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RESEARCH ARTICLE

Development and *In Vitro* Assessment of Cumin Essential Oil Nanoemulsion and Nanoemulgel Formulations for Vaginal Candidiasis

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SUMMARY

Vaginal candidiasis is a common mucosal infection caused by various Candida species, primarily Candida albicans. Increased drug resistance to current antifungal treatments, high recurrence rates, and local side effects necessitate the development of new and effective carrier systems. This study aimed to optimize nanoemulsions containing cumin (Cuminum cyminum) essential oil using a Taguchi Design of Experiments and to convert them into nanoemulgel dosage forms using alginate and carboxymethyl cellulose for the treatment of vaginal candidiasis. The chemical composition of cumin essential oil was determined by GC-FID/MS. Optimization of the nanoemulsions was carried out by targeting droplet size and polydispersity index. The nanoemulsions were additionally characterized by determining zeta potential, pH, and conductivity, and their physical stability was assessed over 30 days. The rheological behavior, spreadability, and ex vivo bioadhesion properties were also investigated. The in vitro antifungal activities of the formulations against Candida species were evaluated by determining their minimum inhibitory concentrations using the microbroth dilution method, and cytotoxicity on Vero cells was also assessed. The results showed that the optimum nanoemulsion exhibited a mean droplet size of 11 nm, a low PDI value, and good physical stability. Nanoemulgels containing 1.5% carboxymethyl cellulose exhibited better spreadability, whereas nanoemulgels containing 4.5% alginate exhibited higher bioadhesion. The nanoemulsion and nanoemulgel formulations were effective against all Candida strains. The toxicity of the nanoemulsion and nanoemulgel formulations was found to be significantly lower compared to free cumin essential oil ($p < 0.001$). In conclusion, the developed cumin essential oil-containing nanoemulsion and nanoemulgel formulations can be considered as promising and safe pharmaceutical candidates for the topical treatment of vaginal candidiasis.

Keywords: *Cumin essential oil, nanoemulsion, nanoemulgel, Taguchi Design of Experiments, vaginal candidiasis.*

Vajinal Kandidiyazis İçin Kimyon Uçucu Yağı Nanoemülsiyon ve Nanoemüljel Formülasyonlarının Geliştirilmesi ve İn Vitro Değerlendirilmesi

ÖZ

Vajinal kandidiyazis, başta Candida albicans olmak üzere çeşitli Candida türlerinin neden olduğu yaygın bir mukoza enfeksiyonudur. Mevcut antifungal tedavilere karşı artan ilaç direnci, yüksek tekrarlama oranları ve lokal yan etkiler, yeni ve etkili taşıyıcı sistemlerin geliştirilmesini gerektirmektedir. Bu çalışma, Taguchi Deney Tasarımı kullanılarak kimyon (Cuminum cyminum) uçucu yağı içeren nanoemülsiyonların optimize edilmesini ve vajinal kandidiyazis tedavisi için aljinat ve karboksimetil selüloz kullanılarak nanoemüljel dozaj formlarına dönüştürülmesini amaçlamıştır. Kimyon uçucu yağının kimyasal bileşimi GC-FID/MS ile belirlenmiştir. Nanoemülsiyonların optimizasyonu, damlacık boyutu ve polidispersite indeksi hedeflenerek gerçekleştirilmiştir. Nanoemülsiyonlar ayrıca zeta potansiyeli, pH ve iletkenlik belirlenerek karakterize edilmiş ve fiziksel stabiliteleri 30 gün boyunca değerlendirilmiştir. Reolojik davranış, yayılabilirlik ve ex vivo biyoadezyon özellikleri de araştırılmıştır. Formülasyonların Candida türlerine karşı in vitro antifungal aktiviteleri, mikrodilüsyon yöntemi kullanılarak minimum inhibitör konsantrasyonlarının belirlenmesiyle değerlendirilmiş ve Vero hücreleri üzerindeki sitotoksisite de incelenmiştir. Sonuçlar, optimum nanoemülsiyonun ortalama 11 nm damlacık boyutuna, düşük PDI değerine ve iyi fiziksel stabiliteye sahip olduğunu göstermiştir. %1,5 karboksimetil selüloz içeren nanoemüljeller daha iyi yayılabilirlik gösterirken, %4,5 aljinat içeren nanoemüljeller daha yüksek biyoadezyon göstermiştir. Nanoemülsiyon ve nanoemüljel formülasyonları tüm Candida suşlarına karşı etkili olmuştur. Nanoemülsiyon ve nanoemüljel formülasyonlarının toksisitesi, serbest kimyon uçucu yağına kıyasla anlamlı derecede daha düşük bulunmuştur ($p < 0,001$). Sonuç olarak, geliştirilen kimyon uçucu yağı içeren nanoemülsiyon ve nanoemüljel formülasyonları, vajinal kandidiyazisin topikal tedavisi için umut vadeden ve güvenli farmasötik adaylar olarak değerlendirilebilir.

Anahtar Kelimeler: *Kimyon uçucu yağı, nanoemülsiyon, nanoemüljel, Taguchi Deney Tasarımı, vajinal kandidiyazis.*

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INTRODUCTION

Vaginal candidiasis is a common mucosal infection caused by various *Candida* species, mainly *Candida albicans*, and affects a large number of women worldwide at least once in their lives (Sobel, 2016). The infection typically occurs during times of weakened immune response, greatly impacting quality of life with sensations including vaginal discharge, burning, stinging, and irritation. Topical azole derivatives such as clotrimazole and miconazole, along with oral fluconazole, are widely used in treating vaginal candidiasis. However, current antifungal drugs face several limitations. Resistance to azole drugs, the biofilm-forming capacity of *Candida* species, high rates of recurrence after treatment, and side effects like local irritation reduce treatment success. Additionally, the limited effectiveness of existing therapies against certain non-albicans species highlights the need for new, more effective antifungal delivery systems (de Cássia Orlandi Sardi et al., 2021).

