

Ag-decorated ZnO/MgO nanocomposite derived from Cinnamomum camphora: Synthesis and functional evaluation

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Abstract

The escalating threat of antimicrobial resistance and the limitations of conventional cancer therapies necessitate the development of novel, multifunctional agents. This study establishes the first eco-friendly protocol for synthesizing a trimetallic Ag/ZnO/MgO nanocomposite (NC) using an aqueous leaf extract of *Cinnamomum camphora* L. Response Surface Methodology (RSM) optimized the synthesis conditions to pH 8.03, 37.8°C, and 3.37 hours. Comprehensive characterization via UV-Vis, FTIR, XRD, SEM-EDX, elemental mapping, TEM, DLS, Zeta potential, and TGA analysis confirmed the formation of a spherical, crystalline nanocomposite with an average size of ~ 48–59 nm and a macroporous structure. Phytochemical profiling revealed the extract's richness in flavonoids, phenolics, and tannins, which facilitated the bio-reduction and capping processes. The biosynthesized Ag/ZnO/MgO NC demonstrated potent, broad-spectrum antibacterial activity against *Staph. aureus*, *Bac. subtilis*, *Esch. coli*, *Kleb. pneumoniae* with minimum inhibitory concentrations (MICs) as low as 62.5 µg/mL. It significantly inhibited biofilm formation in *S. aureus* and *E. coli* (up to 85%) and downregulated associated virulence genes (*icaA*, *icaD*, *papC*, *fimH*). Furthermore, the nanocomposite displayed significant cytotoxicity toward *Mcf-7* breast cancer cells (IC_{50} = 118.0 µg/mL) while exhibiting significantly lower toxicity to normal Vero cells (IC_{50} = 332.7 µg/mL). Mechanistic studies revealed that the nanocomposite exerted its anticancer effect by activating apoptotic pathways and causing cell cycle arrest at the S and G2/M phases. The NC also displayed notable antioxidant (DPPH IC_{50} = 150.1 µg/mL) and lipase inhibitory (IC_{50} = 108.4 µg/mL) properties, indicating anti-obesity potential. These findings underscore the Ag/ZnO/MgO nanocomposite as a promising, multi-target therapeutic agent with significant potential for combating drug-resistant infections, cancer, and oxidative stress-related disorders.

1. Introduction

Antibiotics remain a cornerstone in combating bacterial infections. Nevertheless, their therapeutic utility is severely compromised by the advent of multidrug-resistant (MDR) bacterial strains, which emerge through adaptive mutations. This escalating resistance is a pressing global health concern, as pathogens continuously refine their defensive strategies against conventional antimicrobial agents [1]. A principal defense mechanism is biofilm formation; wherein microbial communities encase themselves within a matrix of extracellular polymeric substances. This biofilm matrix, a complex of biotic and abiotic materials, facilitates robust surface adhesion [2]. The creation of novel agents that disrupt bacterial growth through unique mechanisms of action is widely regarded as an imperative solution to this challenge [3].

Thus, research aims to create novel anti-cancer agents or methods with greater efficacy, reduced toxicity, excellent biocompatibility, and natural degradability. Nanotechnology is becoming a vital field, enabling major progress in drug delivery, bioavailability, imaging, and therapy, while also reducing associated adverse effects [4]. Nanoparticles as anticancer agents provide multiple benefits: they improve drug stability and circulation time [5], enable precise targeting of cancer cells to minimize harm to healthy tissue, and can bypass multi-drug resistance by evading the P-glycoprotein efflux pump. Their unique

properties make nanoparticles an ideal platform for cancer treatment, offering enhanced effectiveness and improved safety [6]. Multimetallic nanoparticles, which incorporate two or more distinct metal elements, represent a promising class of multifunctional agents with demonstrated antimicrobial and anticancer potential [7] [8]. Their efficacy stems from synergistic interactions between the constituent metals, which can simultaneously disrupt bacterial cell membranes, inhibit critical microbial enzymes and metabolic pathways, and generate reactive oxygen species (ROS) that induce oxidative stress [9]. This multi-mechanistic action confers broad-spectrum activity against diverse pathogens, including drug-resistant varieties [10]. In an oncological context, these nanoparticles can be designed to selectively target cancer cells by leveraging differential cellular uptake, impairing mitochondrial function, and initiating programmed cell death. The versatility in their design allows for the precise tuning of physicochemical properties to optimize antimicrobial potency while retaining specific anti-tumor activity [9]. Zinc oxide nanoparticles (ZnONPs) have garnered significant interest due to their cost-effectiveness, straightforward synthesis, and established safety profile in humans and animals [11]. Similarly, magnesium oxide (MgO) has gained widespread attention for its multifaceted biomedical applications. As a common inorganic compound, its distinct properties render it highly valuable across diverse fields including medicine, biochemistry, and physiology [12]. Furthermore, silver nanoparticles (Ag NPs) have emerged as extensively studied metallic nanomaterials over the past two decades, prized for their unique chemical, mechanical, and biological characteristics [13].

For millennia, plants of the *Cinnamomum* genus have been employed both as food additives and in traditional medicine. Recognized globally for their potent antioxidant activities, these herbs are rich sources of diverse polyphenolic compounds [14]. *Cinnamomum camphora* L. Presl, a perennial tree from the Lauraceae family, is native to southern China where it is cultivated for landscaping and serves as an important medicinal herb [15]. Camphor, derived from this tree, is a valuable compound for developing new pharmaceuticals, particularly anti-inflammatory drugs [16].

This work establishes the first environmentally friendly protocol for synthesizing and characterizing Ag/ZnO/MgO nanocomposite utilizing *C. camphora* leaf extract. The investigation additionally sought to analyze the phytochemical profile of the extract and conduct a thorough assessment of the biosynthesized nanocomposites diverse biomedical capabilities, encompassing antioxidant, antibiofilm, antibacterial, anti-virulence, anti-obesity, and anticancer properties.

2. Materials and methods

2.1. Materials

All chemical reagents used in this study were of analytical grade. The precursor salts silver nitrate (AgNO_3), zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), and magnesium nitrate hexahydrate ($\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) along with other required chemicals were purchased from Sigma-Aldrich. Microbiological media, including Nutrient Agar, Nutrient Broth, and Muller Hinton Agar, were obtained from HiMedia. All

chemicals were used as received without further purification. Deionized water was used as the solvent throughout all experimental procedures.

2.2. Source of botanical material and aqueous extraction protocol

Mature, healthy leaves of *C. camphora* were harvested from the botanical garden of Al-Azhar University's Faculty of Science. The collected leaves underwent thorough washing with tap water to remove surface contaminants, followed by rinsing with ultrapure water. After separation from petioles, the drying of the leaf blades was done at about 50°C for 12 h in an electric blast dryer and mechanically fragmented. For aqueous extraction, 8 g of dried leaf material was combined with 100 mL of ultrapure water in an Erlenmeyer flask, brought to boiling for 10 minutes, and subsequently filtered through Whatman No. 1 filter paper. The resulting extract was stored at 4°C for further applications.

2.3. Phytochemical analysis

The quantitative phytochemical profile of the extract, including total phenolic acids, tannins, flavonoids, flavonols, alkaloids, saponins, and steroids, was determined using established spectrophotometric methods as described previously[17].

2.4. Optimization study

2.4.1. Statistical Optimization Employing Central Composite Design (CCD)

A statistical optimization study was performed using Response Surface Methodology (RSM). The Central Composite Design (CCD), recognized as a robust and widely adopted RSM technique, was selected to systematically investigate the relationships between the independent variables specifically, time, pH, and temperature and the response. The CCD employed in this study features three distinct experimental point categories. Cube points are used to estimate linear effects and two-factor interactions, axial points enable the assessment of quadratic effects, and repeated center points are included to quantify pure experimental error and evaluate the overall curvature of the response surface. The resulting data facilitates the generation of optimization curves, which are critical for determining the combination of synthesis parameters (pH, temperature, time) that maximize the yield of the Ag/ZnO/MgO nanocomposite. In this work, a two-level, three-factor full factorial CCD was implemented. The response variables obtained from the experimental runs were fitted using a full quadratic (second-order polynomial) multiple regression model. The model accounts for the three continuous independent variables at their designated levels.

2.4.2. Model Validation

The optimization procedure was executed using the response optimizer tool within the Minitab® Design of Experiments (DoE) package (Version 18, 2017). To determine the ideal synthesis conditions, the

Response Optimizer tool was employed. This method evaluates all possible factor combinations by calculating individual desirability scores (d) for each response and then aggregating them into a composite desirability index (D). Both metrics range from 0 to 1, where the value of 1 indicates a perfect match with the target outcome. The factor settings corresponding to the highest composite desirability were selected as the optimum for maximizing nanocomposite production. Model validation was conducted through the predictive model, followed by a confirmation experiment performed under the identified optimal conditions. All experimental design, data generation, and statistical analyses were performed using the Minitab® software, utilizing its integrated statistical and graphical analysis tools.

2.5. Sequential Phytosynthesis and Calcination for Ag-Decorated ZnO/MgO Hybrid Nanostructures

The Ag/ZnO/MgO nanocomposite was synthesized via a bottom-up approach. Firstly, AgNPs were prepared by reacting a 2 mM aqueous solution of AgNO₃ with 80 mL of *C. camphora* L. extract under continuous stirring for 24 hours. The resulting AgNPs were purified via washing with water and ethanol and then dried. Separately, the MgO/ZnO composite was prepared by dissolving 0.1 M solutions of Mg(NO₃)₂·6H₂O and Zn(NO₃)₂·6H₂O individually in 80 mL of plant extract. At the optimum condition the pre-synthesized AgNPs mixed with the solution of MgO/ZnO NPs at pH 8.0, temperature 38° C for 3.37 h on stirring. The final mixture was calcined in a muffle furnace at 200°C for 3 hours to obtain the Ag/ZnO/MgO nanocomposite, which was then characterized as previous stud with some alteration.

