery with low dose and slow rate to tissues or cells [18-20]. This study aimed to investigate the activity of caprylic acid and nano-encapsulated caprylic acid against *C. albicans* biofilm formation and to evaluate the effect of these compounds on the expression of *EFG1* gene as a transcription factor of biofilm formation in *C. albicans*.

Materials & Methods

This laboratory trial study was carried out at Tarbiat Modares University, Tehran, Iran. In this study, antifungal activity evaluation was done on *C. albicans* (ATCC 10231). The strain was cultured on Sabouraud dextrose agar (Merck, Germany) medium and incubated at 37 °C for 24 hrs. Caprylic acid and encapsulated caprylic acid were purchased from Nano Zino Company, Iran. Caprylic acid (Sigma Aldrich, USA) was prepared at different concentrations in methanol (650, 625, 600, 550, and 500 mg/mL). Nano-encapsulated caprylic acid was also prepared at 50, 25, 12.5, 6.25, 3.1, and 1.5 mg/mL concentrations.

Effect of caprylic acid and nano-encapsulated caprylic acid on biofilm formation:

The inhibitory activity of caprylic acid and nano-encapsulated caprylic acid against *C. albicans* biofilm formation was evaluated according to a previous study by Nikoomanesh et al. (2018) [21]. Briefly, for biofilm formation assay, fungal cells (10^3 cell/mL) were inoculated in 96-well microplates (Nunc), while each well contained 200 μ L of RPMI (Sigma-Aldrich, USA) medium supplemented with different concentrations of caprylic acid (650, 625, 600, 550, and 500 mg/mL) and nano-encapsulated caprylic acid (50, 25, 12.5, 6.25, 3.1, and 1.5 mg/mL). Positive control contained fluconazole, and suspensions with no fungal elements were considered as negative control during tests. The plates were incubated at 35 °C for 24 hrs. Minimum inhibitory concentration (MIC) was determined with the aid of a magnifying mirror. The lowest drug concentration causing a prominent decrease in turbidity (minimum inhibitory concentration that could inhibit 50% of fungal cells growth) was regarded as MIC₅₀, and the growth rate in each well was compared to that of the positive control. Moreover, MIC₉₀ (the MIC which could inhibit 90% of fungal cells growth) was detected. Then to determine the minimum fungicidal concentration (MFC), each well was cultured on SDA and incubated at 37 °C for 24 hrs, and colony count was performed. All experiments were carried out in triplicate.

Real-time PCR (qRT-PCR): To investigate the effect of caprylic acid on gene expression, real-time PCR was performed.

RNA extraction: After obtaining MIC, fresh culture colonies of *C. albicans* at 10^3 cells/mL were prepared and treated with the MIC of caprylic acid and nano-encapsulated caprylic acid at 37 °C for 24 hrs. Wells without caprylic acid/nano-encapsulated caprylic acid were considered as positive

control, and itraconazole-containing wells were considered as negative control. After that the treated fungal cells were collected and washed using phosphate buffered saline (PBS), and total mRNA was extracted by glass bead and lysis buffer as described previously [20].

cDNA synthesized; cDNA was synthesized according to the manufacturer's recommendations using a 2-step cDNA synthesis kit, vivantis (Malassezia) [22]. The PCR primers used to amplify and identify *EFG1* gene were designed by All-ID design software, and the housekeeping gene Act1 was used as an internal control. The primer sequences used in this study are shown in Table 1.

Real-time PCR was accomplished using AMPLIQON (Real Q plus 2 x master mixes Green High Rox) [20]. The reaction mixture, adjusted to a final volume of 20 μ L using DEPC water, contained 10 μ L of master mix (Green High Rox), 0.5 μ L of each specific primer of *EFG1* (10 pmol), and 2 μ L of each cDNA sample. Cycling conditions were as follows: an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s., 58 °C for 30 s, and 72 °C for 30 s.

All reactions were performed in triplicate in the ABI one step (Applied Biosystems; Rotkreuz; Switzerland). The expression level of *EFG1* was evaluated using REST 2009 software Ver. 2.0.13.

Cytotoxicity Assay: The cytotoxicity effect of caprylic acid and nano-encapsulated caprylic acid was assessed by colorimetric MTT assay [20]. In this method, epithelial cell line (SW480) was cultured in DMEM cell culture medium supplemented with 10% fetal bovin serum (FBS), 50 μ L penicillin/streptomycin, and l-glutamine and incubated at 37 °C for 24 hrs with 5% CO₂. Then cells at 10×10^4 cells/mL were seeded into each well of microtiter plates (tissue culture grade, 96 wells, flat bottom, Corning. USA), containing

determined concentrations of caprylic acid and nano-encapsulated caprylic acid. Also, non-treated cells were considered as the control group. Viability of cells was tested by MTT (5 mg/mL, Sigma-Aldrich, USA) at 570 nm using an ELISA reader. Cell viability percentage was calculated and compared to the control group. Cell viability was calculated by the following formula:
 Cell viability = $\text{OD}_{\text{Test}} / \text{OD}_{\text{control}} \times 100$

Statistical Analysis: Data were analyzed using IBM SPSS statistics software Version 23 by employing student-test and one-way ANOVA. A p value $< .05$ was considered as a significant difference between groups.

