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ORIGINAL RESEARCH

Enhancing Therapeutic Efficacy of Cinnamon Essential Oil by Nanoemulsification for Intravaginal Treatment of *Candida* Vaginitis

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Background: Due to its prevalence, recurrence, and the emergence of drug-resistance, *Candida* vaginitis significantly impacts the well-being of women. Although cinnamon essential oil (CEO) possesses antifungal activity, its hydrophobic properties limit its clinical application.

Purpose: To overcome this challenge, a nanoemulsification technology was employed to prepare cinnamon essential oil-nanoemulsion (CEO@NE), and its therapeutic efficacy and action mechanism for *Candida* vaginitis was investigated in vivo and in vitro.

Materials and Methods: CEO@NE, composed of 4% CEO, 78% distilled water, and 18% Tween 80, was prepared by ultrasonic nanoemulsification. The physical properties, anti-*Candida* activity, cytotoxicity, immunomodulatory potential and storage stability of CEO@NE were explored. Subsequently, the effect of intravaginal CEO@NE treatment on *Candida* vaginitis was investigated in mice. To comprehend the possible mechanism of CEO@NE, an analysis was conducted to ascertain the production of intracellular reactive oxygen species (ROS) in *C. albicans*.

Results: CEO@NE, with the droplet size less than 100 nm and robust storage stability for up to 8 weeks, exhibited comparable anti-*Candida* activity with CEO. CEO@NE at the concentration lower than 400 μg/mL had no cytotoxic and immunomodulatory effects on murine splenocytes. Intravaginal treatment of CEO@NE (400 μg/mL, 20 μL/day/mouse for 5 consecutive days) curbed *Candida* colonization, ameliorated histopathological changes, and suppressed inflammatory cytokine production in mice intravaginally challenged with *C. albicans*. Notably, this treatment preserved the density of vaginal lactic acid bacteria (LAB) crucial for vaginal health. Co-culturing *C. albicans* with CEO@NE revealed concentration-dependent augmentation of intracellular ROS generation and ensuing cell death. In addition, co-culturing LPS-stimulated murine splenocytes with CEO@NE yielded a decrease in the generation of cytokines.

Conclusion: This discovery provides insight into the conceivable antifungal and anti-inflammatory mechanisms of CEO@NE to tackle *Candida* vaginitis. CEO@NE offers a promising avenue to address the limitations of current treatments, providing novel strategy for treating *Candida* vaginitis.

Keywords: anti-inflammation, *Candida* vaginitis, cinnamon essential oil, nanoemulsion, reactive oxygen species

Introduction

Candida albicans, a prevalent yeast species, stands as a potential threat due to its ability to induce opportunistic infections. Among the healthy individuals, 39% are carriers of yeast, with 89% being colonized by *C. albicans*.[1] Normally, it maintains a symbiotic relationship with the human body. However, under conditions of compromised immune function, *C. albicans* can overgrow and initiate candidiasis. Notably, *C. albicans* ranks as the most common pathogenic species within the *Candida* genus, and *C. albicans* infections typically manifest in localized mucosal areas such as the oral cavity, esophagus, and vagina. Each year, candidiasis impacts over 250,000 individuals and is linked to

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a mortality rate surpassing 70%.[2] *C. albicans* possesses well-documented pathogenic attributes, including its ability to adhere to epithelial cells, and the formation of hyphae.[3] Upon infecting and adhering to mucosal tissue, *C. albicans* hyphae can infiltrate epithelial cells. Within the tissue, dendritic cells phagocytize the pathogen and present it to $CD4^+$ T helper (Th) cells. This engagement promotes the differentiation of Th1 and Th17 cells, leading to the production of IFN-γ and IL-17, coordinating the eradication of the pathogen.[4] However, *C. albicans* not only inflicts direct damage to vaginal tissue but also incites an inflammatory response. After invading vaginal tissue, the initiation of signaling pathways occurs, resulting in the generation of pro-inflammatory substances like TNF-α and IL-6. Consequently, this triggers the secretion of diverse cytokines, chemokines, and the chemotaxis of neutrophils to the site of infection. If it is failed to effectively combat *C. albicans*, inflammation and tissue damage may intensify.[5] Currently, the management of *Candida* vaginitis predominantly involves oral or topical antifungal medications, with fluconazole being a widely utilized treatment option.[6] Nevertheless, the use of fluconazole potentially yields side effects, such as abdominal pain and diarrhea. Furthermore, investigation involving rodents have indicated embryotoxic and teratogenic effects associated with high-dose fluconazole administration.[7]

In pursuit of effective therapeutic alternatives that mitigate the severe side effects often associated with conventional medications, researchers are increasingly turning to the development of alternative medicines. One such alternative medicine that has garnered significant attention is cinnamon, a commonly used culinary spice with a well-established safety profile and generally recognized as safe. Beyond its culinary utility, cinnamon has emerged as a subject of interest due to its remarkable properties related to blood sugar regulation and its antimicrobial potential.[8,9] Cinnamon essential oil (CEO) has exhibited notable antimicrobial activity against various microorganisms, including *Staphylococcus aureus, Escherichia coli*, and *Candida* species.[10] Specifically, it disrupts the cell wall, leading to intracellular leakage and inhibition of fungal growth.[8] Moreover, CEO could induce accumulation of reactive oxygen species (ROS) within *C. albicans*, ultimately resulting in cell death.[11] Importantly, the development of resistance to antimicrobial effect of CEO appears less likely, making it an attractive candidate for combating microbial infections.[12] Beyond its antimicrobial prowess, CEO demonstrates anti-inflammatory properties by inhibiting the production of inflammatory mediators. It has been shown that CEO can reduce pro-inflammatory cytokine expression and thereby mitigate inflammatory responses, suggesting that CEO hold promise as natural anti-inflammatory agents.[13] Nevertheless, the implementation of CEO encounters hindrances due to certain limitations, notably its reduced stability and lack of solubility in water. Therefore, previous studies have formulated CEO as a nanoemulsion (NE) to improve its stability and solubility.[14,15]

NE, characterized by its composition of oil and water phases stabilized by an emulsifier at the interface, has gained prominence in pharmaceutical research as a versatile drug delivery system. The distinctive properties of NE, including transparency, thermodynamic stability, and nanoscale droplet sizes ranging from 1 to 200 nm, make it an ideal candidate for safeguarding bioactive ingredients against denaturation, degradation, and volatilization under extreme conditions.[16] Its remarkable stability, attributed to the small droplet size, minimizes the impact of gravity, thus preventing oil-water separation or sedimentation during storage. NE has demonstrated its ability to uniformly disperse lipophilic drugs, enhance their water solubility, and improve their stability. Furthermore, NE aids in promoting lymphatic transport, increasing mucosal permeability, and ultimately enhancing the bioavailability of the medicinal components.[17] While a few studies have reported that nanoemulsification improves the in vitro antifungal potency of CEO, there is currently no available study comparing the potency of CEO and CEO@NE against *Candida* vaginitis.[18–20]

Given the unique properties of the vaginal mucosa, including its accessibility and relatively stable pH, it emerges as a promising route for drug delivery, especially in the context of addressing ascending genital tract infections.[21] Previous research has underscored the potential of NE as a mucosal adjuvant for enhancing the penetration of vaccine antigens through mucosal barriers, thereby eliciting robust immune responses.[22] Moreover, intravaginal administration of NE-adjuvanted protein antigens extended the duration of antigen retention in the genital tract, leading to robust systemic and mucosal immune responses.[23] These results underscore the possibility of utilizing NE as a therapeutic alternative for managing genital infections.

In light of anti-inflammatory and anti-*Candida* activities of CEO, this study aimed to overcome the challenges associated with its lipophilic nature by employing an ultrasonic nanoemulsification technology to formulate CEO@NE with Tween 80 as an emulsifier. Subsequently, we evaluated the physical properties, storage stability, and therapeutic

effects, along with the underlying mechanisms, of CEO@NE in the treatment of *C. albicans* vaginal infections using in vitro and in vivo models.

