

Development of a ZnO–chitosan nano-delivery platform encapsulating *Matricaria chamomilla* essential oil for multi-target inhibition of *Candida albicans*

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Article

Keywords: *Candida albicans*, Zinc oxide nanoparticles, *Matricaria chamomilla* essential oil, Antifungal activity, Biofilm formation

Posted Date: May 11th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9466640/v1>

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Additional Declarations: No competing interests reported.

Abstract

The increasing prevalence of candidiasis caused by *Candida albicans* and the limited efficacy of conventional therapies necessitate the development of advanced delivery systems with improved bioavailability and multi-target activity. Nanoencapsulation of essential oils in nano-delivery platform leads to increased solubility, stability, and bioavailability. In this study, a ZnO–chitosan nano-delivery platform encapsulating *Matricaria chamomilla* essential oil (ZnO–CS–MCEO) was developed and evaluated for antifungal and antibiofilm efficacy against *C. albicans*. The nanocomposite was characterized by SEM, XRD, FTIR, and DLS analyses. The formulation exhibited a mean particle size of 98.17 nm, a polydispersity index of 0.362, zeta potential of 25.9 mv and an encapsulation efficiency of 75%, indicating successful loading and colloidal stability. Antifungal activity assessed by broth microdilution revealed MIC and MFC values of 125 and 250 µg/mL, respectively. The nanoplatform inhibited biofilm formation by 71.76% at 3×MIC and reduced ergosterol content by 65.57% at MIC concentration. Mechanistic investigations demonstrated time-dependent potassium leakage (63.80% at 240 min), significant induction of intracellular ROS ($P < 0.001$), and severe ultrastructural damage confirmed by TEM imaging. Cytotoxicity assessment on L-929 fibroblasts showed no toxicity at MIC levels, supporting the biocompatibility of the formulation. These findings are the first to report that the ZnO–CS–MCEO nano-delivery platform exerts multi-target antifungal effects through membrane disruption, oxidative stress induction, and inhibition of biofilm-associated virulence factors, representing a novel, high potential strategy for the management of candidiasis.

1. Introduction

Invasive candidiasis has predominantly been caused by *Candida albicans* and represents a serious global health challenge, with an increasing prevalence despite significant advances in antifungal treatments [1]. Candidiasis accounts for approximately 70% of invasive fungal infections worldwide and imposes a significant burden on healthcare systems, with a high mortality, particularly among immunocompromised individuals such as patients with acquired immunodeficiency syndrome (AIDS), organ transplant recipients, and cancer patients undergoing chemotherapy [2]. Current antifungal drugs have limitations in clinical efficacy, emerging resistance mechanisms, and toxicity of the compounds due to similarities between fungal and human cells [3, 4]. Consequently, there is an urgent need to develop novel therapeutic strategies with multi-target mechanisms of action, reduced toxicity, and improved pharmacokinetic and pharmacodynamic properties [2, 5].

A critical determinant of *C. albicans* pathogenicity is its ability to form biofilms on biotic and abiotic surfaces. Biofilms are highly organized microbial communities embedded within an extracellular polymeric matrix that acts as a physical and biochemical barrier against antifungal agents and host immune defenses [6, 7]. Biofilm development proceeds through sequential stages of adhesion, proliferation, maturation, and dispersal, and is tightly regulated by virulence factors [8]. Overexpression of these genes promotes strong attachment to epithelial tissues and medical devices, contributing to persistent and recurrent infections that are markedly less susceptible to conventional therapy [6, 7].

Nanotechnology offers promising solutions to overcome these therapeutic challenges by enabling targeted delivery, controlled release, and enhanced bioactivity of antimicrobial agents. Zinc oxide nanoparticles (ZnO NPs) have attracted considerable attention due to their broad-spectrum antimicrobial properties, biocompatibility, and ability to generate reactive oxygen species, disrupt membrane integrity, and interfere with microbial metabolism [9, 10]. However, the clinical translation of ZnO NPs alone is limited by aggregation, insufficient stability, and potential cytotoxicity at high concentrations. Incorporation into biopolymer-based carriers such as chitosan can improve dispersion, mucoadhesion, and interaction with fungal cell walls, creating an effective nano-delivery platform.

Matricaria chamomilla (German chamomile) is one of the most widely used medicinal plants with a long history in traditional and modern medicine [11, 12]. Its essential oil is rich in bioactive constituents such as α -bisabolol, chamazulene, and spiroethers, which exhibit pronounced anti-inflammatory, antioxidant, and antimicrobial properties. Several studies have demonstrated that *M. chamomilla* essential oil possesses significant antifungal activity against *Candida* species through disruption of cell membrane permeability, inhibition of ergosterol biosynthesis, and interference with hyphal development. In addition, the natural origin and favorable safety profile of *M. chamomile* make it an attractive alternative to synthetic antifungal drugs. Nevertheless, the clinical application of the essential oil is restricted by poor aqueous solubility, volatility, and limited stability, which necessitate the development of advanced delivery systems to enhance its bioavailability and therapeutic efficacy [13]. Chitosan is considered one of the best biomaterials for the fabrication of nano-delivery platform with various applications due to its biodegradability and non-toxicity [14]. It exhibits antifungal effects against *C. albicans*, *F. solani*, and *A. niger* [15]. Despite the documented antifungal potential of ZnO nanoparticles and *M. chamomilla* essential oil individually, their synergistic integration into a single nano-delivery platform has not been sufficiently explored. Combining the membrane-disruptive and ROS-mediated activity of ZnO with the bioactive phytochemicals of *M. chamomile* may provide a multi-target strategy capable of combating candidiasis. Therefore, the present study aimed to develop and characterize a ZnO–chitosan nanocomposite encapsulating *M. chamomilla* essential oil and to evaluate its antifungal and antibiofilm efficacy, underlying mechanisms, and biocompatibility.

2. Materials and Methods

2.1 Synthesis of Zinc Oxide-*Matricaria chamomilla* essential Oil-Chitosan nanocomposite

(ZnO–CS–MCEO)

2.1.1 Synthesis of Zinc Oxide nanoparticles

First, 2.9 g of zinc acetate was dissolved in 50 mL of double-distilled deionized water and stirred for 1 hour at room temperature. Then, 20 mL of 10% NaOH was slowly added dropwise to the solution and was mixed vigorously for 1 hour. The precipitate was collected by centrifugation ($10,000 \times g$ for 10

minutes), washed three times with distilled water, and finally dried in an oven at 100°C for 1 hour to obtain 0.8 g of ZnO nanoparticles [16].

2.1.2 Preparation of chitosan solution

0.66 g of Chitosan was dissolved in 100 mL of 2% acetic acid solution and stirred for 24 hours at room temperature. After complete dissolution, 2 mL of glycerol was added to the solution.

2.1.3 Synthesis of ZnO–CS–MCEO nanocomposite

Matricaria chamomilla essential oil was purchased from Shimistore company (Tehran, Iran).

0.099 g of zinc oxide powder was dissolved in 1.5 mL of double-distilled deionized water and sonicated for 15 minutes. Then, 200 µL of *M. chamomilla* essential oil dissolved in 400 µL DMSO was added and sonicated for 30 minutes. The solution was added to 10 mL of activated chitosan under vigorous stirring for 1 hour at room temperature [17].

2.2 Antifungal susceptibility testing

Candida albicans ATCC 10231 was obtained by the Pasteur Institute of Iran Culture Collection and was cultured on sabouraud dextrose agar (SDA, Merck, Germany) for 24 hours at 35°C. 100 µL of fungal cell suspension (5×10^6 cells/mL (was cultured on SDA plates.

The minimum inhibitory concentration (MIC) was determined according to CLSI M27-A3 and M27-Ed4 guidelines [18, 19]. Serial dilutions of the tested compounds were prepared in the 96-well micro-plate, then 100 µL of the fungal suspension (2.5×10^3 cells/mL) was added to each well. In this study, RPMI-1640 medium alone and with the inoculum suspensions and fluconazole were included as the negative, positive and drug controls, respectively. The final concentration ranges were as follows: ZnO nanoparticles (1600 – 6.25 µg /mL), ZnO–CS–MCEO nanocomposite (1000 – 3.906 µg /mL), *M. chamomilla* essential oil (1000 – 3.906 µg /mL), chitosan (5000 – 19.53 µg /mL), and fluconazole (64–0.25 µg /mL). Plates were incubated at 35°C with shaking (84 rpm) for 48 hours. MIC was defined as the lowest concentration that inhibited fungal growth.

The minimum fungicidal concentration (MFC) was determined by subculturing 10 µL of any well on SDA plates. Following incubation at 35°C for 24 hours, MFC was expressed as the lowest concentration required for killing at least 99.9% of the primary inoculum [20].

2.3 Structural characterization of ZnO–CS–MCEO nanocomposite

2.3.1 Scanning Electron Microscopy (SEM)

The morphology and size of the ZnO–CS–MCEO nanocomposite and ZnO nanoparticles were analyzed by a Leo 440i scanning electron microscope (SEM; LEO Electron Microscopy Ltd., England) [21].

2.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

The chemical structure of the ZnO–CS–MCEO nanocomposite was characterized using a Jasco FT/IR-4600 spectrometer with wavelength range of 350–7800 cm⁻¹ [22].

2.3.3 X-Ray Diffraction (XRD)

The crystal structure of the ZnO–CS–MCEO nanocomposite was analyzed by a Philips X'Pert MPD diffractometer with a voltage of 40 kV and 40 mA with Cu K α radiation ($\lambda = 1.54056 \text{ \AA}$). The diffraction patterns were recorded for a 2θ range of 5° to 120° with a step size of 0.02°, and crystal size and shape were determined by comparing peak positions with JCPDS standards [23].

2.3.4 Dynamic Light Scattering (DLS)

The particle size distribution and zeta potential of the ZnO–CS–MCEO nanocomposite were measured by a Zetasizer Pro (Malvern Panalytical, Atomika Teknik) [24].

2.4 Quantification of *M. chamomilla* Essential Oil

2.4.1 Standard Curve

A standard calibration curve was constructed by measuring the absorbance of concentrations 5 to 40 $\mu\text{g/mL}$ of *M. chamomilla* essential oil at wavelength of 296 nm using a UV–Vi's spectrophotometer. The linear equation obtained ($R^2 = 0.9943$) was used to quantify the concentrations of essential oil.

2.4.2 Encapsulation efficiency of *M. chamomilla* Essential Oil in ZnO–CS–MCEO

The encapsulation efficiency (EE) of *M. chamomilla* Essential Oil was determined by a dialysis technique and UV-Vis spectrophotometer at a wavelength of 296 nm. EE was calculated as follows:

$$\text{EE (\%)} = (\text{Total amount of drug incorporated} / \text{Total amount of drug added}) \times 100 \text{ [25].}$$

2.4.3 Release kinetics of *M. chamomilla* essential Oil from ZnO–CS–MCEO

The release kinetics of the essential oil was studied by a Franz diffusion cell with a dialysis membrane and spectrophotometric method at a wavelength of 296 nm. [26].