2.6. Characterization of nanocomposite

The biosynthesized Ag/ZnO/MgO nanoparticles were subjected to comprehensive characterization. Ultraviolet-visible (UV-Vis) spectroscopy (JENWAY-6305, UK) was employed to confirm formation, with absorbance scans from 200 to 800 nm. Crystalline structure and phase identification were analyzed by XRD using an X'Pert Pro Philips diffractometer, operating with a 2θ range of 10° to 80°. Bio reduction and capping functional groups were identified by FT-IR spectroscopy (JASCO 3600) in the 400–4000 cm⁻¹ range. Morphological features, particle size, and aggregation state were examined using both SEM, ZEISS EVO-MA10 and TEM, JEM 1230. EDX and elemental mapping were conducted to verify the elemental composition and distribution within the nanocomposite. The surface charge (zeta potential) and hydrodynamic size distribution were determined via dynamic light scattering (DLS) using a HORIBA SZ-100 instrument (HORIBA Ltd., Kyoto, Japan). Thermal degradation behavior was assessed by thermogravimetric analysis (TGA) on a Perkin Elmer instrument, with temperatures ramped from 25°C to 500°C. Textural properties were evaluated using a Belsorp-mini X surface area analyzer (MicrotracBEL Corp., Japan).

Antibacterial effectiveness

The antibacterial potential of the biosynthesized Ag/ZnO/MgO NC was assessed against a panel of standard bacterial strains: *Kleb. pneumoniae* (ATCC 13883), *Bac. subtilis* (ATCC 6633), *Staph. aureus* (ATCC 6538), and *Esch. coli* (ATCC 25922). In the initial screening, the well diffusion assay was utilized.

Bacterial lawns were prepared on Mueller-Hinton agar, and 8 mm wells were filled with 100 μL of the test substance at a concentration of 1000 $\mu\text{g}\cdot\text{mL}^{-1}$. The *C. camphora* leaf extract and norfloxacin served as controls. Following a 24-hour incubation at 37°C, the diameters of the inhibition zones were recorded. For quantitative analysis, the minimum inhibitory concentration (MIC) was determined via the broth microdilution method in 96-well plates. Two-fold serial dilutions of the nanocomposite, ranging from 1000 to 7.8 $\mu\text{g}\cdot\text{mL}^{-1}$, were inoculated with standardized bacterial suspensions. After 24 h at 37°C, the MIC was recorded as the lowest concentration that inhibited visible growth, consistent with Clinical and Laboratory Standards Institute (CLSI) protocols. Subsequently, the MBC was established by subculturing broth from wells without visible growth onto fresh agar plates; the MBC was defined as the lowest concentration from which no colonies grew after subculture[18].

2.7. Evaluation of antibiofilm efficacy

The antibiofilm capacity of Ag/ZnO/MgO NCs against clinical isolates of *E. coli* and *S. aureus* was quantified through microtiter plate methodology [19]. Standardized bacterial suspensions (0.5 McFarland) were incubated with gradient concentrations of the nanocomposite in glucose-enriched Tryptic Soy Broth (1%) for 48 h at 37°C. Following biofilm development, non-adherent cells were eliminated via phosphate-buffered saline (PBS) washing. Biofilms were immobilized using 95% methanol (250 μL /well) and subsequently stained with 0.3% crystal violet solution for 20 min. After removing unbound dye, the adherent stain was eluted by acetic acid (30%). The optical density of eluates was determined spectrophotometrically at 540 nm. Biofilm inhibition percentages were derived by comparing absorbance values between nanocomposite-treated samples and both positive (bacterial inoculum alone) and negative (sterile medium) controls.

2.8. Gene expression analysis

Total RNA was isolated from bacterial cultures utilizing TRIzol reagent (Gibco) per manufacturer specifications, with complementary DNA (cDNA) synthesized using a commercial RT-PCR kit (Clontech, USA). Virulence gene targets (*icaA*, *icaD*, *papC*, *fimH*) and the *recA* housekeeping gene (Table 1) were amplified through conventional reverse transcription PCR using Primer-BLAST-designed primers. Reactions (50 μL) underwent initial denaturation (93°C/4 min), 29 cycles of 93°C (35 s), 50°C (45 s), and 45°C (1 min), concluding with elongation (72°C/6 min). Amplification products were electrophoresed on ethidium bromide-stained 1% agarose gels, visualized under UV illumination, and sized against a 100 bp DNA ladder. Semi-quantitative real-time PCR employed SYBR Green chemistry in 25 μL reactions containing 12.5 μL of 2 $\times$ SYBR Green mix, forward/reverse primers (1 μL each, 25 pmol), cDNA template (1 μL , 50 ng), and nuclease-free water (9.25 μL). Thermal cycling parameters included: initial denaturation (93°C/10 min), 40 cycles of 93°C (15 s), 45°C (35 s), and 72°C (30 s)[20].

Table 1
specific PCR primers were used for amplification of bacterial virulence genes

Primers	sequence 5 – 3	Amplicon size	Reference
Fw- <i>ica A</i>	TCTCTTGCAGGAGCAATCAA	188 bp	[21]
Rw- <i>ica A</i>	TCAGGCACTAACATCCAGCA		
Fw- <i>ica D</i>	ATA AAC TTG AAT TAG TGT ATT	198 bp	
Rw- <i>ica D</i>	ATA TAT AAA ACT CTC TTA ACA		
FW- <i>fimH</i>	GAGAAGAGGTTTGATTAACTTATTG	559 bp	[22]
RW- <i>fimH</i>	AGAGCCGCTGTAGAACTGAGG		
FW- <i>papC</i>	GTGGCAGTATGAGTAATGACCGTTA	205 bp	[23]
RW- <i>papC</i>	ATATCCTTTCTGCAGGGATGCAATA		

2.9. Cytotoxicity and anticancer evaluation

2.9.1. MTT Assay

The nanocomposite's cytotoxic effects were assessed against two human malignant cell lines MCF-7 (mammary carcinoma) and HepG2 (hepatic carcinoma) and normal Vero cells. Cells were plated in 96-well plates at 1×10^5 cells/mL density and cultured for 24 h to achieve monolayer confluence. After incubation, supernatants were aspirated and cell layers were washed twice with buffer solution to remove cellular debris. Test compounds were administered in serum-supplemented RPMI medium at final concentrations ranging from 15.785 to 1000 $\mu\text{g/mL}$. Untreated cells in complete medium functioned as control specimens. After a designated incubation period, cells were first observed microscopically for characteristic morphological alterations indicative of cytotoxicity, including cell rounding, detachment, granulation, and monolayer disintegration. Quantitative assessment of cell viability was subsequently performed using the MTT assay. Following incubation, cell viability was quantified using the MTT assay. In this procedure, viable cells metabolize the MTT reagent to produce insoluble formazan crystals. These crystals were subsequently dissolved using dimethyl sulfoxide (DMSO). The absorbance of the resulting colored solution was measured at 560 nm with a microplate reader, and the absorbance values were directly proportional to the number of viable cells present.

2.9.2. Analysis of apoptosis by annexin V-FITC/Propidium iodide flow cytometry flow

Apoptotic cell death was quantified using a dual-parameter flow cytometry assay with an Annexin V-FITC/Propidium Iodide (PI) kit (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Invitrogen, cat. no. V13241). The assay principle relies on two distinct markers: Alexa Fluor® 488-conjugated Annexin V, which binds specifically to phosphatidylserine externalized on the outer leaflet of the plasma membrane in early apoptotic cells, and the DNA-binding dye propidium iodide (PI), which is membrane-impermeant

and thus stains only the nuclei of late apoptotic and necrotic cells with compromised membrane integrity. Following the manufacturer's staining protocol, the cell suspensions were analyzed using a flow cytometer (Beckman Coulter). Fluorescence was detected using an excitation wavelength of 488 nm, with emissions collected at 530 nm for FITC (Annexin V) and 575 nm for PI. This configuration allowed for the discrimination and quantification of four distinct cell populations: viable (Annexin V⁻/PI⁻), early apoptotic (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺), and necrotic (Annexin V⁻/PI⁺) [24].

2.9.3. Flow Cytometric Cell Cycle Analysis

Following a 72-h treatment with Ag/ZnO/MgO NCs, *Mcf-7* cells were collected for cell cycle analysis. The cells were detached via trypsinization, fixed in cold methanol, and stained with Vybrant® DyeCycle™ Violet stain (Invitrogen, cat. #V35003, ThermoFisher, USA). After incubation in the dark at 37°C for 30 min, the DNA content of the stained cells was analyzed by flow cytometry to determine cell cycle phase distribution. Analysis was performed using a Navios EX flow cytometer (Beckman Coulter, USA) with a 405 nm excitation laser and 440 nm emission detection. The instrument's accompanying software was used to quantify the percentage of cells in the G0/G1, S, and G2/M phases based on DNA content histograms[25].

2.10. Evaluation of antioxidant tests

The radical scavenging capacity of the Ag/ZnO/MgO NCs was determined through DPPH and ABTS assays. In the DPPH protocol, a 1 mM methanolic DPPH solution stabilized in darkness for 30 minutes. Nanocomposite suspensions (15.789–1000 µg·mL⁻¹) were introduced to the radical solution, with ascorbic acid as reference standard. Absorbance reduction at 517 nm, corresponding to radical quenching, was recorded after incubation.

For ABTS analysis, the radical cation was generated through 12–16-h reaction of 7 mmol·L⁻¹ ABTS with 2.4 mmol·L⁻¹ potassium persulfate under dark conditions. The stock solution was ethanol-diluted to 0.70 absorbance at 734 nm. Test samples and ascorbic acid standards (equivalent concentration range to DPPH assay) were combined with the ABTS working solution (1:1 ratio). Absorbance diminution at 734 nm was measured following thermal equilibration at 30°C. Antioxidant efficacy for both methods was computed as percentage absorbance reduction versus negative controls.

2.11. Anti-obesity activity of Ag/ZnO/MgO NPs via lipase suppression

Lipase inhibitory activity was examined according to established protocols [26] to evaluate anti-obesity potential. The investigation employed 4-nitrophenyl butyrate as enzymatic substrate, with Orlistat serving as reference inhibitor. Test concentrations ranged from 3.9 to 1000 µg/mL for both nanocomposite and standard compound. Pancreatic lipase was reconstituted in potassium phosphate buffer (0.1 mM, pH 7.0) at 5 mg/mL concentration. The assay system comprised 220 µL buffer, 35 µL enzyme preparation, 5 µL test compound, and 5 µL substrate solution (15 mM). Following 10-min incubation at 37°C, enzymatic

activity was determined by monitoring 4-nitrophenol liberation at 405 nm wavelength. Percentage inhibition was derived from absorbance reduction relative to inhibitor-free reactions.

2.12. Statistical study

All quantitative data are presented as the mean $\pm$ standard deviation (SD). Statistical significance was determined using GraphPad Prism software (Version 8, San Diego, USA). Inter-group comparisons were performed via one-way or two-way analysis of variance (ANOVA), followed by suitable post-hoc tests. In all analyses, a probability (p) value of less than 0.05 was deemed statistically significant.