Materials and Methods

Materials, Chemicals, Reagents and Detection Kits

CEO was purchased from Mane Kancor (Kerala, India). As provided by the manufacturer, identified eugenol as the primary component, constituting approximately 70–80% of the composition. Other notable constituents included β-caryophyllene, benzyl benzoate, and cinnamaldehyde. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and PanReac AppliChem (Darmstadt, Germany). Reagents for microbial culture were purchased from Difco Laboratories (Detroit, MI, USA). Reagents for cell culture, LIVE/DEAD™ FungaLight™ Yeast Viability Kit and ELISA kits were procured from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation, Characterization and Stability Test of CEO@NE

Tween 80 is advantageous for preparing oil-in-water CEO@NE due to its higher hydrophilic–lipophilic balance value.[24] The emulsion formulation consisted of 40 µL of CEO, 180 µL of Tween 80, and 780 µL of distilled water. After mixing, the emulsion was subjected to ultrasonication (W-380 sonicator Ultrasonic Processor, Heat Systems, USA) at 20 kHz for 20 min. After the process was finalized, it was verified that the mean droplet size of the sample measured below 200 nm, signifying the successful formulation of CEO@NE (Figure 1A). For the analysis of functional groups in the samples, Fourier-transform infrared spectroscopy (FTIR) was employed. The method and procedure for FTIR analysis followed that reported in the previous study, with the exception that each spectrum was acquired in the range of 450–4000 cm^{-1}.[25] The morphology of CEO@NE was examined using the 3D Cell Explorer from Nanolive SA in Tolochenaz, Switzerland, and a liquid transmission electron microscope (TEM) from K-kit at Bio Materials Analysis Technology Inc. in Hsinchu,

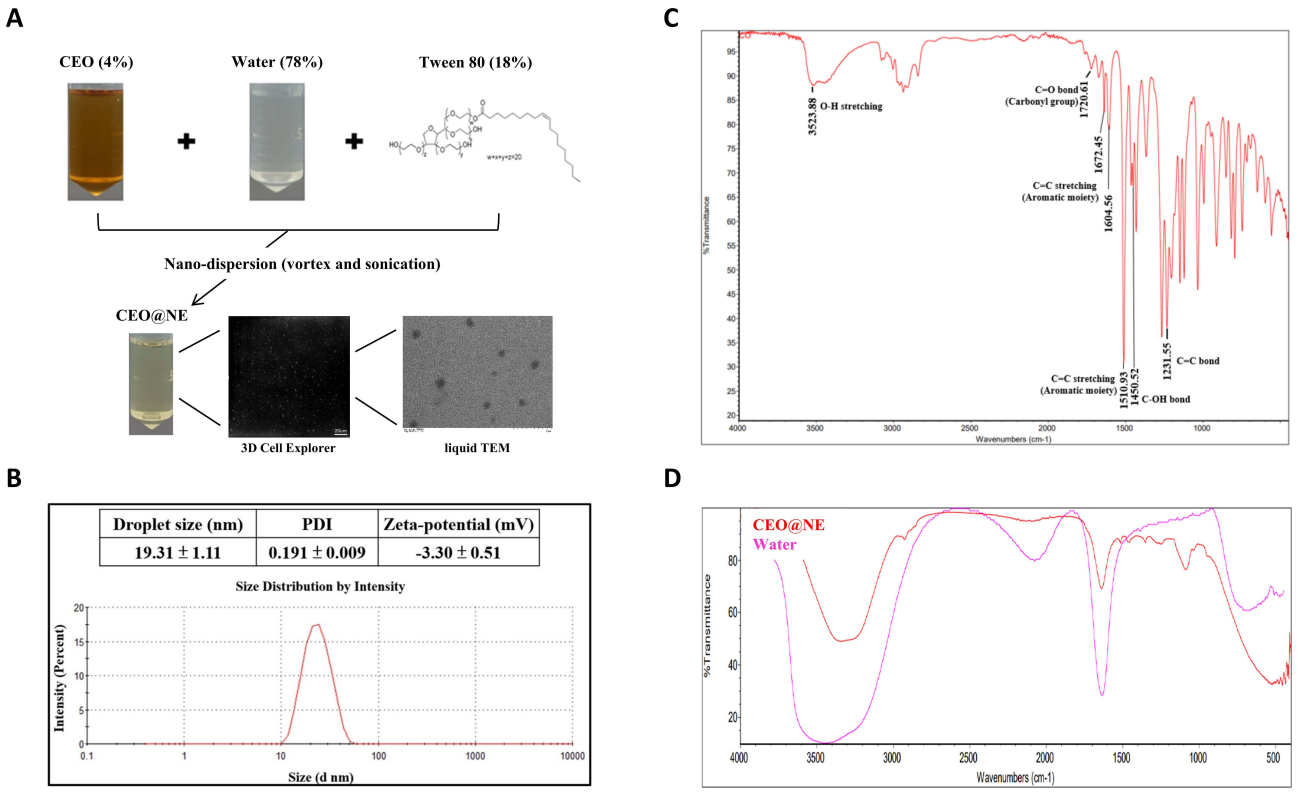

Figure 1 Preparation and characterization of cinnamon essential oil-nanoemulsion (CEO@NE). (**A**) Schematic illustration of CEO@NE preparation and its representative morphology observed by 3D Cell Explorer and liquid TEM. (**B**) Droplet size distribution, polydispersity index (PDI) and zeta potential of CEO@NE analyzed by DLS. FTIR spectrum of (**C**) CEO, (**D**) CEO@NE and pure water.

Taiwan. The droplet size, zeta potential, and polydispersity index (PDI) of CEO@NE were assessed with a dynamic light scattering instrument (DLS, Zetasizer Nano ZS) from Malvern Instruments Ltd. in Worcestershire, UK. CEO@NE samples were stored at 4, 25, and 37°C, and measurements of droplet size, zeta potential, and PDI were conducted once a week for 8 weeks. Simultaneously, a visual observation allowed to understand whether CEO@NE remained stable or water and oil separation.

Evaluation of Minimum Inhibition Concentration (MIC), Minimum Fungicidal Concentration (MFC), Cytotoxicity and Immunomodulatory Activities of CEO@NE

MIC is the lowest concentration of samples that can inhibit fungal growth after 24 h of cultivation. Following the method of El-Baz et al, we prepared a CEO-containing broth solution (8 mg/mL) by adding 0.5% (v/v) Tween 80 to YPD broth to ensure well dissolution of CEO in the broth, and CEO was serially diluted with YPD broth.[26] *C. albicans* ($1–5\times10^6$ cells/mL) was incubated in YPD broth with CEO at concentrations ranging from 62.5 to 4000 μg/mL at 37°C for a duration of 24 h, and the measured absorbance values at a wavelength of 600 nm were measured by using an ELISA reader (Quant, Biotek, Winooski, VT, USA) to determine the MIC. Moreover, MIC of CEO@NE, with the same concentration of CEO as described above, was also investigated by using the same method. MFC is characterized as the lowest concentration of samples necessary to entirely suppress fungal growth. The cultured broth of *C. albicans* with CEO or CEO@NE from the previous MIC test was collected, and 100 μL of the collected broth was spread on YPD plates. Following incubation at 37°C for a duration of 24 h, the lowest concentrations of CEO- and CEO@NE-containing broth at which no visible fungal growth appeared on the surface of YPD plates were considered the MFC. Spleen samples from normal mice were processed to create splenocyte suspensions, achieving a concentration of 6×10^6 cells/mL. The splenocyte suspensions were incubated in the presence of CEO@NE (0–400 μg/mL) for 72 h. Four hours before the end of incubation, MTT reagent (5 mg/mL) was introduced and subsequently incubated for an additional 4 h. Following incubation, dimethyl sulfoxide was added for another incubation for 30 min. Finally, the absorbance values were assessed at a wavelength of 570 nm employing an ELISA reader (Quant, Biotek, Winooski, VT, USA). The absorbance value of the untreated cell suspensions was defined as 100%, and the cell viability for each group was calculated. The supernatants from cultured splenocyte suspensions were gathered and subjected to ELISA for quantifying the levels of IFN-γ and IL-17. The concentration of both CEO and CEO@NE was defined by dividing the weight of cinnamon essential oil by the total volume of cultured broth or medium.

Animal Experiment

Female BALB/c mice, aged six weeks, were procured from the National Laboratory Animal Center in Taipei, Taiwan. They were accommodated at the terrestrial animal center of the National Taiwan Ocean University (NTOU) for a week under controlled conditions, maintaining a constant temperature (24 ± 1°C), humidity (40–60%), a 12-hour light/dark cycle, and providing unrestricted access to food and water prior to the initiation of animal experiments. The murine model of *Candida* vaginitis followed the protocol outlined in a prior study.[27] The mice were segregated into six groups, with each group comprising six mice, including NA group (no treatment), VH group (intravaginally exposed to *C. albicans* challenge and intravaginal treatment with PBS), Flu group (intravaginally exposed to *C. albicans* challenge and oral treatment with fluconazole), CEO group (intravaginally exposed to *C. albicans* challenge and intravaginal treatment with CEO), CEO@NE group (intravaginally exposed to *C. albicans* challenge and intravaginal treatment with CEO@NE), and Ctrl group (intravaginal treatment with CEO@NE but without *C. albicans* challenge). Excluding mice in the NA group, the other mice were injected intraperitoneally with 0.1 mL estrogen (β-estradiol, 0.1 mg/mL) dissolved in sesame oil on the 3rd and 6th days to synchronize the estrous cycle. On the 7th day, the mice were intravaginally challenged with *C. albicans* (20 μL, 5×10^8 cells/mL). From the 10th to the 14th day, mice in CEO and CEO@NE groups received daily intravaginal administration of 20 μL of CEO or CEO@NE at a concentration of 400 μg/mL. On the 10th day, a single oral dose of fluconazole (0.1 mL, 4.9 mg/mL) was administered to mice in the Flu group. The weights of the mice were assessed and documented every three days. On the 15th day, all mice were euthanized using CO_2. Serum samples and vaginal washes were collected for antibodies analysis. Moreover, the vaginal washes underwent

sequential dilution and were cultured on YPD and MRS agar plates at 37°C for 48 h to ascertain the density of yeast and lactic acid bacteria (LAB), respectively. Vaginal tissues were collected for histopathological analysis. Sections stained with hematoxylin and eosin (H & E) and Periodic acid-Schiff (PAS) were inspected using an optical microscope (ZoomKop EZ-20II, Leader scientific, Taiwan). PAS staining is primarily employed in histology to detect carbohydrate components within the samples. The primary polysaccharides in fungal cell walls are β-glucan, and *C. albicans*, when stained, appears purple-red. Area of hyphae to epithelium ratio and epithelial thickness were quantified by ImageJ software in accordance with the methods outlined in the prior investigation.[27] Individually, spleen samples were isolated from mice to generate splenocytes. Cultures of splenocyte suspensions (at a concentration of 6×10^6 cells/mL) were exposed to heat-treated *C. albicans* (HK-CA; 2.5×10^5 cells/mL) for a duration of 72 h. Cell viability was assessed using the MTT assay, and concentrations of IFN-γ and IL-17 in the supernatants were quantified using ELISA, following the previously mentioned procedures.

