Original Article

Effects of *Thymus daenensis* Essential Oil-loaded chitosan Nanoparticles on BCR1 Gene Expression in *Candida Parapsilosis*

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ABSTRACT

Candida parapsilosis is a non-*albicans* species with a high prevalence and potential for nosocomial infections. The BCR1 gene plays a critical role in regulating virulence factors in this species. This study aimed to evaluate the effects of Thymus daenensis essential oil encapsulated in chitosan nanoparticles (TDNs) on the expression of the BCR1 gene in *C. parapsilosis* isolates from animal and human sources. Sixty *C. parapsilosis* isolates (30 from human and 30 from veterinary sources) were screened for the presence of the BCR1 gene via PCR. The TDNs were synthesized and characterized using various techniques. The isolates carrying the BCR1 gene were treated with TDNs to determine the minimum inhibitory concentration (MIC). The expression of the BCR1 gene after treatment with sub-MIC concentrations of TDNs was measured by real-time PCR and compared with the control group. The results were statistically analyzed. Five out of 60 isolates (8.33%) tested positive for the BCR1 gene. The physical properties of TDNs showed that they had a spherical shape, an average size of 92.3 nm, a polydispersity index of 0.129±0.03, a zeta potential of +48.3 mV, and an encapsulation efficiency of 88.6 ± 0.2%. The MIC range for TDNs in these isolates was 0.032-1 µg/ml. Treatment with TDNs significantly reduced the expression of the BCR1 gene in all five isolates compared with the control group ($p=0.012$). TDN has substantial potential for inhibiting the expression of the BCR1 gene, associated with virulence in *C. parapsilosis*. This may enhance the antifungal activity of TDN and reduce the risk of nosocomial infections caused by this species.

Keywords: *Candida parapsilosis*, BCR1, Thymus Daenensis, Nanoparticles, Gene Expression

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1. Introduction

Over the past two decades, the incidence of non-albicans Candida species has increased.Firstly, unlike *Candida albicans*, its transmission route is horizontal, and pre-colonization is not essential.Secondly, its reduced sensitivity to echinocandins, coupled with resistance to azoles, complicates the identification of effective antifungal medications (1, 2). Finally, its ability to rapidly proliferate and form robust biofilms on medical devices, such as urinary and vascular catheters, has resulted in severe invasive bloodstream infections in low-birth-weight newborns and patients receiving intravenous nutrition in intensive care units (ICUs) (3). Numerous reports have documented its significant incidence across various geographical regions, and it is now recognized as one of the most commonly isolated *Candida* species in ICUs (3). In a study of patients with Candida bloodstream infections, 23% were found to be infected with isolates of the C. parapsilosis complex, making it the second most frequently isolated yeast after C. albicans (4). The recent rise in antifungal resistance underscores the necessity for continuous surveillance of antifungal resistance among Candida species. One potential strategy for addressing the challenges posed by antifungal resistance in *Candida* species is to target specific genes involved in their pathogenicity (5). The formation of biofilms is a crucial virulence factor for several Candida species, including C. parapsilosis. Biofilms provide substantial tolerance to antifungal agents and protect yeast cells from the host's immune responses, rendering them a source of persistent infections. BCR1 (Biofilm and Cell wall Regulator 1) is a fungal transcription factor essential for biofilm formation in both C. albicans and C. parapsilosis (5). The transcribed protein targets genes that encode adhesins and cell wall proteins in Candida species, indicating its involvement in the initial adhesion stage of biofilm development, the production of extended pseudohyphae, the regulation of gene expression of other adhesin genes, and the CFEM (common in fungal extracellular membranes) family of proteins, which are involved in iron absorption as a critical virulence factor.Additionally, studies have demonstrated that deletion mutations of the BCR1 gene result in increased susceptibility to antifungal drugs. It appears that the BCR1 gene may represent an Achilles' heel for Candida species, including C. parapsilosis (6). Emergence antimicrobial resistance prompted exploration alternative solutions; green nanoparticles recently gained popularity among researchers as means combat drug resistance. Prior studies indicate green-synthesized nanoparticles potential antifungal agents, but further research needed determine impact green synthesized nanoparticles fungal species. Thyme, traditional herbal medicine, reported significant antimicrobial effects. T. daenensis, endemic Iran, studied various medicinal applications. BCR1 gene crucial role virulence; study aims evaluate effects T. daenensis essential oil encapsulated chitosan nanoparticles (TDN) on expression BCR1 gene C. parapsilosis isolates animal human sources. This study employs thyme-derived T. daenensis essential oil encapsulated chitosan nanoparticles, targeting the BCR1 gene in Cryptococcus parapsilosis isolates from humans and animals. By uncovering the interaction between TDN and a crucial virulence factor, we aspire to introduce innovative antifungal strategies in healthcare.