3. Results and discussion

3.1. Phytochemical analysis of *Cinnamomum camphora* leaves

The efficacy of *C. camphora* as a mediating agent for green synthesis is underpinned by its distinct phytochemical composition. Our analysis demonstrated that its ethanolic leaf extract is a rich source of several key metabolite classes, with high levels of flavonoids, flavonols, phenolic acids, and tannins quantified in triplicate (Tables S1 & S2). This finding places our work in the context of existing research. While a previous report confirmed the presence of tannins and flavonoids but noted the absence of alkaloids and saponins [27], our results show a broader profile. Comparisons with a related species, *Cinnamomum verum*, reveal a shared foundation of flavonoids and tannins, though differences regarding alkaloids and saponins highlight species-specific variations in phytochemical synthesis [28]. Ultimately, the confirmed abundance of potent reducing agents like phenolics and flavonoids strongly correlates with the successful bio reduction and capping observed in the synthesis of the Ag/ZnO/MgO nanocomposite.

Table 1
Analysis of variance for the quadratic model derived from the RSM-
optimization of Ag/ZnO/MgO NC synthesis.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	0.443553	0.049284	6.59	0.003
Linear	3	0.066415	0.022138	2.96	0.084
pH	1	0.003830	0.003830	0.51	0.490
Temp	1	0.043129	0.043129	5.77	0.037
Time	1	0.019457	0.019457	2.60	0.138
Square	3	0.364805	0.121602	16.27	0.000
pH*pH	1	0.144087	0.144087	19.28	0.001
Temp*Temp	1	0.137989	0.137989	18.46	0.002
Time*Time	1	0.155090	0.155090	20.75	0.001
2-Way Interaction	3	0.012333	0.004111	0.55	0.659
pH*Temp	1	0.011881	0.011881	1.59	0.236
pH*Time	1	0.000115	0.000115	0.02	0.904
Temp*Time	1	0.000337	0.000337	0.05	0.836
Error	10	0.074740	0.007474		
Lack-of-Fit	5	0.069157	0.013831	12.39	0.008
Pure Error	5	0.005584	0.001117		
Total	19	0.518293			

3.2. Response Surface Methodology for optimization of Ag/ZnO/MgO nanocomposite synthesis

The statistical analysis of the experimental data for Ag/ZnO/MgO NCs synthesis is summarized in Table 1. The quadratic model was found to be statistically significant, as indicated by an F-value of 6.59 and a corresponding p-value of 0.003. Further analysis of the model terms revealed that the quadratic components specifically pH², Temperature², and Time²—were significant contributors to the model ($p < 0.05$). The coefficient of determination (R^2) for the model was 85.58%, suggesting that 85.58% of the variability in the response can be explained by the model. In statistical modeling, an R^2 value approaching 1 (or 100%) indicates a strong correlation between the predicted and observed values, reflecting model robustness and predictive accuracy[29]. Additionally, the signal-to-noise ratio for the model, represented by an adequate precision value of 31.28, was substantially above the recommended threshold of 4. This confirms a strong and reliable signal within the experimental design, further

validating the model's suitability for navigating the design space and predicting optimal synthesis conditions [30].

The optimization of Ag/ZnO/MgO nanocomposite synthesis was conducted using RSM. Model diagnostics included a normal probability plot to distinguish significant effects from noise. A Pareto chart (Fig. 1a) provided a quantitative ranking of the influence of each model term including main factors (pH, temperature, time), their quadratic forms, and interactions. To visualize the complex variable-response relationships, three-dimensional surface plots and two-dimensional contour plots were generated (Fig. 1b, e), showing the effect of two varying factors while the third was held constant at its central value. The contour lines connect points of equal nanoparticle yield, while the surface plots reveal the response topography, with peaks indicating regions of optimal production. Furthermore, main effects and interaction plots (Fig. 1c, d) were generated. The main effects plots illustrate the average change in the response as each individual factor moves from its low to its high level, revealing significant variations at the extremes of pH, time, and temperature. The interaction plots depict how the effect of one factor depends on the level of another. Based on the predictive model, an optimization curve was generated (Fig. 1f) to identify the optimal parameter settings. The analysis determined that a pH of 8.03, a temperature of 37.8°C, and a stirring time of 3.37 hours constituted the ideal conditions for maximizing biosynthesis. These conditions were subsequently used for confirmatory experiments performed in triplicate. The RSM analysis confirmed the robustness of the model. The normal probability plot of residuals validated the assumption of normality [31]. The significant influence of parameters such as pH and concentration aligns with established literature on nanoparticle synthesis, where these factors are known to critically affect nanoparticle characteristics [32].

3.3. Characterization

3.3.1. UV-Vis, FTIR, and XRD studies

UV-Vis spectroscopy (200–800 nm) confirmed the successful biosynthesis of the Ag/ZnO/MgO nanocomposite at optimum condition, revealing two key absorption features (Fig. 2A). An initial peak at 369 nm corresponds to the semiconductor components (ZnO/MgO), with its position suggesting quantum confinement effects in the nanostructures [33]. A second, distinct maximum at 409 nm is characteristic of the surface plasmon resonance (SPR) from metallic silver nanoparticles, verifying their integration into the composite. The optical band gap, calculated via a Tauc plot $(\alpha h\nu)^2$ vs. $h\nu$, was found to be 3.23 eV (Fig. 2B). This value is notably lower than that reported for similar systems, such as Ag/Zn@Lignin nanoparticles (4.7 eV) [34], highlighting the unique electronic properties of the current nanocomposite.

The functional group composition of the synthesized nanomaterials was characterized using FTIR spectroscopy. The obtained spectrum (Fig. 2C) revealed vibrational modes characteristics of both organic and inorganic components. A broad absorption band at 3407 cm^{-1} confirmed the presence of O-H groups [35], while a peak at 1654 cm^{-1} was assigned to C=O stretching from various carbonyl functionalities. Signatures of an aromatic system were identified through C–C stretching at 1310 cm^{-1}

and a distinct vibration at 992 cm^{-1} [36]. The inorganic framework was evidenced by a metal-oxygen bond vibration at 826 cm^{-1} and bands in the low-frequency region (642 and 453 cm^{-1}), which are diagnostic of the metallic nanocomposite. The overall increase in signal intensity is likely due to van der Waals forces at the bio-metallic interface [37]. Notably, the emergence of intense bands within the $400\text{--}600\text{ cm}^{-1}$ range, consistent with previous reports [38], provides direct evidence of metal-metal interactions, confirming the formation of an integrated nanocomposite structure.

XRD analysis was employed to ascertain the crystalline phases present in the Ag/ZnO/MgO nanocomposite (Fig. 2D). The diffraction peaks were unequivocally assigned by comparison with standard JCPDS files, revealing multi-phase material. The pattern displayed prominent peaks consistent with the hexagonal wurtzite structure of ZnO (JCPDS #01-079-0206), including lattice planes (002), (101), (102), (110), (112), (201), and (202) [39]. Key reflections at 2θ values of 42.59° , 62.92° , and 74.53° were indexed to the (200), (220), and (311) planes of cubic MgO (JCPDS #00-045-0946) [40]. The presence of metallic silver was confirmed by peaks corresponding to the (111), (200), (220), and (311) planes of its face-centered cubic structure (JCPDS #04-0783) [41]. The coexistence of these distinct diffraction patterns confirms the formation of the ternary Ag/ZnO/MgO nanocomposite.

3.3.2. TEM, SEM, DLS, Zeta potential, EDX and elemental Analysis of Ag/ZnO/MgO NCs

The morphology and elemental composition of the Ag/ZnO/MgO nanocomposite were elucidated using SEM and TEM. SEM analysis (Fig. 3A) revealed spherical nanoparticles with an average size of approximately 59 nm (Fig. 3B), a finding corroborated by TEM imaging (Fig. 3C), which showed a similar spherical morphology and a calculated average diameter of 48.3 nm (Fig. 3D). The morphology of the nanocomposite was further characterized by understanding its behavior in suspension [42]. DLS analysis, which measures the hydrodynamic diameter, showed a bimodal size distribution with peaks at 71.4 nm and 426 nm (Fig. 3E). The primary peak aligns with the size range observed by TEM, while the secondary peak indicates the presence of nanoparticle aggregates. The surface charge, a critical factor for colloidal stability and bio-interfacial interactions, was determined by zeta potential measurement (Fig. 3F). A value of -12.2 mV confirmed a net negative surface charge on the particles, which helps stabilize the suspension and may influence initial electrostatic interactions with positively charged bacterial cell membranes [43]. EDX spectroscopy confirmed the presence of the constituent elements Carbon, Oxygen, Silver, Zinc, and Magnesium (Fig. 4A) [44]. Furthermore, elemental mapping (Fig. 4B) demonstrated a uniform spatial distribution of these components throughout the nanocomposite structure.

3.3.3. TGA Analysis

The TGA was performed on the synthesized Ag/ZnO/MgO NCs to evaluate their thermal stability and quantify the loss of volatile constituents. As depicted in Fig. 5A, the TGA profile exhibits distinct regions of mass loss, with the corresponding percentages detailed in Fig. 5B. The initial region, up to 100°C ,

shows a mass reduction of 6.1%, assigned to the desorption of surface-bound water and the thermal degradation of volatile organic constituents. This is further supported by DTG peaks observed at 72°C and 196°C. A subsequent, more pronounced mass loss of 17.3% occurs between 200 and 500°C, coinciding with a sharp differential thermal analysis peak at 344°C. The observed mass reduction in this temperature range is attributed to the decomposition of volatile organic functional groups. These groups originate from the phytochemical constituents of the *C. camphora* leaf extract, which facilitated nanoparticle bio reduction and stabilization [45]. The collective TGA/DTA data indicates that the biosynthesized Ag/ZnO/MgO nanocomposites are thermally stable, exhibiting a total mass loss of 23.4% over the analyzed temperature range.