Evaluation of ROS-Mediated Cell Death of C. albicans by CEO@NE

Utilizing the procedures outlined in the prior study, *C. albicans*, previously incubated at 37°C for 24 h, underwent centrifugation and was subjected to two washes with 1 mL of sterile PBS.[11] *C. albicans* (1×10^6 cells/mL) were incubated with CEO@NE (0–400 μg/mL) for 8 h. *C. albicans* treated with H_2O_2 (0.3 mg/mL) or heated at 80°C for 10 min were utilized as control groups. Subsequently, the *C. albicans* cells were stained with dichlorofluorescin diacetate (DCFH-DA; 20 μM) or propidium iodide (PI; 20 mM) for 30 min and then analyzed using inverted fluorescence microscope (ZoomKop EZ-20II, Leader scientific, Taiwan) and flow cytometer (Thermo Fisher Scientific, Attune™ NxT Flow Cytometer). The morphology of CEO@NE-treated *C. albicans* cells was observed by liquid TEM.

Assessment of Anti-Inflammatory Activity of CEO@NE on Lipopolysaccharide (LPS)-Treated Murine Splenocytes

As described above, splenocyte suspensions, with a concentration of 6×10^6 cells/mL, derived from normal mice were cultured with varying concentrations of CEO@NE (ranging from 0 to 400 μg/mL) in the presence of LPS (1 μg/mL) for a duration of 72 h. The supernatants of cultured splenocyte suspensions were collected and assayed using ELISA to measure the levels of IFN-γ, IL-17, TNF-α and IL-6.

Statistical Analysis

To evaluate the variance across different groups, an analysis of variance (ANOVA) was executed. In cases where the F-value derived from the ANOVA was deemed significant, the least significant difference (LSD) test was employed for discerning significant differences among individual groups. A significance level of $P < 0.05$ was adopted, indicating statistically noteworthy distinctions.

Results

Physical Characterization and Storage Stability of CEO@NE

The morphology of CEO@NE observed by the 3D Cell Explorer and liquid TEM reveals uniform and nanosized droplets well dispersed in the aqueous continuous phase (Figure 1A). Results of DLS analysis reveal that CEO@NE exhibited favorable droplet size, PDI, and zeta potential values, measuring at 19.31 ± 1.11 nm, 0.191 ± 0.009, and −3.30 ± 0.51 mV, respectively (Figure 1B). These results not only met the requirements for the size of nanodroplet but also indicated a low PDI, underscoring its suitability as an optimal delivery carrier characterized by a uniform droplet size distribution.

FTIR analysis was performed to elucidate the functional groups of both CEO and CEO@NE. The FTIR spectrum of CEO revealed prominent signals in the wavelength range of 3300–3600 cm^{-1}, corresponding to -OH functional groups, and signals in the range of 1648–1746 cm^{-1}, indicative of C=O bond functional groups, primarily associated with aldehydes of saturated fatty acids, including compounds like coumarin. Signals were also detected at 1450 cm^{-1} (C-OH bond bending), 1231 cm^{-1} (phenolic C-OH groups), and several characteristic peaks in the 720–1250 cm^{-1} range associated with C=C bonds, likely attributed to eugenol, the predominant compound in the CEO. Additionally, peaks

at 1672, 1604, and 1510 cm^{-1} were observed, attributed to the stretching of C=C bonds in the aromatic ring of eugenol. These peaks indicated the rich presence of various phenolic and aromatic compounds within CEO (Figure 1C). Comparatively, FTIR analysis of CEO@NE demonstrated a pronounced signal for –OH stretching in the wavelength range of 3300–3600 cm^{-1}, potentially attributable to the presence of water. Signals at 2900 cm^{-1} (CH_2 stretching) and 1655 cm^{-1} (HOH bending) corresponded to the functional groups of Tween 80, the emulsifier used in the CEO@NE formulation. Furthermore, signals in the 720–1250 cm^{-1} range indicated C=C functional groups, characteristic of eugenol.[28] Notably, when comparing the FTIR spectrum of CEO with that of CEO@NE, several signals disappeared after the nanoemulsification process (Figure 1C and D). Furthermore, the similarity of the CEO@NE spectrum to the standard spectrum of water affirmed the successful preparation of an oil-in-water NE.

To evaluate the storage stability of CEO@NE under different temperature conditions, CEO@NE was stored separately at 4, 25, and 37°C for 8 weeks. Measurements were conducted weekly to monitor changes in the size of droplet, PDI, and zeta potential. Remarkably, the results demonstrated that CEO@NE maintained its droplet size at nanoscale, and changes in storage temperatures did not induce significant alterations in both the PDI and zeta potential, affirming its robust storage stability (Table 1). In parallel, no significant signs of separation and precipitation were observed.

In vitro Antifungal Effect of CEO@NE Without Obvious Cytotoxic and Immunomodulatory Activities

The MIC of both CEO and CEO@NE was 1 mg/mL. Concordantly, the MFC of both CEO and CEO@NE was 2 mg/mL (Figure 2A), suggesting that nanoemulsification did not yield a better antifungal effect but also did not compromise the fungicidal effectiveness of CEO. To investigate the cytotoxicity and immunomodulatory properties of CEO@NE, primary splenocyte suspensions prepared from normal mice were cultured in the presence of CEO@NE (20 to 400 μg/mL). After culture for 72 h, there was no significant decrease in cell viability among cultures treated with CEO@NE, indicating that concentrations of CEO@NE ranging from 20 to 400 μg/mL did not induce cytotoxicity (Figure 2B). Furthermore, the expression of cytokines related to the defense against *Candida* infection, namely IFN-γ and IL-17A, was quantified. The results demonstrated that no significant changes were observed in the expression of these cytokines when treated with CEO@NE at concentrations ranging from 20 to 400 μg/mL (Figure 2C and D), revealing that CEO@NE could not exert significant immunostimulatory or immunosuppressive effects on murine splenocytes. Accordingly, the concentration of 400 μg/mL was employed for the animal experiment to achieve the 3R principle.

Therapeutic Effect of Intravaginal CEO@NE Treatment on Candida Vaginitis

To investigate the therapeutic potential of CEO@NE against *Candida vaginitis*, estrus synchronized mice were intravaginally challenged with C. *albicans* followed by intravaginal treatment of CEO@NE once daily for a continuous span of 5 days (Figure 3A). The mice in treatment groups displayed consistent and steady increases in body weight throughout the experimental period (Figure 3B). Their overall appearance and activity remained normal, suggesting that the treatments did not induce significant side effects. After euthanasia, mice in the VH group exhibited notable cloudy and excessive swelling in both the fallopian tubes and uterus, indicating severe inflammation. Conversely, mice in the treatment groups displayed less cloudy and swelling in the fallopian tubes and uterus. Particularly, the appearance of a fallopian tubes and uterus of mice in Flu and CEO@NE groups was almost the same with that in the NA group. However, mice in the CEO group showed partial relief of swelling and still exhibited noticeable inflammatory signs, indicating a relatively less effective treatment outcome (Figure 3C). To assess whether the treatments reduced the quantity of *C. albicans* in the vaginal tract, vaginal washes were prepared for yeast colony counts. Compared to that of the NA group, a significant increase in yeast density of the VH group was observed. However, a significant decreased yeast density was observed in the Flu and CEO@NE groups (Figure 3D). In healthy women, vaginal microbiota primarily consists of LAB, crucial for maintaining microbial balance and inhibiting pathogenic microorganisms. However, during microbial infections, this balance is disrupted, resulting in a decrease in LAB and disease development.[29] In comparison to the NA group, the density of LAB in the vaginal wash of the VH group was diminished.

