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ORIGINAL ARTICLE

Inhibitory effect of *Nigella sativa* oil loaded to liposomal nanocarriers on *Candida parapsilosis* isolates

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ABSTRACT

Background and Objectives: *Candida parapsilosis* is the second most common species causing infectious diseases and can lead to biofilm resistance. This study aims to adjust and synthesize a liposomal compound of *Nigella sativa* and evaluate its antifungal properties against *C. parapsilosis* isolates.

Materials and Methods: The liposomal formulation of *N. sativa* was optimized through the utilization of transmission electron microscopy (TEM), particle size analysis, zeta potential measurement, and UV-visible spectrophotometry. Furthermore, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was conducted on peripheral blood mononuclear cells (PBMCs). The antifungal efficacy was evaluated in accordance with the M27-A3 guideline.

Results: The minimum inhibitory concentrations (MICs) of *N. sativa* oil and the liposomal formulation on *C. parapsilosis* isolates ranged from 128 to 8 µg/mL and from 250 to 31.25 µg/mL, respectively. The MIC_{50} and MIC_{90} values of *N. sativa* oil and the liposomal formulation were 125, 187, and 32, 96 µg/mL, respectively. The viability percentage of cells treated with the liposomal formulation and free *N. sativa* oil was 91% and 85%, respectively.

Conclusion: The cytotoxicity of free *N. sativa* was significantly reduced when using nanoliposomes. The liposomal form of *N. sativa* showed greater antifungal properties compared to the free *N. sativa* extract against *C. parapsilosis* isolates.

Keywords: *Candida parapsilosis*; Isolates; Antifungal; Nanoliposomes; *Nigella sativa*

INTRODUCTION

Candida parapsilosis is the most common cause of nosocomial bloodstream infection (BSI) among non-*C. albicans* species (1). It has been found to exhibit widespread resistance to antifungal agents. Recently, there have been reports of drug resistance in different species of *Candida* among hospitalized patients who are receiving continuous antifungal treatment for *Candida* infections (2). The transcription factors BCR1 and EFG1 play a major role in both *C. albicans* and *C. parapsilosis.* In particular, BCR1 is an essential transcription factor in the early stages of adhesion and biofilm formation in both *C. albicans*

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and *C. parapsilosis* (3, 4). The frequency of drug-resistant *Candida* species is increasing, especially in patients affected by non-*albicans Candida* (NAC) species (1, 5, 6). Some species have a high degree of adaptability, which enables them to transition from being commensal organisms to becoming pathogens. This transition is facilitated by various virulence factors, with biofilm formation being particularly significant (7). Currently, *C. parapsilosis* has been recognized as a prevalent fungal pathogen. It possesses various pathogenic factors, including the ability to form biofilms which contribute to internal drug resistance. Additionally, this fungus is inherently resistant to antifungal drugs and demonstrates limited susceptibility to conventional treatments, owing to the presence of biofilm cell wall regulators (8). EFG1 is another transcription factor that is necessary for hyphal growth in the biofilm formation of *C. parapsilosis* (9). Among the countless herbal medicines available, *Nigella sativa*, also known as black seed, is an annual herbaceous plant belonging to the Ranunculaceae family. This miraculous plant has a rich history and religious background, leading many to consider it a herbal drug with vast potential and a wide range of medicinal benefits (10). Liposomes have been utilized to enhance drug absorption, minimize metabolism and toxicity, and extend the biological half-life of drugs. Additionally, they effectively prevent the occurrence of pyrogenic or antigenic reactions (11). Hence, the present study aimed to synthesize and investigate a new combination of nanoliposomes with *N. sativa* against *C. parapsilosis* isolates and evaluate its antifungal properties.

MATERIALS AND METHODS

Ethical approval. The project received financial support from the Faculty of Medical Medicine at Tarbiat Modares University (Date: 2022-05-02, No: 89166). All experiments were conducted according to the regulations and guidelines established by the Biomedical Research Ethics Committee of the Faculty of Medical Medicine at Tarbiat Modares University in Tehran, Iran (IR.MODARES.REC.1401.025).