Plant-derived essential oils (EOs) have recently attracted attention as alternative or complementary strategies to traditional treatments due to their antimicrobial properties (Rasheed et al., 2023). *Cuminum cyminum* (cumin) EO, thanks to its primary component, cuminaldehyde, exerts its antifungal effect by disrupting membrane integrity, which leads to the loss of ion balance, generation of cellular apoptosis, and reactive oxygen species (ROS) (Behbahani et al., 2019). The main limitations of EOs in clinical use include low water solubility, volatility, physicochemical instability, and poor penetration into mucosal surfaces (Cimino et al., 2021). However, despite their promising biological activities, the clinical translation of essential oils remains limited due to their complex multicomponent nature (Garcia et al., 2022), which complicates their physicochemical characterization and evaluation of release and permeation behavior. Therefore, the development of alternative formulation strategies focusing on stability and local efficacy is of particular importance.

Nanoemulsions are colloidal systems with droplet sizes ranging from 10 to 200 nm, offering benefits such as high surface area, enhanced solubility, increased bioavailability, and protection of the active compound from environmental factors (Kumar et al., 2019). Nanoemulgel systems, meanwhile, combine a nanoemulsion with a gel matrix, providing both mucosal adhesion and ease of application, making them a suitable pharmaceutical carrier for the mucosal area (Donthi et al., 2023). In this context, formulating herbal antifungal agents with nanocarriers presents a promising approach to improve efficacy and reduce systemic side effects. In vaginal drug delivery, nanoemulsion and nanoemulgel systems offer additional advantages by enhancing the distribution of active compounds over the mucosal surface and promoting closer contact with fungal cells at the nanoscale, which may improve antifungal efficacy (Basu et al., 2025; Vanić et al., 2021). Furthermore, nanoemulgels provide increased mucosal residence time through bioadhesion, which is essential for the effective local treatment of vaginal infections (Campaña-Seoane et al., 2019).

Taguchi Design of Experiments allows for systematic determination of the most effective parameters while reducing the number of experiments in formulation development (Fukuda et al., 2018). Compared to conventional experimental approaches, the Taguchi method provides an efficient and robust statistical framework that enables the evaluation of multiple formulation variables with a reduced number of experimental runs, thereby saving time and resources (Hisam et al., 2024). This approach is particularly advantageous for complex systems such as nanoemulsions, where multiple formulation parameters simultaneously influence critical quality attributes (Shahavi et al., 2016). Therefore, the Taguchi design was selected for this study to achieve an optimized formulation in a systematic, cost-effective manner. The critical quality attributes of nanoemulsions, such as droplet size and PDI, can be optimized with minimal experimentation (Katata-Seru et al., 2017).

In the literature, cumin EO nanoemulsions were prepared, and their anticancer and antibacterial properties were evaluated (Nirmala et al., 2020). Another study by Mostafa et al. (2015) examined cumin EO nanoemulsions for their antioxidant and hepatoprotective effects in transdermal applications. Additionally, a cumin EO nanoemulgel was formulated and shown to be a potential penetration enhancer to enhance the transdermal bioavailability of drugs (Morteza-Semnani et al., 2022). To date, no research has been published investigating the optimization of cumin EO nanoemulsions using the Taguchi Design of Experiments, nor examining the *in vitro* effects of both nanoemulsion and nanoemulgel formulations on vaginal candidiasis or their cytotoxicity on Vero cells. Moreover, although essential oils have been widely studied for their antimicrobial properties, their formulation into nanoemulsion and nanoemulgel systems specifically for vaginal candidiasis remains largely unexplored (Esmaeili et al., 2025). In particular, no study has combined formulation optimization using a systematic design approach with antifungal and cytotoxicity evaluation of essential oil-based nanoformulations for vaginal application.

In this context, the present study aims to develop, for the first time, nanoemulsion and nanoemulgel formulations of cumin essential oil for the local treatment of vaginal candidiasis. To achieve this, biocompatible and mucoadhesive polymers such as alginate (ALG) and carboxymethyl cellulose (CMC) were selected as gelling agents due to their ability to enhance viscosity and improve retention at the site of application (Osmałek et al., 2021). These systems are designed to improve antifungal activity through increased interaction with fungal cells and reduce the toxicity of the essential oil, as demonstrated on Vero cells representing vaginal mucosal epithelium. Additionally, the incorporation of the nanoemulsion into a gel matrix is expected to improve mucosal bioadhesion and prolong local residence time.

In this study, nanoemulsions containing cumin EO were optimized using a Taguchi Design of Experiments and subsequently formulated into nanoemulgels. The developed formulations were physicochemically characterized, and the flow properties were assessed through rheological analysis, while their spreadability and bioadhesion behavior were evaluated by texture analysis. Additionally, the *in vitro* antifungal activities and cytotoxicity profiles on Vero cells were examined to develop potential nanoemulsion and nanoemulgel systems for use in vaginal candidiasis.

MATERIALS AND METHODS

Materials

Cumin seed EO (hydrodistillation) was obtained from Orlife Global International Trade Company (Istanbul, Türkiye). Capryol® 90 was kindly provided by Gattefossé (Lyon, France). Tween® 20, ALG (15-25 cP, 1% in H_2O), CMC sodium salt (400-800 cP, 2% in H_2O), phosphate buffered saline (PBS, pH 7.2-7.6), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium, morpholine propane sulfonic acid buffer (MOPS), resazurin and fluconazole were supplied by Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Sabouraud dextrose agar (SDA) were obtained from Difco Laboratories (Detroit, MI, USA). Vero CCL-81 cell line, *Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 750, and *Candida parapsilosis* ATCC 22019 were purchased from the American Type Culture Collection (ATCC, USA). A total of five clinical isolates, including three *Candida albicans*, one *Candida glabrata*, and one *Candida tropicalis*, were obtained from the Microbiology Laboratories of Florence Nightingale Hospital (Istanbul, Türkiye). The Vitek®-2 automated identification system and API 20C AUX® yeast identification kit were obtained from BioMérieux (Marcy-l'Étoile, France). The Merck Millipore Milli-Q® ultrapure water system (Merck Millipore, France) provided the distilled water. The remaining chemicals were all analytical grade and used as received.