Table 1 Changes in Droplet Size, PDI, and Zeta Potential of Cinnamon Essential Oil Nanoemulsion (CEO@NE) at 4, 25, and 37°C

		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
4°C	Particle size (nm)	19.31 ± 1.11	20.13 ± 1.33	20.01 ± 1.00	21.59 ± 1.54	18.56 ± 2.31	16.60 ± 2.25	19.45 ± 1.41	18.78 ± 2.14	21.45 ± 2.94
	PDI value	0.191 ± 0.01	0.199 ± 0.02	0.197 ± 0.01	0.217 ± 0.01	0.216 ± 0.01	0.223 ± 0.02	0.198 ± 0.02	0.279 ± 0.01	0.273 ± 0.07
	Zeta potential (mV)	−2.97 ± 0.55	−3.07 ± 0.71	−3.24 ± 0.10	−3.15 ± 0.93	−4.69 ± 0.50	−2.20 ± 0.31	−1.49 ± 0.40	−2.82 ± 0.65	−2.11 ± 0.58
25°C	Particle size (nm)	21.91 ± 2.82	19.31 ± 2.34	17.79 ± 1.52	22.53 ± 3.56	23.02 ± 4.38	17.93 ± 3.37	19.29 ± 1.82	22.69 ± 2.46	21.23 ± 1.02
	PDI value	0.204 ± 0.01	0.178 ± 0.02	0.162 ± 0.02	0.116 ± 0.02	0.215 ± 0.04	0.236 ± 0.04	0.241 ± 0.03	0.276 ± 0.02	0.243 ± 0.05
	Zeta potential (mV)	−3.30 ± 0.51	−3.06 ± 0.72	−3.22 ± 0.53	−2.38 ± 0.57	−3.16 ± 0.37	−2.38 ± 0.94	−1.94 ± 0.71	−2.22 ± 0.33	−2.18 ± 0.67
37°C	Particle size (nm)	20.49 ± 1.33	25.31 ± 7.54	33.06 ± 12.82	39.39 ± 15.14	57.07 ± 14.81	39.66 ± 5.59	42.65 ± 9.96	49.09 ± 9.03	51.17 ± 11.07
	PDI value	0.195 ± 0.01	0.203 ± 0.01	0.191 ± 0.03	0.203 ± 0.04	0.225 ± 0.05	0.199 ± 0.02	0.213 ± 0.05	0.307 ± 0.07	0.313 ± 0.05
	Zeta potential (mV)	−3.01 ± 0.61	−2.88 ± 0.77	−2.79 ± 0.38	−3.93 ± 0.29	−3.71 ± 0.92	−2.11 ± 0.30	−1.98 ± 0.52	−1.87 ± 0.64	−2.01 ± 0.34

Note: Each value is mean ± SEM (n = 3).

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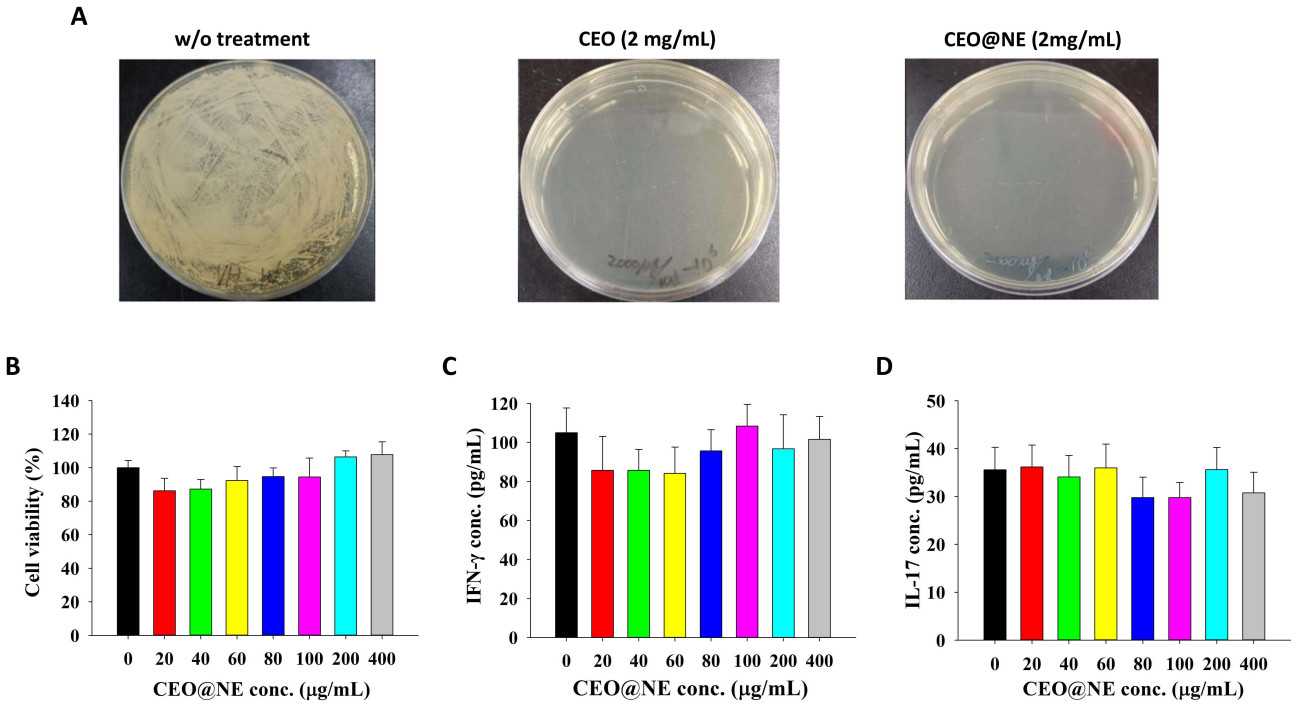

Figure 2 The minimum fungicidal concentration (MFC) of CEO@NE against C. albicans and the impact of CEO@NE on primary murine splenocytes. (**A**) Representative images of *C. albicans* cultured without or with either CEO or CEO@NE (2 mg/mL). (**B**) Cell viability, (**C**) IFN-γ and (**D**) IL-17 of primary murine splenocytes cultured in the presence of CEO@NE (0–400 μg/mL) for 72 h. The results are provided in the form of mean ± SEM (n = 3), representing a typical dataset from two separate experiments.

Treatment of Flu, CEO, and CEO@NE reversed the density of LAB, although the density of vaginal LAB in the treatment groups was still less than that of the NA group (Figure 3E). It is worth noting that intravaginal treatment of CEO@NE to normal mice did not alter the density of vaginal LAB (Figure 3E). Taken together, these results indicate that CEO@NE effectively reduced the density of vaginal *C. albicans*, and its antifungal activity did not negatively impact the density of vaginal LAB.

In order to investigate whether the treatments were able to effectively mitigate tissue damage caused by intravaginal *C. albicans* challenge, the vaginal tissues were collected and subjected to histopathological examination. In contrast to the NA group, the vaginal epithelium of mice in the VH group was almost entirely eroded and colonized by *C. albicans* as evidenced by numerous signals of purple-red *C. albicans* hyphae. However, the structure of epithelium of mice in the Flu and CEO@NE groups was retained and similar to that of the NA group, and almost no *C. albicans* hyphae was observed (Figure 4A and B). Notably, mice in the CEO group exhibited sporadic purple-red signals on the epithelium, indicating significant *C. albicans* hyphae attachment to the vaginal epithelium and a poorer treatment outcome (Figure 4A and B). In the VH group, noticeable cellular infiltration and microabscess formation were observed (Figure 4C), which are typical signs of vaginal mucosal tissue following fungal infection.[30] However, cellular infiltration and microabscess formation were attenuated in mice of the Flu and CEO@NE groups (Figure 4C). Furthermore, quantification of the vaginal epithelial layer thickness revealed that the epithelial layer was almost completely eroded in the VH group, with a thickness decrease of approximately 90% compared to the NA group. However, while not fully returning to the NA thickness, mice in the Flu and CEO@NE groups showed a significant improvement of epithelial thickness (Figure 4D).