***C. parapsilosis* and reference isolates.** The materials used in this study were DPPC (Cat. No. 850355P; Alabaster, Alabama, USA), cholesterol (Cat. No. 700100P; Alabaster, Alabama, USA), and *N. sativa* extract (Cat. No. SC-215986; Sigma Chemical Company, Supelco). MOPS (Sigma Chemical Co., St. Louis, USA) was also included. A reference strain of *C. parapsilosis* (ATCC 22019) along with fifteen isolates of *C. parapsilosis* obtained from hospitalized patients affected by candidemia in Mashhad City, Iran, was examined. These clinical isolates were correctly identified using the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) method, as described previously (1).

Preparation and characterization techniques of nanoliposomes. The liposomal formulations were modified, and *N. sativa* liposomes were created using traditional thin-film hydration methods. DPPC, cholesterol, and *N. sativa* were dissolved in a 1:1 mixture of chloroform and methanol. All components, with a drug-to-lipid ratio of 1:10, were placed in a round-bottom flask. The solvents were evaporated with a rotary evaporator to produce a thin lipid film. This dried film was then hydrated with 2 ml of sterile normal saline and subjected to brief sonication. Finally, the best nanoliposomal formulation was selected by considering the characteristics of the lipid structure as a whole, such as size, zeta potential, homogeneity, and polydispersity index (Table 1). Additionally, free *N. sativa* was separated by centrifuging the liposomal formulation at 14,000 rpm. Briefly, a small aliquot (~50 ml) of liposomal *N. sativa* was disrupted in DMSO, and its amount was calculated using the standard curve of the drug. The entrapment efficiency was calculated by measuring the amount of *N. sativa* associated with the liposomes out of the total *N. sativa* originally added to the lipids. In the end stage, the amount of *N. sativa* incorporated in liposomes was estimated by determining its absorbance at 330 nm using a UV-visible spectrophotometer.

% N. Sativa entrapment efficiency = *N. sativa* entrapped in the Liposomes / Total amount of *N. sativa* X 100

Liposomes are considered an effective drug delivery

Table 1. Characterization of the *N. sativa* nanoliposomal formulation.

Test	Average
Zeta average	131 ± 1
Zeta potential (mv)	- 51 ±1.5
Encapsulation efficiency	64 ± 7 %
Poly dispersity index	0.3

system because they have the ability to encapsulate both lipophilic and hydrophilic drugs. Their capacity to interact with cell membranes aids in delivering the contents into cells. *N. sativa*, a lipophilic drug with poor solubility in aqueous solutions, required enhancement for increased effectiveness. To improve the activity of *N. sativa*, a liposomal formulation of the drug was prepared. The *N. sativa*-liposomes were examined using a transmission electron microscope (TEM). The analysis of the nanoliposomes revealed the presence of both multi-lamellar and unilamellar vesicles, consistent with previous findings (12). The lamellarity and size of the liposomes were determined using the negative staining method. A drop of the liposomal preparation was placed on a platform-coated grid for 10-15 minutes. Then, around 10-15 drops of the negative stain (2% uranium acetate at pH 7.0) were flushed over the grid and allowed to dry. The grid was viewed using a transmission electron microscope and images were acquired using a digital camera. The size of the placebo liposomes ranged between 50 and 120 nm, while the *N. sativa*-loaded liposomes showed a slightly larger size (50-200 nm) (12).