Gas chromatography-flame ionization detector/mass spectrometry (GC-FID/MS) analysis of cumin EO

The chemical composition of cumin EO was determined by GC-FID/MS with slight modifications from the method of Karik et al. (2021). Before analysis, the EO was diluted to 10% (v/v) in n-hexane. An Agilent gas chromatograph was connected to an Agilent mass-selective detector (Agilent Technologies, CA, USA). A DB-WAX capillary column (60 m, 0.25 mm, 0.25 μm) was used. The oven temperature was set to 220 °C at 10 °C/min, then to 240 °C at 1 °C/min. The carrier gas, helium, was employed at a rate of 1.5 mL/min. A 1 μL sample was injected in split mode with the injector temperature of 250 °C. The mass spectra were recorded in electron ionization mode. The relative composition of the components was expressed as percentage peak areas, and the analysis was considered semi-quantitative.

Preparation and optimization of nanoemulsions by Taguchi Design of Experiments

Optimization of nanoemulsion formulations was conducted using the L9(3^3) Taguchi Design of Experiments in Minitab® 19 (Minitab, LLC, USA) to assess how three independent formulation parameters influence nanoemulsion properties. The study focused on 3 factors: total water content (%), EO fraction in the oil phase (%), and the total oil-to-surfactant ratio. Each factor was tested at three different levels. These factors were selected because they are crucial for determining the physicochemical stability and key properties of nanoemulsions, such as droplet size. The factors and levels used in the Taguchi design are listed in Table 1. Also, L9(3^3) Taguchi experimental design for nanoemulsion formulations is given in Table 2.

Table 1. Factors and levels used in L9(3^3) Taguchi experimental design

Factors	Level 1	Level 2	Level 3
(A) Water (%)	85	90	95
(B) EO fraction in oil phase (%)	10	20	30
(C) Total oil to surfactant ratio	1:19	1:9	1:4

Table 2. L9(3^3) Taguchi experimental design for nanoemulsion formulations

Formulation code	Water (%)	EO fraction in oil phase (%)	Total oil to surfactant ratio
F1	85	10	1:19
F2	85	20	1:9
F3	85	30	1:4
F4	90	10	1:9
F5	90	20	1:4
F6	90	30	1:19
F7	95	10	1:4
F8	95	20	1:19
F9	95	30	1:9

A low-energy spontaneous emulsification method was used to produce nanoemulsions (Roozitalab et al., 2022). The oil and surfactant were first stirred using a magnetic stirrer, then slowly added to the aqueous phase. This method was chosen because EOs are sensitive to heat, so high temperatures or energy were avoided. The oil phase, which included cumin EO and Capryol® 90 as the carrier oil, was mixed with Tween® 20 surfactant at specified ratios on a magnetic stirrer at 1000 rpm for 30 minutes. Distilled water was included gradually, and stirring continued for an additional 10 minutes. The nanoemulsions were then allowed to equilibrate at ambient conditions.

The average droplet size and PDI of the nanoemulsions were measured using dynamic light scattering (DLS) on a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). In the Taguchi experimental design, droplet size (Y_1) and PDI (Y_2) were defined as the response variables, representing critical quality attributes of the nanoemulsion system. To achieve smaller droplet sizes and a narrow size distribution, the "smaller-the-better" quality characteristic was applied. The signal-to-noise (S/N) ratio was calculated for each experimental run, and the main effects of the formulation factors were evaluated to identify the optimal levels. Furthermore, analysis of variance (ANOVA) was performed in Minitab 19 to assess the statistical significance and the contribution of each factor to the variability in the responses. A significance level of $p < 0.05$ was used, and the optimal formulation conditions were determined accordingly.

Characterization of the optimum nanoemulsion

The optimum nanoemulsion formulation was tested for droplet size, PDI, pH, electrical conductivity, and macroscopic appearance. Each analysis was conducted at room temperature (25 ± 1 °C). The average droplet size and PDI of the optimum nanoemulsion were analysed using DLS and Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Measurements were performed at 25 °C using water as the dispersant. Samples were analyzed without dilution to avoid altering the nanoemulsion structure. Each sample was measured in triplicate (n = 3), and the average values were reported. For each measurement, multiple runs (20 runs) were performed to ensure the reliability of the results. A digital pH meter and a conductometer (Mettler Toledo SC S210-Uni, Switzerland) were used to test pH and electrical conductivity. The macroscopic properties were evaluated for color, transparency, and signs of instability, including phase separation, sedimentation, and creaming.

Stability study of the optimum nanoemulsion

The optimum nanoemulsion formulation was first put through centrifugation testing as part of the accelerated stability studies (Vargas-Escobar et al., 2025). 1 mL of the nanoemulsion was placed in a tube and centrifuged at 3000 rpm for 30 minutes at room temperature. The nanoemulsions were then examined for physical instabilities, including phase separation, creaming, sedimentation, and turbidity. Also, the optimum nanoemulsion was stored for 30 days at room temperature (25 ± 2 °C) and in a refrigerator (4 ± 1 °C) to evaluate its long-term stability. Droplet size, PDI, zeta potential, pH, and electrical conductivity were measured at intervals of 0, 15, and 30 days. Additionally, they were evaluated macroscopically for appearance, color, transparency, homogeneity, and potential phase separation.

Furthermore, freeze-thaw stability was evaluated through repeated temperature cycling to assess the resistance of the system to extreme temperature stress. Each cycle consisted of a freezing phase at -18 °C for 20 h followed by a thawing phase at 40 °C for 2 h. The samples were subjected to consecutive 10 cycles (Donsì et al., 2011). After each cycle, the formulations were visually inspected for signs of instability such as phase separation, creaming, or turbidity. In addition, droplet size and PDI were measured to monitor any changes in the physicochemical properties. All measurements were performed in triplicate.

In addition, the thermal stability of the optimum nanoemulsion was evaluated using a heating–cooling (thermal cycling) test to assess stability under moderate temperature fluctuations. The samples were incubated at 30 °C for 8 h, followed by cooling at 4-8 °C for 16 h, constituting one cycle. This cycle was repeated for up to 14 days. Samples were withdrawn at predetermined time intervals (7 and 14 days) and analyzed (Prasetyo et al., 2018). After each interval, the formulations were visually inspected for any signs of instability, and droplet size and PDI were determined. All measurements were performed in triplicate.