Influence of CEO@NE on the Modulation of Antibody and Splenic Cytokine Production in Mice Exposed to C. albicans Challenge

Antibodies and cytokines are crucial components of the immune system, each playing distinct but equally important roles in the immune response. Antibodies can recognize and tag pathogens, preventing them from entering cells, while cytokines can stimulate the proliferation and differentiation of immune cells, enhancing their ability to combat pathogens

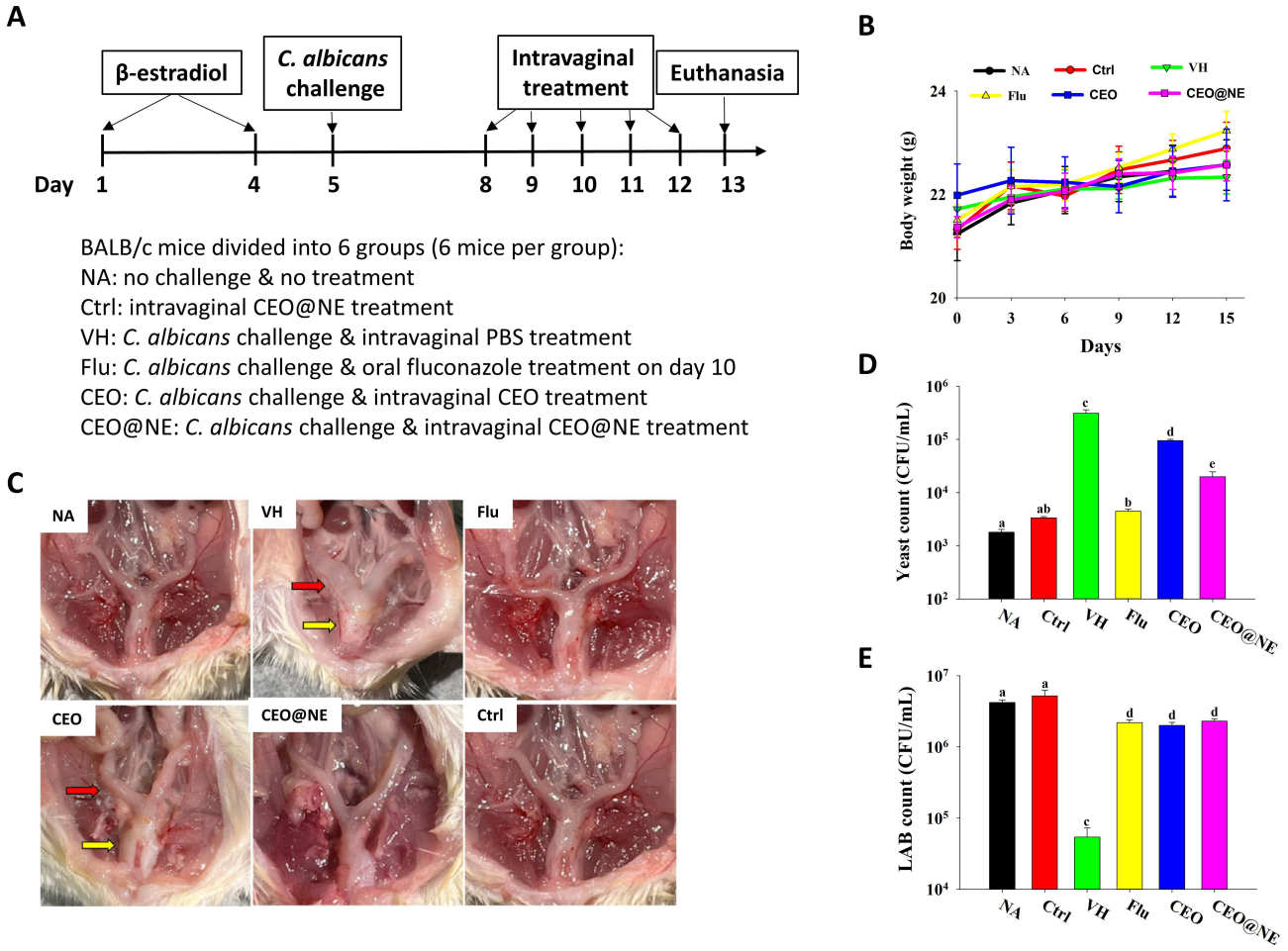

Figure 3 Attenuation of *Candida* vaginitis by intravaginal treatment of CEO@NE in mice. (**A**) Protocol of the animal experiment. (**B**) Body weights of mice throughout the experiment. (**C**) The pathological change of uterus and fallopian tube in mice intravaginally challenged with *C. albicans*. The red arrows indicate swollen and inflammation in the fallopian, and the yellow arrows indicate erosion and inflammation in the uterus. The density of (**D**) yeast and (**E**) lactic acid bacteria (LAB) in the vaginal washes of mice in each group. The data is displayed as mean ± SEM (n = 6), showcasing a typical dataset from two separate experiments. Significance, denoted by distinct letters (a–e), indicates a noteworthy difference ($p < 0.05$) among the values.

and regulating inflammatory responses.[31] Serum IgG antibodies can bind to antigens on the surface of pathogen, thereby inducing the immune system to eliminate the pathogen, controlling the spread and worsening of the infection.[31] As shown in Figure 5A, noteworthy elevation in serum IgG production was noted in the VH group as compared to the NA group, while Flu and CEO@NE treatment significantly suppressed IgG production. IgA antibodies are primarily found on mucosal surfaces, which can prevent the invasion and attachment of microorganisms on mucosal surfaces, thereby inhibiting the growth and proliferation of pathogen.[31] As shown in Figure 5B, a significant increase in vaginal IgA production of the VH group was observed, while only CEO@NE treatment significantly suppressed IgA production. Antigen-specific antibodies can bind to extracellular specific pathogens or cell surface proteins already infected by microorganisms, triggering immune responses to clear specific antigens. Consistently, similar results of *C. albicans*-specific IgA production in the vaginal washes were observed to that of total IgA production, suggesting that intravaginal CEO@NE treatment effectively suppressed *C. albicans* colonization and infection, and thus prevented against *C. albicans*–induced antibody production (Figure 5C).

A moderate immune response can help eliminate pathogens within the body. However, an excessive immune response can lead to a cytokine storm, which not only clears invading pathogens but also causes damage or failure of the organs of host, and even death.[32] Therefore, the impact of CEO@NE on *Candida*-induced cytokine production was investigated. To confirm whether CEO@NE treatment altered splenocytes activity, the MTT assay was employed. No noteworthy

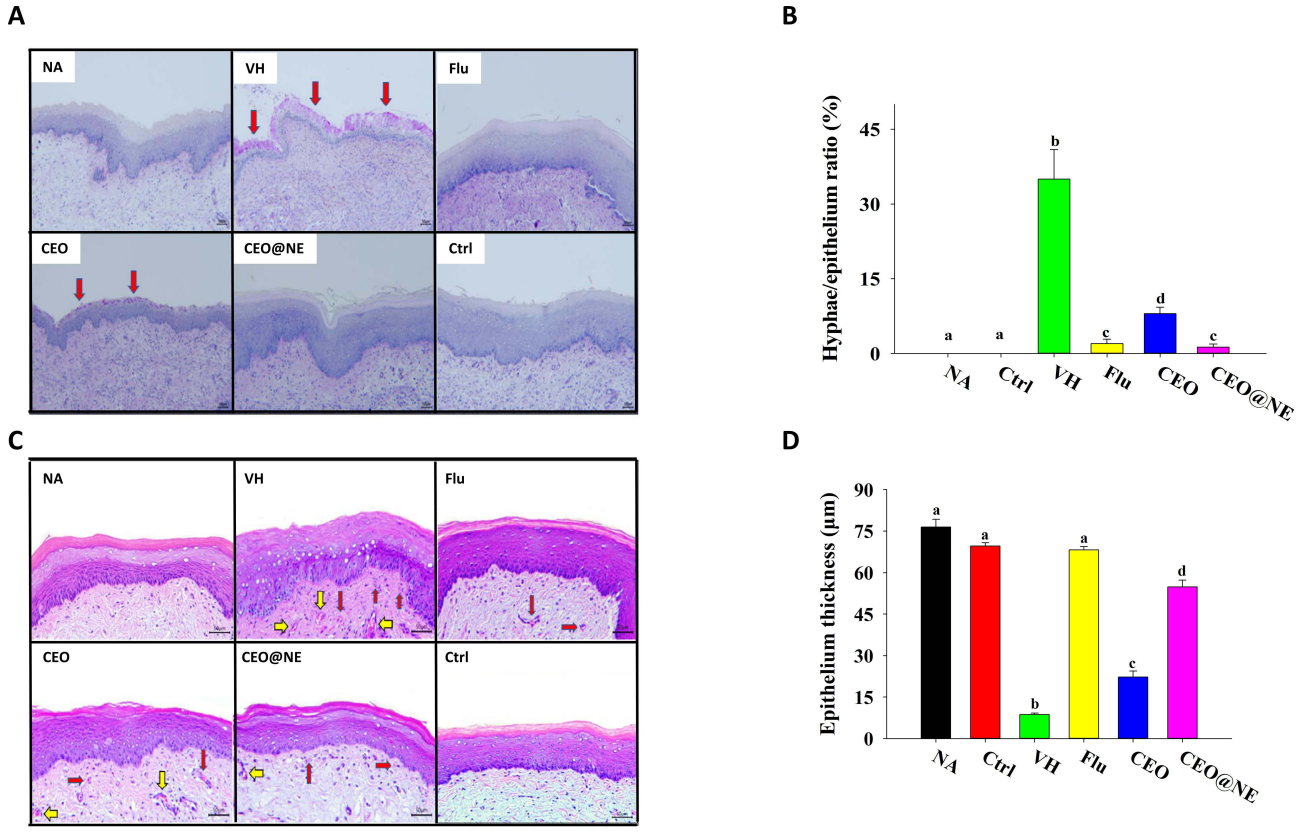

Figure 4 Histopathological analysis of vaginal tissues. (**A**) Representative image of PAS staining of vaginal tissues isolated from mice of each group. The purple signals (red arrows) indicate the candidal hyphae. (**B**) The ratio of hyphae positive area to vaginal epithelial area was quantified by ImageJ software. (**C**) Representative image of H & E staining of vaginal tissues isolated from mice of each group. Yellow arrows indicate the formation of microabscesses, and red arrows indicate neutrophil infiltration. (**D**) The thickness of vaginal epithelium of mice from each group was quantified using ImageJ software. The data is displayed as mean ± SEM (n = 6), showcasing a typical dataset from two separate experiments. Significance, denoted by distinct letters (a-d), indicates a noteworthy difference ($p < 0.05$) among the values.

changes in cell viability were observed among the groups, indicating that the treatments did not affect lymphocyte viability (Figure 5D). The VH group exhibited significantly elevated expression of IFN-γ and IL-17 compared to the NA group. However, CEO@NE treatment could suppress the expression of both cytokines (Figure 5E and F). The results indicate that CEO@NE effectively mitigated the immune response caused by *Candida* infection, potentially avoiding the generation of a cytokine storm.