2. Materials and Methods

2.1. *C. Parapsilosis* Isolates

In this study, we utilized a collection of 60 C. parapsilosis isolates sourced from the Pasargad laboratory in Tehran, Iran. These isolates were obtained from human and animal sources (30 isolates each). These samples were collected by physicians or veterinarians in compliance with established bioethical guidelines (IR.IAU.QODS.REC.1401.001). The isolates were cultured on Potato-dextrose agar (PDA) (Himedia, India) supplemented with 50 mg of chloramphenicol and incubated at 30°C for 48-72 hours. After being cultured on Potato-dextrose agar, the fresh and pure colonies were transferred to Candida CHROMagar medium for additional phenotypic confirmation tests. The plates were subsequently incubated at 30°C for 48 hours, and the colony color was examined to confirm the isolates and differentiate them. Tryptic Soy Broth (TSB) supplemented with 10% glycerol was used to store the isolates at -80°C for future investigations.

2.2. DNA Extraction and Molecular Detection of the BCR1 Gene

The BCR1 gene in C.parasilopsis was identified by extracting genomic DNA from pure colonies of Candida fungus using the phenol-chloroform method. DNA quantity and quality were assessed using the OD 260/280 ratio (Nanodrop) and agarose gel electrophoresis, respectively. Primers specific to the BCR1 gene in C. parasilopsis had the forward and reverse sequences: 5'-CCATTAACCGGGTTGCTATT3'- and 5'-GAGTCCGTTATCGCCAATGT-3'. The resulting amplicon had a size of 177 bp. A pair of specific primers was designed using the NCBI website's Pickprimer tool based on the highly conserved sequences previously reported for C. parapsilosis. The reaction used 20 µL of AccuPower® PCR Premix (Bioneer) and 0.2 µL (10 ng) of DNA template with each primer and other required reagents for the PCR. The PCR thermal program consisted of a 3-minute denaturation step at 95°C followed by 35 cycles of 30-second denaturation at 95°C, 30-second annealing at 52°C, 30-second elongation at 72°C, and a 5-minute final extension at 72°C. Each reaction containing 20 µL of AccuPower® PCR Premix (Bioneer) and 0.2 µL (10 ng) of DNA template also used PCR-specific positive and negative controls. Electrophoresis analysis was conducted on all 60 isolates for the presence of BCR1 gene.

2.3. Preparation and Physicochemical Characterization of TDNs

According to previous studies, T. daenensis essential oil encapsulated in chitosan nanoparticles was prepared by extracting the essential oil from thyme plants collected in the Dena region of the Zagros Mountains, Iran, during the spring. The extract was obtained by the percolation method, and Hamouda et al.'s (10) protocol was followed for creating thyme nano-emulsion essential oil, followed by the addition of oil phase components from Sigma-Aldrich, France. The ionic gelation method, described in a previous study (11), was utilized to prepare chitosan nanoparticles containing T. daenensis essential oil. For this process, 1 g of low molecular weight chitosan (Merck, Germany) was dissolved in a 50 mL solution of 1% acetic acid, which was subsequently stirred magnetically at 100 rpm for 5 hours at a temperature of 25°C. Following this, 0.5 g of T. daenensis essential oil was mixed with this chitosan solution for 60 minutes. Upon completion of the ionic gelation process, the resulting chitosan nanoparticles containing T. daenensis essential oil were subjected to centrifugation for 15 minutes to separate the supernatant. The nanoparticles were then dried at 40°C. Their characteristics were analyzed through scanning electron microscopy (SEM), and further parameters such as surface charge, size distribution, scattering index (PDI), and average particle size were determined using the dynamic light scattering (DLS) technique. Furthermore, the encapsulation efficiency was assessed by employing UV-Vis-NIR spectroscopy.