Preparation and optimization of nanoemulgels

Nanoemulgel formulations were prepared using the optimized nanoemulsion as the dispersion me-

dium instead of distilled water to ensure uniform distribution of the oil phase within the gel matrix and to enhance formulation stability. Biocompatible and mucoadhesive polymers, ALG and CMC, were employed as gelling agents due to their ability to increase viscosity and improve mucosal retention. ALG was added to the optimal nanoemulsion formulation at 3.0%, 4.5%, and 6.0% (w/w), while CMC was added at 1.0%, 1.5%, and 2.0% (w/w). For the preparation of nanoemulgels, the required amount of polymer was gradually added to the optimized nanoemulsion under continuous stirring to ensure a homogeneous gel structure. The mixture was stirred at 200 rpm at room temperature for 2 hours until complete hydration and uniform gel formation were achieved. Viscosity measurements of the prepared nanoemulgel formulations were conducted to identify the optimal polymer concentrations, based on ease of application and suitable viscosity. A 20 g sample of each formulation was placed in a container and tested using a Brookfield DV2T viscometer (Brookfield, USA) with an S04 spindle at 20 rpm. Measurements were done at 25 ± 2°C and 60 ± 5% relative humidity. These measurements were used for preliminary viscosity evaluation during formulation development. Compatibility between the essential oil and formulation components was assessed based on the absence of any visible instability, such as phase separation, creaming, or precipitation, during preparation and storage. The formulations also exhibited a homogeneous appearance and consistent viscosity, indicating good compatibility between the essential oil and the selected excipients.

Evaluation of rheological properties

The rheological behavior of the optimum nanoemulsion and nanoemulgel formulations was assessed using a Thermo Scientific Haake RheoStress 1 rheometer (Thermo Fisher Scientific, Germany). The rheometer was employed for detailed rheological characterization under controlled shear conditions. Measurements were done using a cone-plate geometry (C35/1° Ti L, 35 mm diameter, 1° cone angle, and 0.053 mm gap). The cone was gradually lowered to contact the sample after approximately 1.0 g of sample was placed on the lower plate at 25.0 ± 0.2°C (Kalami et al., 2023). By controlled shear rates, shear stress (τ, Pa) and viscosity (η, Pa·s) were determined, and the flow curves were used to evaluate shear-thinning or Newtonian flow behavior.

Evaluation of spreadability and *ex vivo* bioadhesion by texture analysis

First, the spreadability properties of the optimum nanoemulgel formulations were assessed using a texture analysis method adapted from Kakuda et al. (2024). A TA.XT Plus Texture Analyzer (Stable Micro Systems, Surrey, UK) and a TTC Spreadability Rig apparatus were used. Tests were done at 25 °C using a test speed of 3.0 mm/s in compression mode. Each nanoemulgel formulation was placed on the rig platform in the appropriate amount, and the spreading test was performed by lowering the conical probe onto the sample. Secondly, an *ex vivo* bioadhesion study was carried out to evaluate the adhesion properties of the optimum nanoemulgel formulations to the vaginal mucosa, using a method modified from the protocol reported by Tomás et al. (2023). Fresh sheep vaginal mucosa, obtained from a local slaughterhouse, was gently washed with isotonic saline before use to preserve surface moisture. The study utilized the TA.XT Plus Texture Analyzer, equipped with a mucoadhesive test holder and a P/10 (10 mm diameter) cylindrical probe. Tests were conducted at 37°C to mimic physiological conditions. After fixing the tissue to the testing platform, the nanoemulgel sample was applied to the underside of the probe. The probe was kept in contact with the mucosa under a force of 0.2 N for 150 seconds, then pulled upward at 1 mm/s. The force-distance curves recorded by the device were analyzed using Exponent Connect software (Stable Micro Systems, UK) to determine the work of shear and work of adhesion values for each nanoemulgel formulation.

Determination of *in vitro* antifungal activities

Minimum inhibitory concentrations (MICs) of

the nanoemulsion and nanoemulgel formulations, and cumin EO were obtained using the microbroth dilution method as outlined by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute (CLSI), 2017, 2024) to evaluate the antifungal activity of the formulations against standard strains and five clinical *Candida* isolates. The clinical isolates were identified using the Vitek®-2 system diagnostic procedure and confirmed with the API 20C AUX® test kit. Blank gel formulations (Blank CMC Gel and Blank ALG Gel), prepared without the nanoemulsion, were used as controls. Fungal inocula were prepared as 0.5 McFarland standards using overnight cultures to achieve a concentration of 1×10^7 colony-forming units (cfu/mL). In a 96-well U-shaped microtiter plate, two-fold serial dilutions were prepared in RPMI-1640 medium buffered to pH 7.0 with MOPS. After that, 50 µL of yeast culture was added to each well, resulting in a final concentration of 5×10^3 cfu/mL. To prevent evaporation, the plates were enclosed in plastic bags and incubated for 24 to 48 hours at 35°C. The colony counts were done using SDA. The lowest concentrations that totally stopped visible growth were known as the MICs. When necessary, resazurin was used as a viability indicator, and the appearance of a pink color was considered indicative of microbial growth. Fluconazole was employed as a reference antifungal for the standardization of the study.

Evaluation of *in vitro* cytotoxicity

To evaluate the *in vitro* cytotoxicity of the nanoemulsion and nanoemulgel formulations, each well of a 96-well flat-bottom microplate was filled with 100 µL of a suspension containing 1×10^5 Vero-CCL-81 cells/mL in RPMI medium with 10% FBS. For 72 hours, the cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. The media was removed from the wells after the test samples had been incubated and serially diluted. As controls, the final two columns of the microplate were filled with medium only. In addition, blank gel formulations without nanoemulsion were also included as controls. The test samples were made as serial dilutions and then incubated for an additional day at 37°C in 5% CO_2. Subsequently, the contents of each well were collected, and the wells were rinsed with PBS. To assess cell viability, each well received MTT solution, which was then incubated for 3 hours at 37°C in 5% CO_2. A BioTek Eon High-Performance Microplate Spectrophotometer (Winooski, VT, USA) was used to measure absorbance at 570 nm. By comparing the absorbance of the treated wells to that of the control group, cell survival was evaluated. Cell viability (%) was calculated using Equation 1, where OD570s in the formula represent the mean optical density of the sample, while OD570c is the mean optical density of the cell control (Catunda et al., 2017):

$$Viability(\%) = 100 \times \frac{OD570s}{OD570c} \quad (1)$$

Statistical analysis

Three experiments were used to average the data, which were then presented as the mean ± standard deviation. Statistical significance was assessed by one-way ANOVA at $p < 0.05$, using GraphPad Prism 7.0a software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