2.5 Quantification of Zinc Content

The content of zinc in the nanocomposite was quantified via atomic absorption spectroscopy (AAS) using a Varian spectrometer.

2.6 Biofilm formation using XTT assay

The biofilms of *C. albicans* were formed in a 96-well micro-plate by a volume of 100 μL cell suspension (1×10^6 cells/mL). After 48 hours incubation at 35°C, wells were washed twice with PBS (PH 7.4) to remove nonadherent cells. Serial dilutions of the concentration of 3 $\times$ MIC (1000 μg /mL) of the ZnO–CS–MCEO nanocomposite were prepared in RPMI-1640 medium and added to the washed-biofilms, followed by incubation at 35°C for 24 hours. Wells in other rows of the plate also treated with ZnO nanoparticles, *M. chamomilla* essential oil, chitosan, fluconazole. The untreated biofilm and medium-only were included as controls. Each well was washed again with PBS. The biofilm formation activity was quantified via adding 100 μL of XTT-menadione solution (0.5 g/L XTT in PBS with 10 mM menadione in acetone) to the washed-biofilm plate. The plate was incubated at 35°C for 3 hours in the dark. The absorbance of plate was measured at 490 nm using a micro-plate reader [27]. All experiments were performed in triplicate.

2.7 Quantification of cell membrane ergosterol content

The ergosterol content in the plasma membrane of *C. albicans* was quantified using spectrophotometric method. Briefly, 200 μL of a fungal suspension (0.5 McFarland, $\sim 5 \times 10^6$ cells/mL) inoculated into 20 mL of sabouraud dextrose broth containing different concentrations of the ZnO–CS–MCEO nanocomposite ($\times 0.5$, $\times 1$, $\times 2$, and $\times 4$ MIC), ZnO nanoparticles, *M. chamomilla* essential oil, chitosan, and fluconazole at based on MIC concentrations. All cultures were incubated at 35°C with shaking (120 rpm) for 48 hours. The cells were harvested by centrifugation at 5000 g for 5 minutes, washed twice with sterile distilled water, dried at 70°C for 2 hours. The dry weight of the cell pellets was determined. 3 mL of 25% alcoholic potassium hydroxide was added to each pellet and incubated at 90°C for 1 hour in a water bath. The mixture of 1 mL of distilled water and 3 mL of n-hexane was added to each tube. The upper n-hexane phase was collected and stored at -20°C for 24 hours. Then, the n-hexane phase diluted fivefold with absolute ethanol. The quantification was performed by measuring absorbance at 230 nm and 281.5 nm using a UV-Vis spectrophotometer. The calculation was based on the following equation [28]:

$$\% \text{ Ergosterol} = [(A_{281.5} / 290) / \text{pellet weight}] - [(A_{230} / 518) / \text{pellet weight}].$$

2.8 Extracellular potassium leakage

The potassium leakage assay was performed by measuring the extracellular potassium concentration released from *C. albicans* cell suspension, using a previously described method with minor modifications. Briefly, the cultured *C. albicans* cells were harvested by centrifugation at 5000 g for 5 minutes, then washed three times, and resuspended in sterile deionized water. 2 mL of the fungal cell suspension was mixed with 2 mL of sterile deionized water containing different concentrations of the ZnO–CS–MCEO nanocomposite ($\times 1$, $\times 2$, and $\times 4$ MIC), and MIC concentrations of ZnO nanoparticles, *M. chamomilla* essential oil, chitosan, and amphotericin B. Samples were incubated at 35°C for 0, 30, 60, 120, and 240 minutes. After centrifugation at 6000 g for 15 minutes, the supernatant was measured for percentage of potassium released using atomic absorption spectroscopy (AAS) [29]. The data of three independent experiments are reported in this study.

2.9 Intracellular ROS induction

The intracellular reactive oxygen species (ROS) was measured using the colorimetric ROS Assay Kit (Karmania Pars Gene, Iran). *C. albicans* cells (1×10^6 cells/mL) were exposed to concentrations of MIC the ZnO–CS–MCEO nanocomposite, ZnO nanoparticles, *M. chamomilla* essential oil, chitosan, and fluconazole. All treatments were incubated at 35°C with shaking (120 rpm) for 24 hours. After incubation, the cells were harvested and washed three times with phosphate-buffered saline (PBS pH 7.4; Gibco, USA). Cell lysates were prepared in the 96-well microplate using two parallel wells (A ROS-quenched blank and a ROS-reaction well). Following a 10 min incubation at 37°C, TMB substrate was added and reactions were stopped after 15 min. Absorbance was read at 450 nm using a microplate reader [30]. The ROS concentration (μM) was calculated by the following formula:

$$\text{ROS } (\mu\text{M}): (A_{\text{sample}} - A_{\text{Blank}}) \times 24.858116.$$

All experiments were performed in triplicate.

2.10 Fungal morphological changes Using Transmission Electron Microscopy (TEM)

To determine morphological changes of *C. albicans*, transmission Electron Microscopy was used. After treating *C. albicans* cells with a concentration of MIC the ZnO–CS–MCEO nanocomposite, the pellet was fixed with 3% glutaraldehyde overnight at 4°C. Post-fixation was done with 1% osmium tetroxide in PBS for 70 minutes. Then, the samples were dehydrated in gradually increasing acetone solutions (30–100%) and embedded with Eon 812. The ultrathin sections were cut using an RMC MT-X ultramicrotome. Thin sections were transferred to copper grids. Uranyl acetate and lead citrate were used for staining, and finally Images were observed using an Ara-80 transmission electron microscope (Germany) at 80 kV [31].

2.11 Cytotoxicity assay

The cytotoxicity assay of the ZnO–CS–MCEO nanocomposite was performed on murine fibroblast cell line L-929 using MTT method according to Saharkhiz et al. with minor modifications. The L-929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and maintained at 37°C with 5% CO₂ in a humidified incubator. Subsequently, the cells (1×10^4 cells /well) were seeded in 96-well plates, and incubated for 24 hours. Next, the cells were treated with a serial dilution concentration of the nanocomposite (7.81–250 $\mu\text{g/mL}$) and incubated for 24 and 48 hours. The Untreated cells were used as the negative control. Following incubation, 10 μL of MTT solution was added to each well, and the plates were incubated at 37°C for 4 hours. Then, the supernatants were discarded, and 100 μL of DMSO (dimethyl sulfoxide) was added to the wells with gentle shaking to dissolve the formazan crystals. Finally, the OD of each well was measured at 570 nm using a microplate reader [32]. The cell viability was estimated as follows:

$$\text{Cell viability (\%)} = (\text{Abs control} - \text{Abs treated} / \text{Abs control}) \times 100.$$

2.12. Statistical Analysis

All data were calculated as mean $\pm$ standard deviation of three experiments. Statistical analysis was performed using one-way ANOVA. P values < 0.05 were considered statistically significant.

3. Results

3.1 Antifungal susceptibility testing

MIC and MFC

In vitro antifungal activity profiles of ZnO–CS–MCEO nanocomposite and ZnO nanoparticles, MCEO, and chitosan against *C. albicans* are described in Tables 1. The MIC and MFC values of the nanocomposite for *C. albicans* were 125 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$, respectively. The MIC values of the ZnO nanoparticles, MCEO, chitosan, and fluconazole for *C. albicans* were 400 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$, 625 $\mu\text{g/mL}$, and 1 $\mu\text{g/mL}$ respectively.

Table 1
MIC and MFC values ($\mu\text{g/mL}$) of the ZnO–CS–MCEO nanocomposite, chitosan, ZnO NPs, and *M. chamomilla* EO against *C. albicans* by broth microdilution method.

Agent	MIC ($\mu\text{g/mL}$)	MFC ($\mu\text{g/mL}$)
Nanocomposite	125	250
ZnO nanoparticles	400	1600
MCEO	500	1000
Chitosan	625	1250
Fluconazole	1	2
MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration.		

3.2 Structural characterization of the ZnO–CS–MCEO nanocomposite

3.2.1 X-Ray Diffraction (XRD)

The XRD pattern of ZnO nanoparticles (Fig. 1) showed a pure hexagonal wurtzite structure (JCPDS 36-1451), with sharp peaks at $2\theta = 31.8^\circ$ (100), 36.3° (101), 34.4° (002), 47.5° (102), 56.6° (110), 62.9° (103), 68.0° (200), and 77.0° (201). The sharpness of the peak indicated high crystallinity of ZnO nanoparticles. The XRD pattern of ZnO–CS–MCEO nanocomposite (Fig. 2) showed reduced peak intensity and broadening of the peaks for ZnO. Additionally, a broad amorphous peak ($2\theta = 10\text{--}30^\circ$) observed due to chitosan in the nanocomposite.

3.2.2 Fourier Transform Infra-Red Spectroscopy (FTIR)

The FTIR spectroscopy (Fig. 3) showed interactions between the different components in the ZnO–CS–MCEO nanocomposite including: Chitosan displayed bands corresponding to –OH and –NH stretching ($3200\text{--}3600\text{ cm}^{-1}$), C–H stretching ($2800\text{--}3000\text{ cm}^{-1}$), C = O (amide I, 1650 cm^{-1}), and C–O–C vibrations ($1000\text{--}1200\text{ cm}^{-1}$). ZnO nanoparticles exhibited Zn–O stretching vibrations at $400\text{--}600\text{ cm}^{-1}$ and surface –OH groups near 3400 cm^{-1} . The *M. chamomilla* EO spectrum showed peaks for C–H ($2850\text{--}2960\text{ cm}^{-1}$), aromatic C = C ($1400\text{--}1600\text{ cm}^{-1}$), and C–O stretching ($1000\text{--}1300\text{ cm}^{-1}$). The nanocomposite spectrum retained the characteristic Zn–O band but exhibited shifted –OH and –NH stretching frequencies, confirming hydrogen bonding between chitosan, EO, and, ZnO.

3.2.3 Scanning Electron Microscopy (SEM)

The morphologic characteristics of ZnO nanoparticles and ZnO–CS–MCEO nanocomposite were determined by SEM. The ZnO nanoparticles (Fig. 4) were observed to be spherical to rod-like particles with smooth surfaces and minimal aggregation, ranging from 50 nm to 200 nm in diameter. The ZnO–CS–MCEO nanocomposite (Fig. 5) displayed a porous sponge-like structure morphology with uniform dispersion of ZnO throughout the polymeric matrix.