2.4. Minimum Inhibitory Concentration (MIC) assessment of TDNs

In this study, the Minimum Inhibitory Concentration (MIC) of TDNs was assessed for 5 Candida isolates, which had the BCR1 gene, via the microdilution broth method. TDNs with a concentration of 1 microliter per milliliter (mL) were diluted with distilled water to concentrations ranging from 0.032 to 32 microliters per milliliter (µL/mL). For the evaluation, a standard concentration of 1.5 x 108 CFU/mL of Candida suspension was added to each well of the microplate, followed by adding 100 µL of the respective dilutions of the TDNs, 100 µL of RPMI1640 media (Gibco, USA) and 100 µL of sterile media (without loaded chitosan nanoparticles) in control wells. After 48 hours of incubation at 35°C, the turbidity of the wells was measured at a wavelength of 540 nm and the lowest concentration that exhibited fungistatic activity was deemed the MIC for that isolate and was reported in micrograms per milliliter.

2.5. Real-Time PCR

In this study, RNA was extracted from isolates treated with a sub-MIC concentration of thyme nanoparticles using an RNA extraction kit (Sinaclon, Iran) according to the manufacturer's instructions. Following the removal of DNA contamination, the purity of the extracted RNA was assessed. Complementary DNA (cDNA) was then synthesized using a cDNA synthesis kit (Sinaclon, Iran) following the provided guidelines. The beta-actin gene (ACT1) was used as a reference gene to normalize the results. Real-time PCR was performed using the Bioneer Exicycler 96 (South Korea) with a total reaction volume of 25µL. The reaction mixture comprised 12.5µL of 2X master mix, 10µL of SYBR Green I (Genet Bio, USA), 5 pmol of each primer (final concentration of 1µM), 9.5µL of RNase-free water, and 100ng of sample cDNA. The PCR conditions were as follows: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. All qPCR assays were conducted in duplicate and included standard curves and controls.

2.6. Statistics Analysis

Data analysis was conducted using REST 2009 and SPSS version 16 software. Results were analyzed using one-way ANOVA, and differences in target gene expression between control and treated samples were assessed using Tukey's HSD post hoc test. Data are presented as mean ± standard deviation (SD), and a p-value of < 0.05 was considered statistically significant. Real-time PCR data analysis was based on the comparison of threshold cycles, with the difference between the threshold cycles of treated and control samples calculated.

3. Results

3.1. Candida Isolates and Detection of the BCR1 Gene

Culturing isolates on Candida CHROMagar medium, as a phenotypic test, confirmed that all isolates were *Candida parapsilosis*. PCR testing with the specified primers revealed that the frequency of the BCR1 gene in this population was 8.33% (5 isolates), all of which were derived from human sources. The amplicon size of this gene, amplified with the specified primer, was 177bp. In the subsequent step, these five isolates were selected to evaluate the effects of thyme nanoparticles on the expression of this gene.

3.2. Characterization of Synthesized TDNs

Examination of the data obtained from the study of physical properties revealed that the size of *T. daenensis* encapsulated in chitosan nanoparticles ranged from 58.1 nm to 110.12 nm, with an average size of 92.3 nm. The polydispersity index (PDI) of these nanoparticles was 0.129 ± 0.03. The zeta potential of the nanoparticles measured +48.3 mV (Figure 1). The encapsulation efficiency of the nanoparticles was 88.6 ± 0.2%. Scanning electron microscopy (SEM) images confirmed that the nanoparticles were successfully synthesized and exhibited a spherical shape.