GC-FID/MS analysis of cumin EO

According to GC-FID/MS analysis, the EO contained 93.5% of the identifiable components in total. Cumin aldehyde was identified as the main component of the oil, comprising up to 38.6% of its total composition. The other components were γ-terpinene (16.7%), p-mentha-1,3-dien-7-al (15.2%), p-cymene (12.0%), β-pinene (9.4%), and β-acoradiene (1.6%). This component distribution reveals the characteristic monoterpene and aldehyde-rich chemical structure of cumin EO. The main ingredient in cumin oil that gives it its distinctive aroma and antimicrobial properties is cumin aldehyde (Wanner et al., 2010). The amount found in our study aligns with the cumin aldehyde content reported in the literature, which ranges from 19% to 53% (Baser et al., 1992; Bettaieb et al.,

2011; Wanner et al., 2010). Monoterpene components such as γ-terpinene, p-cymene, and p-mentha-1,3-dien-7-al, which are present in significant amounts in cumin EO, exert effects primarily on oxidative stress and microbial inhibition (Xu et al., 2021). These substances increase the biological activity of cumin EO. Additionally, the presence of components such as β-pinene supports the antimicrobial potential of the EO (da Silva Rivas et al., 2012).

Preparation and optimization of nanoemulsions by Taguchi Design of Experiments

Droplet size (Y1) and PDI (Y2) values for nine nanoemulsion formulations prepared according to the L9(3^3) Taguchi orthogonal design are presented in Table 3. The droplet sizes ranged from 11.23 to 31.21 nm, and the PDI values ranged from 0.19 to 0.68.

Table 3. Responses of cumin EO nanoemulsions

Experimental run number	Responses	
	Y1*	Y2*
1	31.21	0.23
2	11.35	0.35
3	13.29	0.29
4	26.22	0.68
5	11.24	0.35
6	14.14	0.27
7	17.01	0.23
8	14.79	0.38
9	11.82	0.19

* Y1 (droplet size (nm)) and Y2 (PDI).

The main effects plot for S/N ratios is shown in Figure 1. The main effects plots showed that increasing the EO ratio from 10% to 20% significantly increased the S/N ratio, but a slight decrease was observed at the 30% level. Similarly, increasing the total oil-to-surfactant ratio to 1:4 provided the highest S/N value, thereby improving droplet size. These results showed that the EO fraction in the oil phase was the most important parameter for droplet size and distribution. Increasing the EO fraction in the oil phase (%) to 20% level significantly reduced the droplet size. This may be due to the more effective distribution of oil molecules in the interphase and the more efficient organization of the surfactant at the interface. However, the increase in droplet size at the highest level (30%) of the EO fraction in the oil phase (%) may have been due to the increased oil-phase charge, which prevented the surfactant from providing sufficient interfacial stabilization. A 1:4 total oil-to-surfactant ratio with a higher surfactant content reduced interfacial tension more effectively, resulting in smaller and more stable droplets (Barzegar et al., 2018). While the water percentage had the least effect, increasing the water ratio facilitated nanoemulsion formation and provided a more homogeneous distribution. The mean S/N ratios for each factor level are presented in Table 4, highlighting the relative influence of formulation parameters on droplet size and PDI.

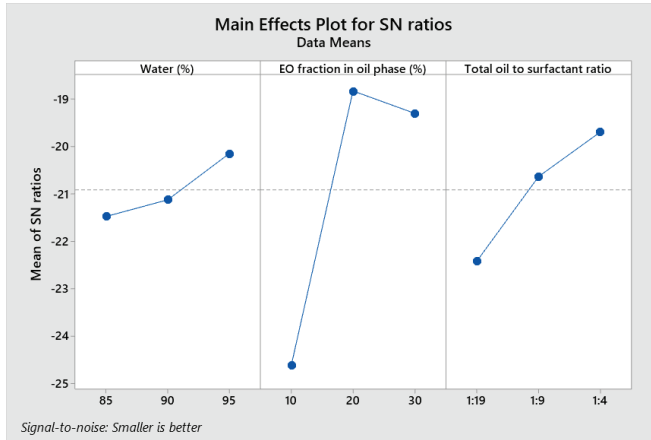


Figure 1. Main effects plot for S/N ratios for cumin EO nanoemulsion droplet size and PDI

Table 4. Mean S/N ratios for each level of formulation parameters for cumin EO nanoemulsion (droplet size and PDI)

Level	Factors		
	Water (%)	**EO fraction in oil phase (%)**	**Total oil to surfactant ratio**
1	-21.48	-24.61	-22.42
2	-21.12	-18.83	-20.63
3	-20.15	-19.30	-19.69
Delta	1.33	5.78	2.73
Rank	3	1	2

Based on the highest S/N ratios, droplet size (Y_1) and PDI (Y_2) were defined as response variables, and the optimal factor combination was determined as A3B2C3, corresponding to 95% water, 20% EO fraction in the oil phase, and a total oil-to-surfactant ratio of 1:4. This optimum nanoemulsion formulation was coded as NE, and the experimentally obtained droplet size (11.68 nm) and PDI (0.24) confirmed the suitability of the design.

The optimization of the nanoemulsion formulation was carried out using the Taguchi L9(3^3) experimental design based on signal-to-noise (S/N) ratio analysis employing the "smaller-the-better" criterion. The predicted S/N ratio for the optimum formulation was calculated using the Taguchi additive model as given in Equation 2:

$$(S/N)\,pred = A3 + B2 + C3 - 2T \quad (2)$$

where A3, B2, and C3 represent the mean S/N ratios at the selected factor levels, and T represents the overall mean S/N ratio. The response values were estimated using the standard S/N relationship (Equation 3.):

$$S/N = -\,20\log(Y) \quad (3)$$

where Y represents the response variable (droplet size or PDI). The experimental results confirmed the suitability of the selected factor combination for improving the formulation characteristics.