3.4. Antibacterial efficacy of Ag/ZnO/MgO NCs

The bio fabricated Ag/ZnO/MgO NCs shown a substantial antimicrobial action toward various bacterial pathogenic strains. Inhibition zone measurements showed concentration-dependent antibacterial effects, with optimal activity at 1000 µg/mL producing zones of 21.0 mm (*E. coli*), 16.5 mm (*S. aureus*), 16.3 mm (*K. pneumoniae*), and 15.4 mm (*B. subtilis*) as shown in Fig. 6A. MIC analysis yielded values of 62.5 µg/mL for *E. coli*, 125 µg/mL for both *K. pneumoniae* and *S. aureus*, and 250 µg/mL for *B. subtilis*. Corresponding MBC were determined as 250 µg/mL (*E. coli* and *K. pneumoniae*), 500 µg/mL (*S. aureus*), and 1000 µg/mL (*B. subtilis*). These results correspond with established antimicrobial characteristics of metallic components. Silver nanoparticles are widely documented for their extensive antimicrobial spectrum and human safety profile, while plant-mediated zinc and magnesium nanoparticles show notable antibacterial performance against various bacterial types [46]. The enhanced activity observed in our trimetallic nanocomposite, compared to individual metal nanoparticles, is likely due to synergistic effects. This aligns with previous findings on similar composites, such as ZnO-MgO-Ag, which showed pronounced effects against Gram-negative bacteria [47]. Comparative studies have shown ZnO nanoparticles to possess strong inherent growth inhibition, which is significantly augmented by incorporating silver or gold. For instance, Ag-ZnO nanocomposites exhibit robust activity against antibiotic-resistant *S. aureus* and *E. coli* [48]. To visualize the antibacterial effect of the synthesized Ag/ZnO/MgO NC, TEM analysis was performed on treated *Esch. coli* and *Staph. aureus* cells. Representative TEM micrographs (Fig. 6B) revealed profound morphological damage in both bacterial types compared to their smooth, intact control counterparts. This damage is best explained by a synergistic mechanism involving ROS-generated oxidative stress, Ag⁺ ion toxicity, and direct physical membrane disruption, highlighting the superior antimicrobial potential of this ternary nanocomposite[6].

(B) via Ag/ZnO/MgO NCs

3.4. Antibiofilm and anti-virulence gene activity of Ag/ZnO/MgO NCs

Evaluation of the nanocomposite's antibiofilm activity revealed dose-responsive inhibition against *S. aureus*, achieving 69.49% suppression at 1000 µg·mL⁻¹ and 17.0% at the minimum tested concentration of 15.78 µg·mL⁻¹ (Fig. 7A). Notably, this biofilm suppression occurred without affecting bacterial growth.

In contrast, the nanocomposites showed higher efficacy against the biofilm-producing *E. coli* strain, achieving a maximum inhibition of only 83.92% at 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ reach to 23.3 at 15.78 $\mu\text{g}\cdot\text{mL}^{-1}$. The precise mechanism by which the Ag/ZnO/MgO NPs disrupt biofilm architecture requires further validation. The antibiofilm efficacy of tri-metallic NCs versus *S. aureus* and *E. coli* was quantified using a crystal violet assay. Results demonstrated a dose-dependent relationship, where higher nanocomposite concentrations correlated with increased biofilm inhibition. Specifically, maximum inhibition rates of 69% for *S. aureus* and 83% for *E. coli* were observed at elevated nanocomposite doses [49]. This trend is attributed to the inherent antibiofilm properties of the nanocomposite, whereby a greater quantity of tri-metallic nanocomposite leads to more effective suppression of biofilm formation in both strains. These findings align with the work of Garza-Cervantes et al., who similarly documented a progressive decline in biofilm development with increasing concentrations of composite nanoparticles [50].

The formation of bacterial biofilms is facilitated by specific virulence genes. In *Staphylococcus aureus*, essential genes include *ica* ADBC, *eno* (which encodes a laminin-binding protein), *ebps* (elastin-binding), and *fib* (fibrinogen-binding) [51]. Conversely, in *Escherichia coli*, cellular attachment is mediated by type I fimbriae from the *fim* ABCDEFGH cluster [52] and by P pili from the *pap* operon. These P pili are heteropolymers composed of roughly 1,000 major PapA pilin subunits, with the *papC* gene encoding a protein critical for their polymerization [53]. This research marks the first investigation into the effectiveness of a Phyto fabricated Ag/ZnO/MgO nanocomposite against key virulence genes specifically *icaA*, *icaD*, *papC*, and *fimH* in *S. aureus* and *E. coli*, respectively using a concentration (1/2 MIC). According to Fig. 7B, the nanocomposite suppressed the expression of *papC*, *fimH*, *icaA*, and *icaD* by 40.9%, 44.5%, 36.3%, and 33.7%, respectively. Previous reports align with these findings; for instance, Ag/ZnO at 1/2 MIC notably down-regulated a suite of genes (*spaP*, *gbpB*, *gtfB*, *gtfC*, *ldh*, *comD*, *comE*, *luxS*) in *S. mutans* [54].

3.5. Cytotoxicity and anticancer capability of Ag/ZnO/MgO nanocomposite

3.5.1. MTT assay

The cytotoxicity screening of new materials on healthy cells is considered a primary step for appraising their potential for future applications. This work employed Vero cells to examine the toxicity of an Ag/ZnO/MgO nanocomposite across a range of concentrations, shown in Fig. 8B. Results revealed that low doses of the nanocomposite (15.78 and 31.25 $\mu\text{g}\cdot\text{mL}^{-1}$) produced no cytotoxic effect on the normal cells. The anticancer potential of the Ag/ZnO/MgO nanocomposite was investigated against breast adenocarcinoma (MCF-7) cell lines. Data showed the nanocomposite possessed notable anticancer efficacy, with IC₅₀ values of 118.0 $\mu\text{g}\cdot\text{mL}^{-1}$ against MCF-7, respectively; this compares to a Vero cell IC₅₀ of 332.7 $\mu\text{g}\cdot\text{mL}^{-1}$ (Fig. 9B), confirming the nanocomposite's safety, since an IC₅₀ under 90 $\mu\text{g}\cdot\text{mL}^{-1}$ denotes a non-toxic substance[55]. Inverted microscopy images confirmed that morphological damage to cells was contingent on the Ag/ZnO/MgO nanocomposite concentration (Fig. 8A). The produced Ag/ZnO/MgO nanocomposite displayed considerable action against cancerous

cells at non-toxic levels, suggesting its promise as a secure chemotherapeutic agent pending in vivo studies. Existing literature has established that bimetallic NPs possess anticancer activity against various cancer cell lines[56]. Earlier studies have demonstrated that bimetallic nanoparticles show significant anticancer effects, and it was discovered that the synergistic action of silver and zinc oxide enhanced toxicity toward A549 lung cancer cells [57].

3.5.2. Ag/ZnO/MgO NCs induce apoptosis in *Mcf-7* Cells

To elucidate the pro-apoptotic mechanism underlying the anticancer action of the Ag/ZnO/MgO NCs, we estimated its effect on cell death pathways in *Mcf-7* breast cancer cells using Annexin V-FITC/PI dual staining. The quantitative analysis revealed a distinct shift in the mode of cell death upon treatment. Untreated control cells exhibited a baseline level of 6.0% early apoptosis and 1.7% necrosis (Fig. 9A). In contrast, treatment with the NCs at their IC₅₀ concentration for 72 h extensively encouraged the population of early apoptotic cells to approximately 21.6%, while necrosis remained minimal at 0.5% (Fig. 9B). These results indicate that the Ag/ZnO/MgO NCs exert their cytotoxic effect primarily by inducing programmed cell death (apoptosis) rather than causing indiscriminate necrotic cell death. The significant induction of early apoptosis (a more than threefold increase relative to the control) provides strong evidence that the cytotoxic effect is mediated via the triggering of apoptotic signaling mechanisms, which may include both the intrinsic (mitochondrial) and extrinsic (death receptor) pathways [58]. This is a favorable therapeutic outcome, as apoptosis is a controlled process that minimizes inflammatory responses often associated with necrosis. The minimal increase in necrosis further confirms that the NCs do not cause significant membrane damage or acute toxicity at the IC₅₀ dose under these conditions [59]. This apoptotic induction is likely a key contributor to the overall anticancer efficacy of the Ag/ZnO/MgO nanocomposite against MCF-7 cells.

3.5.3. Analysis of cell cycle progression in treated *Mcf-7* cells

To further delineate the mechanism by which the Ag/ZnO/MgO NCs inhibits cancer cell proliferation and induces apoptosis, we investigated its impact on the cell cycle dynamics of *Mcf-7* breast cancer cells, as analyzed by flow cytometry.. Given the intrinsic link between cell cycle regulation and programmed cell death, this analysis provides critical insight into the NC's mode of action. Cell cycle distribution analysis revealed a profound disruption upon treatment. Untreated control cells (Fig. 10A) displayed a typical distribution, with the majority of the population in the G0/G1 phase. In stark contrast, treatment with the NCs at the IC₅₀ concentration for 72 hours (Fig. 10B) induced a significant arrest, predominantly in the S phase (72.4% of cells compared to ~ 80.4% in controls) and the G2/M phase (26.3% compared to ~ 18.2% in controls). Concurrently, a marked decline in the cell population in the G1 phase was observed.

The observed cell cycle arrest, specifically at the S and G2/M checkpoints, provides a mechanistic explanation for the nanocomposite's anti-proliferative and pro-apoptotic effects [60]. Arrest in the S phase suggests interference with DNA replication, potentially through the induction of DNA damage or

the inhibition of key synthesis enzymes, which triggers checkpoint activation to prevent the propagation of genetic errors. The concurrent accumulation in the G2/M phase indicates an additional blockade prior to mitosis, which can be a cellular response to unrepaired DNA damage or disrupted microtubule dynamics [61]. This coordinated arrest and apoptosis induction underscore the nanocomposite's efficacy in targeting fundamental cancer hallmarks uncontrolled proliferation and evasion of cell death.

3.6. Antioxidant and anti-obesity properties capacity of Ag/ZnO/MgO NCs

The biosynthesized Ag/ZnO/MgO nanocomposite's biological profile is defined not only by its cytotoxicity but also by its significant antioxidant potential, as established through DPPH and ABTS radical scavenging assays. This antioxidant activity, which increased with concentration, adds a critical dimension to its overall functionality. Quantitative analysis revealed the nanocomposite's half-maximal inhibitory concentration (IC_{50}) was $150.1 \mu\text{g}\cdot\text{mL}^{-1}$ for DPPH, indicating superior scavenging efficiency compared to its ABTS IC_{50} of $173.3 \mu\text{g}\cdot\text{mL}^{-1}$ (Fig. 11A). This concentration-dependent neutralization of the DPPH and ABTs radical across a 15.78 to $1000 \mu\text{g}\cdot\text{mL}^{-1}$ range is shown in Fig. 10B and C. These results align with previous findings; for instance, Ag@MgO/ZnO showed notable activity ($IC_{50} = 23.35 \mu\text{g/mL}$) [62], and an MgO/ZnO composite reported an IC_{50} of $175 \mu\text{g/mL}$ in the DPPH assay [63].