3.3. Minimum Inhibitory Concentration of TDNs

To evaluate the antimicrobial activity of *T. daenensis* essential oil nanoencapsulated in chitosan nanoparticles, we employed the broth dilution method using various concentrations of the oil, ranging from 0.032 to 32 µg/mL. The results demonstrated that the minimum inhibitory concentration (MIC) varied from 0.032 to 1 µg/mL (Table 1). In contrast, the control group exhibited a high and consistent growth rate throughout the experiment.

3.4. Gene expression

We employed the Real-Time PCR method to measure the relative expression of the BCR1 gene in *C. parapsilosis* isolates. Complementary DNA (cDNA) was synthesized, and a Real-Time PCR reaction was performed using

BCR1-specific primers, with ACT1 serving as the reference gene. The specificity of the amplification was confirmed by analyzing the melting curve for each gene. The BCR1 gene exhibited a melting temperature of 84.25°C, while the ACT1 gene had a melting temperature of 84.90°C (Figure 2). The results indicated that the threshold cycle (Ct) value of the BCR1 gene increased following exposure to a sub-MIC concentration of synthesized nanoparticles in all five isolates, suggesting a decrease in its expression level relative to the reference gene (ACT1) (Table 2). The bar chart in Figure 3 illustrates that this reduction in gene expression was statistically significant (p = 0.012).

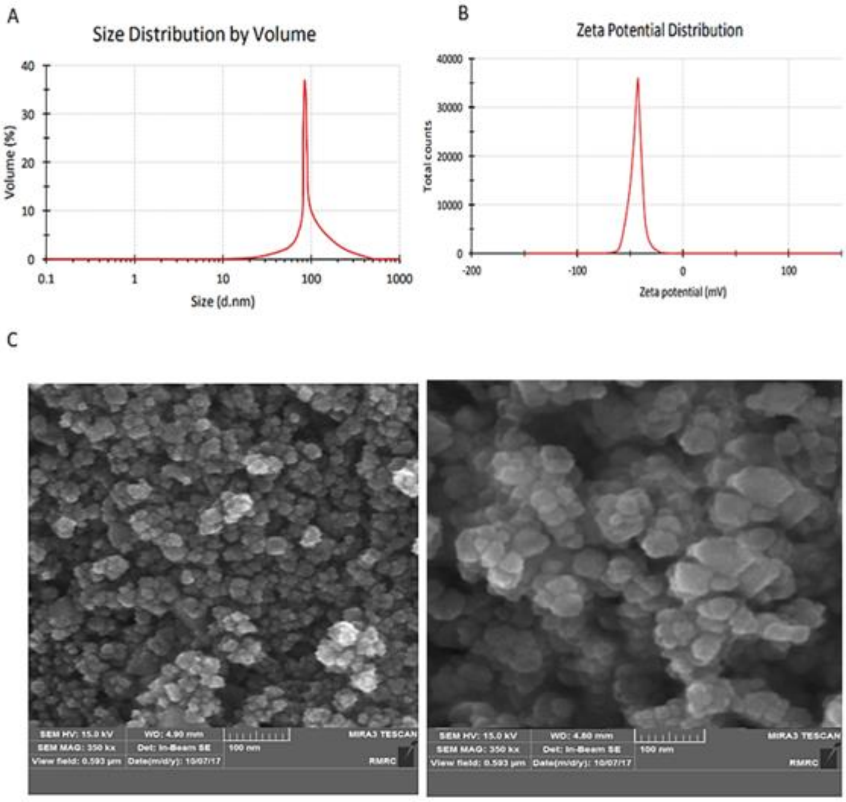


Figure 1. Characterization of the prepared nanoparticles. Particle size (A), zeta potential distribution (B), and scanning electron microscope image (C) of T. daenensis essential oil nanoencapsulated in chitosan nanoparticles.