Induction of Intracellular ROS Generation and Cell Death of C. albicans by CEO@NE

DCFH-DA staining, combined with flow cytometry and fluorescence microscopy analysis, was employed to evaluate the generation of ROS within *C. albicans* cells in response to CEO@NE treatment. The results showed that treatment with 0.3 mg/mL of H_2O_2 or 100–400 μg/mL of CEO@NE induced higher levels of intracellular ROS generation than that without treatment. Notably, CEO@NE treatment elevated the percentage of DCFH-DA positive cells and the mean DCFH-DA fluorescence intensity per cell in a manner that depended on the concentration (Figure 6A and B). Concordantly, the observation of DCFH-DA fluorescence under fluorescence microscopy was consistent with the trends detected by flow cytometry (Figure 6C). Consistent with the results of ROS production, CEO@NE treatment induced PI fluorescence expression in a manner that depended on the concentration (Figure 7A and B). The observation of red fluorescence under fluorescence microscopy was consistent with the trends detected by flow cytometry (Figure 7C). Moreover, thickening of the cell wall, disorganized cytoplasmatic organelles, and absence of clear nuclear shape was observed in *C. albicans* cells treated with CEO@NE at 400 μg/mL (Figure 7D). This indicates that CEO@NE-induced cell death of *C. albicans* was closely associated with its effect on eliciting intracellular ROS generation.

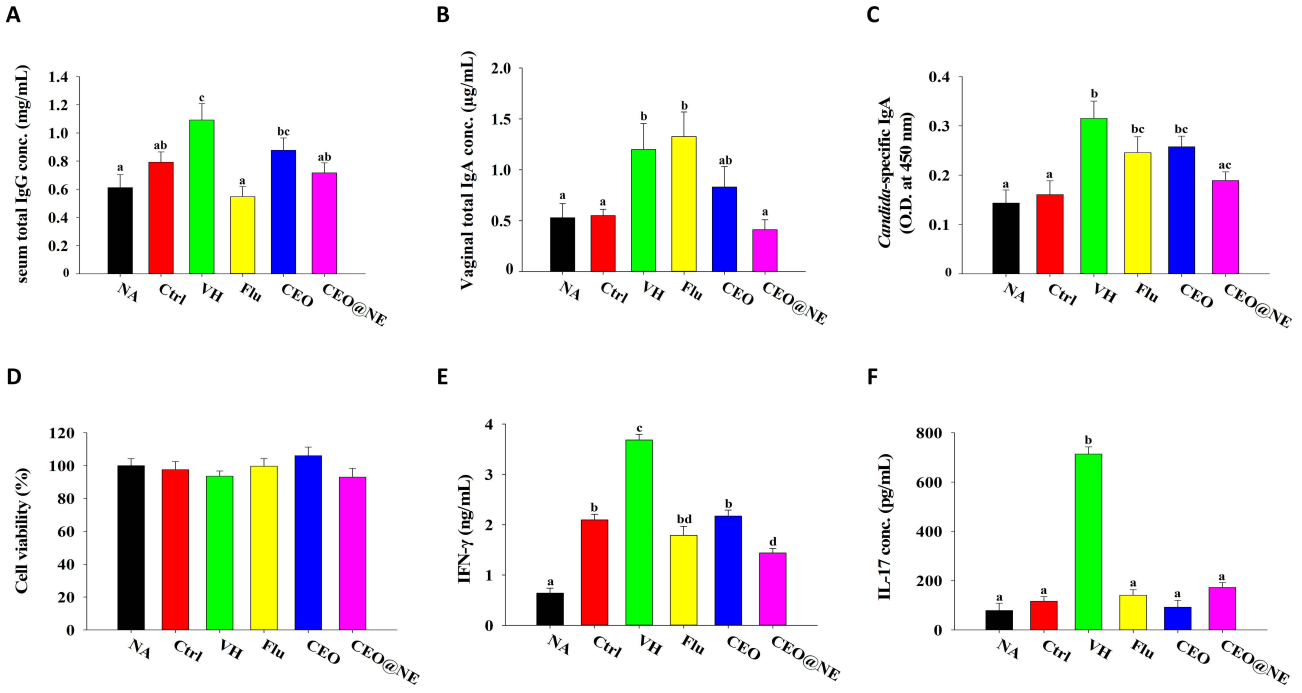

Figure 5 Effect of intravaginal CEO@NE treatment on modulating antibody and cytokine production in mice with candidal vaginitis. Following euthanasia, samples of serum, vaginal wash, and spleen were obtained. The concentrations of (**A**) total IgG in serum, (**B**) total IgA, and (**C**) *Candida*-specific IgA in vaginal washes were assessed using ELISA. Spleen cell suspensions were processed and cultured for 72 h, wherein (**D**) cell viability was determined through MTT assay, and the levels of (**E**) IFN-γ and (**F**) IL-17 in the supernatants were quantified by ELISA. The data is displayed as mean ± SEM (n = 6), showcasing a typical dataset from two separate experiments. Significance, denoted by distinct letters (a–d), indicates a noteworthy difference ($p < 0.05$) among the values.

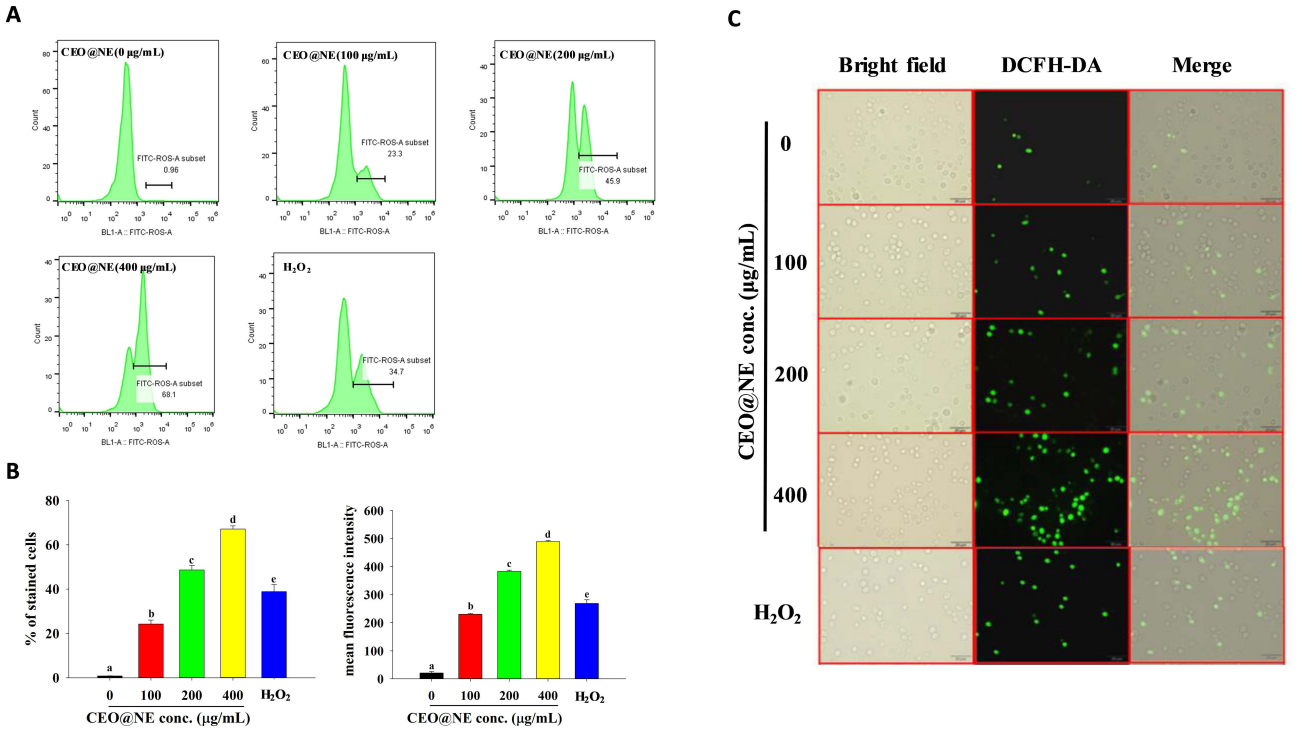

Figure 6 CEO@NE-induced ROS production in *C. albicans* cells. *C. albicans* cells were incubated in the presence of CEO@NE (0–400 μg/mL) or H_2O_2 (0.3 mg/mL), and the production of intracellular ROS was investigated by DCFH-DA fluorescent probe and analyzed by flow cytometry and fluorescence microscopy. (**A**) Representative histograms of flow cytometry analysis are shown, and (**B**) the percentage of DCFH-DA stained cells and mean fluorescence intensity were compared between the groups. (**C**) Representative images of DCFH-DA stained cells observed by fluorescence microscopy. The data is displayed as mean ± SEM (n = 6), showcasing a typical dataset from two separate experiments. Significance, denoted by distinct letters (a–e), indicates a noteworthy difference ($p < 0.05$) among the values.