Release studies. The release profile of *N. sativa* nanoliposomes was investigated in simulated gastric fluid (SGF) and phosphate saline buffer (PSB) to determine the stability of nanoliposomes in SGF and whole blood cells. To prepare the SGF, a solution of hydrochloric acid (0.2 N, 39 ml) was added to a solution of sodium chloride (0.2 N, 250 ml). Then, 600 ml of deionized water (DW) was added, and the pH was adjusted to 2.2. The final volume was adjusted to 1000 ml with DW. PSB was prepared by dissolving KH_2PO_4 (6.8 g) in 250 ml of DW. This solution was mixed with a solution of NaOH (0.2 N, 77 ml). Subsequently, 600 ml of distilled water (DW) was added, and the pH was adjusted to 6.8. The total volume was then brought to 1000 ml with DW. The nanoliposomes were diluted in simulated gastric fluid (SGF) and phosphate-buffered saline (PBS) at a ratio of 1:10 and placed in an incubator at 37 ± 1.0°C. Samples were collected at various time intervals of 0.5, 1, 2, 4, 6, 12, 24, and 48 hours. To isolate the released nanoliposomes, the samples were filtered through a 0.22 µm microbial filter. The quantification of nanoliposomes in the filtered samples was conducted using spectrophotometry. For the spectrophotometric analysis, 20 µl of nanoliposomes (diluted to a final concentration of 30 to 50 µg/ml) was assessed (Fig. 1).

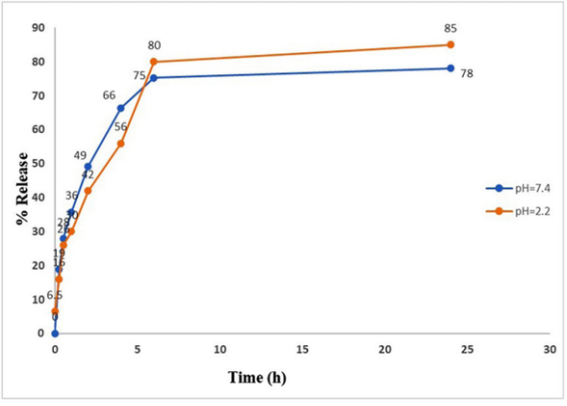

Fig. 1. Release profiles of *N. sativa* in nanoliposomes were investigated in different media: simulated gastric fluid (SGF) with pH 2.2, whole blood cell (WBC), and phosphate buffer saline (PBS) with pH 7.4. The data presented in this study represent the means ± standard deviation (SD) of three independent experiments (n=3). For each experiment, nanoliposomes were diluted 1:10 in the respective media, and samples were taken at time points of 0.5, 1, 2, 4, 6, 12, 24, and 48 hours. The concentration of nanoparticles was determined using spectrophotometry.

Minimum inhibitory concentration (MIC) evaluation using CLSI M27 A3 protocol. The MICs of *N. sativa* and its liposomal formulation were assessed using the broth dilution antifungal susceptibility method, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines, specifically following the M27 A3 protocol. This protocol is a widely accepted method for conducting antifungal susceptibility testing, ensuring standardized procedures for precise determination of MIC values (1, 13). Stock solutions of nanoliposomes were prepared in DMSO and diluted with RPMI 1640 culture medium supplemented with glutamine without bicarbonate buffered to a pH of 7.4 with MOPS (Bio Basic, Canada). The final concentrations of *N. sativa*-Lip-NPS ranged from 0.97 to 500 µg/mL, and free *N. sativa* ranged from 0.5 to 256 µg/mL. Antifungal susceptibility testing was conducted in 96-well micro-titration plates. The *C. parapsilosis* inoculate was prepared in 5% dextrose and diluted in RPMI medium to achieve a final inoculum concentration of 5 × 10^3 cells per ml. The plates were incubated at 37°C for 48-72 h, and the optical density was measured at 530 nm. The MICs were determined as the lowest drug concentrations that resulted in a 50% growth reduction compared to the drug-free growth control (Tables 2 and 3).