Table 1: Minimum inhibitory concentration of T. daenensis essential oil nanoencapsulated in chitosan nanoparticles against *Candida parapsilosis* species.

Strain code	**Minimum inhibitory concentration (µg/ml)**
Candida parapsilosis 7	1
Candida parapsilosis 11	0.25
Candida parapsilosis 16	1
Candida parapsilosis 52	0.032
Candida parapsilosis 58	0.25

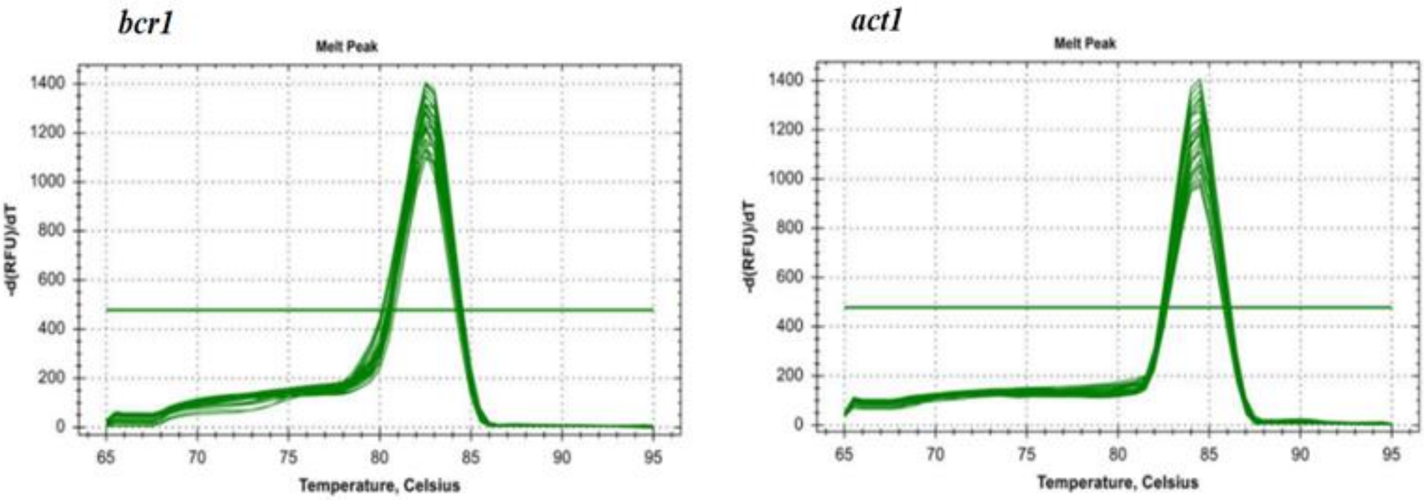
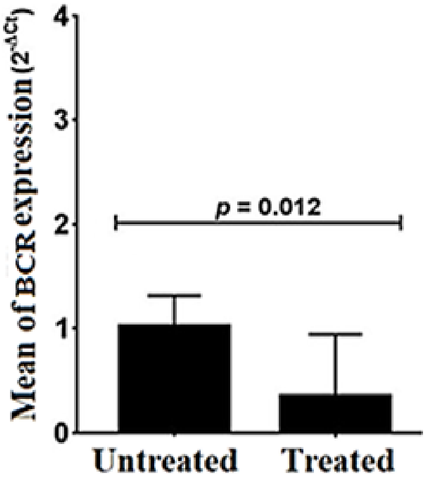


Figure 2. Melting curve results of Real-Time PCR for *BCR1* and *ACT1* gene

Table 2: Results of *BCR1* gene expression changes compared to *ACT1* in *Candida parapsilosis* strains.