3.2.4 Dynamic Light Scattering (DLS)

The results of DLS, a monomodal distribution with mean diameter approximately 100 nm was shown. $D_n50\% = 98.17\text{ nm}$; $D_n10\% = 93.73\text{ nm}$; $D_n90\% = 107.68\text{ nm}$, the polydispersity index of 0.362, and zeta potential of 25.9 mv was revealed in Fig. 6.

3.3 Quantification of *M. chamomilla* Essential Oil and Zinc content

The standard curve of *M. chamomilla* essential oil ($\lambda_{\text{max}} = 296\text{ nm}$; $5\text{--}40\text{ }\mu\text{g/mL}$ in 96% ethanol) was shown in Fig. 7 ($R^2 = 0.998$). Encapsulation efficiency of *M. chamomilla* essential oil (MCEO) in the nanocomposite was measured 75%.

The release of MCEO from ZnO–CS–MCEO has been shown in Fig. 8. The released amount of MCEO was measured at 296 nm at different times. The release profile of CEO from the ZnO–CS–MCEO exhibited a biphasic pattern, with a sudden primary phase followed by a gradual plateau over 240 min.

The Zinc concentration was $1687/33\text{ }\mu\text{g/mL}$ by atomic absorption Spectroscopy.

3.4 Biofilm formation assay

The biofilm inhibition percentage of the ZnO–CS–MCEO nanocomposite, ZnO, MCEO, and chitosan increased in a concentration-dependent manner. (Table 2; Fig. 9). The nanocomposite at a concentration of $1000\text{ }\mu\text{g/mL}$ ($\times 3\text{ MIC}$) had an inhibition percentage of $71.76 \pm 3.2\%$ that it was higher than ZnO ($54.45 \pm 2.8\%$ at $3200\text{ }\mu\text{g/mL}$), MCEO ($49.99 \pm 2.5\%$ at $2000\text{ }\mu\text{g/mL}$), chitosan ($70.84 \pm 3.0\%$ at $5000\text{ }\mu\text{g/mL}$) compared to fluconazole ($49.85 \pm 2.1\%$ at $1024\text{ }\mu\text{g/mL}$) as a positive control.

Table 2

The biofilm inhibition Percentage (%) the ZnO–CS–MCEO nanocomposite, chitosan, ZnO NPs, and *M. chamomilla* EO in highest Concentration tested against *C. albicans* with XTT assay. (mean $\pm$ SD, n = 3).

Concentration ($\mu\text{g/mL}$)	Nanocomposite	ZnO NPs	MCEO	Chitosan	Fluconazole
Highest Tested	71.76 $\pm$ 3.2	54.45 $\pm$ 2.8	49.99 $\pm$ 2.5	70.84 $\pm$ 3.0	49.85 $\pm$ 2.1

3.5 Quantification of ergosterol content

Figure 10 demonstrates the effects of the ZnO–CS–MCEO nanocomposite, ZnO, MCEO, chitosan, and fluconazole on ergosterol content in *C. albicans*. Ergosterol content of *C. albicans* membranes after treatment with the concentration based on 1 MIC of the ZnO–CS–MCEO nanocomposite, ZnO, MCEO, chitosan, and fluconazole were 0.0303 ± 0.0005 $\mu\text{g/mg}$, 0.038 ± 0.0010 , 0.0448 ± 0.0015 , 0.048 ± 0.0018 , 0.032 ± 0.0012 , respectively.

As shown in the Fig. 11, percentage of reduction of membrane ergosterol for the ZnO–CS–MCEO nanocomposite, ZnO, MCEO, chitosan, and fluconazole were 65.57%, 56.82%, 49.1%, 45.46%, 63.64%, respectively.

3.6 Extracellular potassium leakage

The potassium leakage test was carried out to determine probable membrane permeability in *C. albicans*. The release of potassium increased over 240 min (Fig. 12). The ZnO–CS–MCEO nanocomposite at MIC concentration showed 63.80% of potassium release after 240 min, which was higher than ZnO (13.66%), MCEO (8.08%), and chitosan (58.58%). Amphotericin B induced 79.41% of potassium release after 240 min incubation, as positive control. The extracellular concentration of potassium in the untreated *C. albicans* increased slowly over time in the control group, reaching 1.2% after 240 minutes.

3.7 Intracellular ROS induction

The intracellular ROS production was measured using the colorimetric ROS assay in *C. albicans* cells after treatment with the ZnO–CS–MCEO nanocomposite, ZnO nanoparticles, MCEO, chitosan, and fluconazole at 125, 400, 500, 625, and 1 $\mu\text{g/mL}$ respectively for 24 h. As shown in Table 3, and Fig. 13 treatment with the ZnO–CS–MCEO nanocomposite significantly increased intracellular ROS ($P < 0.001$), rising from 0.938 ± 0.021 $\mu\text{M/mL}$ in the untreated control to 2.434 ± 0.105 $\mu\text{M/mL}$, fluconazole at 1 $\mu\text{g/mL}$ raised ROS to 1.938 ± 0.084 $\mu\text{M/mL}$, ZnO nanoparticles, MCEO, and chitosan induced ROS of 2.212 ± 0.098 , 2.038 ± 0.089 , and 1.466 ± 0.067 $\mu\text{M/mL}$, respectively. These results indicate that nanocomposite produced intracellular ROS, leading to intracellular oxidative damage and possibly membrane damage.

Table 3

The intracellular ROS($\mu\text{M}/\text{ml}$) in *Candida albicans* after 24 h exposure to MIC concentration of the ZnO–CS–MCEO nanocomposite, chitosan, ZnO NPs, and *M. chamomilla* EO Data are presented as mean $\pm$ SD (n = 3). ***P < 0.001 vs. untreated control (one-way ANOVA followed by Dunnett's post-test).

Treatment	Concentration ($\mu\text{g}/\text{mL}$)	ROS ($\mu\text{M}/\text{mL}$)
Untreated	–	0.938 ± 0.021
ZnO–CS–MCEO nanocomposite	125	$2.434 \pm 0.105^{***}$
MCEO	500	$2.038 \pm 0.089^{***}$
Chitosan	625	$1.466 \pm 0.067^{***}$
ZnO nanoparticles	400	$2.212 \pm 0.098^{***}$
Fluconazole	1	$1.938 \pm 0.084^{***}$

3.8. Transmission Electron Microscopy (TEM)

To determine the effect of ZnO–CS–MCEO nanocomposite on *C. albicans*, the ultrastructure of fungal cells was analyzed by transmission electron microscopy (TEM). The ultrastructure of the untreated control *C. albicans* cells showed normal morphology, with a thick and uniform cell wall, intact plasma membrane closely associated with the cell wall, and a dense homogeneous cytoplasm. In contrast, the exposure to the nanocomposite showed morphological changes, including detachment of the plasma membrane from the cell wall, decreased cytoplasmic density, leakage of intracellular contents. These results indicate significant cellular damage, which could lead to cell death (Fig. 14).

3.9. Cytotoxicity assay

The cytotoxicity effects of the ZnO–CS–MCEO nanocomposite, ZnO, MCEO, chitosan on L-929 cells by MTT assay after 24, 48 h of treatment are shown in Fig. 15. After 24,48 h of incubation, no significant cytotoxicity has been detected. The cell viability of L929 cells in the MIC concentration of the ZnO–CS–MCEO nanocomposite (125 $\mu\text{g}/\text{mL}$) remained at 85% and 75% after 24 and 48 h, respectively. Also, the cell viability of MCEO was significantly reduced after loading into the nanocomposite.

4. Discussion

The rising incidence of candidiasis caused by *Candida albicans*, associated with high mortality rates in immunocompromised patients and the limitations of current antifungal therapies, highlights the critical need for innovative nano delivery platform that can overcome *candida* infections through multi-target mechanisms while improving biocompatibility and controlled release profiles [2, 33, 34].

In the present study, we successfully synthesized a porous ZnO–CS–MCEO nanocomposite. Structural characterization (SEM, XRD, FTIR, and DLS) confirmed that ZnO nanoparticles were effectively incorporated into the chitosan matrix and the loading of *M. chamomilla* essential oil (MCEO) with an encapsulation efficiency of 75%, and essential oil was released in a controlled manner from the nanocomposite. The porous structure and relatively uniform particle size distribution of 98 nm, zeta potential of 25.9 mv, and polydispersity index (PDI) of 0.362, which may improve biocompatibility and enhance antifungal activity Compared to previous ZnO-chitosan systems. According to the size, PDI, EE%, and zeta potential, ZnO–CS–MCEO nanocomposite showed more compatible properties than many other formulations. The zeta potential of the nanocomposite provides adequate colloidal stability. The MCEO was released in a controlled manner from the ZnO–CS–MCEO, which is a suitable platform for its efficiency in prolonging the half-life with keeping fungal growth inhibitory activity. Increased solubility of the encapsulated MCEO could also be a reason for the greater efficacy of the nanocomposite. Another advantage of nanoformulations is the ability to release the drug slowly and steadily. This allows for maintaining the appropriate concentration of the drug for a relatively longer period of time. For example, in the study by Garcia et al. chitosan microparticles loaded with essential oils showed regular distribution and spherical shape (1–15 μm), without any morphological and physical modifications after EO incorporation, and only 12–39% encapsulation efficiency of essential oils [35].

The ZnO–CS–MCEO nanocomposite demonstrated higher antifungal activity than its individual components. The nanocomposite revealed lower MIC and MFC values than ZnO nanoparticles, MCEO, and chitosan. It revealed that encapsulation enhanced the antifungal activity of nanocomposite compared to its components. These findings are consistent with previous studies on ZnO-based nanocomposites. Nabil Y et al. emphasize the effectiveness of the chitosan/essential oil/ZnO nanobiocomposites in wound healing and combating resistant pathogens [36]. Yassin et al. reported the synergistic effects of ZnO nanoparticles and fluconazole against *C. albicans* [37]. Similarly, Motelica et al. developed a chitosan nanocomposite with zinc oxide nanoparticles and *citronella* essential oil that exhibited a zone of inhibition comparable to our ZnO- *M. chamomilla*-chitosan nanocomposite [17]. Liu and colleagues reported chamomile nanocapsules with maximum inhibition zones of 15.7 mm at higher concentrations (1.0 g/100g) [38].