Findings

Activity of caprylic acid and nano encapsulated caprylic acid on biofilm formation: Antifungal activity of caprylic acid and nano-encapsulated caprylic acid was examined against biofilm formation ability of *C. albicans*. The findings indicated that *C. albicans* in the presence of caprylic acid and nano-encapsulated caprylic acid at 550 and 6.25 $\mu\text{g/mL}$ concentrations showed the least growth, respectively. Moreover, caprylic acid and nano encapsulated caprylic acid at 450 and 3.1 $\mu\text{g/mL}$ concentrations were able to inhibit the growth of this organism. (Table 2).

EFG1 gene expression: RNA was extracted from *C. albicans* before and after treatment with MIC values of caprylic acid and nano-encapsulated caprylic acid. The effect of caprylic acid and nano-encapsulated caprylic acid on the expression of *EFG1* gene was evaluated, and the results showed a significant decrease in the expression of *EFG1* gene in *C. albicans* treated with nano-encapsulated caprylic acid compared to the caprylic acid-treated and control groups. Gene expression analysis showed that treatment of *C. albicans* with caprylic acid and nano-encapsulated caprylic

acid significantly decreased *EFG1* gene expression compared to the non-treated group ($p < .01$) (Figure 1).

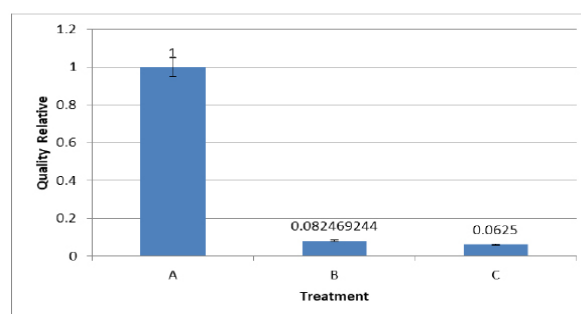


Figure 1) *C. albicans* *EFG1* gene expression before and after treatment with MIC values of caprylic acid and nano-encapsulated caprylic acid. Act1 was used as internal control. A: non-treated sample; B: caprylic acid treated group; C: nano-encapsulated caprylic acid treated group.

Cytotoxicity Assay: The cytotoxicity effect of caprylic acid and nano-encapsulated caprylic acid was examined on SW480 cell line using MTT test. Cells treated with caprylic acid at 450 $\mu\text{g/mL}$ concentration showed higher viability compared to those treated with nano-encapsulated caprylic acid at 3.1 $\mu\text{g/mL}$, and this difference was significant (p value = .15, Figure 2). The results showed that cells treated with caprylic acid represented higher viability than those treated with nano encapsulated caprylic acid.

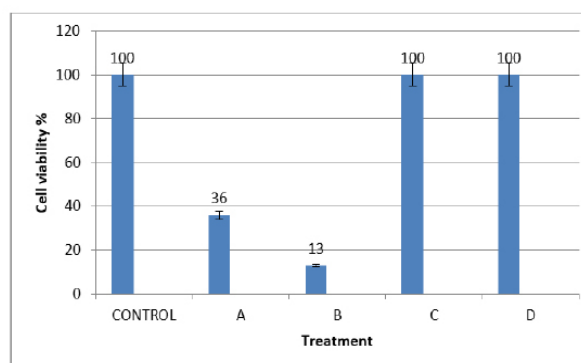


Figure 2) Comparison of the viability of SW480 cell line treated with different concentrations of caprylic acid. A: nano-encapsulated caprylic acid at 3.1 $\mu\text{g/mL}$, B: nano-encapsulated caprylic acid at 6.2 $\mu\text{g/mL}$, C: caprylic acid at 500 $\mu\text{g/mL}$, D: caprylic acid at 450 $\mu\text{g/mL}$

Table 1) Primer sequences used in this study for real-time PCR assay

Primer Name	Sequence (5' to 3')	PCR Product Size(bp)
EFG1-F EFG1-R	TATGCCCCAGCAAAGTCTG TTGTTGTCCTGCTGTCTGTC	140
ACT1-F ACT1-R	TGCCTCTGGTCGTACCACTG GCGAAACCTTCGTAGATGGG	110

Table 2) The MIC and MFC values of caprylic acid and nano-encapsulated caprylic acid against *C. albicans*.