Table 2. The MIC results of *N. sativa* on the growth of clinical isolates and reference strain of *C. parapsilosis.*

No	256 µg/ml	128 µg/m	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml	0.5 µg/ml	PC	NC
C1	S	S	S*	R	R	R	R	R	R	R	+	-
C2	S	S	S	S*	R	R	R	R	R	R	+	-
C3	S	S	S*	R	R	R	R	R	R	R	+	-
C4	S	S	S	S	S*	R	R	R	R	R	+	-
C5	S	S	S*	R	R	R	R	R	R	R	+	-
C6	S	S	S	S*	R	R	R	R	R	R	+	-
C7	S	S	S*	R	R	R	R	R	R	R	+	-
C8	S	S	S	S*	R	R	R	R	R	R	+	-
C9	S	S*	R	R	R	R	R	R	R	R	+	-
C10	S	S	S	S	S*	R	R	R	R	R	+	-
C11	S	S	S*	R	R	R	R	R	R	R	+	-
C12	S	S	S	S*	R	R	R	R	R	R	+	-
C13	S	S	S	S	S	S*	R	R	R	R	+	-
C14	S	S	S*	R	R	R	R	R	R	R	+	-
C15	S	S	S	S*	R	R	R	R	R	R	+	-
ATCC 22019	S	S	S	S*	R	R	R	R	R	R	+	-

S, Sensitive; R, Resistance; *, the wells which represents the lowest concentration that inhibited 50% of the *Candida* growth; MIC, minimum inhibitory concentration; PC, positive control; NC, negative control.

Table 3. The minimum inhibitory concentration (MIC) results of the N. sativa liposomal nanoparticles on the growth of clinical isolates and reference strain of *C. parapsilosis.*

No	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml	31.25 µg/ml	15.6 µg/ml	7.81 µg/ml	3.90 µg/ml	1.95 µg/ml	0.97 µg/ml	PC	NC
C1	S	S	S	S*	R	R	R	R	R	R	+	-
C2	S	S*	R	R	R	R	R	R	R	R	+	-
C3	S	S	S*	R	R	R	R	R	R	R	+	-
C4	S	S	S	S*	R	R	R	R	R	R	+	-
C5	S	S	S*	R	R	R	R	R	R	R	+	-
C6	S	S	S	S*	R	R	R	R	R	R	+	-
C7	S	S	S*	R	R	R	R	R	R	R	+	-
C8	S	S	S*	R	R	R	R	R	R	R	+	-
C9	S	S	S	S*	R	R	R	R	R	R	+	-
C10	S	S	S*	R	R	R	R	R	R	R	+	-
C11	S	S	S	S*	R	R	R	R	R	R	+	-
C12	S	S	S	S	S*	R	R	R	R	R	+	-
C13	S	S	S*	R	R	R	R	R	R	R	+	-
C14	S	S*	R	R	R	R	R	R	R	R	+	-
C15	S	S	S	S*	R	R	R	R	R	R	+	-
ATCC 22019	S	S	S	S*	R	R	R	R	R	R	+	-

S, Sensitive; R, Resistance; *, the wells which represents the lowest concentration that inhibited 50% of the *Candida* growth; MIC, minimum inhibitory concentration; PC, positive control; NC, negative control.

Evaluation of PBMC viability to liposomal formulation (MTT assay procedure). In the MTT assay, peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood cells of a healthy human donor. Initially, the cells were cultured in a complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin. Subsequently, the cells were plated in a 96-well microplate and incubated overnight at 37°C with 5% CO_2. Subsequently, cultured cells were exposed to liposomal formulations containing *N. sativa* and free *N. sativa* at concentrations ranging from 50 to 800 µg/ml for 48 hours in triplicate. Following the incubation period, 5 mg/ml of MTT was introduced to each well, and the cells were further incubated for 4 hours. Subsequently, 200 µL of dimethyl sulfoxide (DMSO) was added to each well. After agitation for 45 minutes in the absence of light, the purple coloration of formazan crystals within viable cells was quantified at 560 nm. The calculation for cell viability was determined using the following formula:

Cell viability (%) = Absorbance of the test / Absorbance of the untreated control cells × 100

Statistical analysis. All experiments were analyzed using the Npar Test, and the results were obtained as the average of the measurements. Moreover, the data analysis was performed using SPSS Version 23 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp). The normality of quantitative variables was determined using the Kolmogorov-Smirnov test. Descriptive analysis was expressed as median (interquartile range) or median (standard deviation) for quantitative variables and percent for frequency of qualitative variables. For the comparison of variables between the two groups, the Mann-Whitney test was used for non-normal quantitative variables. The significance level was considered a p-value less than 0.05.