Lipases constitute a class of enzymes which catalyze the breakdown of triglycerides into fatty acids and glycerol. These lipid-hydrolyzing enzymes are found in blood, gastric juices, and pancreatic secretions [64]. The inhibition of lipase is a widely studied mechanism for assessing new drugs developed for managing obesity. Lipase suppression was recorded following exposure to the Ag/ZnO/MgO nanocomposite (Fig. S2). A reduction in enzymatic activity was seen at elevated concentrations of the nanomaterial. According to the present results, the Ag/ZnO/MgO nanocomposite had an IC_{50} of $108.4 \mu\text{g/mL}$ for lipase inhibition, compared to $9.4 \mu\text{g/mL}$ for orlistat. Similar results consistent with this work show a ZnO/CuO/Se nanocomposite from *Schinus terebinthifolia* biomass had excellent lipase inhibition, showing an IC_{50} of $41.66 \mu\text{g/mL}$ [65]. One study reported that CuO-NPs at $200 \mu\text{g/mL}$ produced an 80.5% lipase inhibition [66].

4. Conclusion

In conclusion, this study successfully developed a novel, environmentally benign route for synthesizing a trimetallic Ag/ZnO/MgO NC using *C. camphora* leaf extract as a proficient reducing and stabilizing agent. Optimization via RSM yielded a well-characterized nanocomposite with demonstrated multifunctional biomedical potential. Broad-spectrum antibacterial efficacy was a key characteristic of the nanocomposite, which inhibited planktonic growth, disrupted biofilm architecture, and attenuated virulence gene expression in clinically significant bacterial strains, highlighting its potential as a comprehensive anti-infective agent. Furthermore, the nanocomposite showed selective anticancer activity against *Mcf-7* breast cancer cells by inducing apoptosis and cell cycle arrest, while displaying significantly lower toxicity toward normal cells. Its additional antioxidants and lipase-inhibitory

properties further broaden its therapeutic scope. The synergistic interplay between the metallic constituents (Ag, Zn, Mg) and the phytochemical capping agents underpins its enhanced bioactivity. These compelling *in vitro* results position the Phyto fabricated Ag/ZnO/MgO NC as a promising candidate for advanced nanotherapeutic applications in antimicrobial, anticancer, and metabolic disorder treatments, warranting further *in vivo* investigations to validate its efficacy and safety profile.

Declarations

Author Contribution

Munirah F. Aldayel: Resources, Investigation, Visualization, Project administration, Funding acquisition. Nada H. Aljarba: Data curation, Writing – original draft, Software, Project administration, Funding acquisition. Abdulkarim S. Binshaya: Methodology, Formal analysis, Visualization. Adil Abalkhail: Writing – review and editing, Validation, Visualization. Mohamed K. Y. Soliman: Supervision, Investigation, Software, Validation, Conceptualization

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Figures

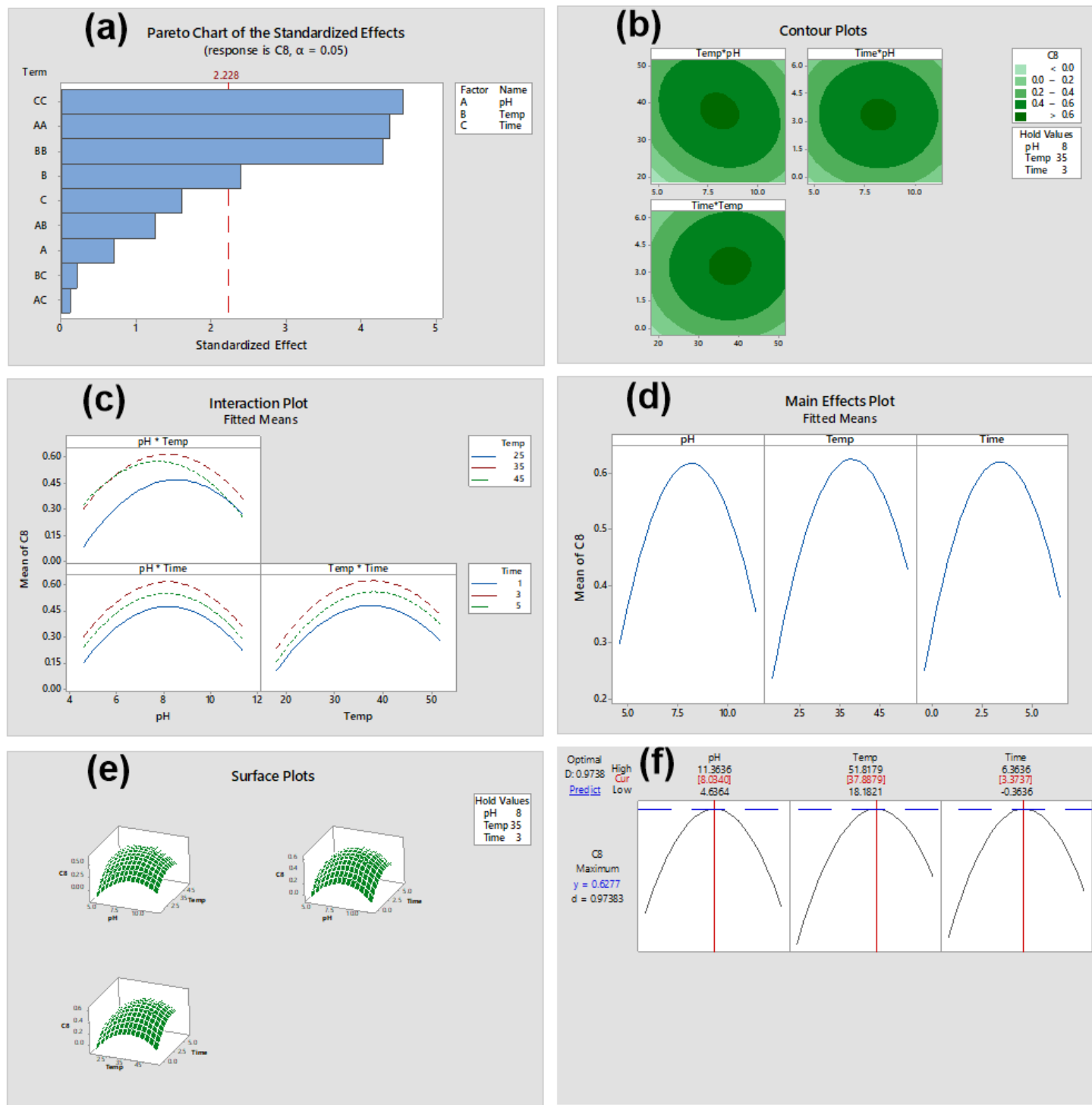


Figure 1

RSM analysis showing Pareto chart (a), Contour Plot (b), Interaction plot (c), Main effects plot (d), Surface plot (e) and Optimal predictor (f)

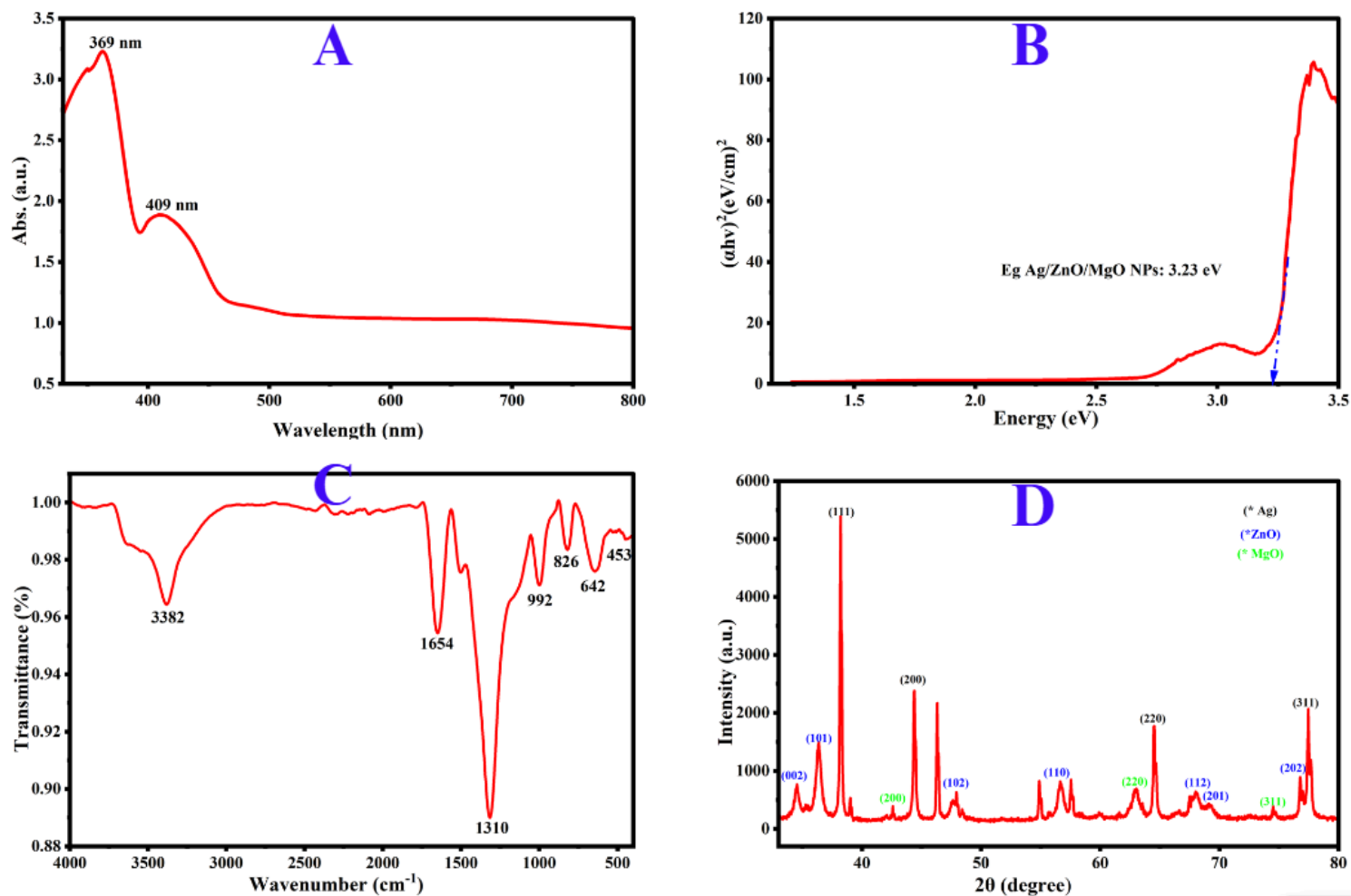


Figure 2

Graphs of UV–Vis (A), bandgap (B), FTIR graph (C) and XRD pattern (D) of Ag/ZnO/MgO NCs