The ANOVA results for droplet size and PDI are presented in Table 5. For droplet size, the EO fraction in the oil phase showed the highest contribution to the variability (Adj SS = 290.54), indicating its dominant influence on the response. The remaining factors exhibited comparatively lower contributions. A similar trend was observed for PDI, where the effects of the studied factors were relatively limited. The high coefficient of determination (R^2 = 92.38%) further suggests that the model adequately explains the variability in droplet size.

Table 5. ANOVA results for (a) droplet size and (b) PDI of cumin EO nanoemulsions

(a) Mean droplet size	DF*	Adj SS*	Adj MS*	F-Value*	P-Value*
Water (%)	2	25.65	12.83	0.83	0.55
EO fraction in oil phase (%)	2	290.54	145.27	9.41	0.10
Total oil to surfactant ratio	2	58.14	29.07	1.88	0.35
Error	2	30.88	15.44		
Total	8	405.22			
R^2=92.38%					
(b) Mean PDI	2	0.05	0.02	0.78	0.56
Water (%)	2	0.03	0.01	0.46	0.68
EO fraction in oil phase (%)	2	0.025	0.01	0.39	0.72
Total oil to surfactant ratio	2	0.06	0.03		
Error	8	0.17			
Total	2	0.05	0.02	0.78	0.56
R^2=61.98%					

*DF (Degrees of Freedom), Adj SS (Adjusted Sum of Squares), Adj MS (Adjusted Mean Square), F-Value (F-test statistics), and P-Value (Level of significance)

Characterization of the optimum nanoemulsion

Organoleptic examination revealed that the formulation was transparent and showed no phase separation. The mean droplet size was 11.68 ± 0.08 nm, and the PDI value was within a narrow range of 0.24 ± 0.01. The measurements were repeated under identical conditions to confirm reproducibility. The low zeta potential value (-2.24 ± 0.11 mV) indicates limited electrostatic stabilization. However, the physical stability of the formulation is attributed to steric stabilization provided by the nonionic surfactant (Tween® 20). At the same time, the conductivity value of 0.19 ± 0.001 mS/cm indicated that the continuous phase was water and that the system had a typical O/W nanoemulsion structure. The optimum formulation had a pH of 3.90 ± 0.02. The small droplet size of the optimum nanoemulsion offers a significant advantage by increasing drug diffusion across the vaginal mucosa, as nanometric droplets have a large surface area and enhance mucosal permeability (Smoleński et al., 2021). Also, a pH close to natural vaginal acidity reduces the risk of potential irritation and provides a suitable environment for topical application. The absence of phase separation in the centrifuge test indicated that the nanoemulsion possessed high physical stability. The stability of droplet size, PDI, pH, and conductivity values throughout the storage studies indicated that no coalescence, Ostwald ripening, or intercomponent degradation occurred in the system. Despite the low zeta potential values, which indicate limited electrostatic stabilization, the system remained physically stable. This behavior can be attributed to steric stabilization provided by the nonionic surfactant (Tween® 20), which is known to effectively prevent droplet aggregation (Perugini et al., 2018). All findings demonstrated that the optimum cumin oil nanoemulsion maintained its stability and is a suitable carrier system for subsequent nanoemulgel development.

Stability study of the optimum nanoemulsion

Centrifugation testing revealed that the optimum nanoemulsion showed no phase separation, turbidity, or sedimentation. Pre- and post-centrifugation images (Figure 2.) showed that the formulation maintained its transparency, confirming the resistance of the sys-

tem to centrifugation stress. The droplet size, PDI, and zeta potential values of the nanoemulsion did not exhibit significant changes over time in the long-term stability assessment (Table 6.), which was monitored for 30 days at two distinct storage temperatures (4 °C and 25 °C). The conductivity and pH values of the formulation also did not change during the 30-day storage period; conductivity remained constant at around 0.20 mS/cm, and pH was around 4.0, indicating that the system maintained its stability. Also, there was no phase separation, and the nanoemulsions were transparent in both conditions. All these results indicate that the optimum nanoemulsion exhibits high physical stability under both short-term stress and long-term storage conditions.

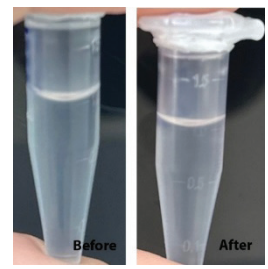


Figure 2. Optimum nanoemulsion appearance before and after centrifugation

Table 6. Long-term stability study results of the optimum nanoemulsion

Parameters	25 ± 2 °C conditions			4 ± 1 °C conditions		
	Beginning	**15th day**	**30th day**	**Beginning**	**15th day**	**30th day**
Organoleptic properties	Transparent and no phase separation	Transparent and no phase separation	Transparent and no phase separation	Transparent and no phase separation	Transparent and no phase separation	Transparent and no phase separation
Droplet Size (nm)	11.47 ± 0.08	11.28 ± 0.46	11.05 ± 0.84	11.47 ± 0.08	11.56 ± 0.11	15.96 ± 0.45
PDI	0.23 ± 0.1	0.26 ± 0.47	0.29 ± 0.10	0.23 ± 0.1	0.29 ± 0.02	0.32 ± 0.26
Zeta potential (mV)	-2.42 ± 0.75	-2.26 ± 0.23	-1.06 ± 0.15	-2.42 ± 0.75	-2.70 ± 0.88	-1.34 ± 0.20
Conductivity (mS/cm)	0.19 ± 0.001	0.19 ± 0.02	0.20 ± 0.001	0.19 ± 0.001	0.19 ± 0.04	0.20 ± 0.06
pH	3.90 ± 0.02	4.00 ± 0.01	4.20 ± 0.01	3.90 ± 0.02	3.90 ± 0.004	4.00 ± 0.01

Following the freeze-thaw test, no visible instability (phase separation, creaming, or turbidity) was observed. The mean droplet size and PDI were 12.86 ± 0.06 nm and 0.27 ± 0.04, respectively, with no significant differences compared to the initial values ($p > 0.05$). Similarly, after the heating-cooling (thermal cycling) test, no macroscopic instability was detected. On day 7, the mean droplet size was 12.34 ± 0.03 nm, and the PDI was 0.26 ± 0.03, while on day 14, the mean droplet size was 13.01 ± 0.05 nm and the PDI was 0.29 ± 0.05. No significant changes in droplet size or PDI were observed over time ($p > 0.05$), indicating that the nanoemulsion remained stable under thermal cycling conditions.