RESULTS

Characterization techniques of NPs. The synthesized *N. sativa*-Lip-NP was approved using TEM, particle size, zeta potential, and UV-Vis. Size measurements were carried out in triplicate, and the average of the recordings was reported (Table 1).

Evaluation of particle size (Zave) and Zeta potential. The average Zave results of *N. sativa*-Lip-NP and the polydispersity index (PDI) range showed a good mean average size and polydispersity in the liposomal formulation (Fig. 2). The mean average of zeta potential with -51 (mv) showed a good negative voltage charge with no agglutination and aggregation of nanoliposomes (Fig. 3).

Encapsulation efficiency (EE%). The encapsulation efficiency of *N. sativa*-Lip-NP was 64 ± 7% (Table 1).

Release profile. The release efficiency of *N. sativa* liposomal nanoparticles in acidic media like gastric fluid was 85%, which is higher than in buffer media (PV < 0.5) (Fig. 2).

The cell viability percentages of cells treated with *N. sativa*-Lip-NPs and free *N. sativa*. The viability percentage results of cells treated with liposomal formulation and free *N. sativa* were 91% and 85%, respectively. Therefore, the liposomal formulation showed low toxic effects and a dose-dependent range on the viability of PBMCs (Fig. 4).

The minimum inhibitory concentrations (MIC) range of free *N. sativa* and liposomal formulation. The MICs of *N. sativa* oil and liposomal formulation on *C. parapsilosis* isolates were found to be 128 to 8 and 250 to 31.25 µg/mL, respectively. The MIC_{50} and MIC_{90} values of *N. sativa* oil and liposomal formulation were also determined to be 125, 187, and 32, 96 µg/mL, respectively. This showed an inhibitory effect on fungal cell growth and significant susceptibility of isolates to the liposomal formulation of *N. sativa* (Tables 2 and 3).

DISCUSSION

Nowadays, the emergence of drug-resistant species has led to the development of new drugs to address this issue. Recently, bloodstream infection (BSI) caused by non-*C. albicans* species, particularly *C. parapsilosis*, has been increasing in frequency (1). This rise in infections has been associated with higher morbidity and mortality rates, posing challenges in the treatment with antifungal agents. Additionally, there has been a significant increase in antifungal resistance among *Candida* isolates, compared to

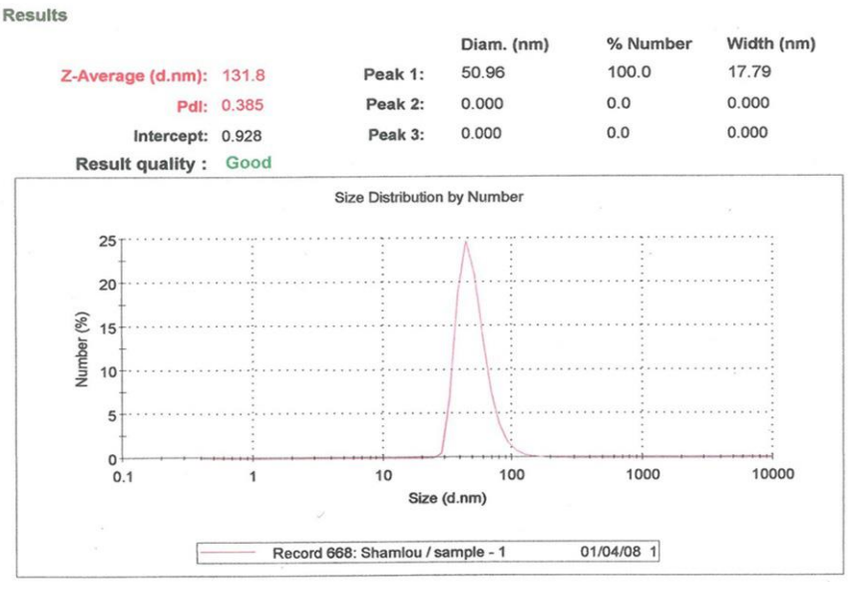

Fig. 2. The average particle size (ZAve) and polydispersity index (PDI) of *N. sativa* liposomal nanoparticles (Ns-Lip-NP)