Substance	MFC (µg/mL)	MIC ₉₀ (µg/mL)	MIC ₅₀ (µg/mL)
Caprylic acid	600	550	450
Nanocapsulated caprylic acid	12.5	6.25	3.1

Discussion

In the present study, the antifungal activity of caprylic acid and nano-encapsulated caprylic acid as well as their effects on the expression of *EFG1* gene of *C. albicans* were investigated. The obtained results showed that caprylic acid at MIC₅₀ value exhibited antifungal effect on *C. albicans* (ATCC10231). Also, nano-encapsulated caprylic acid at identified concentration inhibited the growth of *C. albicans* and the expression of *EFG1* gene. These findings demonstrated that both nano-encapsulated caprylic acid and caprylic acid were effective against *C. albicans* biofilm formation. Candidiasis still remains as one of the most important opportunistic fungal infections in immunocompromised patients worldwide [1]. Multidrug resistance in *Candida* strains against common antifungal agents has been reported to be increasing. However, the existing antifungal agents are widely used in the treatment of fungal infection [3-6]. Nowadays, the use of new drug delivery strategies to deliver antifungal agents in the treatment of fungal infections has received much attention. Therefore, the effectiveness of natural-based antifungal agents would be increased by employing new drug delivery systems, such as nano-encapsulated system [23-25].

Fatty acids penetrate the cell membrane of fungi, thereby leading to the disruption of cell membrane integrity [15]. Previous studies have confirmed the antimicrobial effect of fatty acids; in a study conducted by Avis et al. (2001), medium-chain fatty acids were shown to induce membrane dysfunction and cell death [26]. Similarly, Bergson et al. (2001) indicated caprylic acid antifungal properties [27]. Takahashi et al. (2012) showed caprylic acid inhibitory activity against *C. albicans* growth and its effectiveness in the treatment of oral candidiasis in rats [28]. Therefore, in this study for the first time, nano-encapsulated caprylic acid was designed and synthesized to investigate its antifungal properties. These findings revealed that the use of nanostructure caused caprylic acid to inhibit *Candida* growth at a lower concentration than caprylic acid without a nanostructure; in addition, nano-encapsulation improved the antifungal activity of caprylic acid. Therefore, the design of drug delivery systems based on nano compounds has several advantages as follows: overcoming defects in pharmaceutical formulation, increasing drug retention time at the desired site, decreasing drug administration time, and uniform drug distribution [29]. On the other hand, according to the cytotoxicity examination results, caprylic acid at MIC₅₀

value showed the lowest cytotoxicity effect against SW480 cell line; however, at higher concentrations the viability of cells decreased; therefore, it acted in a dose-dependent manner.

Interestingly, the viability of cells in the presence of nano-encapsulated caprylic acid decreased compared to caprylic acid. In a previous study conducted by Carballeira et al, (2008), caprylic acid at different concentrations showed no cytotoxicity effect on the epithelium cell line ^[16]. As a result, the form of nanocapsulation probably has a toxic effect on the cell line. In this regard, the silica used in the structure of nanocapsule in combination with caprylic acid is likely to induce a toxic effect.

According to the obtained findings, nano-encapsulated caprylic acid significantly down regulated *C. albicans EFG1* gene expression. It is well-known that *EFG1* gene is a major transcription factor responsible for pathogenesis as well as transformation of yeast to hyphae; adhesion and hyphae formation are the most important virulence factors of *C. albicans* ^[30].

A previous study explained that in mutant strains of *C. albicans*, the pathogenicity and tissue invasion significantly diminished through defects in *EFG1* gene expression. Overall, it seems that the design of drug delivery systems based on nanostructure increases the efficacy of drugs while reducing their dose. Also, it could reduce the pathogenicity of *C. albicans* by inhibiting the related genes involved in the progression of *Candida* infections. In any case, in addition to the drug efficacy and drug delivery system, it is necessary to pay attention to the side effects of the strategy used, including nano-carriers, which are interestingly investigated today.

Conclusion

The present study showed that caprylic acid

and nano-encapsulated caprylic acid might be mentioned as efficient agents against candidiasis. Hence, it is suggested that more comprehensive studies be conducted on other natural antifungal agents and drug delivery strategies that have antifungal properties to fight against fungal infections.

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Ethical Permissions: This study was approved by Ethics Committee of Tarbiat Modares University, Tehran, Iran (IR.TUM.REC.1394.207).

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Authors' Contribution: Conceptualization: RZ; Data curation and formal analysis: RZ, FN; Investigation: RZ; Methodology and project administration: SHR; Supervision: SHR; Validation: MR; Writing of original draft: RZ; Writing, reviewing, and editing: FN,PA,SY.

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