We exhibited that the nanocomposite effectively inhibited biofilm formation, and gradually reduced ergosterol biosynthesis. Additionally, ZnO–CS–MCEO nanocomposite induced potassium release from *C.albicans* cells at higher concentrations is similar to manner amphotericin B causes potassium leakage. These effects indicate disruption of cell membrane integrity, probably due to a synergistic interaction. ZnO nanoparticles generate oxidative stress through the production of ROS, and hydrophobic constituents in *M. chamomile* essential oil, particularly α -bisabolol, reduce ergosterol biosynthesis. Moreover, the amine groups in chitosan enhance electrostatic interactions with the fungal cell wall and increase membrane permeability [39, 40, 41]. Consistent with these finding, Garcia et al. achieved up to 84% biomass reduction in mature *C. albicans* biofilms with chitosan microparticles loaded with essential oils, and Joshi and co-workers reported more than 90% biofilm inhibition at concentrations of lignin-coated ZnO nanoparticles [35, 42]. Additionally, α -bisabolol has been shown to inhibit yeast-to-hyphae

transformation and biosynthesis of ergosterol in *C. albicans* [40]. The nanocomposite also significantly increased intracellular ROS levels, in line with the oxidative effects of zinc oxide nanoparticles [39].

Transmission electron microscopy showed severe morphological changes in *C. albicans* cells. The TEM results align with those reported by Saha et al., who noted comparable cell wall rupture and complete membrane depolarization in fungal cells subjected to MoSe₂/chitosan nanosheets, highlighting the morphological deformities of metal-chitosan combinations [31].

The ZnO–CS–MCEO nanocomposite exhibited no cytotoxic effects on L-929 fibroblast cells. Notably, the incorporation of MCEO into the nanocomposite significantly reduced the toxicity of *M.chamomile* essential oil, as demonstrated by higher percentage of cell viability compared to MCEO alone, which is likely due to the protective role of the chitosan matrix.

This is the first study that evaluates the antifungal and antibiofilm efficacy of the ZnO–chitosan nano-delivery platform encapsulating *Matricaria chamomilla* essential oil, demonstrating multi-target antifungal effects against *C. albicans* through susceptibility tests, biofilm inhibition, membrane disruption, ROS induction, morphological changes, and HWP1 and ALS3 gene expression. Overall, these results support the concept that incorporating plant-derived essential oils into metal oxide-polymer nanocomposites represents a promising approach to treat candidiasis and improve biocompatibility. The simplicity of synthesis, low production cost, and favorable physicochemical properties of this ZnO-CS-MCEO nanocomposite could make it an attractive candidate for combating *Candida* infections.

Conclusion

In conclusion, the ZnO–CS–MCEO nanocomposite is a biocompatible, multi target antifungal system that can manage *C. albicans* infections through multiple synergistic mechanisms, highlighting its potential application in the formulation of efficient antifungal drugs, including mouthwashes, ointments, lotions, and creams for the effective treatment of candidiasis.

Declarations

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors consent to the publication of this manuscript.

Conflict of interest

The authors declare no competing interests.

Funding

This work was financially supported by the Research Deputy of Tarbiat Modares University.

Research reported in this publication was supported by Elite Researcher Grant Committee under award number 1403.030 from the National Institute for Medical Research Development (NIMAD), Tehran, Iran.

Author Contribution

Iman Torabi carried out the experiments and wrote the original draft of manuscript. Masoomeh Shams-Ghahfarokhi conceived, designed and supervised the study. Iman Torabi, Masoomeh Shams-Ghahfarokhi and Mehdi Razzaghi-Abyaneh carried out the data analysis. All authors revised and approved the final version of manuscript.

Acknowledgement

Not Applicable.

Data Availability

All the important data relevant to this study was reported in the manuscript.

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Figures

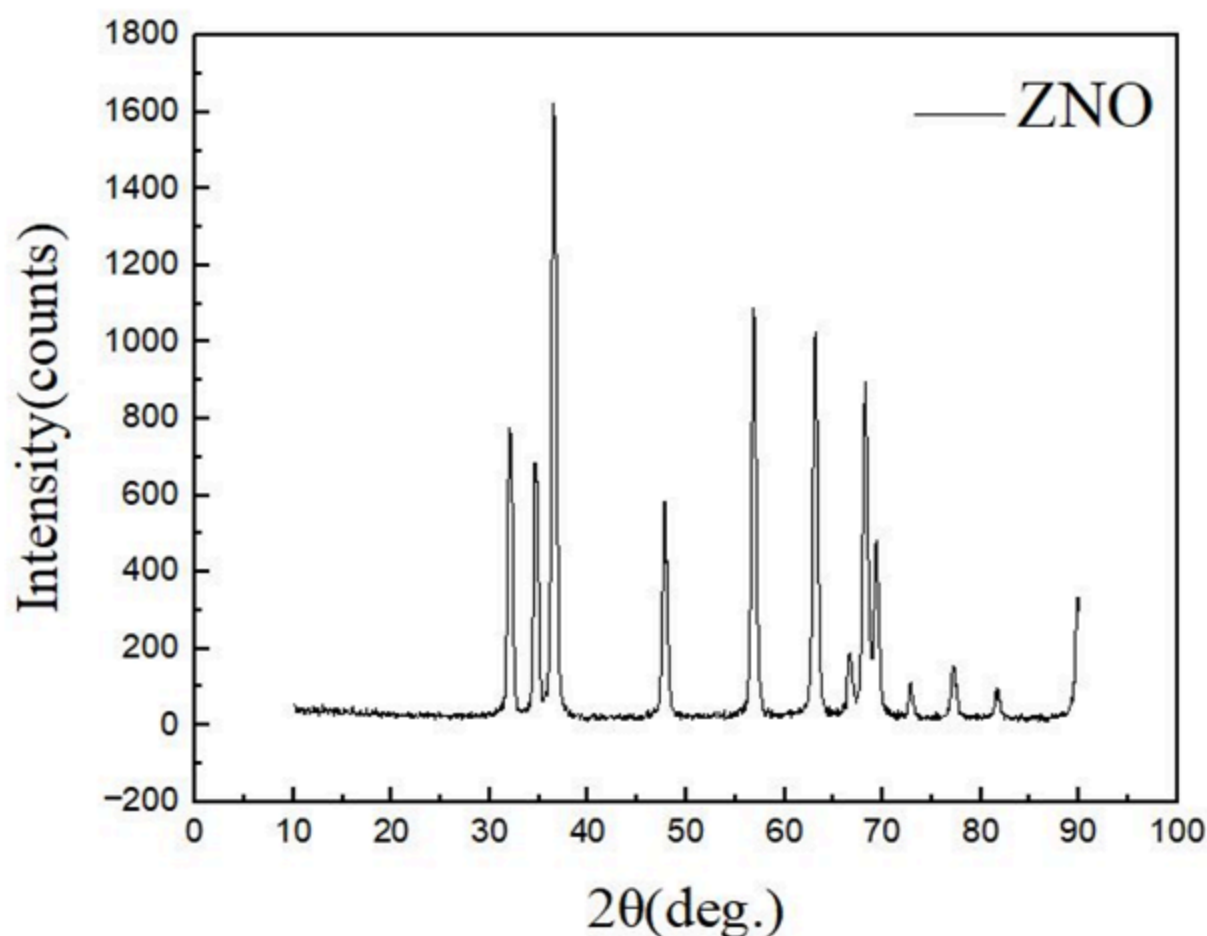


Figure 1

The X-ray diffractogram analysis of pure ZnO nanoparticles (wurtzite structure).

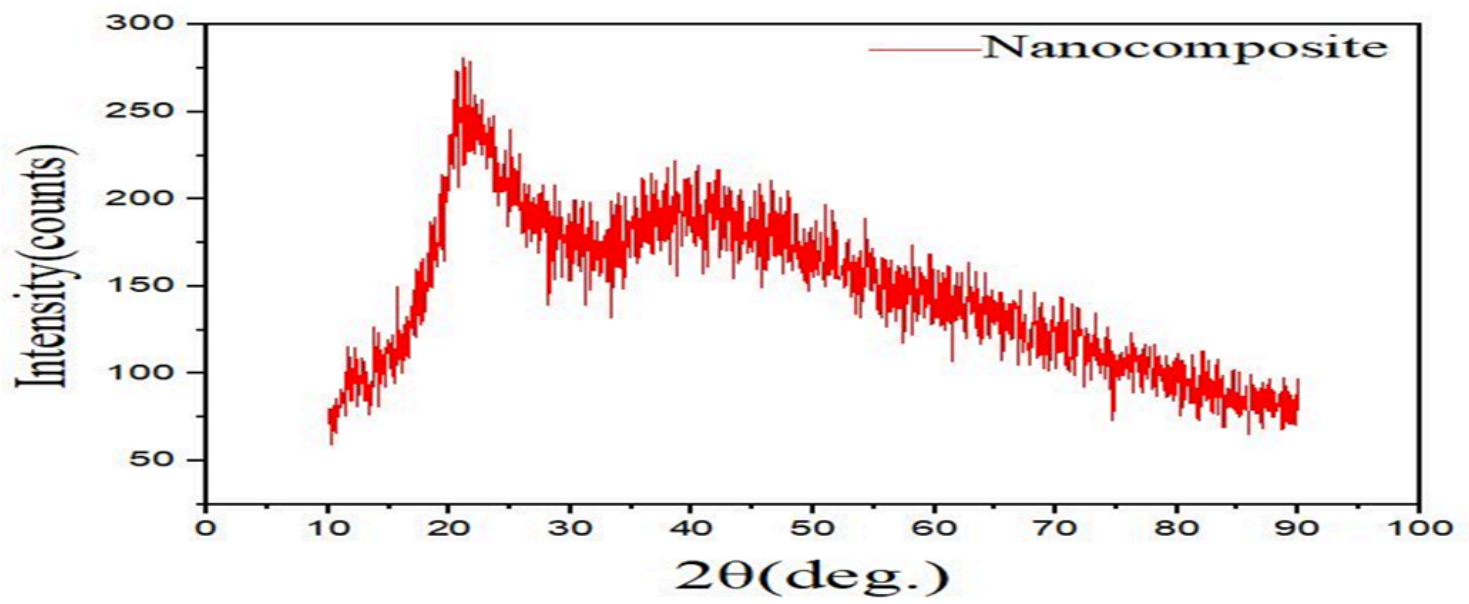


Figure 2

The X-ray diffractogram analysis of the ZnO–CS–MCEO nanocomposite.