Strain	**Control**		**Treated with TDNs**			**Comparing the results**	**P-value**
	Average ct for *ACT1*	Average ct for *BCR1*	Average ct for *ACT1*	Average ct for *BCR1*	Changes of the BCR1 expression	ACT1 gene expression level	
C. parapsilosis 7	18.8	20.8	19.7	26.7	-0.023	1	0.02
C. parapsilosis 11	19.5	19.65	20.2	23.6	-0.085	1	0.01
C. parapsilosis 16	19	20.4	20.3	26.95	-0.014	1	0.05
C. parapsilosis 52	18.9	20.55	19.6	25.25	-0.051	1	0.05
C. parapsilosis 58	19.2	18.65	19.8	22.3	-0.109	1	0.00

Figure 3: Expression of BCR1 gene in *Candida parapsilosis* isolate before and after treatment with *T. daenensis* essential oil nanoencapsulated in chitosan nanoparticles.



4. Discussion

Candida parapsilosis is one of the most common Candida species isolated from nosocomial infections, particularly in patients with catheters or prosthetic devices. It differs from *Candida albicans* in terms of virulence, antifungal resistance, and associated risk factors. Therefore, findings from studies on *C. albicans* cannot be easily generalized to other species. *C. parapsilosis* has higher minimum inhibitory concentrations for echinocandins compared to other Candida species and can also develop resistance to azoles (2). Additionally, *C. parapsilosis* can form biofilms, complicating the treatment and management of these infections.Recently, essential oils encapsulated in nanoparticles have been explored as a potential alternative for combating microbial infections. Thyme is a herb widely recognized for its therapeutic properties, and nanoparticles containing its essential oil have demonstrated antimicrobial activity against various pathogens (12). This study presents a novel approach to addressing *Candida parapsilosis*, a rising threat in nosocomial infections, by targeting a key virulence factor, the BCR1 gene. Unlike previous research that focused on inhibiting fungal growth, this work investigates the potential of *Thymus daenensis* essential oil-loaded chitosan nanoparticles to downregulate BCR1 expression, potentially reducing fungal pathogenicity.The use of nanoparticles enhances the delivery and targeting of the essential oil, while the focus on BCR1 represents a new direction in controlling *C. parapsilosis*. This strategy holds promise for developing therapeutic alternatives that address fungal virulence mechanisms, potentially leading to more effective and targeted antifungal therapies. BCR1 is a common gene found in *C. albicans* and *C. parapsilosis* that is involved in biofilm production. Previous studies have identified BCR1, FKS1, and EFG1 as the three genes associated with biofilm formation in both *C. parapsilosis* and *C. albicans* species (13). Further investigations have revealed that BCR1 serves as the key transcription factor that mediates early adhesion in both species. Although some studies have reported that *C. parapsilosis* can form biofilms in a BCR1-dependent or -independent manner, BCR1 still plays other important roles in *C. parapsilosis* (14). Therefore, we hypothesized that targeting the expression of this gene could affect the pathogenicity of Candida species, and we examined the effect of T. daenensis essential oil-loaded chitosan nanoparticles (TDNs) on the expression level of this gene. We found that the BCR1 gene was present in 5 out of 60 (8.33%) isolates, all of which were derived from human sources. This prevalence rate may be related to the genetic diversity within the *C. parapsilosis* complex, which may exhibit different gene contents. Modiri et al. (15) reported that planktonic isolates of *C. parapsilosis* were less likely to form biofilms, which may also depend on their genetic content. However, we had limited information regarding the sources of these isolates, which could influence biofilm formation. Some studies suggest that the site and tissue of infection affect biofilm formation (16). For instance, some researchers argue that isolates from blood are more prone to form biofilms (17). Since our primary goal was to evaluate the effects of TDN on the BCR1 gene, we used these five isolates for this pilot study. However, we recommend conducting epidemiological studies to determine the frequency of virulence genes. In this study, the minimum inhibitory concentration (MIC) of TDN ranged from 0.032 to 32 µg/mL. The real-time PCR results indicated that isolates with higher BCR1 expression exhibited higher MIC values. This finding is consistent with the results of Modiri et al. (15), who reported that isolates with higher BCR1 expression had increased biofilm formation and MIC levels compared to those with lower BCR1 