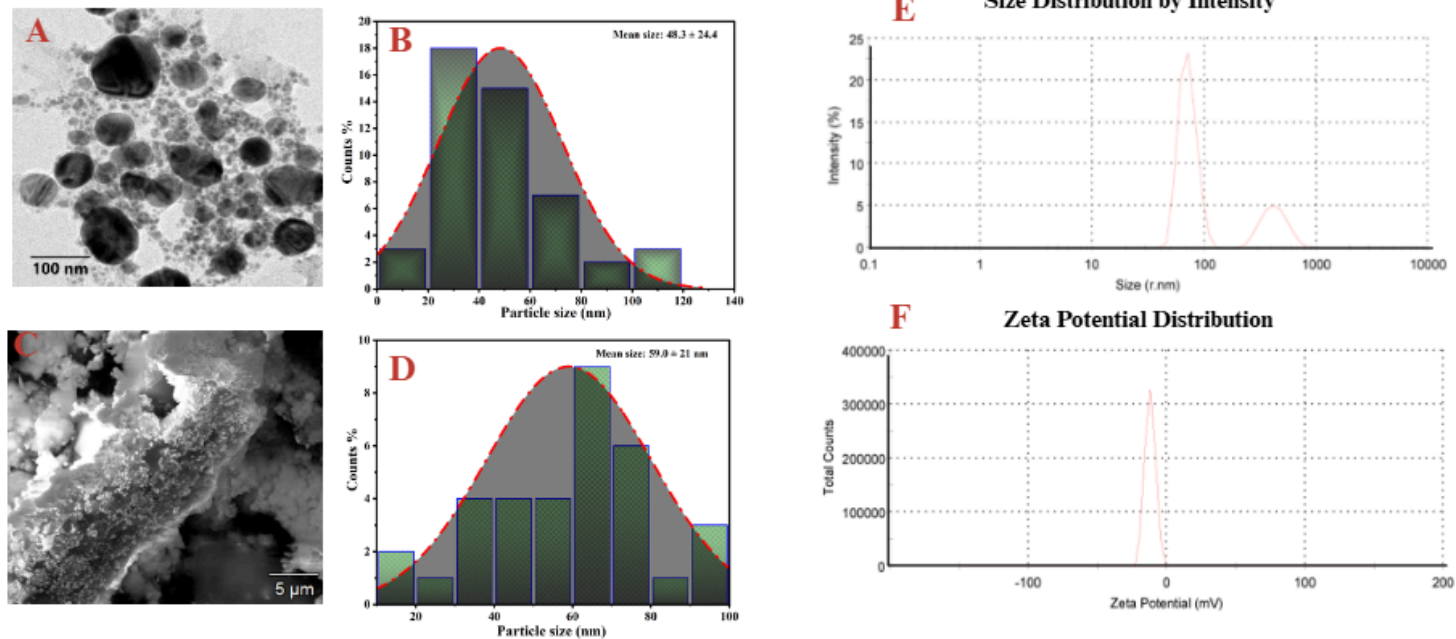


Figure 3

TEM (A) and PSA of TEM (B) SEM (C), PSA of SEM (D), DLS (E) and Zeta potential (F) of Ag/ZnO/MgO NCs