Preparation and optimization of nanoemulgels

Although nanoemulsions naturally have low viscosity, which can be a drawback for topical use, the addition of appropriate gelling agents can transform them into easily applied and effective formulations (Gorat et al., 2025). It was observed that the viscosity of nanoemulgels increased with increasing polymer concentrations. The viscosities of nanoemulgels containing ALG at 3.0%, 4.5%, and 6.0% were 920 ± 20 cP, 3650 ± 45 cP, and 8123 ± 202 cP, respectively. The literature recommends a viscosity range of 2000-4000 cP for gel dosage forms (Nurman et al., 2019). The formulation containing 4.5% ALG provided the

optimal viscosity within this range. Also, the viscosities of nanoemulgels containing CMC at 1.0%, 1.5%, and 2.0% were 1066 ± 38 cP, 2896 ± 50 Cp, and 7213 ± 147 cP, respectively. It was determined that the formulation containing 1.5% CMC was within the optimal viscosity range. Mucosal retention may be reduced at very low viscosities, while ease of application may be reduced at extremely high viscosities. Therefore, nanoemulgel formulations containing 4.5% ALG and 1.5% CMC were found to provide the appropriate viscosity range for vaginal application and were selected as the optimum nanoemulgel systems, coded ALG-NEG and CMC-NEG, respectively.

Evaluation of rheological properties

Rheological analysis revealed that the structural properties of nanoemulgel systems vary significantly depending on the polymer type used. Shear stress curves showed that both nanoemulgel systems produced significantly higher shear stress compared to the nanoemulsion (Figure 3.). The ALG-NEG formulation exhibited the highest shear stress values, indicating a stronger and more resilient structure. In contrast, CMC-NEG exhibited moderate shear stress, resulting in a structure that spread more readily during application. The shear-thinning behavior observed in both gel systems provides advantages for vaginal application. This maintains adhesion to the mucosa at low shear rates, while increasing shear rates during application increase fluidity and spreadability (Aleanizy et al., 2021). In contrast, the NE formulation exhibited a profile similar to that of Newtonian flow, with very low shear stress, indicating that vaginal retention will be limited in the absence of gelation and highlighting the need for the development of a nanoemulgel. ALG-NEG and CMC-NEG formulations exhibited a significant decrease in viscosity values with increasing shear rate and exhibited shear-thinning (pseudoplastic) behavior. In contrast, NE has a very low viscosity and is unaffected by shear rate.

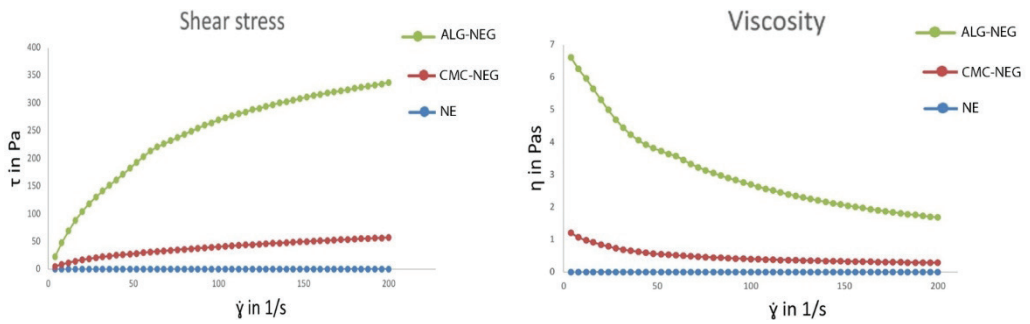


Figure 3. Rheograms of shear stress and viscosity profiles of the optimum nanoemulsion and nanoemulgel formulations at 25 °C ± 0.2 °C

Evaluation of spreadability and *ex vivo* bioadhesion by texture analysis

The setups for the spreadability and *ex vivo* bioadhesion studies are shown in Figure 4. According to the results, while the CMC-NEG formulation offered lower resistance and higher spreadability with a firmness value of 49.15 ± 0.07 g and a work of shear value of 25.36 ± 1.10 g·s, potentially allowing easier application, the ALG-NEG formulation showed a more viscous and denser structure with a firmness value of 147.08 ± 20.43 g and a work of shear value of 68.64 ± 11.24 g·s. According to the *ex vivo* bioadhesion test results, both formulations showed significant adhesion ability to the sheep vaginal mucosa. ALG-NEG formulation showed a higher work of adhesion value of 0.180 ± 0.06 N·s, indicating stronger adhesion to the mucosa. In contrast, CMC-NEG showed a lower but still important adhesion value of 0.163 ± 0.02 N·s. The ALG-NEG formulation may have a significant advantage in vaginal applications, as it can remain in the mucosa for longer periods, thereby increasing its biological efficacy (Rahman & Ahmed, 2019).