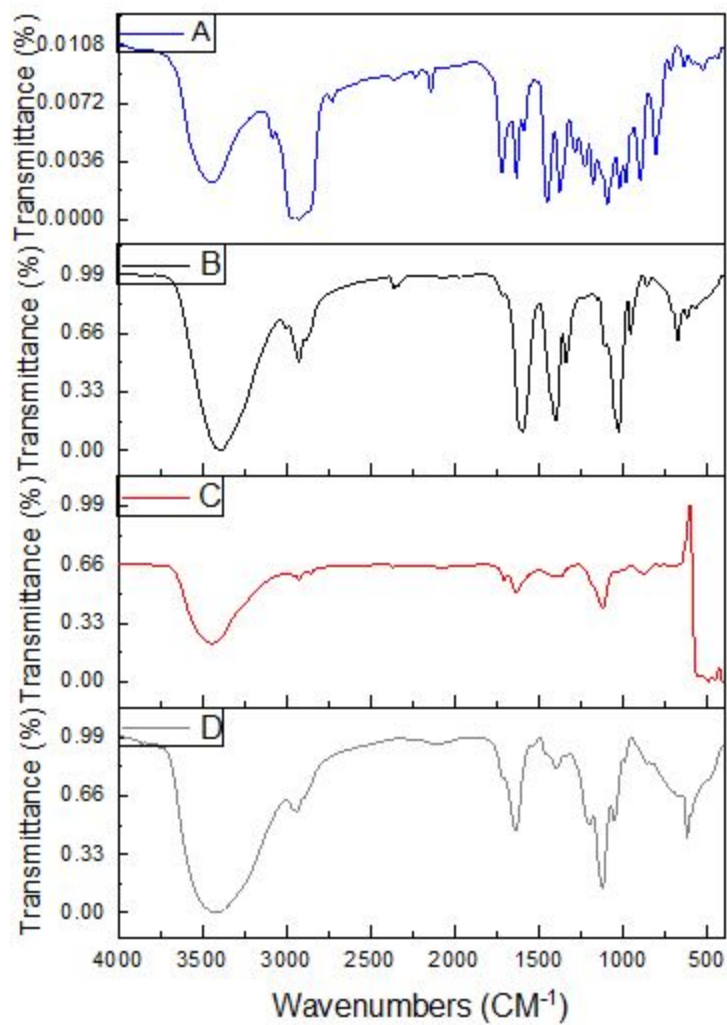
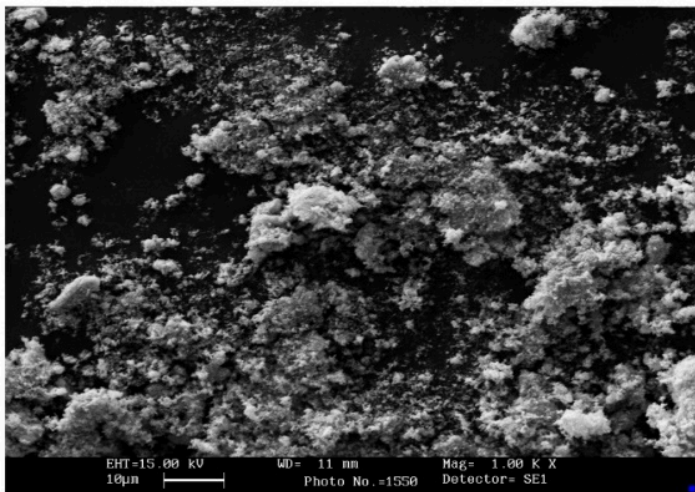


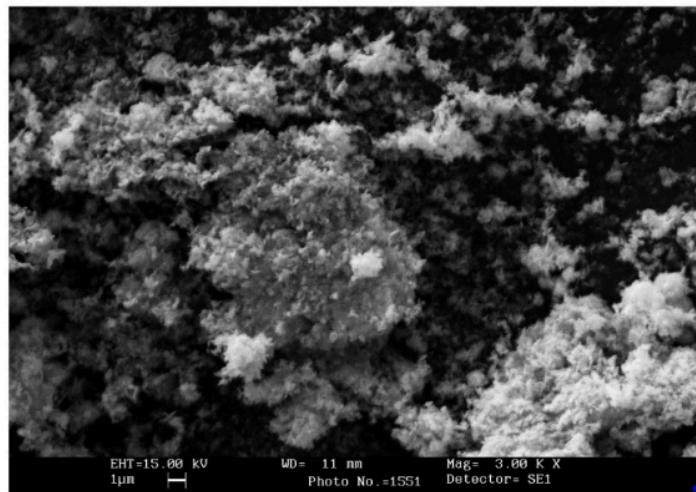
Figure 3

The FTIR spectroscopy of the *M. chamomilla* EO(A), ZnO-CS-MCEO nanocomposite (B), ZnO (C), and chitosan(D).

(a)



(b)



(c)

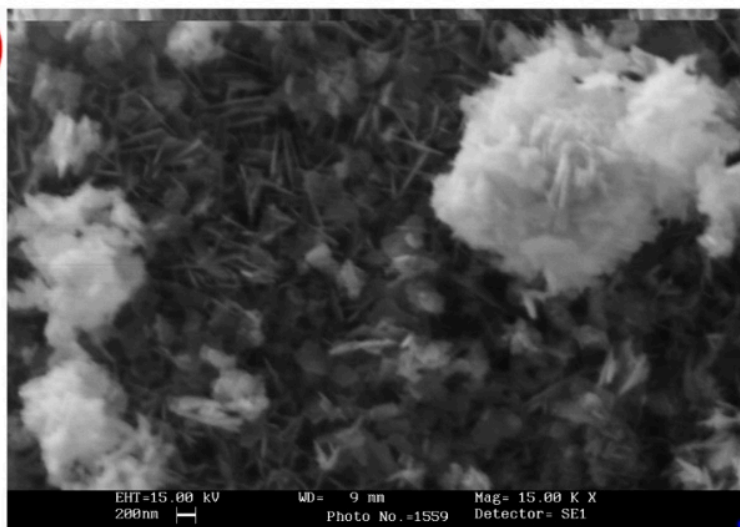
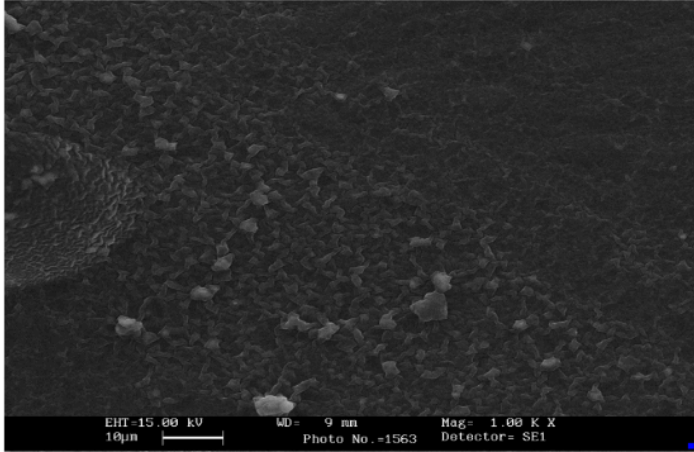


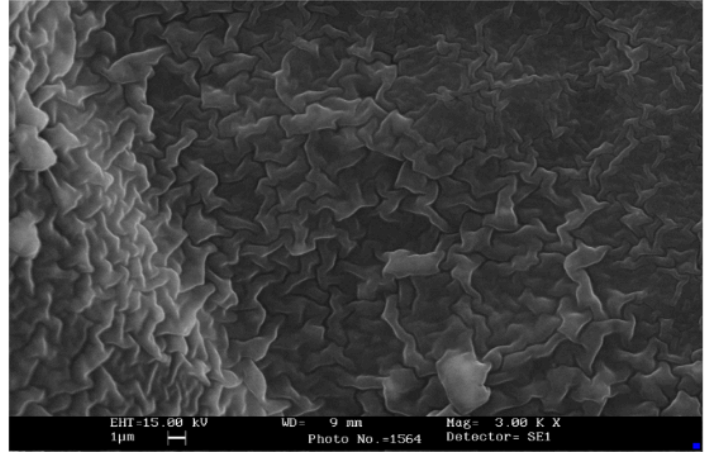
Figure 4

SEM images of ZnO nanoparticles: (a) 1000 $\times$, (b) 3000 $\times$, (c) 15000 $\times$. Scale bars: 10 μ m, 5 μ m, 2 μ m.

(a)



(b)



(c)

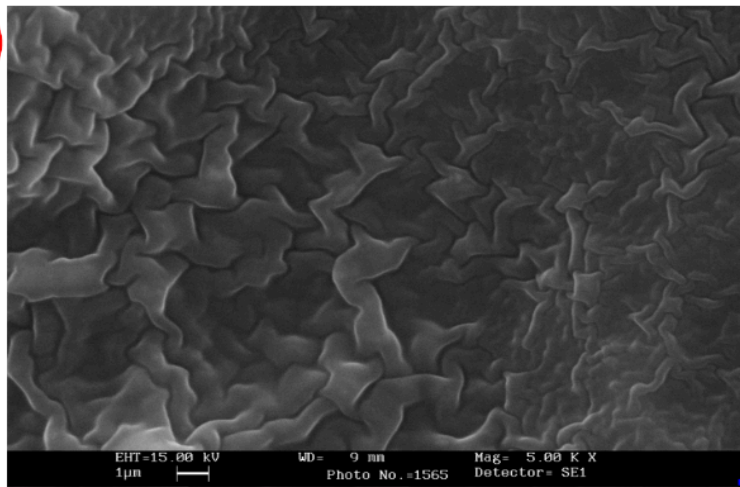
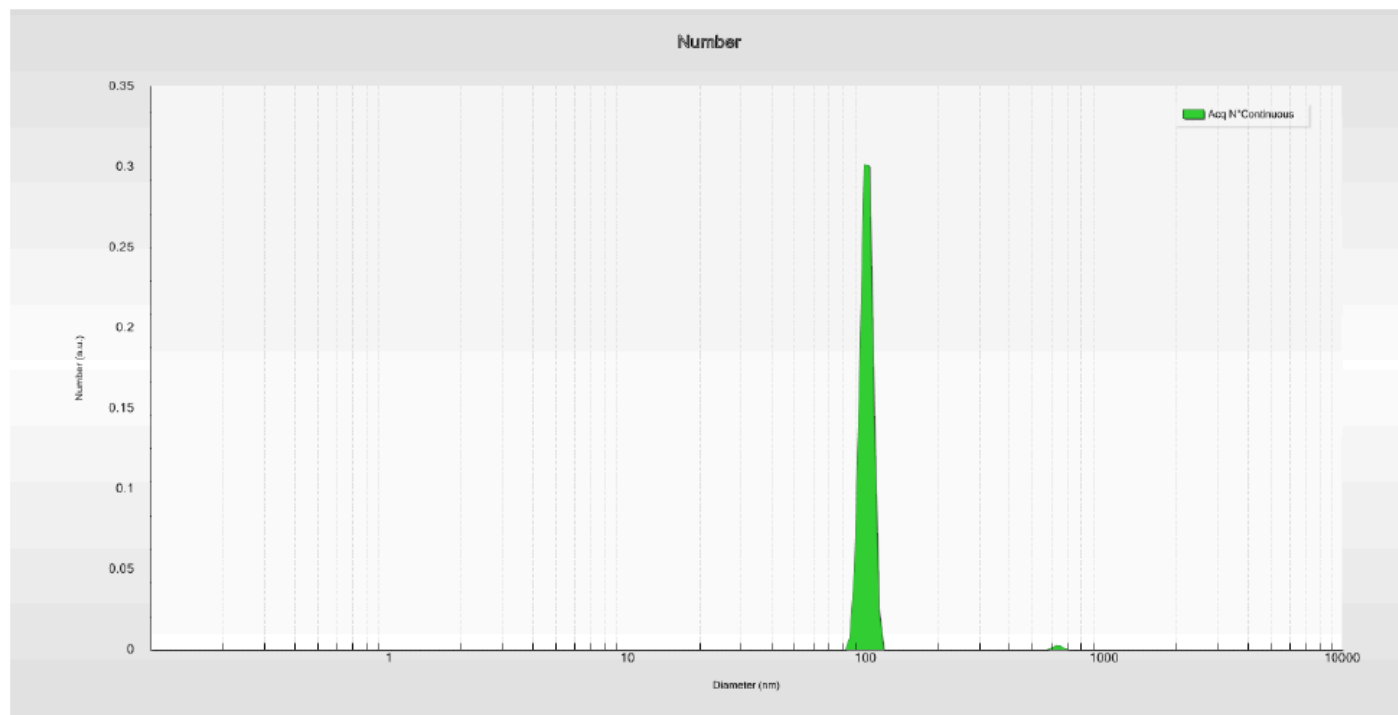


Figure 5

SEM images of ZnO-CS-MCEO nanocomposite: (a) 1000 $\times$, (b) 3000 $\times$, (c) 5,000 $\times$. Scale bars: 10 μm , 5 μm , 500 nm.