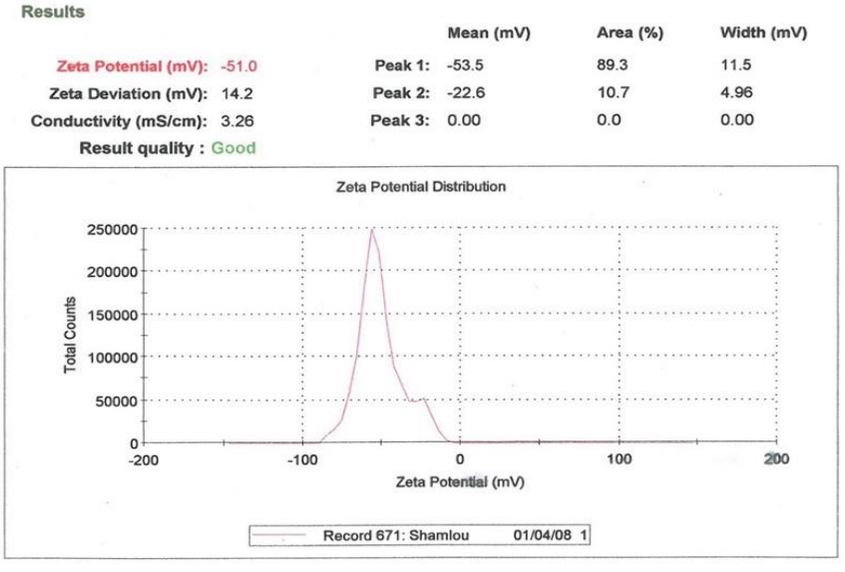

Fig. 3. The average zeta potential of liposomal nanoparticles derived from *N. sativa* (Ns-Lip-NP)

previous studies (14). Recent research has indicated that leveraging nanotechnology can enhance the efficacy of antimicrobial agents. This development holds significant promise, especially in combating drug-resistant strains (15). Furthermore, nanoliposomes can enhance drug delivery by encapsulating drugs and facilitating their entry into cells. This can help mitigate drug resistance. Currently, a wide range of antifungal drugs, including fluconazole, itraconazole, and echinocandins, are available for treating *Candida* infections, both locally and systemically. However, the long-term use of these drugs can lead to toxicity, side effects, and drug resistance, not to mention their high cost. As an alternative, the combination of plant extracts and compounds derived from plants can be effectively employed to address these concerns (16, 17). Black seed oil and its important compounds, particularly thymoquinone (TQ), have inhibitory properties against *Candida* infections and filamentous fungal agents that produce aflatoxins. Consequently, at a concentration of 9 µg/mL, TQ makes peripheral blood macrophage cells 42% viable. In our research, we discovered that a concentration of 12 µg/mL of *N. sativa* oil resulted in periph-