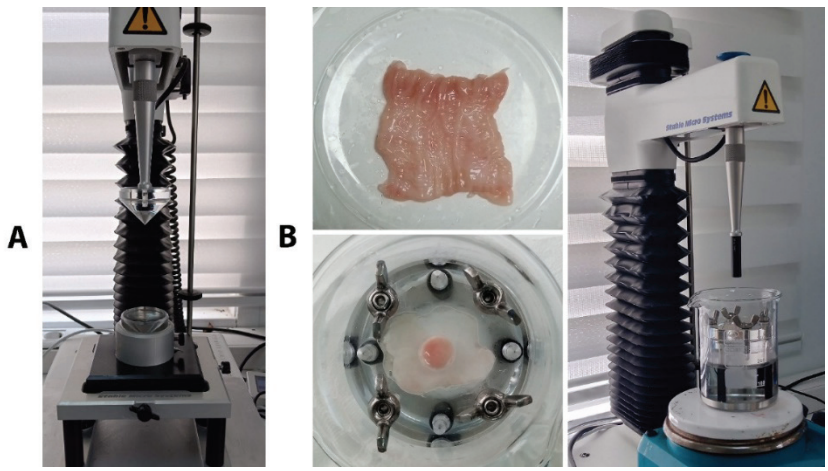


Figure 4. A) Spreadability and B) *ex vivo* bioadhesion test setups

Determination of *in vitro* antifungal activities

All formulations containing cumin EO exhibited significant antifungal activity against both standard ATCC strains and clinical isolates (Table 7.). NE showed the highest antifungal activity, being effective at 1/32 or 1/16 dilutions against all strains. The activities of the CMC-NEG and ALG-NEG were generally 2 or 4-8 times lower than those of the NE, respectively. These results indicate that the effect of cumin NE was decreased in gel formulations, especially in ALG-NEG. These activities did not differ significantly across species or between clinical isolates and reference strains, indicating that the effect of the formulations was similar across *Candida* species and was not affected by antifungal susceptibility. The developed systems showed potential not only against reference strains but also against clinically significant *Candida* isolates. The effectiveness of NE against all tested *Candida* strains at the most diluted concentrations (1/32-1/16) is attributable to the small droplet size, which enables the drug to penetrate easily into the fungus by enhancing interaction with the cell wall/membrane (Pongsumpun et al., 2020). Studies in the literature have also shown that the presence of EO in the oil phase of NE can inhibit fungal growth by interacting more easily with ergosterol in the cell membrane (Hussain et al., 2016). A decrease in antifungal activity of nanoemulgel formulations has also been reported in the literature (Elsayed et al., 2025). The lower MIC values of the CMC-NEG compared with ALG-NEG may be due to the CMC gel matrix providing a less effective barrier to the diffusion of active compounds. In the ALG-NEG, the denser gel structure, although it increased mucosal adhesion, may have partially restricted the free diffusion of active compounds, leading to higher MIC values. Additionally, blank CMC gel and blank ALG gel, which were empty gel formulations without any active agent, exhibited no antifungal activity, confirming that the observed effect is entirely due to the cumin EO and the nanoformulation structure.

Table 7. MIC results of the formulations (as dilution rates)

Yeasts	Blank CMC Gel	Blank ALG Gel	CMC-NEG	ALG-NEG	NE
C. albicans ATCC 10231	-	-	1/16	1/8	1/32
C. tropicalis ATCC 750	-	-	1/16	1/4	1/32
C. parapsilosis ATCC 22019	-	-	1/16	1/8	1/32
C.I. 1*	-	-	1/8	1/4	1/16
C.I. 2*	-	-	1/16	1/8	1/32
C.I. 3*	-	-	1/16	1/8	1/32
C.I. 4*	-	-	1/16	1/4	1/32
C.I. 5*	-	-	1/8	1/4	1/32

*C.I. 1-3: Clinical *C. albicans* isolates 1-3, C.I. 4: Clinical *C. tropicalis* isolate, C.I. 5: Clinical *C. parapsilosis* isolate, -: no activity.

Evaluation of *in vitro* cytotoxicity

The results of MTT analysis performed on the Vero cell line are shown in Figure 5. Cumin EO alone significantly reduced cell viability (average 55%). In contrast, the cell viabilities of the NE, CMC-NEG, and ALG-NEG were 68%, 70%, and 75%, respectively, and all formulations provided significantly higher cell viability than the free EO ($p < 0.001$). This may be due to the entrapment of the EO within the nanostructure. In a study by Carvalho et al. (2023), copaiba EO nanoemulsions reduced the cytotoxicity of the EO in Vero cells. Furthermore, the gel matrix of biocompatible polymers, such as ALG and CMC, enhances the biocompatibility of the formulations. The difference between NE, CMC-NEG, and ALG-NEG was not statistically significant ($p > 0.05$). The polymers themselves (CMC and ALG) did not adversely affect cell viability, with 100% viability observed.

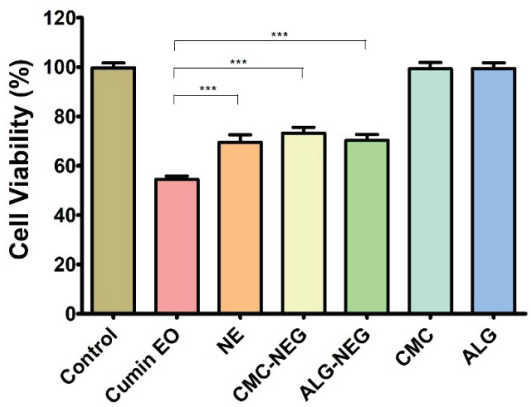


Figure 5. *In vitro* cytotoxicity of the formulations on Vero cells

CONCLUSION

This study demonstrated that cumin EO can be formulated effectively and safely into nanoemulsion and nanoemulgel systems for the treatment of vaginal candidiasis. The nanoemulsion obtained through Taguchi design of experiments optimization exhibited high antifungal activity, attributable to its small droplet size and narrow droplet size distribution. Among the nanoemulgels developed to increase the viscosity of the nanoemulsion, CMC-NEG offered better spreadability and ease of use. At the same time, ALG-NEG offered advantages in mucosal retention due to its high bioadhesion capacity. Additionally, all formulations demonstrated acceptable cytotoxicity profiles for vaginal application. Although *in vitro* release and permeation studies were not included within the scope of the present study, these evaluations are important for a more comprehensive understanding of formulation performance and are planned as part of future work. In this context, *in vitro* release and permeation studies, together with quantitative analysis by GC-MS, will be performed to better elucidate the delivery behavior of the active components. Furthermore, *in vivo* antifungal efficacy and safety evaluations in a vulvovaginal candidiasis model are also planned to confirm the therapeutic potential of the developed formulations under physiological conditions. These results suggest that nanoemulsion and nanoemulgel systems containing cumin EO are promising options for the topical treatment of vaginal candidiasis.

AUTHOR CONTRIBUTION STATEMENT

Design of the study, methodology, data acquisition, data analysis, writing manuscript, critical revision of manuscript (IEG), data acquisition, data analysis (SP), data acquisition, data analysis (AGY), data acquisition, data analysis (LA), data acquisition, data analysis (CÖ), data analysis, critical revision of manuscript (MSE), data analysis, critical revision of manuscript (SD).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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