**Distribution statistics**

Dn 10%: 93.73 nm
Mean Number: 103.65 nm
Solution index: 13

Dn 50%: 98.17 nm

Dn 90%: 107.68 nm

Peak : 1 | **Mode:** 98.17 nm **Mean:** 100.16 nm **Dev.:** 6.63 % **Intensity.:** 100 %

Figure 6

The DLS results of the ZnO-CS-MCEO nanocomposite.

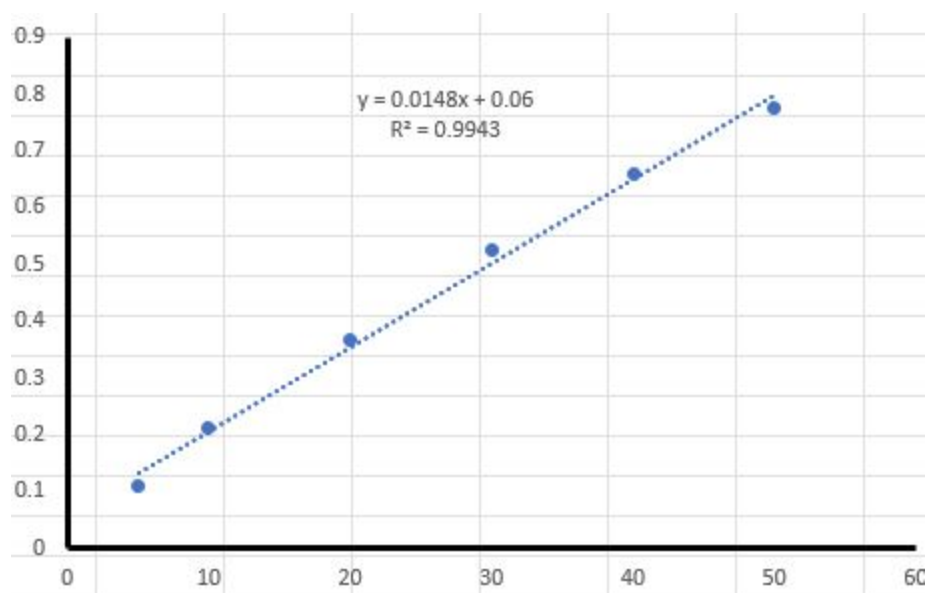


Figure 7

Standard Curve of *M.chamomilla* EO (y=296 nm)

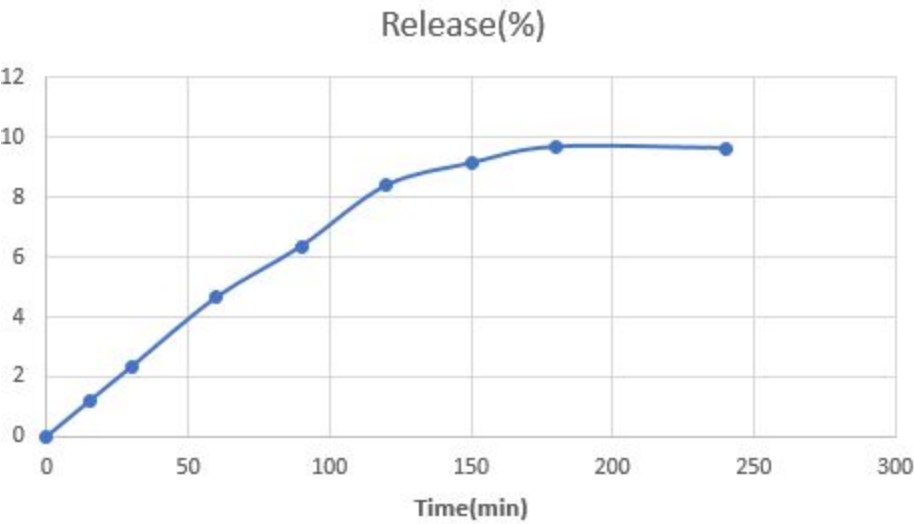


Figure 8

Release kinetics of the *M.chamomile* EO from ZnO–CS–MCEO nanocomposite

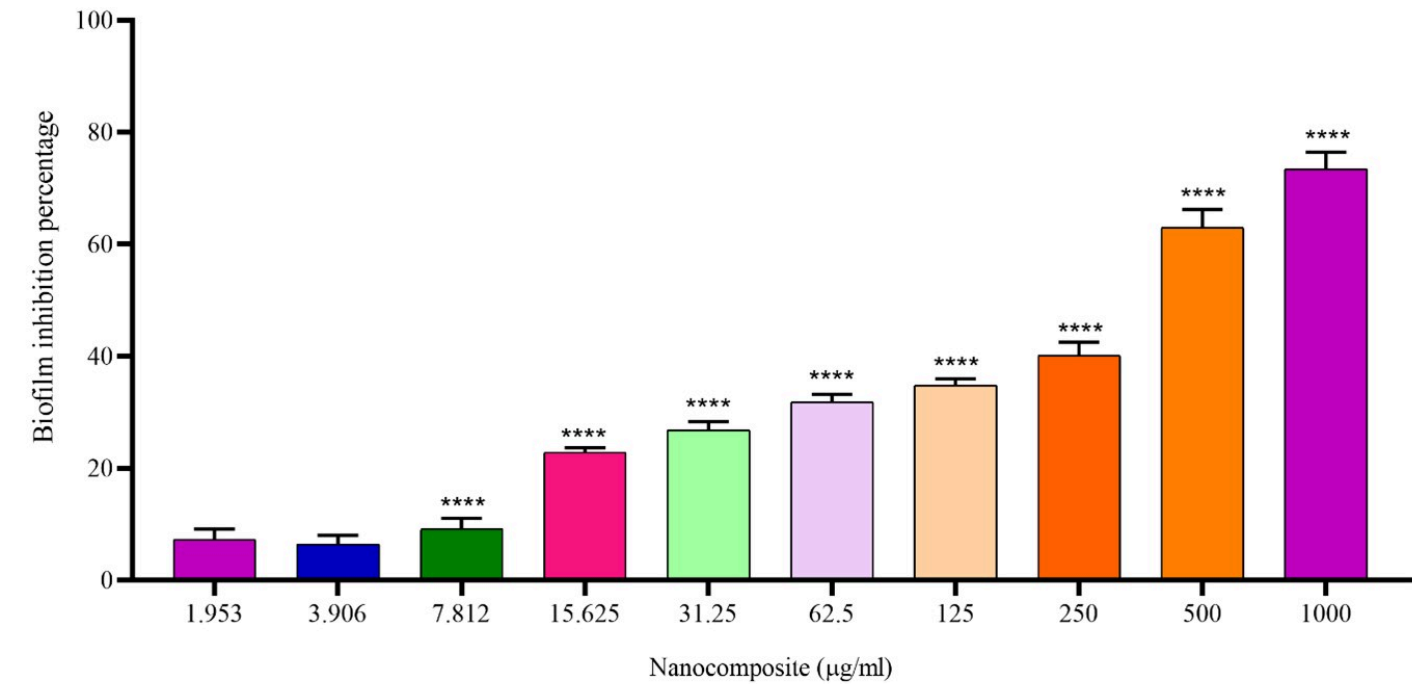


Figure 9

The biofilm inhibition Percentage (%) of the ZnO–CS–MCEO nanocomposite on *C. albicans* by XTT assay. (mean $\pm$ SD, n=3).