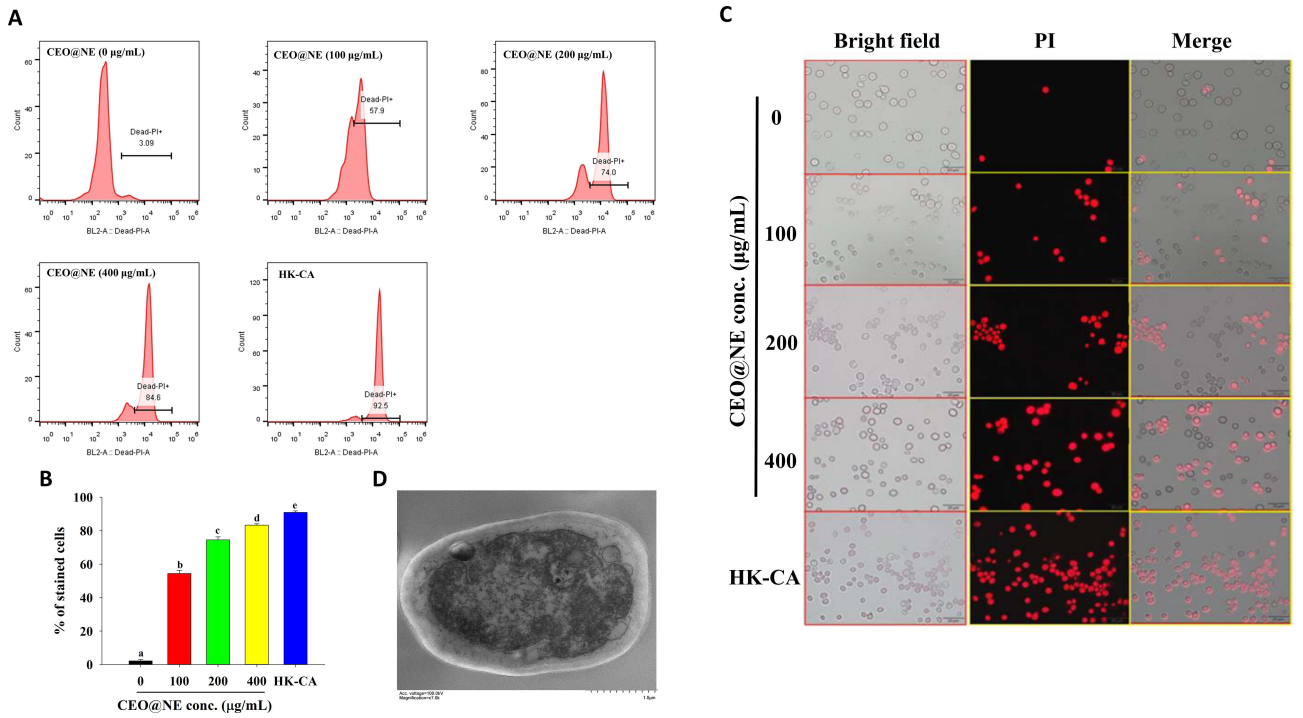

Figure 7 CEO@NE-induced cell death in *C. albicans*. *C. albicans* cells were incubated in the presence of CEO@NE (0–400 μg/mL), and cell death of incubated *C. albicans* and heat-killed *C. albicans* (HK-CA) was investigated by propidium iodide (PI) staining and analyzed by flow cytometry and fluorescence microscopy. (**A**) Representative histograms of flow cytometry analysis are shown, and (**B**) a comparison was made between the groups based on the percentage of PI stained cells. (**C**) Representative images of PI stained cells observed by fluorescence microscopy. (**D**) Representative image of CEO@NE (400 μg/mL)-treated *C. albicans* cell observed by liquid TEM. The data is displayed as mean ± SEM (n = 6), showcasing a typical dataset from two separate experiments. Significance, denoted by distinct letters (a–e), indicates a noteworthy difference ($p < 0.05$) among the values.

Anti-Inflammatory Activity of CEO@NE in LPS-Stimulated Splenocytes

LPS is commonly used to simulate the inflammatory response during pathogen invasion. LPS can activate immune cells by binding to toll-like receptor 4 to stimulate the production of pro-inflammatory cytokines. Compared to that without treatment, the secretion of IFN-γ, IL-17, TNF-α and IL-6 were significantly increased from splenocytes stimulated by LPS (Figure 8A–D). However, the concentrations of these cytokines were significantly decreased by CEO@NE treatment, confirming the direct effect of CEO@NE at concentrations of 100–400 μg/mL on suppressing inflammation and potentially inhibiting the activation of Th1 and Th17 cells (Figure 8A–D).

Discussion

In the current study, the MIC for CEO against *C. albicans* was 1 mg/mL, categorizing it as a moderately effective antifungal agent, consistent with previous research.[8] However, CEO faces challenges like poor stability and low water solubility, rendering it ineffective for in vivo application. Moreover, high concentration of CEO can lead to discomfort and local irritation.[33] Therefore, we attempted to prepare CEO@NE using various proportions of CEO, Tween, and Span emulsifiers, along with distilled water, through vortexing, homogenization, and ultrasonication. Among the tested formulations and processes, the selected formulation contained the highest proportion of CEO and the lowest proportion of emulsifier. The employed process was found to require the least time and power to formulate stable CEO@NE with a diameter less than 100 nm. Further investigation may employ response surface methodology as a tool to optimize the formulation and nanoemulsification process of CEO@NE.

In this study, the ultrasonic nanoemulsification process successfully prepared CEO@NE to efficiently disperse and deliver CEO to the vaginal mucosa, enhancing therapeutic effects against *Candida* vaginitis. NE offer key advantages in preparation and customization, allowing researchers to tailor emulsifiers, ratios, and oil-phase compositions for specific drug release profiles, stability, and compatibility with various compounds.[34] NE, suitable for large-scale production, is

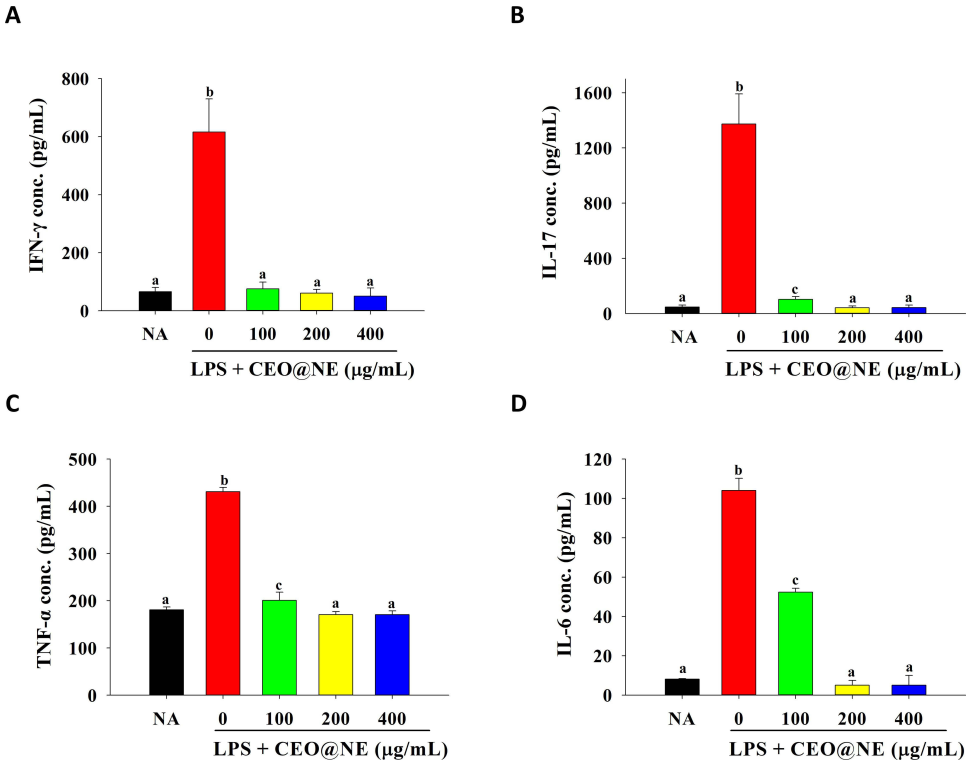

Figure 8 Effect of CEO@NE on suppressing LPS-induced cytokine production from murine splenocytes. Splenocytes isolated from normal mice (6×10^6 cells/mL) were cultured in the presence of LPS (1 μg/mL) and CEO@NE (0–400 μg/mL) for 72 h. The supernatants from these cultured splenocytes were gathered and subjected to ELISA to quantify the concentrations of (**A**) IFN-γ, (**B**) IL-17, (**C**) TNF-α, and (**D**) IL-6. The data is displayed as mean ± SEM (n = 6), showcasing a typical dataset from two separate experiments. Significance, denoted by distinct letters (a–c), indicates a noteworthy difference ($p < 0.05$) among the values.