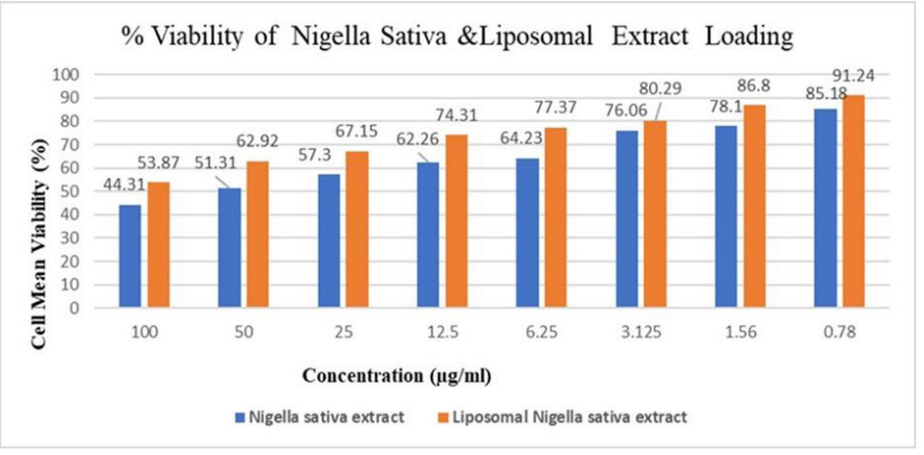

Fig. 4. Assessment of the cytotoxic effects of *N. sativa*'s liposomal formulation and its free compound extract. The viability percentages of PBMCs exposed to *N. sativa*-Lip-NPS and free *N. sativa* during 24 hours of incubation are presented. The viability percentage of cells treated with *N. sativa*-Liposomal nanoparticles and free *N. sativa* was 85% and 91%, respectively. *N. sativa*-Lip-NPS exhibited low toxicity and a dose-dependent effect on the viability of PBMCs.

eral blood cells being 64% viable. This concentration was found to be lower than that reported in the study by Mosbah et al. (18). The aqueous extract of *N. sativa* seeds exhibited an inhibitory effect against candidiasis in the groups of animals that were post-treated with the plant extract. The results were confirmed through histopathological examination of the organs that were examined (19). The inhibitory effect on animals was confirmed by observing its impact on neutrophils and the production of nitric oxide (NO) synthesis in a separate study. In that study, the addition of a polyethylene glycol carrier to TQ increased the viability of macrophage cells by 80%. However, in the present study, the percentage of viable cells reached 90% by adding nanoliposomal carriers. TQ, which is one of the main active components of N. sativa oil, induces the death of fungal cells through the production of oxidants and their agents (20). Black seed plant extracts and their components directly stimulate granulocytes and monocytes, inducing the production of nitric oxide, which creates antifungal properties. Beta-sitosterol and oleic acid, two oils that are the main composition of black seeds, along with long-chain fatty acids, have been found to have antifungal effects against certain strains of *Candida*, including *C. parapsilosis*. Additionally, these black seed compounds show antifungal properties against other *Candida* species (21). TQ with a concentration of 9 µg/mL makes 42% of peripheral blood macrophage cells viable. In our study, a concentration of 12 µg/mL of *N. sativa* oil makes 64% of peripheral blood cells viable. However, TQ alone has no inhibitory effect against *Candida* isolates in systemic infections. This is because its activity will be diminished by serum proteins (22). Therefore, the use of essential oils, such as TQ, as major components of *N. sativa* improves the effectiveness of antifungal agents and reduces antifungal resistance (23). In addition, various strategies are planned to fight infections caused by different microorganisms, including the development of new families or classes of antimicrobial agents (24). Liposomal *N. sativa* is slowly released, with bioactive phytochemical effects and numerous pharmacological therapies, such as its inhibitory properties on *C. parapsilosis* isolates. Importantly, it has no side effects (25). Therefore, the use of herbal agents as medical aids in treating *Candida* infections, particularly against common antifungal drugs like azoles, has been explored. This is due to the active role of liposomal compounds in enhancing the chemical and physical properties of these agents. In the conducted assays, *N. sativa*-Lip-NPs demonstrated favorable antifungal effects on *C. parapsilosis*, indicating their potential as a viable option for the development of new antifungal medications. However, it is important to note that this study had some limitations. These included a few clinical isolates of *C. parapsilosis* and a limited focus on related *Candida* species. Therefore, further studies are needed, particularly with a larger number of clinical isolates, including other non-*C. albicans* more prevalent species.

CONCLUSION

The results showed that the cytotoxicity of free *N. sativa* was significantly decreased when using nanoliposomes. *N. sativa*-Lip-NPs were found to be more biocompatible, safer, and more effective compared to the free *N. sativa* extract. Therefore, they could serve as a suitable alternative to azole drugs, which often come with numerous side effects. Nonetheless, additional *in vitro* and *in vivo* studies are required to assess their efficacy.

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