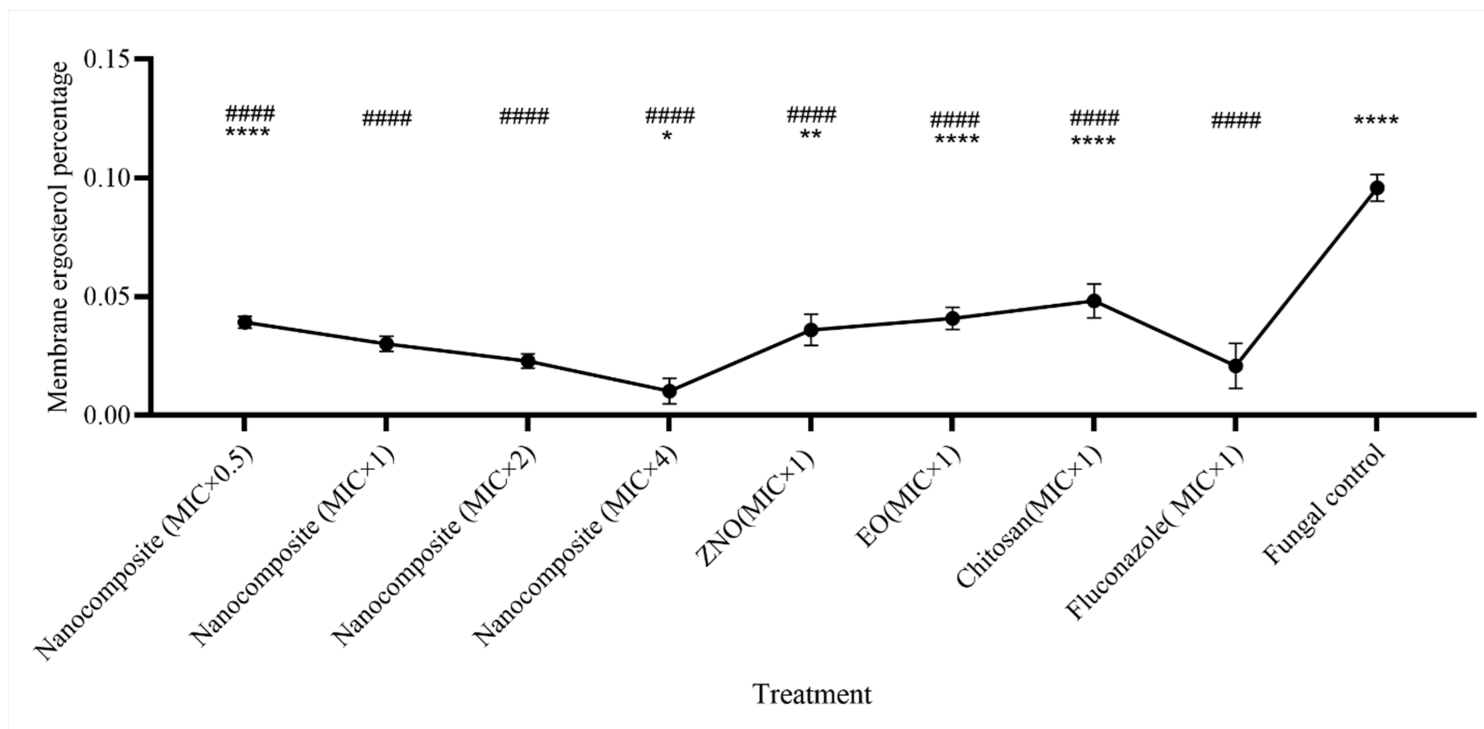


Figure 10

The ergosterol content of *C. albicans* after 24 h treatment with different concentrations of the ZnO–CS–MCEO nanocomposite, ZnO, *M. chamomilla* EO, chitosan, and fluconazole based on MIC.

(*p < 0.05, **p < 0.01, ***p < 0.0010, **** P Value <0.0001).

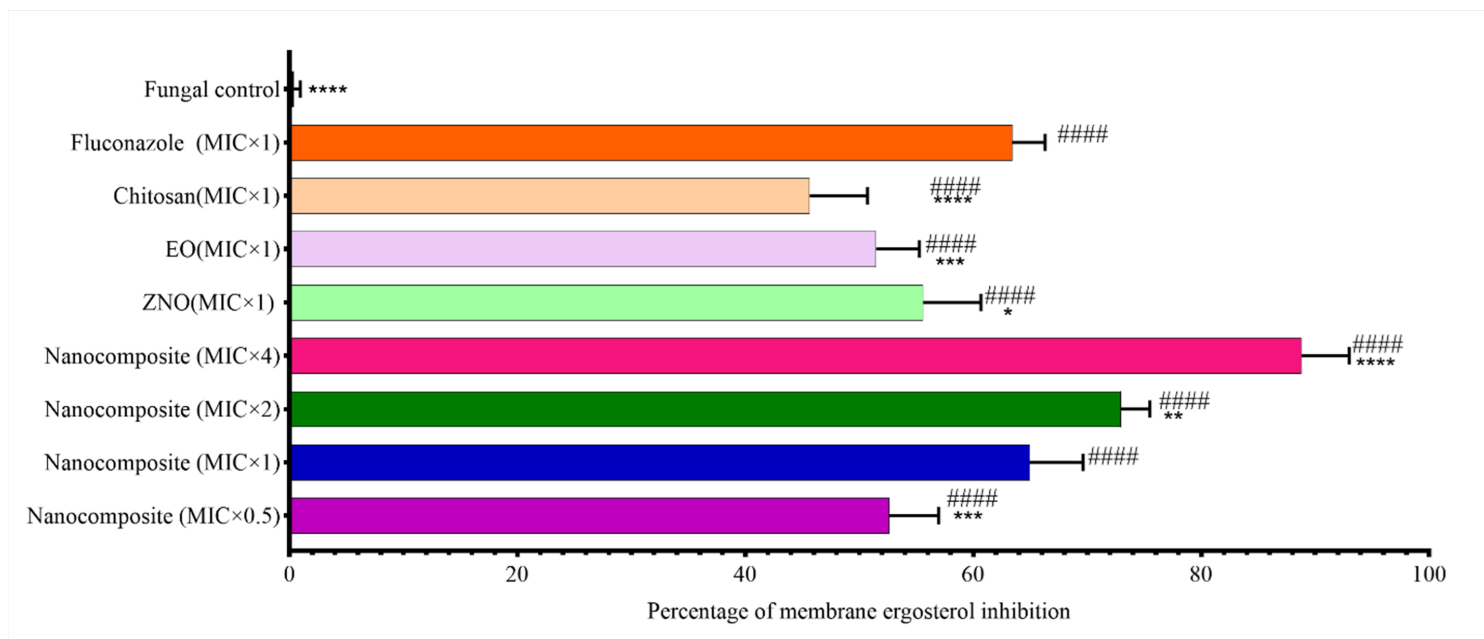


Figure 11

The percentage of ergosterol inhibition of *C. albicans* after 24 h treatment with different concentrations of the ZnO–CS–MCEO nanocomposite, ZnO, *M. chamomilla* EO, chitosan, and fluconazole based on MIC

(*p < 0.05, **p < 0.01, ***p < 0.0010, **** P Value <0.0001).

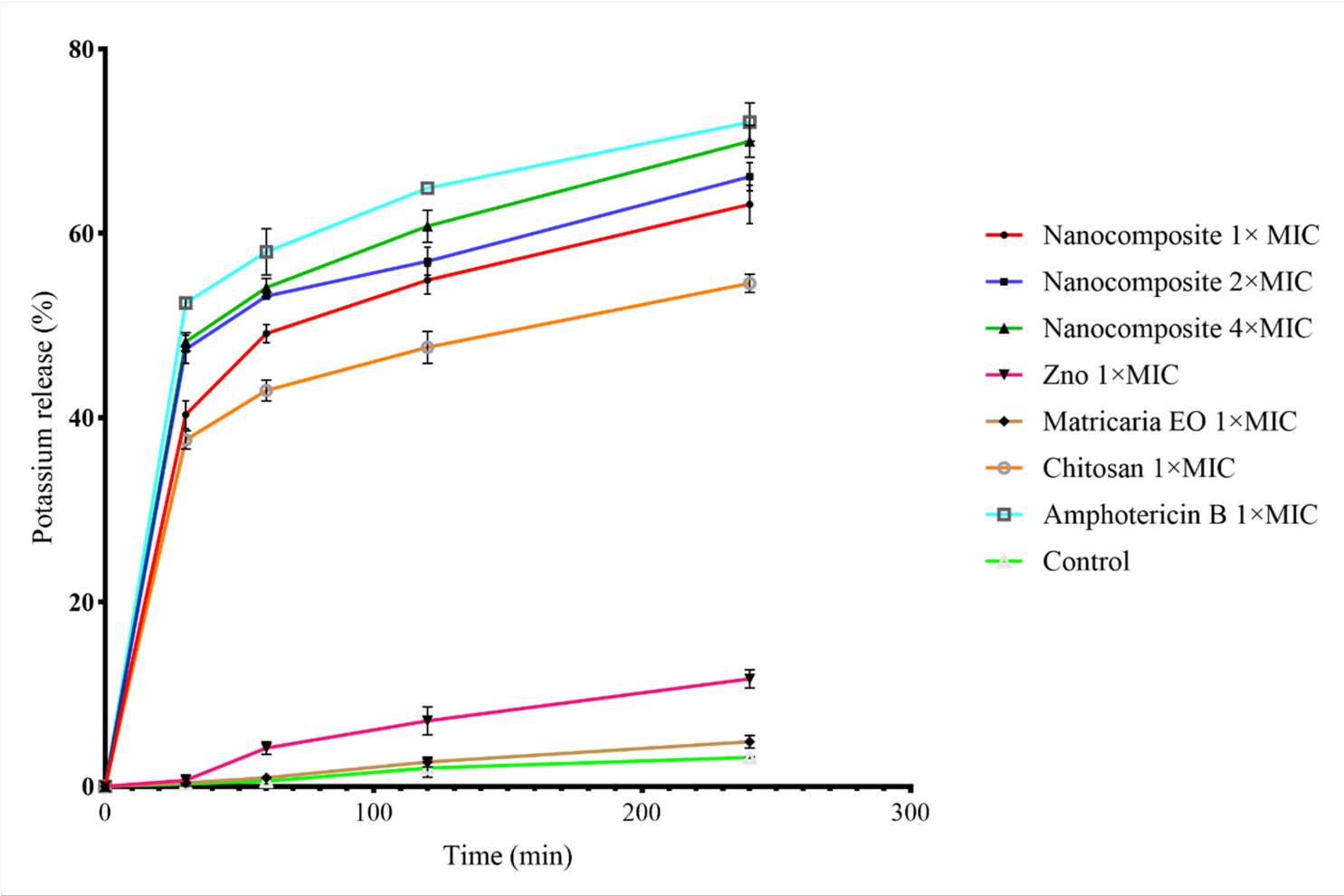


Figure 12

Effect of the ZnO–CS–MCEO nanocomposite, ZnO, *M. chamomilla* EO, chitosan, and fluconazole on the potassium leakage of *Candida albicans* for 240 minutes.