expression. Thymol, a phenolic compound found in the medicinal herbs of the Thymus genus, possesses potent antimicrobial effects. It can inhibit ergosterol biosynthesis in fungi (18), which may also reduce the expression of various virulence genes, including those associated with mycotoxins and drug resistance (19). Anvar et al. (2021) assessed the effects of thyme on the expression of the Nor1 gene in *Aspergillus flavus* and found that thyme essential oil could downregulate this gene. However, their MIC range was from 200 to 400 µg/mL (20), which was significantly higher than ours. This discrepancy may be attributed to the advantageous properties of chitosan nanoparticles. Moazeni et al. used *Thymus vulgaris* essential oil nanoemulsion as an antifungal agent for *C. albicans* and *C. glabrata* isolates, reporting MIC values between 0.031 µg/mL and 0.0625 µg/mL for both species (21), which are very similar to our results. They also applied the same nanoemulsion against *Aspergillus fumigatus* isolates and obtained an MIC of 0.016 µg/mL (21). This suggests that thyme compounds have significant potential as antimicrobial agents, regardless of the target organism. Moreover, utilizing them in nanoparticle form can dramatically enhance their efficacy. Our findings indicated that the average Ct values for the target gene in all five strains significantly increased, suggesting downregulation of the gene after treatment with synthesized TDNs. Compared to the control group, this reduction in gene expression was statistically significant ($p = 0.012$). Furthermore, it is noteworthy that medicinal herbs from different geographical regions may exhibit varying medicinal effects (22). In the current study, we utilized *Thymus* plants from the Dena area in Iran, which demonstrated considerable effectiveness against *C. parapsilosis*. However, we recommend conducting comparative studies between thyme herbs from different parts of the world to determine any significant differences. This study demonstrated that TDNs significantly reduced the expression levels of the BCR1 gene in *C. parapsilosis* strains. The fold change in BCR1 expression ranged from 0.014 to 0.109. Similarly, Erfaninejad et al. reported that zinc oxide nanoparticles drastically decreased the expression levels of the Hwp1 gene in *C. albicans* isolates (23). However, their reduction in gene expression was slightly higher than ours, which may be related to the specific gene and type of nanoparticle used. They suggested that zinc oxide exhibits reactive radical oxygen effects for microbial cytotoxicity. Therefore, we propose using TDN in combination with zinc oxide against Candida isolates to investigate their potential synergistic effects. In conclusion, we tested the antifungal and anti-biofilm effects of *T. daenensis* essential oil nanoencapsulated in chitosan nanoparticles against *C. parapsilosis*. We found that TDN was highly effective in inhibiting the growth of *C. parapsilosis* and reducing BCR1 gene expression, which is involved in biofilm production. TDN may serve as a potential treatment for *C. parapsilosis* infections; however, further studies are needed to elucidate its mechanism of action and assess its safety. Additionally, we recommend studying other virulence genes in the *C. parapsilosis* complex and their responses to TDN.

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Authors' Contribution

Conceptualization, methodology, validation, and formal analysis A.F., Z.H., and M.P.; investigation and resources, data curation, A.F., and M.P.; writing—original draft preparation; writing—review and editing, A.F.; visualization, Z.H; supervision, A.F.; project administration, A.F.; funding acquisition, Z.H.”.

Ethics

The current research study has been subjected to an ethical review and approval process in accordance with the university's guidelines for ethical research practices set forth by the Islamic Azad University, Science and Research Branch. All experimental procedures were conducted in accordance with the utmost respect for the principles of ethical research, with the utmost respect for the welfare and safety of the participants.

Conflict of Interest

The authors certify that they have no competing financial interests or personal relationships that could potentially influence the outcome of this research study. The study was not sponsored by any commercial entity. The authors assert that they have maintain complete independence in their research and conclusions.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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