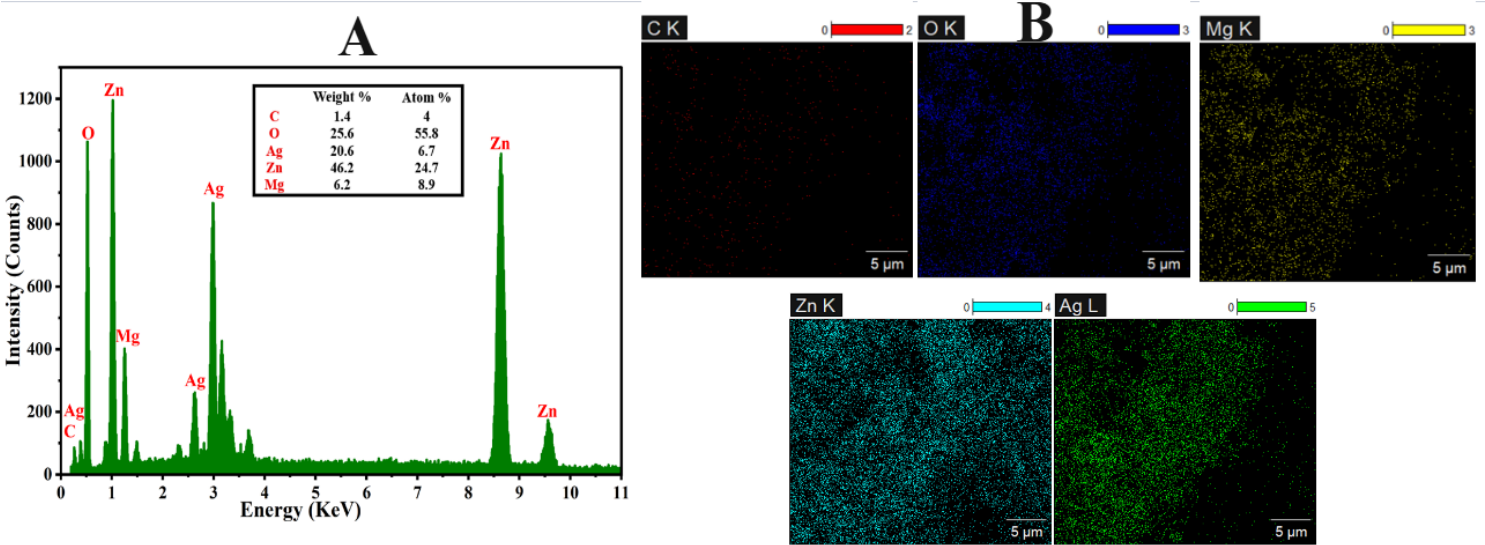


Figure 4

EDX analysis (A) and elemental mapping (B) of Ag/ZnO/MgO NCs

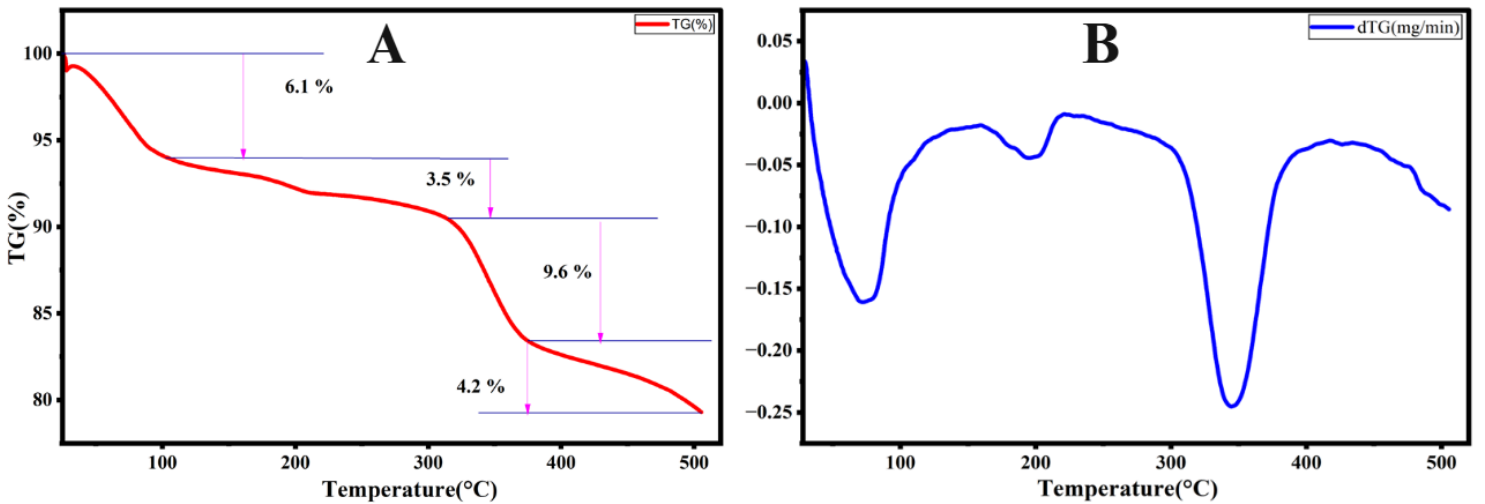


Figure 5

TGA (A) and DTA (B) analysis profiles of the biosynthesized Ag/ZnO/MgO NCs

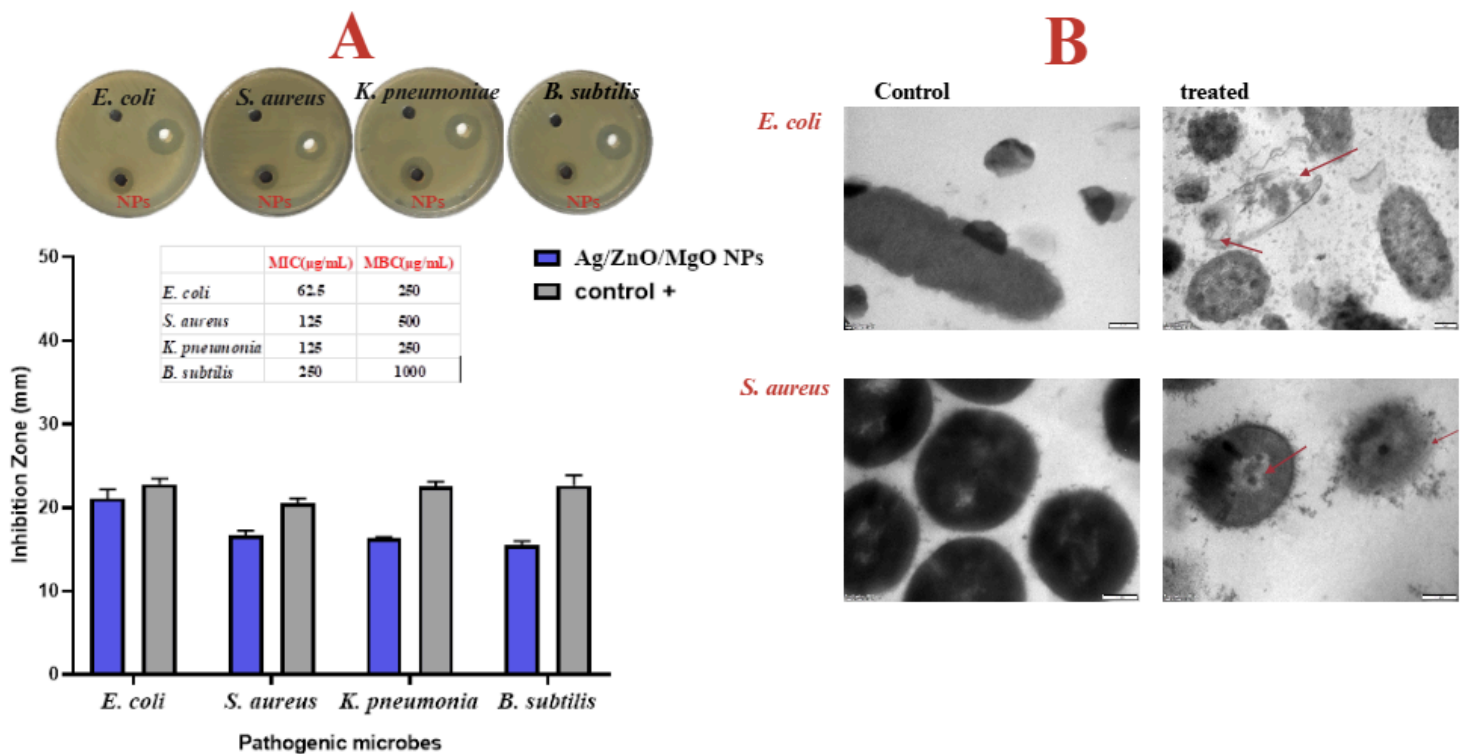


Figure 6

Antibacterial assay of Ag/ZnO/MgO NCs (A), TEM images of *E. coli* and *S. aureus* treated with Ag/ZnO/MgO NCs (B) show morphological damage, including cell surface deformation and disruption

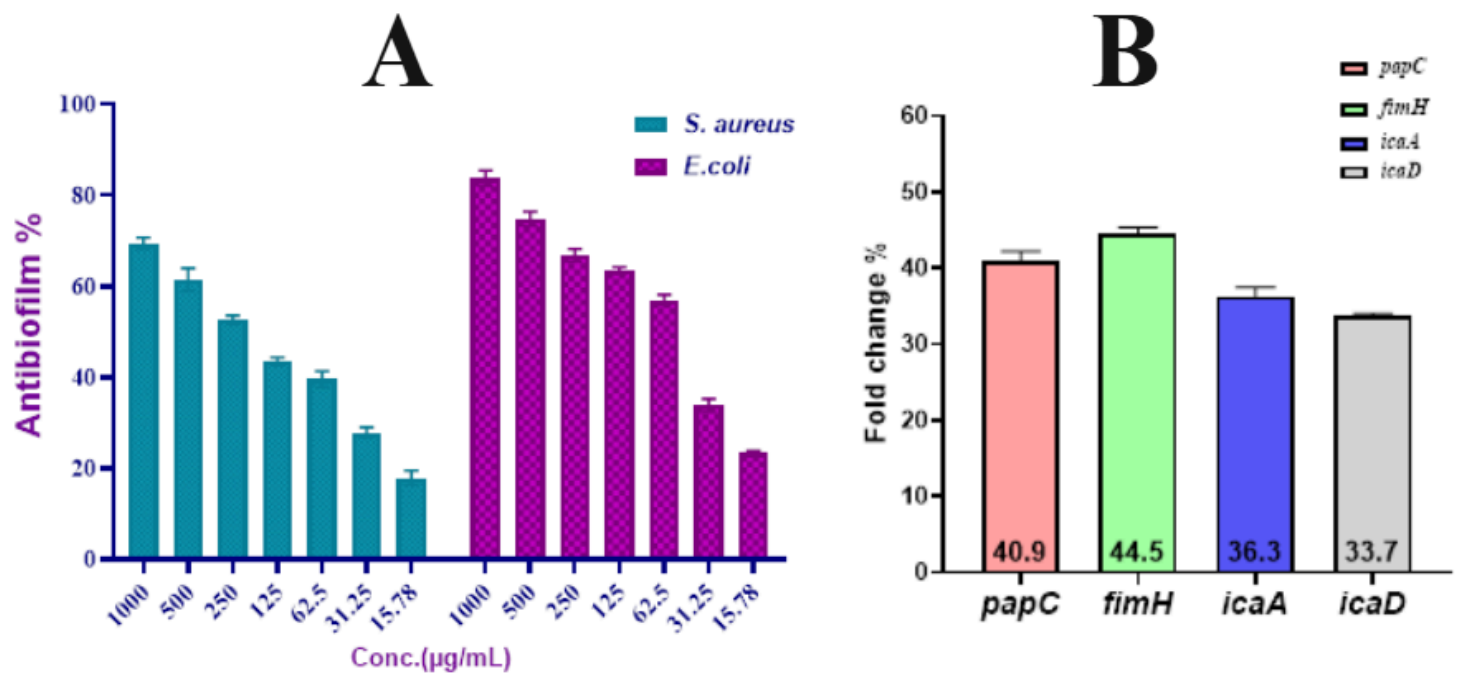


Figure 7

Antibiofilm (A) and Folds of gene expression *papC*, *fimH*, *icaA*, and *icaD* (B) via Ag/ZnO/MgO NCs

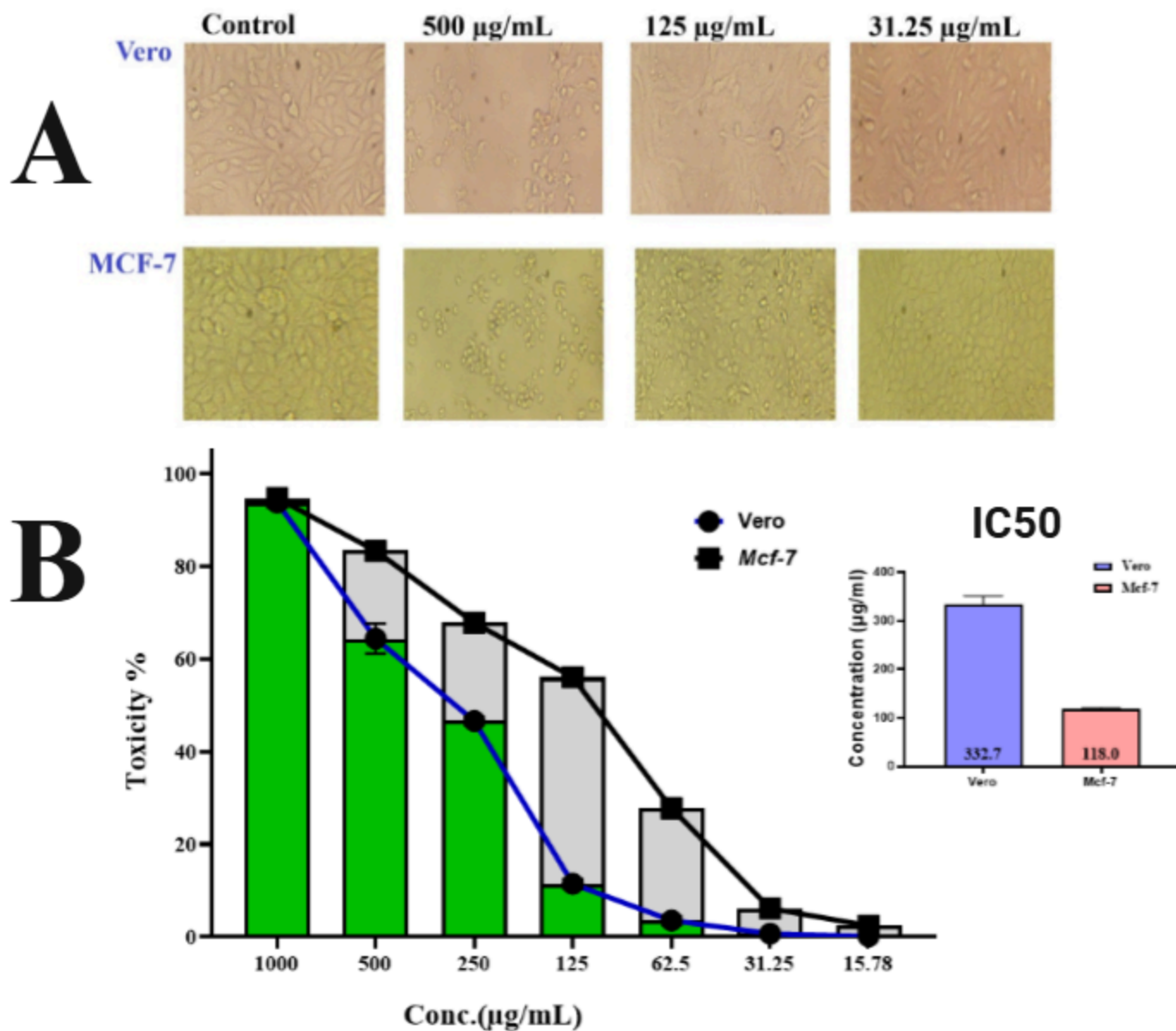


Figure 8

Effect of Ag/ZnO/MgO NCs on the morphology of Vero and MCF-7 cells (A) and the cytotoxicity and anticancer potential of Ag/ZnO/MgO NCs (B)

Quadrant	Cell population	Percentage (%)	
		Control	Ag/ZnO/MgO NCs
F3	Alive cells	92.2%	77.8%
F4	Early Apoptotic	6.0%	21.6%
F1	Late Apoptotic	0.1%	0.0%
F2	Necrotic	1.7%	0.5%