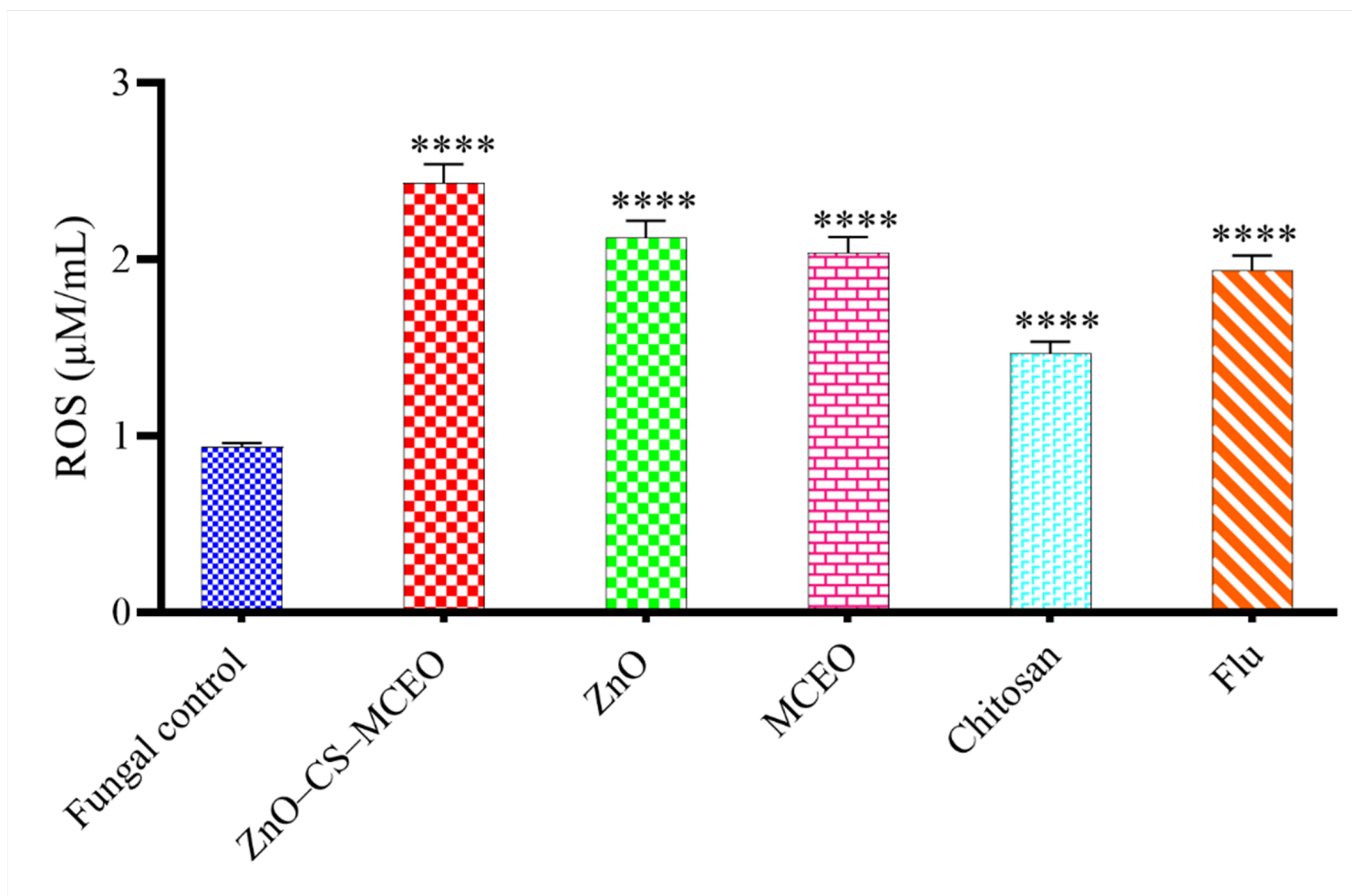


Figure 13

Effect of the ZnO-CS-MCEO nanocomposite, ZnO, *M. chamomilla* EO, chitosan, and fluconazole after 24 h treatment based on MIC on ROS generation in *C. albicans*.

(**** P Value < 0.0001)

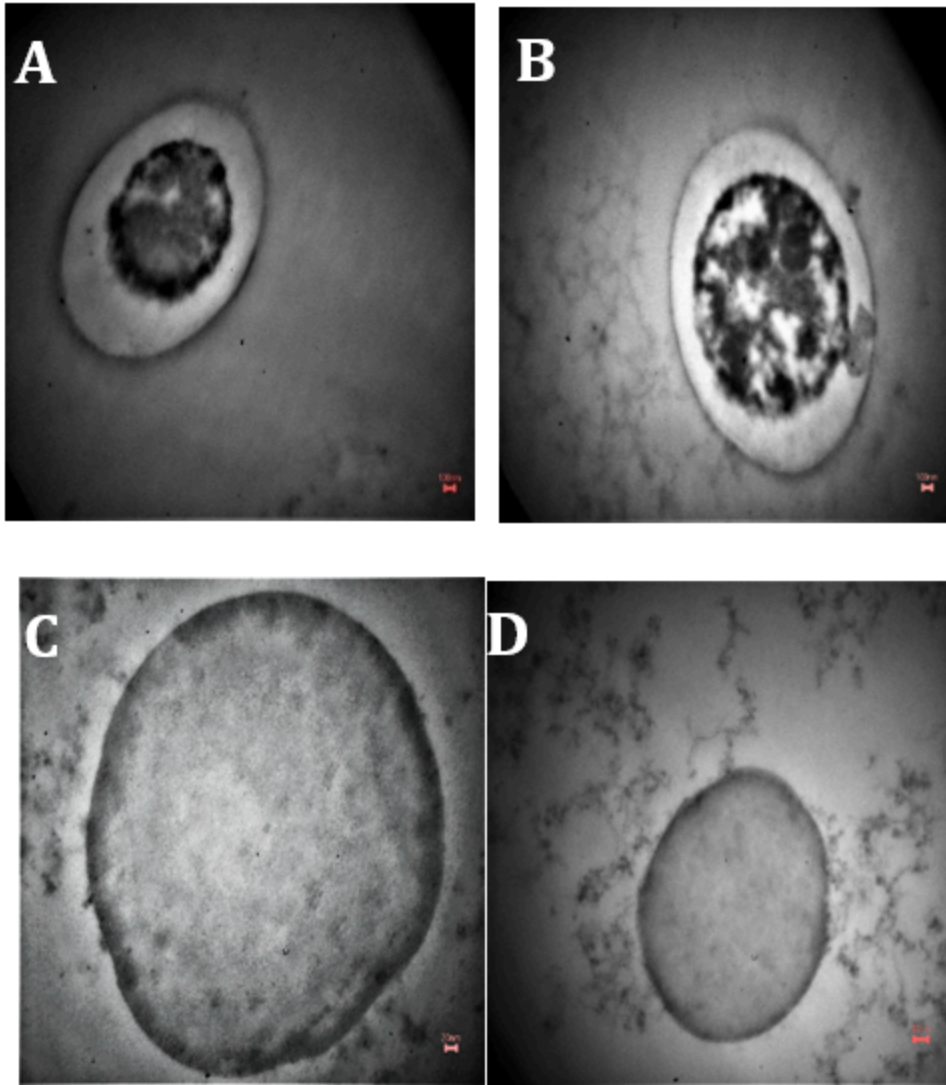


Figure 14

Transmission electron microscopy of *Candida albicans* cells treated with the nanocomposite and control. (A–B) Untreated control cells showed normal ultrastructure: thick and uniform cell wall, intact plasma membrane closely apposed to the cell wall, and dense, homogeneous cytoplasm. (C–D) Cells treated with ZnO–CS–MCEO nanocomposite (125 µg/mL, 48 h) exhibited severe ultrastructural damage, including detachment of the plasma membrane from the cell wall, marked reduction in cytoplasmic density, leakage of intracellular contents.

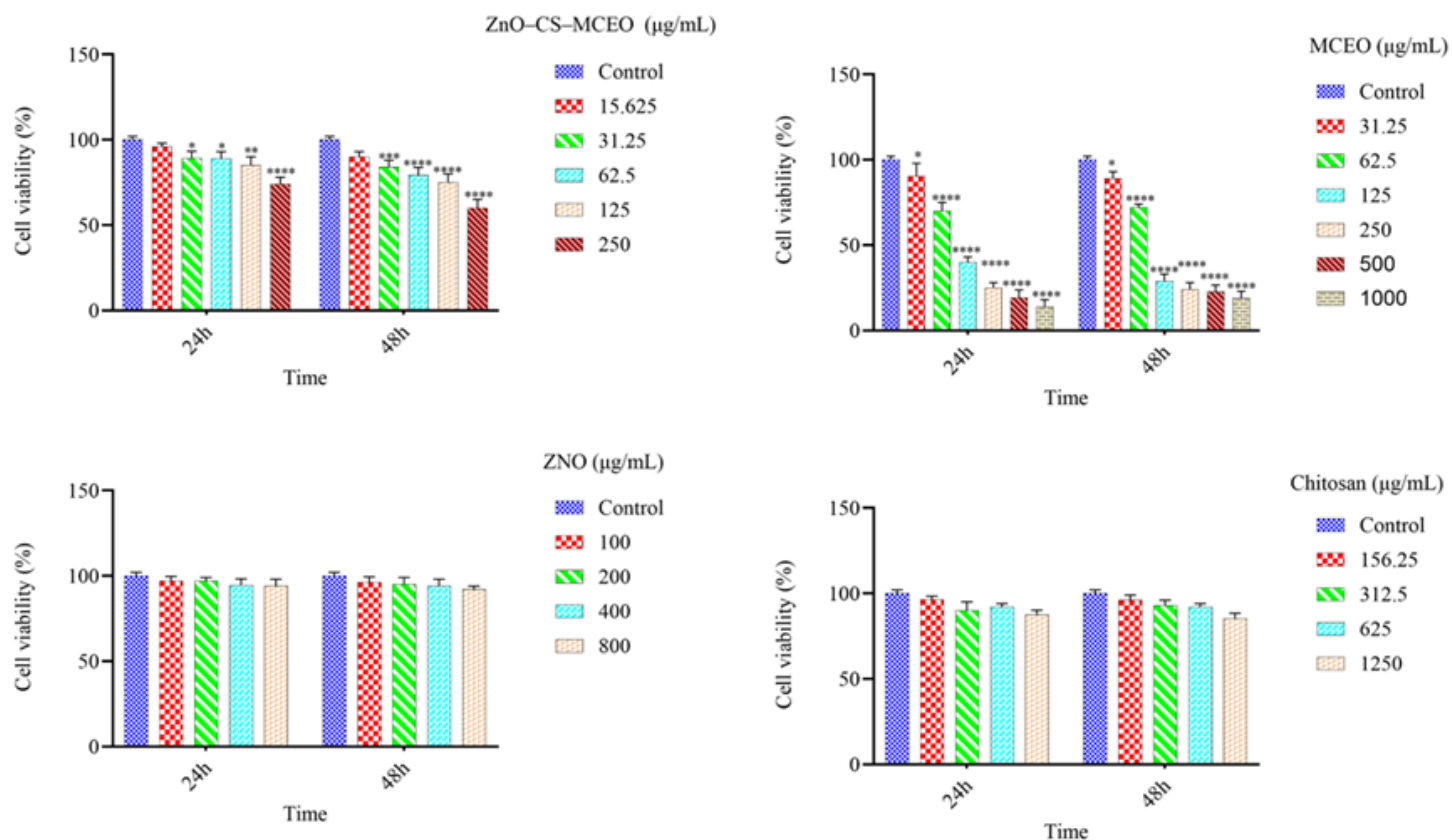


Figure 15

Cytotoxicity of the ZnO-CS-MCEO nanocomposite, chitosan, ZnO NPs, and *M. chamomilla* EO on L929 fibroblast cells.

Data are presented as the mean $\pm$ SD of three independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Supplementary Files

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