cost-effective and scalable for drug delivery. The small droplet size provides superior physical stability due to Brownian motion counteracting gravitational or viscous forces, aligning with the reported excellent physical stability in previous research.[35] The PDI of CEO@NE confirmed a uniform and stable droplet size distribution. Beyond physical stability, NE enhances bioavailability compared to conventional emulsions, attributed to smaller droplet size and increased surface area, facilitating rapid and efficient drug absorption. The ability of NE to disperse lipophilic compounds makes it suitable for diverse drugs and applications, potentially improving therapeutic efficacy.[36]

At the moment, there is a lack of research investigating the effectiveness of CEO@NE in treating *Candida* vaginitis, although a few studies have demonstrated that nanoemulsification improves the antifungal properties of CEO. For example, a CEO-based NE, consisting of CEO, deionized water, and Tween 80, formed using an ultrasonic bath and an ultrasonic probe, exhibited better antifungal activity against *Aspergillus niger, Rhizopus arrhizus, Penicillium* sp., and *Colletotrichum gloeosporioides* compared to CEO coarse emulsion.[18] Naserzadeh et al prepared a CEO-based NE consisting of CEO, water, Tween 80, Span 80, and Lecithin by ultrasonication. The CEO-based NE exhibited significantly increased antifungal activity against *Rhizopus stolonifera* and *Botrytis cinerea*. Furthermore, the CEO-based NE at a concentration of 0.2% demonstrated a significant effect in reducing fruit decay and showed the lowest fruit infection.[19] Recently, a cinnamon bark oil-based NE was prepared by ultrasonication using Span 80 and Tween 80 as emulsifiers. Although the MIC of cinnamon bark oil and the NE did not differ significantly, the antifungal activity of the NE was evidenced by the inhibition of *Saccharomyces cerevisiae* with a 2 log reduction within 60 minutes of incubation with 10% (v/v) NE compared to cinnamon bark oil alone.[20] In the current study, the ultrasonic process was also employed to prepare CEO@NE. Notably, a better therapeutic potency of CEO@NE than CEO was demonstrated in a murine model of *Candida* vaginitis, further indicating the potential for developing CEO@NE as a nanomedicine against *Candida* infection.

The antifungal effect of most lipophilic components of essential oils is commonly through disrupting cell walls and membranes, causing substance leakage, coagulation, inhibiting DNA, RNA, protein, and polysaccharide synthesis, and hindering enzyme production.[37] A recent study revealed compelling findings, demonstrating that 100% inhibition of *Raffaelea quercus-mongolicae* was accomplished with 5 mg per paper disc of cinnamon bark essential oil and 2.5 mg per paper disc of eugenol. Both ROS generation and disruption of the cell membrane were observed in the treated fungal cells.[38] In another study, cinnamaldehyde displayed robust antifungal efficacy, with a MIC of 0.26 mg/mL for managing *C. albicans*. As the cinnamaldehyde concentration increased, the intracellular ROS levels rose, reaching a significant 55.2 ± 16.23% at 520 μg/mL.[11] In the current study, CEO@NE at the concentration of 400 μg/mL exhibited a 68.87% ROS generation within *C. albicans* cells. The heightened ROS production coincided with an increase in PI expression, indicating a strong correlation between ROS accumulation and the antifungal activity of CEO@NE. Nevertheless, additional research is imperative to elucidate potential mechanisms, including the liberation of cytochrome c from the mitochondria, depolarization of mitochondrial membrane potential, and activation of metacaspase.

The intact epithelial barrier of the vagina can maintain a balance between symbiotic microorganisms and the host. In hosts without *C. albicans* infection, the vaginal epithelial layer and mucosa should remain intact without inflammation. Conversely, severe damage to the vaginal mucosa was evident in H&E staining, accompanied by epithelial layer loss, cell infiltration, and inflammatory responses in infected hosts.[39] Moreover, the formation of micro-abscesses resulting from the aggregation of neutrophils and lymphocytes is a typical symptom after fungal infection.[30] This study utilized H&E and PAS staining to observe histopathological changes of vaginal tissues. Intravaginal *C. albicans* challenge elicited noticeable filamentous adhesion, cell infiltration, a decrease in epithelial layer thickness, and the abundant formation of micro-abscesses. However, the damage and inflammation in mice treated with CEO@NE, demonstrating an effective therapeutic outcome. Notably, CEO treatment partially alleviated vaginal lesions associated with *C. albicans* infection. This result suggests that CEO without nanoemulsification may not uniformly and stably act in the vaginal cavity after intravaginal administration, leading to poor therapeutic effects.

Inhibiting the excessive expression of cytokines to prevent cytokine storms is a primary goal in treating various diseases.[32] A previous investigation revealed that the essential oil of *C. osmophloeum*, at a concentration of 60 μg/mL, markedly suppressed the secretion of TNF-α, IL-1β, and IL-6 from J774A.1 cells stimulated with LPS.[40] In this study, CEO@NE exhibited a significant inhibitory effect on the secretion of IL-6, IFN-γ, TNF-α, and IL-17A in splenocytes stimulated with LPS. The levels of these cytokines decreased to that comparable to the unstimulated group. Importantly, the suppressive impact of CEO@NE on cytokine expression was not accompanied by a decrease in cell viability. The in vitro anti-inflammatory activity of CEO@NE further supports its potential as an agent to treat *Candida* vaginitis.

The vaginal microbiota not only influences mucosal integrity but also functions as the primary barrier against opportunistic pathogens. The lactic acid produced by vaginal microbes contributes to maintaining host health and physiological functions. When LAB dominates the vaginal microbiota, they generate substantial amounts of lactic acid, acidifying the vaginal pH to strong acidity, thereby preventing most microbial infections.[41] In addition, LAB is recognized for its pivotal role in preserving vaginal health.[42] In this study, CEO@NE was administered into the vaginal tract of non-infected mice to assess its potential impact on LAB in the vaginal microbiota. The current results suggest that intravaginal treatment of CEO@NE would not affect the abundance of LAB in the vaginal tract, thereby avoiding related side effects induced by alterations in the vaginal LAB. However, these results provide a preliminary assessment, and further research using advanced techniques, such as 16S rRNA gene sequencing, is needed to identify and explore changes in the vaginal microbiota.

Conclusion

Results of this study indicate that CEO@NE effectively treated vaginal *C. albicans* infection in mice, evidenced by reducing *C. albicans* colonization, alleviating vaginitis, restricting immune response, and limitedly altering vaginal LAB. Induction of intracellular ROS production is considered one of the key mechanisms for the anti-*Candida* activity of CEO@NE. While the nanoemulsification process did not alter the MIC and MFC, it did facilitate the efficient delivery and dispersion of CEO to the vaginal mucosa, thereby enhancing its therapeutic effects. The ability of CEO@NE to reduce *C. albicans* colonization and inflammation, as well as its favorable stability and safety profile, make it a valuable candidate for further clinical research and development.

Abbreviations

CEO, cinnamon essential oil; CEO@NE, cinnamon essential oil-nanoemulsion; DCFH-DA, 2',7'-Dichlorofluorescin diacetate; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; FTIR, Fourier-transform infrared spectroscopy; H&E, hematoxylin and eosin; IFN-γ, interferon gamma; IL-6, interleukin 6; IL-17, interleukin 17; LAB, lactic acid bacteria; MFC, minimum fungicidal concentration; MIC, minimum inhibition concentration; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NE nanoemulsion; PAS, Periodic acid-Schiff; PDI, polydispersity index; PI, propidium iodide; ROS, reactive oxygen species; Th cell, T helper cell; TNF-α, tumor necrosis factor alpha.

Ethical Statements

Every procedure and protocol adhered strictly to the principles set forth in the National Research Council's Guide for the Care and Use of Laboratory Animals. Approval for the experimental protocols was obtained from the NTOU Institutional Animal Care and Use Committee (NTOU IACUC-109057).

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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