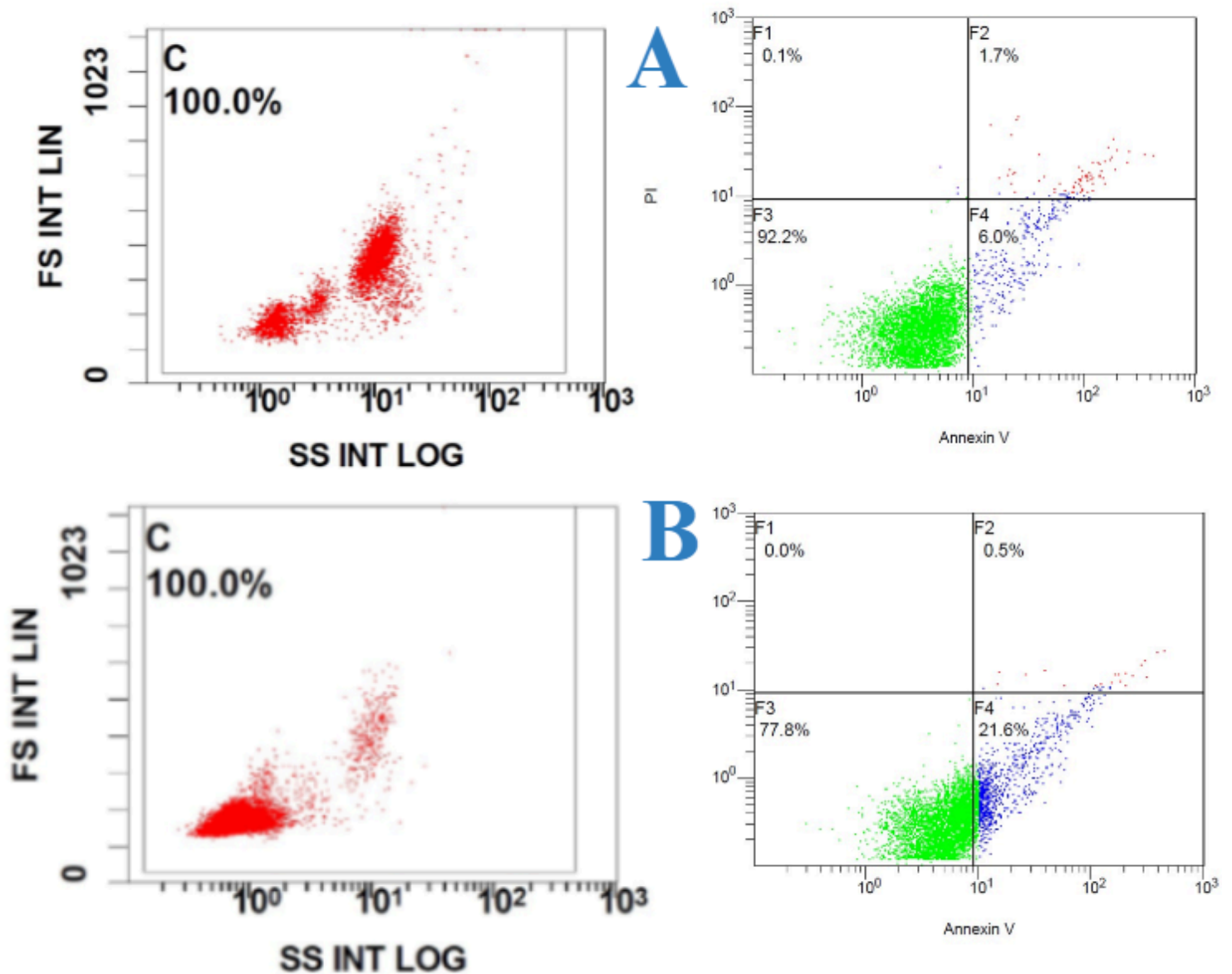


Figure 9

Flow cytometric analysis of apoptosis in *Mcf-7* cells using Annexin V-FITC/PI staining. Representative dot plots show (A) untreated control cells and (B) cells treated with the Ag/ZnO/MgO nanocomposite

Cell population	Percentage (%)	
	Control	Ag/ZnO/MgO NCs
G0 phase	0.2%	0.0%
G1 phase	0.5%	1.0%
S phase	80.4%	72.4%
G2M phase	18.2%	26.3%

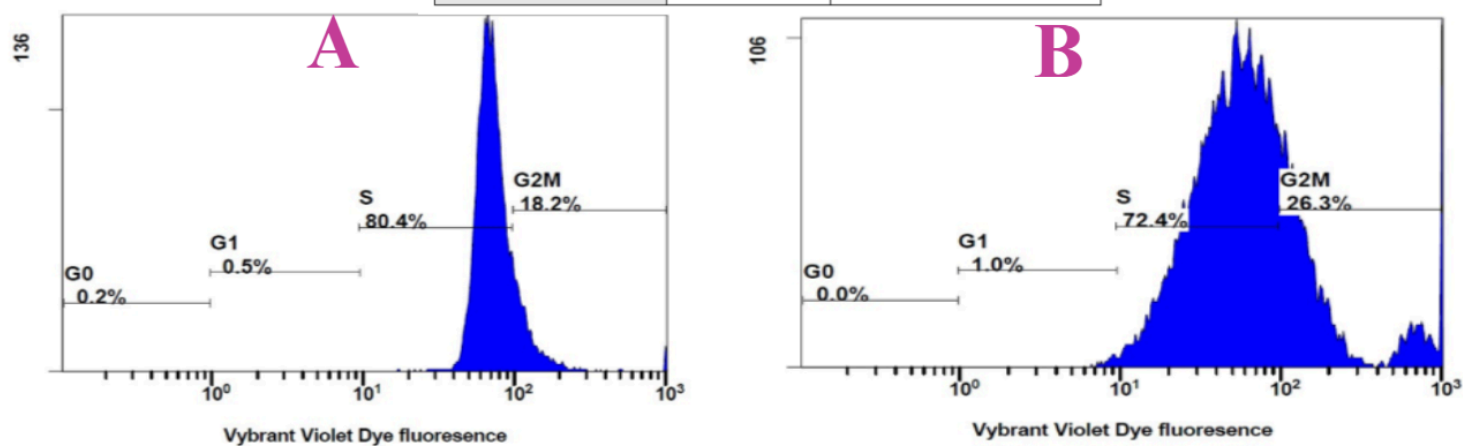


Figure 10

Induction of S and G2/M phase cell cycle arrest in MCF-7 cells (A) untreated and (B) treated by Ag/ZnO/MgO NCs.

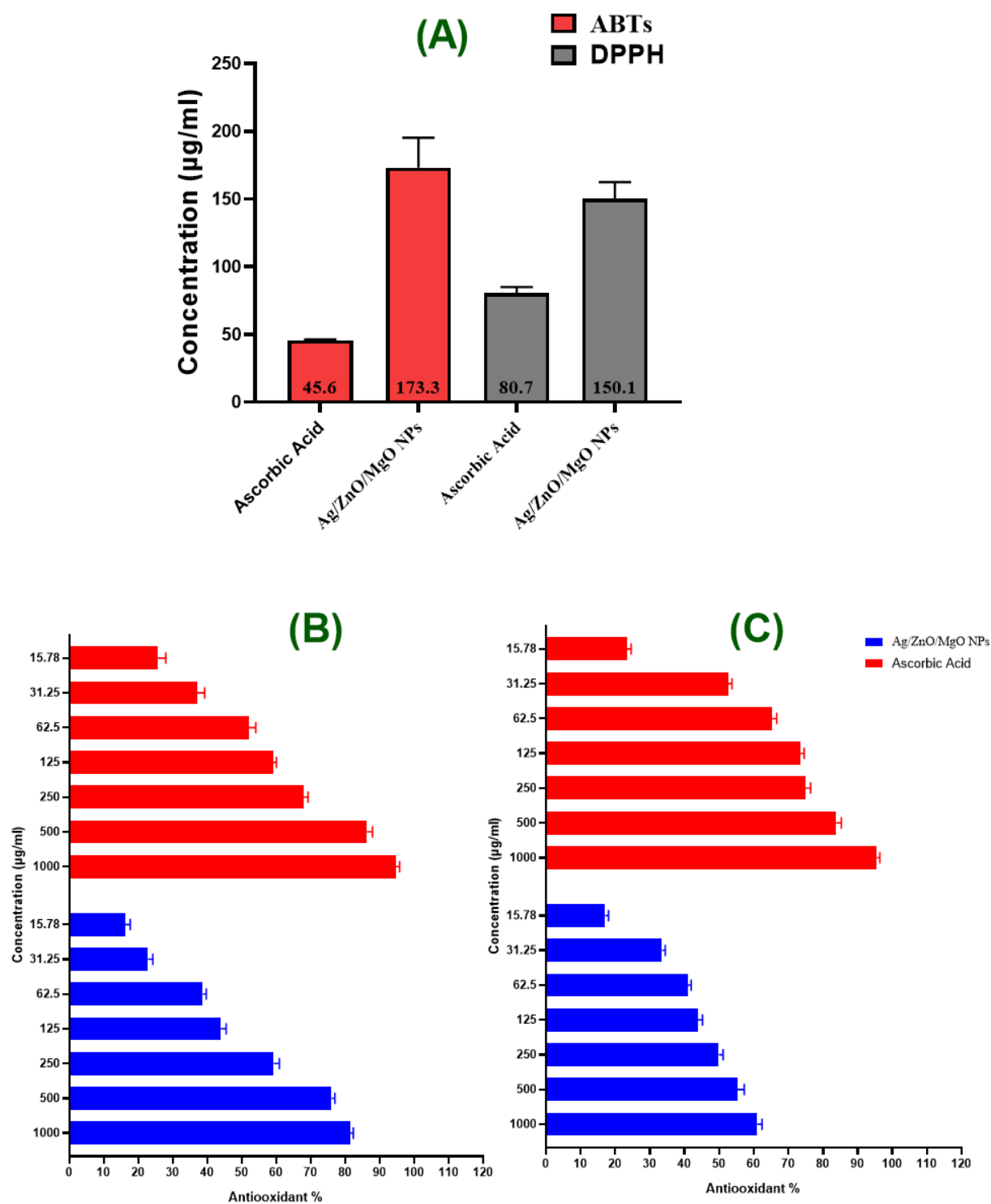


Figure 11

Antioxidant potential of Ag/ZnO/MgO NCs; IC₅₀(A), DPPH (B) and ABTs (C)

Supplementary Files

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- [image1.png](#)